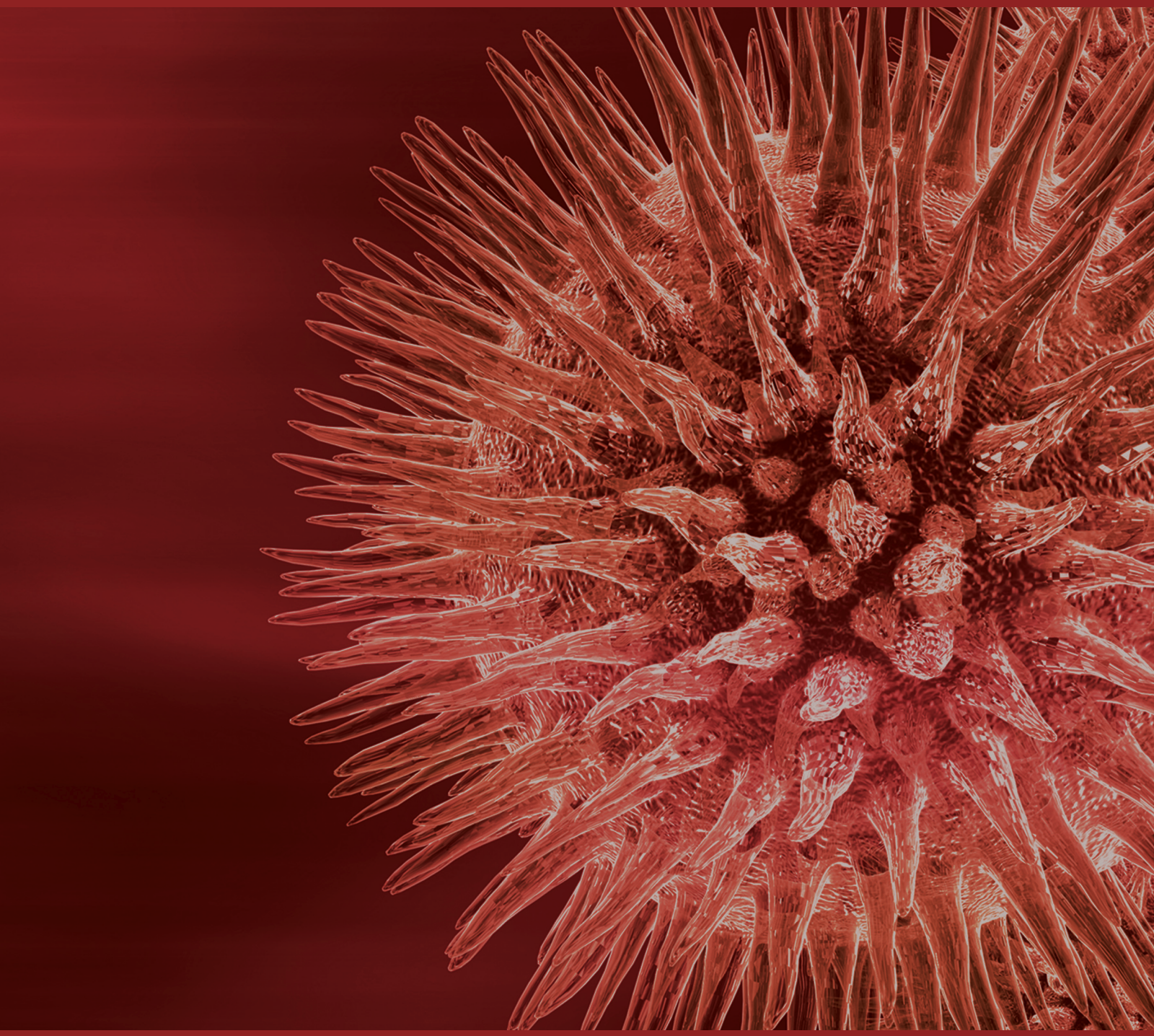
Journal of Biomedicine and Biotechnology

Cytotoxic T Lymphocytes and Vaccine Development

Guest Editors: Zhengguo Xiao, Kim Klonowski, and Hanchun Yang



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Virology

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Editorial

Cytotoxic T Lymphocytes and Vaccine Development

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Along with the development of new technology, the molecular mechanisms of the generation of functional CTLs have been elucidated over the last several years which together will undoubtedly enhance our progression towards the development of successful vaccines for the many chronic viral infections and cancer. It has been shown that the activation of naïve CTLs requires all three signals to be fully activated: antigen, costimulation, and third signal, which can be provided by inflammatory cytokines such as IL-12 and type I IFNs. In addition, differentiation of memory CTLs requires survival signals and γ_c-cytokines such as IL-2, IL-7, and IL-15. With the booming information on CTLs activation and their application, we felt it was in urgent need to launch a special issue focusing on the most recent progress on CTL research.

We expected an active response to our call for this special issue and indeed are satisfied with the large number of submissions and quick and accurate response of authors to all the reviews and editorial comments. The selected research manuscripts cover a broad area of CTL research, including studies to define new strategies for vaccination against devastating chronic infections like HIV, tumor therapies defining novel tumor-specific epitopes for CTLs, and strategies to enhance cancer control. Interesting attempts have been made to indentify nontraditional adjuvants such as bacteriophages and β-glucan oligosaccharides while other groups have used unique vectors and cytokines for vaccination studies. Interestingly, this issue attracted a large number of review articles which address the mechanisms of CTL activation, new function of CTLs, vaccine development for viral infections, new adjuvant exploration, and the most up-to-date progress in cancer vaccine and cancer therapy.

We thank all of the authors for their enthusiasm in submitting their manuscripts to this special issue and appreciate their efforts and time in process of their manuscripts. Acknowledgement should certainly go to all of the reviewers, most of whom reviewed at least two versions of the same manuscript. Their sacrifice of research time is highly appreciated. We hope the original scientific discoveries and summarized information within this special issue will be helpful and promote new avenues of exploration for scientists studying CTLs in the context of vaccine development.

Zhengguo Xiao
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Research Article

Immunization with a Mixture of HIV Env DNA and VLP Vaccines Augments Induction of CD8 T Cell Responses

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The immune response induced by immunization with HIV Env DNA and virus-like particle (VLP) vaccines was investigated. Immunization with the HIV Env DNA vaccine induced a strong CD8 T cell response but relatively weak antibody response against the HIV Env whereas immunization with VLPs induced higher levels of antibody responses but little CD8 T cell response. Interestingly, immunization with a mixture the HIV Env DNA and VLP vaccines induced enhanced CD8 T cell and antibody responses. Further, it was observed that the mixing of DNA and VLP vaccines during immunization is necessary for augmenting induction of CD8 T cell responses and such augmentation of CD8 T cell responses was also observed by mixing the HIV Env DNA vaccine with control VLPs. These results show that immunization with a mixture of DNA and VLP vaccines combines advantages of both vaccine platforms for eliciting high levels of both antibody and CD8 T cell responses.

1. Introduction

Since the identification of the human immunodeficiency virus (HIV) as the causative agent of AIDS twenty-five years ago, tremendous amount of effort has been poured into vaccine development. However, despite the significant progress that has been made over the last two decades, an efficacious AIDS vaccine strategy is still elusive. Earlier clinical trials using HIV Env-based subunit vaccines failed to show significant protection against HIV infection or disease progression [1–3]. Subsequent studies in nonhuman primates showed that induction of a strong cellular immune response against SIV and HIV antigens, particularly a strong cytotoxic CD8 T cell response, was able to exert a successful control of disease progression and AIDS development [4–7]. However, despite the promising results obtained in animal studies, the results from a recent clinical trial of a T-cell-based vaccine regimen dealt another setback to AIDS vaccine development [8]. The disappointing outcomes from these vaccine trials further reinforce the notion that an effective AIDS vaccine should be able to induce both strong antibody and cytotoxic T cell responses against HIV [9–12].

A number of studies have shown that DNA vaccines can effectively induce both antibody and T cell responses against their encoded antigens [13, 14]. DNA immunization induces immune responses through both direct transfection of antigen presenting cells (APCs) and cross priming of APCs [14, 15] and offers several advantages over other vaccine platforms. First, the direct in vivo expression of antigens by DNA vaccination renders it more effective in eliciting cellular immune responses than protein-based vaccines, as in vivo synthesized antigens are processed and presented through both major histocompatibility complex I and II for inducing both CD4 and CD8 T cell responses. Second, expression of the antigens over a long period of time after DNA vaccination may provide sustained stimulation of the immune system for

inducing long lasting immune responses [16]. Third, DNA vaccines can be applied repeatedly without inducing immune responses against the vector in contrast to recombinant viral-vector-based vaccines.

Virus-like particles (VLPs) represent another attractive concept for vaccine development [17–19]. VLPs share with DNA vaccines the ability to be administered repeatedly to vaccinated individuals. The nonreplicative nature of VLPs and their lack of viral genomic RNA make them safe for broad and repeated application. Since the assembly and arrangement of viral glycoproteins in VLPs resemble intact virions, they are likely to be more effective in inducing neutralizing antibodies as compared with soluble antigens. Earlier studies have shown that a viral glycoprotein presented in a highly repetitive form in virus particles is more potent in inducing B cell response and antibody production than the same antigen presented in a poorly organized form [20, 21]. In several studies, HIV VLPs have been shown to induce both neutralizing antibodies and CTL responses to HIV antigens [22, 23].

While both the HIV DNA and VLP vaccines can induce antibody as well as cytotoxic T cell responses [12, 24, 25], DNA vaccines induce immune responses through direct in vivo antigen synthesis whereas VLP vaccines directly present viral glycoproteins on the surface of a particulate antigen. As a result of their different properties, immune responses induced by these two vaccine platforms are likely to be different. In this study, we compared the immunogenicity of HIV Env-DNA and VLP vaccines and investigated whether a combination of these two vaccine platforms may complement each other when given as a mixture for inducing both antibody and CD8 T cell responses.

2. Materials and Methods

2.1. Preparation of DNA and VLP Vaccines. The HIV 89.6 Env718Tr/Y710S DNA construct, designated as Env-DNA, has been described in previous studies [26] For large-scale preparation, the plasmids were amplified in E. coli DH5α and purified with a Qiagen Endo-Free Megaprep kit. The plasmids were then resuspended at 1 μg/μl in sterile PBS and stored at -80°C until used for immunization. Construction of recombinant baculoviruses expressing the SIV Gag (rBV-Gag) and the HIV 89.6 Env protein (rBV-Env) has also been described previously [27, 28]. SHIV 89.6 VLPs were produced by coinfection of Sf9 insect cells with rBV-Gag and rBV-Env at a multiplicity of 2 and 5, respectively. At 60 hours post infection, medium from infected cells was collected and clarified of cell debris by centrifuge at 7,000 rpm in a Sorvall SS-34 rotor for 20 minutes. The VLPs in the supernatant were concentrated by ultracentrifugation at 28,000 rpm for 1 hour in a Beckman SW28 rotor, resuspended in PBS, and further purified by centrifugation through a discontinuous sucrose gradient (20%, 35%, and 50% layers) at 30,000 rpm for 1 hour in a Beckman SW41 rotor. The band that contained VLPs (between 35% and 50% layers) was collected, concentrated, and then resuspended in PBS at a final concentration of 2 μg/μl. The integrity of purified VLPs was examined by electron microscopy and the presence of SIV Gag and HIV 89.6 Env proteins in VLP preparations was determined by western blot analysis as described previously [28]. Protein concentration of the VLP preparation was determined by a BCA protein assay (Pierce Biotechnology, Rockford, IL) and the amount of HIV 89.6 Env proteins in the VLP preparation were determined by a sandwich ELISA using purified HIV 89.6 gp120-Histag as standards. The VLP preparation was stored in -80°C until immunization and the same VLP preparation was used in the immunization experiments throughout this study. The SIV Gag only VLPs were produced by infection of Sf9 cells with rBV-Gag at the multiplicity of 2 and purified similarly as described above.

2.2. Immunization of Mice. Female BALB/c mice (H-2d) 6–8 weeks of age were purchased from Charles River Laboratory. Mice were housed in the animal facility operated by the Division of Animal Resources at Emory University. All procedures were carried out following animal research guidelines and approved by IACUC. For immunization with DNA or VLP vaccines alone, groups of mice (six mice per group) were immunized with 50 μg DNA or VLPs dissolved in 100 μl PBS per mouse by intramuscular injection in both side quadriceps with 50 μl of the preparations at each side. For immunization with a mixture of DNA and VLPs, 50 μg DNA and 50 μg VLPs were mixed in 100 μl PBS prior to immunization and then administered to mice by intramuscular injection in both side quadriceps with 50 μl of the preparations at each side. For immunization with both DNA and VLP vaccines at separate sites, 50 μg DNA or 50 μg VLPs were each dissolved in 50 μl PBS and administered to mice by intramuscular injection in separate side quadriceps respectively. Mouse blood samples were collected by retro-orbital bleeding at 14 days after each immunization, heat-inactivated, and stored at -80°C until analysis. All mice received a boosting immunization at 4 weeks after priming following the same procedure.

2.3. Flow Cytometry Analysis of CD8 T Cell Responses. Analysis of cellular immune responses by intracellular cytokine staining and flow cytometry was carried out as described previously [29, 30]. Immunized mice were sacrificed two weeks after the second immunization and mouse splenocytes were prepared with lysis of red blood cells with ammonium chloride and washed twice with RPMI 1640. The cells were resuspended in complete culture medium (RPMI 1640 plus 10% fetal calf serum, 50 μM beta-mercaptoethanol, and antibiotics mix) and the cell viability was determined by trypan blue exclusion. Cells (10^6) were cultured in the presence of brefeldin A (Sigma, 10 μg/mL), with a stimulating peptide corresponding to a CTL epitope of the HIV89.6 Env protein (IGPGRARYAR, 10 μg/mL) or an irrelevant peptide corresponding to a segment in the HIV Gag protein (AMQMLKETI, negative control) for 6 hours. After stimulation with peptide, the cells were washed twice with PBS containing 3% fetal calf serum and then stained with FITC-conjugated rat anti-CD8 and PerCP-conjugated rat anti-CD4 antibodies (Pharmingen). Cells were then fixed and permeabilized with cytofix buffers (Caltag) and then

stained for intracellular IFNγ using APC conjugated rat anti-IFNγ antibody (Pharmingen). Flow cytometry analysis was performed on a BD FACSCalibre with CELLQuest software.

2.4. ELISA. ELISA plates were coated with purified HIV-1 89.6 gp120-Histag (prepared by Nicald affinity purification using a Qiagen kit, at 2 μg/mL in borate-buffered saline, pH 8.5, 100 μl per well) at 4°C overnight and blocked with PBS-T-BSA (phosphate-buffered saline, 0.1% tween-20, 3% bovine serum album) for 3 hours at 37°C. Serial dilutions of mouse serum were then added to each well in triplicate and incubated at 37°C for 3 hours. HRP-conjugated secondary antibody (Sigma) against mouse IgG, IgG1, or IgG2a was added for 2 hours at 37°C. After a final wash, ABTS (2,2′-azino-bis(3-ethybenz-thiazoline-6-sulfonic acid), Sigma) dissolved in citrate phosphate buffer (3 mg ABTS in 10 mL CPB, pH 4.2, plus 10 μl H_2O_2) was added at 100 μl/well for developing color and read by an ELISA reader at 405 nm. A standard curve for absorbance and the amount of mouse antibody absorbed to the well were obtained by coating ELISA plates with serial 2-fold dilutions of purified mouse antibodies followed by addition of HRP-conjugated secondary antibody and development of color. Data were analyzed by a Microsoft Excel program and presented as the equivalent amount of Env-binding antibodies in mouse sera (ng/mL) and statistical analysis was carried out using Student t-test.

2.5. Sedimentation Assay. To determine whether the DNA and VLPs bind to each other in the DNA and VLP mixture, we carried out a sedimentation study. The HIV Env-DNA (50 μg) and DNA and VLP mixture (50 μg DNA plus 50 μg VLP) were loaded onto a two-layer sucrose cushion, with 500 μl 30% sucrose at bottom and 2 mL 20% sucrose above, followed by centrifugation at 30,000 RPM for 30 minutes in a Beckmann SW55Ti rotor. To determine whether the integrity of VLPs is necessary for binding to DNA molecules, we also included a control sample in which VLPs (50 μg) were lysed by 1% Triton X-100 prior to mixing with the HIV Env-DNA (50 μg). VLPs (50 μg) only were also included as a negative control. After centrifugation, top layers were removed from the tube and the concentration of DNA molecules in the 30% sucrose cushion was determined by UV spectrometry at OD260. The relative amount and mobility of DNA in the 30% sucrose cushion was also examined by electrophoresis in an agarose gel.

2.6. Bone Marrow-Derived Dendritic Cell (BMDC) Preparation and Stimulation. Mouse BMDCs were prepared following procedures as reported by Lutz et al. in their studies [31]. Briefly, femurs were obtained from sacrificed mice and the marrows were flushed out with FACS medium and then filtered through a cell strainer. After lysis of red blood cells, bone marrow leukocytes were resuspended in FACS medium and cell numbers were counted by trypan blue staining. The leukocytes were then seeded in 100 mm bacteriological petri dishes at 2×10^6 per dish in 10 mL FACS medium containing 20 ng/mL recombinant mouse GM-CSF. On day 3, another 10 mL FACS medium containing 20 ng/mL recombinant mouse GM-CSF was added to each dish. On day 6, half of the culture supernatant was collected, centrifuged, and the cell pellet resuspended in 10 mL fresh FACS medium containing 20 ng/mL recombinant mouse GM-CSF and then added back into the original dish. Analysis by flow cytometry shows that BMDCs prepared by this method are over 70% CD11c positive. On day 7 of culture, 10^5 BMDCs were added to each well in a 96-well plate and stimulated in vitro with HIV Env-DNA (50 μg/mL), SHIV 89.6 VLPs (10 μg/mL), a mixture of DNA (50 μg/mL), and VLPs (10 μg/mL), as well as with LPS (10 ng/mL, positive control) or mock-treated media (negative control) in triplicates. In parallel, the samples were also heated at 100°C for 30 minutes before being added to DCs in the stimulation assay. Supernatants were harvested after 24 hours of incubation at 37°C in 5% CO_2, and cytokine levels in cell supernatants were measured by ELISA in duplicates using commercially available kits (eBioscience) for IL-6, IL-12, as well as TNF-alpha according to the manufacturer's instruction. For detection of CD80 and CD86 surface expression, BMDCs were harvested after stimulation by DNA or VLP vaccines as described above and the cells were washed twice with PBS containing 3% fetal calf serum. After wash, the cells were stained with PE conjugated rat anti-CD11c and APC conjugated rat anti-CD80 (or anti-CD86) antibodies (Pharmingen). Cells were then fixed with cytofix buffers (Caltag) and then analyzed by flow cytometry on a BD FACSCalibre with CELLQuest software.

3. Results

3.1. HIV Env-DNA and VLP Vaccines Exhibit Different Abilities to Induce CD8 T Cell and Antibody Responses. Construction and characterization of the HIV 89.6 Env718Tr/Y710S DNA vaccine (designated as the Env-DNA in this study) has been described previously [30]. SHIV 89.6 VLPs were produced by coinfection of Sf9 insect cells with recombinant baculoviruses expressing the HIV 89.6 Env and SIVmac239 Gag proteins, respectively. As shown in Figure 1(a), both HIV 89.6 Env and SIV Gag proteins were detected in SHIV 89.6 VLPs by Western blot. Examination of purified VLPs by electron microscopy showed that they exhibit a uniformed spherical morphology that is about 150 nm in diameter and similar in size to HIV virions (Figure 1(b)). The amount of HIV Env proteins in SHIV 89.6 VLP preparations was further determined by a sandwich ELISA using purified HIV Env gp120 as a standard and the results showed that there is about 100–130 ng HIV Env proteins in 5 μg of three different SHIV 89.6 VLP preparations (Figure 1(c)). After production and characterization, the SHIV 89.6 VLPs were used in immunization studies in comparison with the HIV Env-DNA vaccine as outlined in Figure 2. The plasmid DNA vector pCAGGS and SIV Gag-VLPs that were produced by infection of Sf9 insect cells with recombinant baculoviruses expressing the SIVmac239 Gag protein were used as control DNA and VLPs, respectively, in the immunization studies.

As shown in Figure 3, immunization with the Env-DNA vaccine alone (Group 2) induced significant levels of CD8 T cell responses against the HIV Env with an

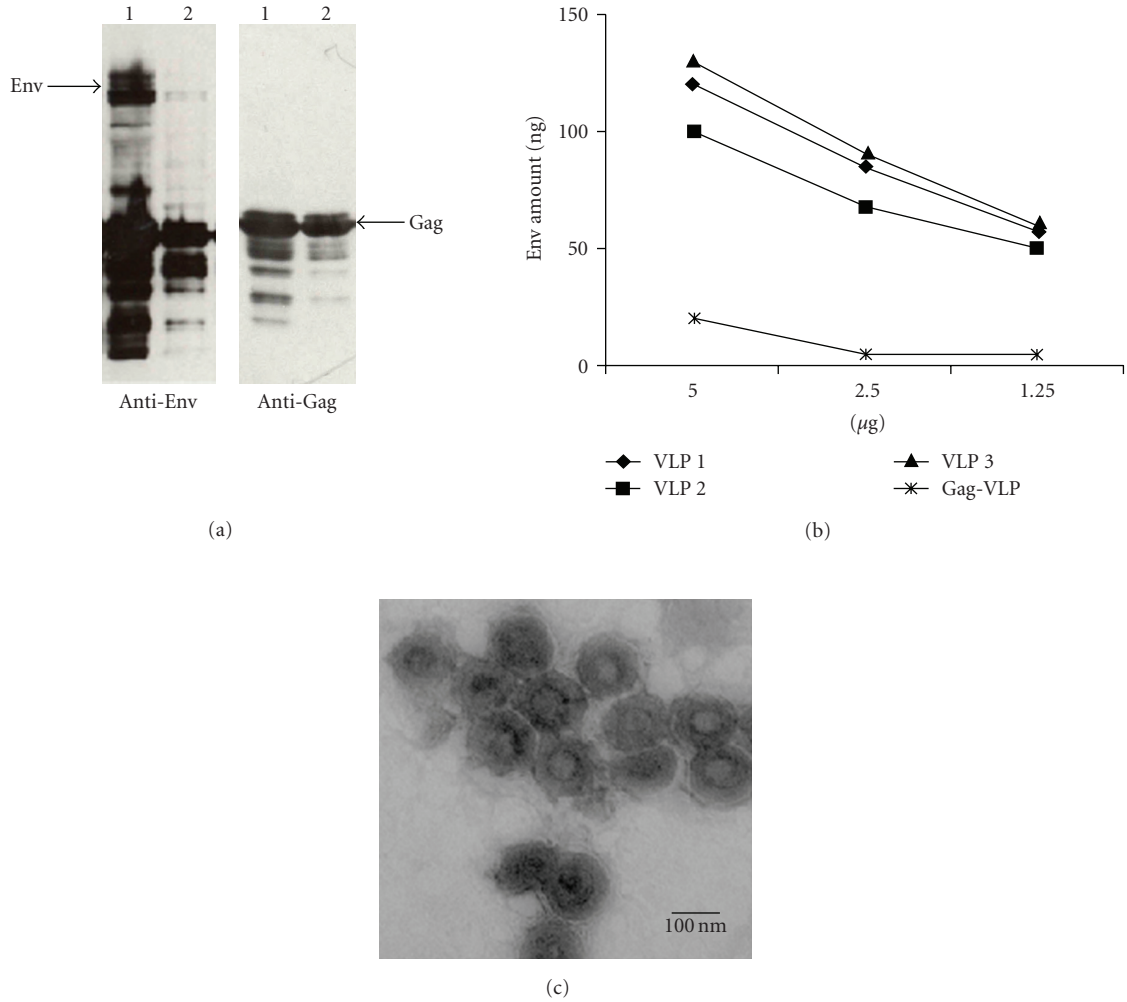

Figure 1: Characterization of SHIV 89.6 VLPs. SHIV 89.6 VLPs were produced by coinfection of Sf9 cells with rBVs expressing HIV 89.6 Env and SIVmac239 Gag proteins at the MOI (multiplicity of infection) of 5 and 2, respectively, and purified as described in Section 2. SIV Gag VLPs were produced by infection of Sf9 cells with rBV expressing SIVmac239 Gag proteins and purified similarly. (a) *Characterization of SHIV 89.6 VLPs by Western blot.* 5 μg total proteins were taken from each VLP preparation and analyzed by SDS-PAGE followed by Western blot using rabbit-anti-gp120 antibody for detection of HIV Env proteins and monkey-anti-SIV fordetection of the SIV Gag proteins. Lanes 1, SHIV 89.6 VLP; 2, SIV Gag-only VLP. (b) *Comparison of HIV 89.6 Env amount in VLP preparations by a quantitative ELISA.* The amount of HIV Env proteins in SHIV 89.6 VLP preparations was determined by a sandwich ELISA. ELISA plates were coated with a sheep-anti-gp120 antibody as the capture antibody, followed by addition of serial 2-fold dilutions of SHIV 89.6 VLPs lysed by 1% Triton X-100. The amount of HIV Env bound to the plate was then detected by sera from HIV-infected patients as the detecting antibody, followed by addition of HRP-conjugated Goat-antihuman antibody and development of color. Lysed SIVmac239 Gag VLPs were used as controls. A standard curve for the amount of HIV Env was obtained by adding purified HIV 89.6 Gp120 mixed with SIVmac239 Gag VLP lysed by 1% Triton X-100 to the sheep-anti-gp120 antibody-coated ELISA plate. The amount of HIV Env proteins was then calculated based on the obtained standard curve and then expressed as nanograms (ng) of HIV Env in 1 μg VLP preparation. VLP1, VLP2, and VLP3 represent SHIV 89.6 VLPs produced from three different batches and Gag-VLP represents the control SIVmac239 Gag only VLP preparation. (c) *Negative staining and EM examination of SHIV 89.6 VLPs.* Purified SHIV 89.6 VLPs were stained with 1% uranyl acetate followed by examination under a transmission electron microscope.

average of about 0.8% total CD8 T cells stimulated to produce interferon-gamma by a peptide corresponding to a dominant epitope in the HIV Env protein. In contrast, no significant level of CD8 T cell response against the HIV Env was induced by immunization with SHIV 89.6 VLPs alone (Group 3) compared to the control group that was immunized with a mixture of plasmid DNA vector pCAGGS and SIV Gag-VLPs (Group 1). On the other hand, immunization with SHIV 89.6 VLPs induced higher levels of antibody responses against the HIV Env gp120, which is almost twofold higher on average compared to the HIV Env-DNA vaccine (Figure 4(a)). We further compared the levels of IgG1 and IgG2a antibody subtypes against the HIV Env gp120 induced by the HIV Env-DNA and VLP vaccines. As shown in Figures 4(b) and 4(c), immunization with the HIV Env-DNA and SHIV 89.6 VLPs induced similar levels

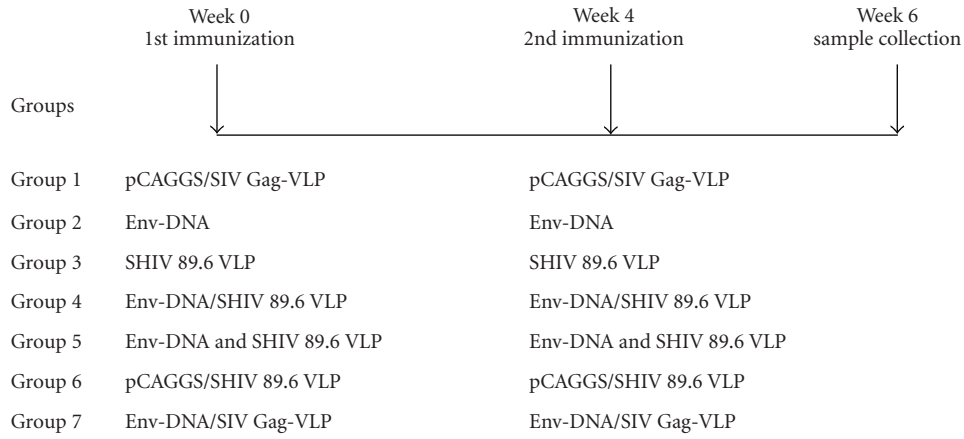

Figure 2: Schematic diagram of immunization study design. Six groups of mice (groups of 6) were immunized with different vaccine preparations as indicated. Group 1: a mixture of 50 μg DNA vector pCAGGS and 50 μg SIV Gag-VLPs; Group 2: 50 μg HIV Env-DNA; Group 3: 50 μg SHIV 89.6 VLPs; Group 4: a mixture of 50 μg HIV Env-DNA and 50 μg SHIV 89.6 VLPs; Group 5: simultaneous injection with 50 μg HIV Env-DNA and 50 μg SHIV 89.6 VLPs at separate sites; Group 6: a mixture of 50 μg pCAGGS DNA vector and 50 μg SHIV 89.6 VLPs; Group 7: a mixture of 50 μg HIV Env-DNA and 50 μg SIV Gag-VLPs. Mice were immunized twice by intramuscular injections at weeks 0 and 4, and sacrificed at week 6 (two weeks after the second immunization) to collect blood samples and prepare splenocytes for analysis of immune responses.

of IgG1 antibodies against the HIV Env gp120. However, the level of IgG2a antibodies induced by SHIV 89.6 VLPs was significantly higher than that induced by the Env DNA vaccine. Taken together, these results show that the HIV Env-DNA vaccine is more potent in eliciting CD8 T cell responses whereas the SHIV 89.6 VLPs is more effective in eliciting antibody responses against the HIV Env.

3.2. Immunization with a Mixture of the HIV Env-DNA and VLP Vaccines Induces Enhanced CD8 T Cell Responses. Comparison of immune responses induced by HIV Env-DNA and VLP vaccines showed that these two different vaccine platforms exhibit different abilities in eliciting CD8 T cell and antibody responses. We therefore further investigated whether a combination of these two vaccines may complement each other. As outlined in Figure 2, mice in Group 4 were immunized with a mixture of HIV Env-DNA and VLP vaccines. In addition, we also included three additional groups that were immunized by simultaneous injection of the HIV Env-DNA vaccine and SHIV 89.6 VLPs at separate sites (Group 5), by a mixture of SHIV 89.6 VLPs and the DNA vector pCAGGS (Group 6), and by a mixture of the HIV Env-DNA vaccine and the control SIV Gag-only VLPs (Group 7), respectively, for comparison. As shown in Figure 3, immunization with a mixture of HIV Env-DNA and VLP vaccines (Group 4) induced an average of about 2% total CD8 T cells against the Env, more than twofold higher than the level induced in Group 2 mice that were immunized by the HIV Env-DNA vaccine alone ($P < .05$). On the other hand, immunization by separate injections of the HIV Env-DNA and SHIV 89.6 VLPs (Group 5) induced an average of about 0.9% total CD8 T cells against the HIV Env, similar to the level induced by immunization with the HIV Env-DNA vaccine alone (Group 2). Moreover, immunization with a mixture of the HIV Env-DNA vaccine and control SIV Gag-VLPs (Group 7) induced an average of about 1.6% total CD8 T cells against the HIV Env that is also significantly higher ($P < .05$) compared to the levels induced by immunization with the HIV Env-DNA vaccine alone (Group 2) or by separate injections of HIV Env-DNA and SHIV 89.6 VLPs (Group 5). In contrast, no significant CD8 T cell response was induced by immunization with a mixture of SHIV 89.6 VLPs and the DNA vector pCAGGS (Group 6) similar as observed for mice immunized with SHIV 89.6 VLPs alone (Group 3), further demonstrating the weakness of VLPs for eliciting CD8 T cell responses. These results indicate that VLPs may exert an adjuvant activity and augment induction of CD8 T cell responses by the HIV Env-DNA vaccine. Moreover, administering the HIV Env-DNA and VLPs in a mixture is necessary for eliciting enhanced CD8 T cell responses.

3.3. Induction of Higher Levels of Antibody Responses Does Not Require Mixing of the HIV Env DNA and VLP Vaccines during Immunization. Analysis of antibody responses revealed a different outcome compared to the CD8 T cell responses. As shown in Figure 4, immunization with the Env-DNA and SHIV 89.6 VLP mixture (Group 4) induced higher levels of antibody responses on average than immunization with either the HIV Env-DNA (Group 2) or the SHIV 89.6 VLP vaccine (Group 3) alone. However, statistical analysis showed that the antibody response induced by the vaccine mixture (Group 4) is only significantly higher than the antibody responses induced by the HIV Env-DNA vaccine (Group 2). Further, while immunization by separate injections of the HIV Env-DNA and SHIV 89.6 VLPs (Group 5) did not augment induction of CD8 T cell responses, it induced higher levels of antibody responses against the HIV Env compared to immunization with the HIV Env-DNA vaccine alone (Group 2), similar to those induced by immunization with

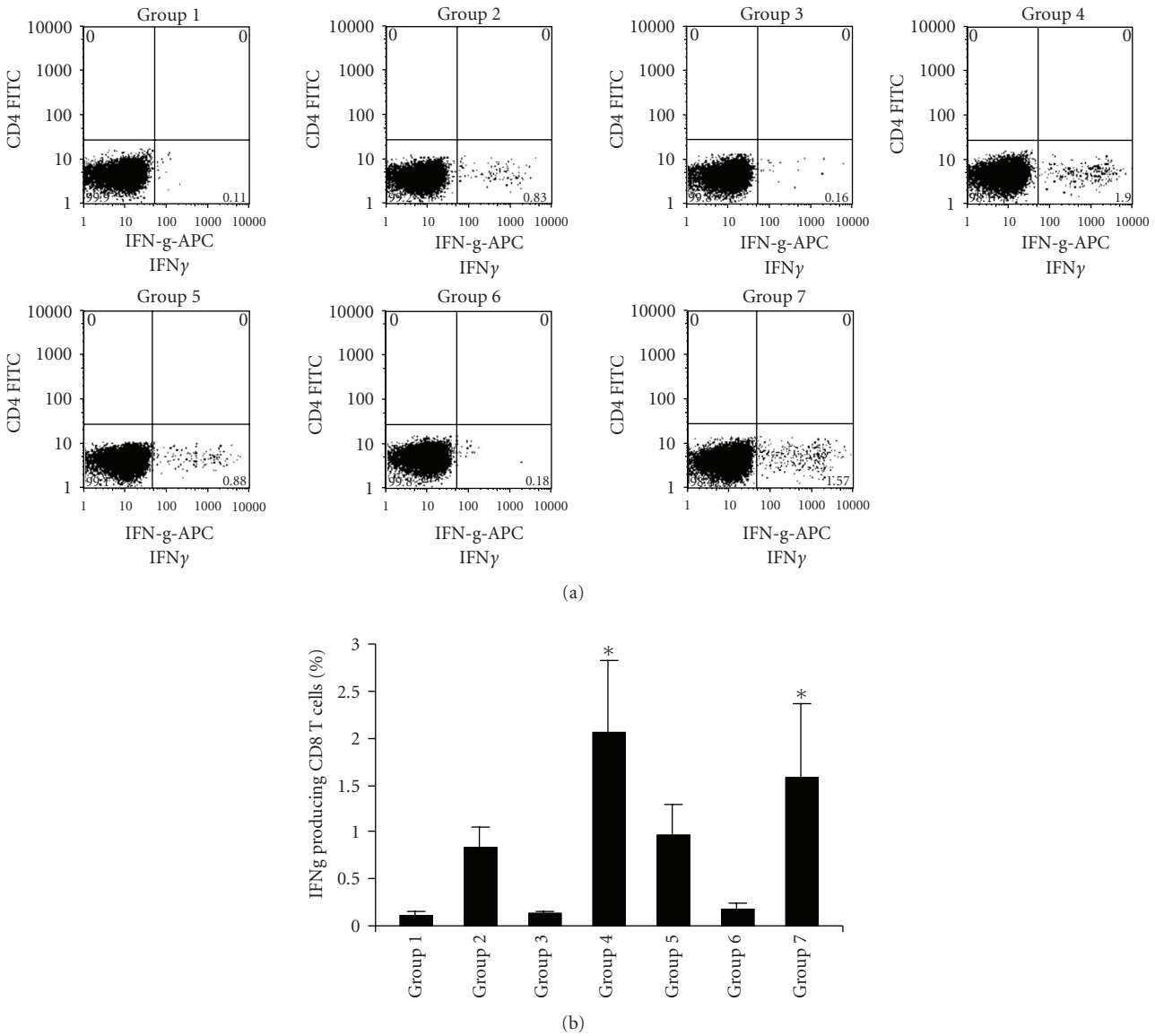

Figure 3: Enhanced CD8 T cell responses by immunization with a mixture of HIV Env-DNA and SHIV 89.6 VLPs but not by simultaneous injection at separate sites. Groups of mice (6 per group) were immunized by intramuscular injection at weeks 0 and 4 with different vaccine preparations as shown in Figure 2. Two weeks after the second immunization, mouse splenocytes were prepared and stimulated by the peptide IGPGRAFYAR corresponding to the dominant CD8 epitope in the HIV Env for Balb/c mice. The percentages of IFNγ-producing CD8 T cells were analyzed by intracellular-cytokine staining and flow cytometry. (a) Representative results of FACS analysis for IFNγ-producing CD8 T cells from each immunization group stimulated with the peptide IGPGRAFYAR. Numbers in lower-right boxes represent percentages of IFNγ staining positive CD8 T cells. Background levels of IFNγ-producing CD8 T cells similar to Group 1 were obtained for all samples stimulated with an irrelevant peptide AMQMLKETI (data not shown). (b) Percentages of IFNγ staining positive CD8 T cells for each immunization group after stimulation with the peptide IGPGRAFYAR. Error bars represent standard deviations for each group. $*$ indicates the groups with significantly higher levels of CD8 T cell responses than Group 2 that received the HIV Env-DNA vaccine only ($P < .05$).

the mixture of HIV Env-DNA and VLP vaccines (comparing Group 4 and Group 5). On the other hand, while immunization with a mixture of the HIV Env-DNA vaccine and SIV Gag-VLPs induced enhanced CD8 T cell responses, it did not augment induction of antibody responses against the HIV Env. In fact, the average level of antibody response induced by immunization with a mixture of the HIV Env-DNA vaccine and SIV Gag-VLPs is lower compared to that induced by immunization with the HIV Env-DNA vaccine alone for both IgG1 and IgG2a antibodies (comparing Group 7 with Group 2). Moreover, the antibody response against the HIV Env induced by immunization with a mixture of SHIV 89.6 VLPs and the control DNA vector pCAGGS is similar to that induced by immunization with SHIV 89.6 VLPs alone (compare Group 3 and Group 6). Taken together, these results indicate that in contrast to induction of CD8 T cell responses,

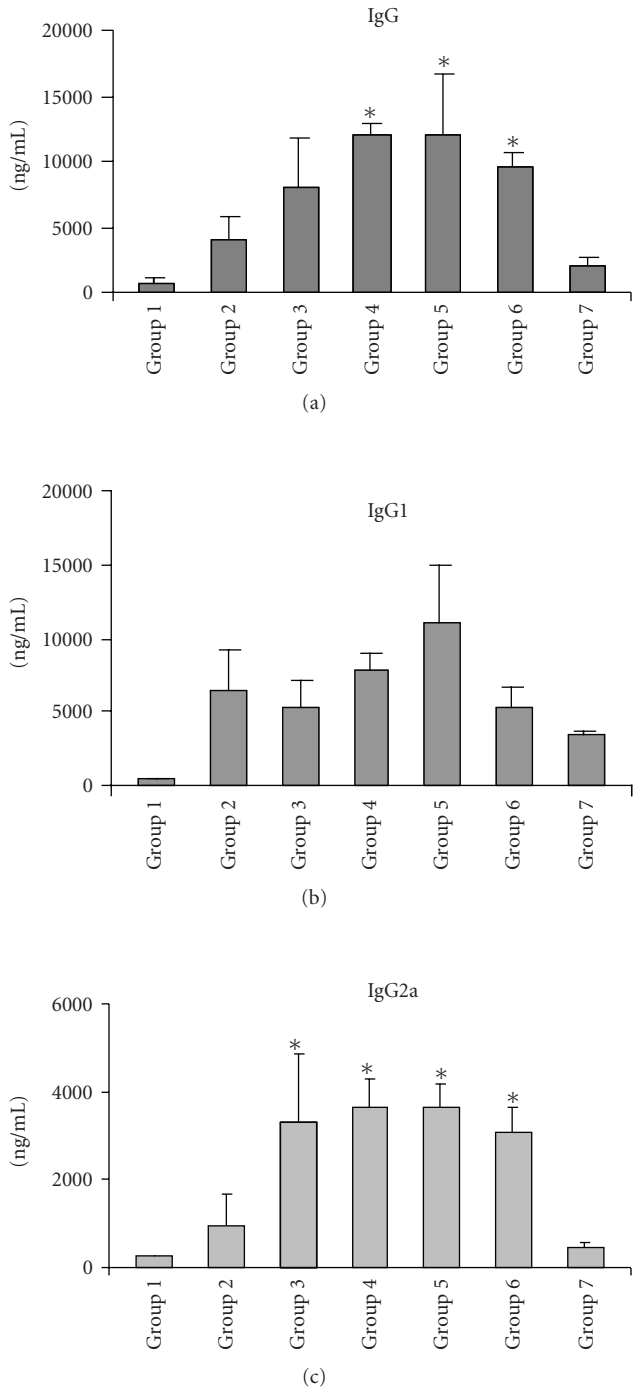

Figure 4: Comparison of antibody responses induced by immunization with the HIV Env-DNA and SHIV 89.6 VLP vaccines in different combinations. Mice were immunized by different vaccine preparations as described in Figure 2. Sera were collected at 2 weeks after the second immunization and analyzed for antibodies specific for HIV 89.6 gp120 by ELISA. The levels of antibody responses are expressed as the quantity (ng) of antibodies binding to HIV 89.6 gp120 in 1 mL sera from each mouse. (a) Total IgG antibodies against gp120. (b) IgG1 subtype antibodies against gp120. (c) IgG2a subtype antibodies against gp120. Error bars indicate the standard deviations for each immunization group. ∗ indicates the groups with significantly higher levels of antibody responses than Group 2 that received the HIV Env-DNA vaccine only ($P < .05$).

the HIV Env-DNA and VLP vaccines do not exert an adjuvant activity to each other for eliciting antibody responses. Of note, while both SHIV 89.6 VLPs and HIV Env-DNA vaccines induced similar levels of IgG1 antibody responses, immunization with VLPs induced significantly higher levels of IgG2a antibodies that are over 4-fold higher compared to the HIV Env-DNA vaccine ($P < .05$). Moreover, this is similarly observed for all groups that received SHIV 89.6 VLPs in immunization (Groups 3, 4, 5, and 6) versus those that received HIV Env-DNA but no SHIV 89.6 VLPs in immunization (Groups 2 and 7). These results demonstrate that the profiles of the antibody response induced by the HIV Env-DNA and VLP vaccines are different and the IgG2a antibodies were primarily induced by VLPs in immunization with a mixture of HIV Env-DNA and SHIV 89.6 VLP vaccines.

3.4. VLPs Bind to DNA Molecules in the DNA and VLP Mixture and Exhibit Dendritic Cell- (DC-) Stimulating Activities. The results from above studies showed that VLPs augmented induction of CD8 T cell responses by the HIV Env-DNA vaccine and the adjuvant effect was only observed when VLPs and HIV Env-DNA are given as a mixture during immunization. To delineate the underlying mechanism, we first investigated whether DNA molecules bind to VLPs in the mixture by using a sedimentation assay. The HIV Env-DNA, SHIV 89.6 VLP, or their mixture was overlaid on top of a 30% sucrose cushion, and after centrifugation, the relative amount of DNA in the bottom 30% sucrose cushion was examined by electrophoresis in an agarose gel. To determine whether the integrity of VLPs affects binding with DNA molecules, we also included a mixture of HIV Env-DNA and lysed-VLPs for comparison. As shown in Figure 5(a), the amount of DNA detected in the sample containing the DNA and VLP mixture is much higher compared to those detected in samples containing DNA only or DNA mixed with lysed VLPs. Further, the DNA molecules in the sample containing the DNA and VLP mixture also exhibited a slower mobility, which may result from the formation of aggregates through binding to VLPs. These results indicate that DNA and VLPs are bound to each other in the mixture and the binding is dependent on the integrity of VLPs.

Previous studies have shown that SHIV 89.6 VLPs exhibit DC-stimulating activities [32]. We thus further investigated whether SHIV 89.6 VLPs stimulate cytokine secretion by DCs when mixed with DNA molecules. As shown in Figure 5(b), incubating DCs with SHIV 89.6 VLPs or a mixture of HIV Env-DNA and SHIV 89.6 VLPs effectively stimulated secretion of cytokines IL-6, IL-12, as well as TNF-alpha to similar levels as incubating DCs with bacteria LPS. On the other hand, incubating DCs with HIV Env-DNA molecules only did not stimulate significant levels of cytokine secretion as compared to unstimulated DCs. The stimulation of DCs by SHIV 89.6 VLPs is not due to contamination of bacteria endotoxin as boiled-SHIV 89.6 VLPs or HIV Env-DNA mixed with boiled-SHIV 89.6 VLPs did not stimulate cytokine secretion. In contrast, boiling does not affect the ability of LPS to stimulate cytokine secretion by DCs. We further determined surface expression of costimulatory molecules CD80 and CD86 on DCs after

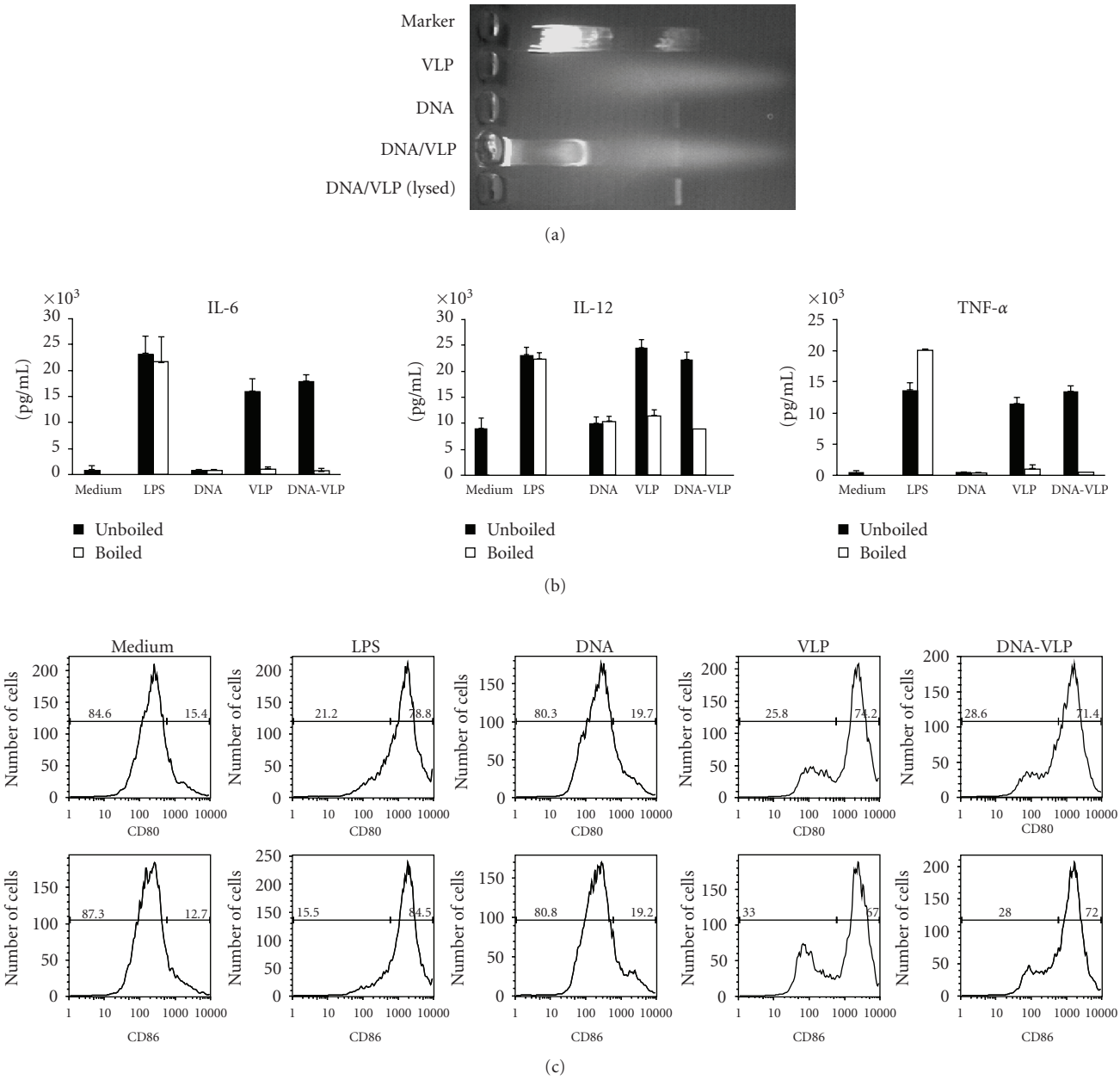

Figure 5: (a) *DNA molecules bind to VLPs in the DNA/VLP mixture.* HIV 89.6 Env-DNA, SHIV 89.6 VLPs, or their mixtures were loaded onto a two-layer sucrose cushion, with 500 μl 30% sucrose at bottom and 2 mL 20% sucrose above, followed by ultracentrifugation. After centrifugation, 10 μl from the 30% cusion of each sample was loaded onto a 1% agarose gel, and after electrophoresis, the gel was stained with Ethidium Bromide followed by destaining and then visualization of DNA by UV light. Marker: DNA molecular weight marker (lambda DNA Hind III digest); VLP: 50 μg SHIV 89.6 VLPs; DNA: 50 μg HIV Env-DNA; DNA/VLP: 50 μg HIV Env-DNA mixed with 50 μg SHIV: 89.6 VLPs; DNA/VLP (lysed): 50 μg HIV Env-DNA mixed with 50 μg SHIV 89.6 VLPs that were lysed with 1% Triton X-100. (b) *SHIV 89.6 VLPs stimulate cytokine secretion by BMDCs.* BMDCs were prepared as described in Section 2 and incubated with different stimulants in triplicates. DCs incubated with culture medium only were used as negative controls and DCs incubated with LPS (10 ng/mL) were used as positive controls. To ensure that stimulation of DC by DNA or VLPs is not due to contamination of endotoxin, the DCs were also incubated with the same stimulants that have been heat-treated at 100°C for 30 minutes. Cell-free supernatants were harvested 24 hours after incubation at 37°C in 5% CO_2 and assayed for the levels (pg/mL) of IL-6, IL-12, and TNF-alpha by ELISA. Error bars represent standard deviations and the results shown represent typical results obtained from two different stimulation experiments. Medium, cell culture medium (negative control): LPS, 10 ng/mL (positive control); DNA: HIV Env-DNA (50 μg/mL); VLP: SHIV 89.6 VLPs (10 μg/mL); DNA-VLP: a mixture of HIV Env-DNA (50 μg/mL) and SHIV 89.6 VLPs (10 μg/mL). (c) *Maturation of BMDCs after stimulation by SHIV 89.6 VLP vaccines.* BMDCs were incubated with different stimulants as described above. After stimulation, the BMDCs were stained for surface expression of CD11c, CD80, and CD86 and then analyzed by flow cytometry. The results are presented as histograms for CD80 (upper panel) and CD86 (lower panel) for CD11c positive cells. Medium: cell culture medium (negative control); LPS: 10 ng/mL (positive control); DNA: HIV Env-DNA (50 μg/mL); VLP: SHIV 89.6 VLPs (10 μg/mL); DNA-VLP: a mixture of HIV Env-DNA (50 μg/mL) and SHIV 89.6 VLPs (10 μg/mL).

stimulation. As shown in Figure 5(c), stimulation of DCs by VLPs or the DNA-VLP mixture significantly increased the surface expression of CD80 and CD86 that is comparable to stimulation by LPS. In contrast, incubation of DCs with DNA vaccine alone did not induce upregulation of CD80 and CD86 surface expression similar to the use of medium alone. These results show that the VLPs are highly potent in DC-stimulation compared to DNA molecules, and this property of VLPs may also be responsible for their adjuvant activity to augment induction of CD8 T cell responses by the HIV Env-DNA vaccine when given as a mixture.

4. Discussion

The advancement in molecular biology and biomedical research has led to the development of new vaccine strategies against virus infection. Among these are DNA and VLP vaccines that both have attracted great interest for HIV vaccine development [33]. Both DNA and VLP vaccines share the ability to be administered repetitively for boosting induction of immune responses without the concern for preexisting or induced antivector immune responses. However, information on direct comparison of these two different vaccine platforms for inducing antibody and CD8 T cell responses is still lacking. In this study, we evaluated immune responses induced by the HIV Env-DNA and SHIV 89.6 VLP vaccines for comparison and investigated the effectiveness of their combination for inducing both antibody and CD8 T cell responses.

The results from this study showed that immunization with the HIV Env-DNA vaccine induced significant levels of CD8 T cell responses, similar as observed in our previous studies [30]. The potency of DNA vaccines to induce CD8 T cell responses is probably due to direct in vivo antigen synthesis after DNA immunization, which are more efficiently presented to the MHC I antigen presentation pathway for eliciting CD8 T cell responses [14]. In contrast, immunization with SHIV 89.6 VLPs induced minimal to undetectable levels of CD8 T cell responses against the HIV Env, indicating that the VLPs are relatively poor inducers of CD8 T cell responses similar as inactivated virus vaccines as well as other protein-based vaccines [2]. Of note, previous studies have shown the induction of CTL responses against the HIV Env by HIV or SHIV VLPs [24, 25, 32]. However, the immune response induced by VLPs was not compared with other vaccine platforms such as DNA vaccines in those studies and the CTL responses were assessed by assay for cytolytic activity of T cells after in vitro stimulation. Thus, it is possible that VLPs can elicit low levels of CD8 T cell responses that become detectable after in vitro stimulation as shown in early studies [24, 25, 32]. On the other hand, the CD8 T cell responses were investigated in this study by direct ex vivo stimulation with a peptide corresponding to the dominant CD8 epitope in the HIV Env. Moreover, previous studies in nonhuman primates have shown that DNA vaccines are more effective than VLPs for induction of T cell responses against the HIV Gag protein [34]. Taken together, these results show that the HIV DNA vaccines are more effective than the VLPs for eliciting CD8 T cell responses.

On the other hand, SHIV 89.6 VLPs were found to induce higher levels of antibody responses compared to the HIV Env-DNA vaccine. Interestingly, further analysis of antibody responses showed that the profiles of antibody responses induced by the HIV Env DNA vaccines and VLPs are different. While both SHIV 89.6 VLPs and the the HIV Env-DNA vaccine induced similar levels of IgG1 antibody responses, immunization with VLPs induced significantly higher levels of IgG2a antibodies that are over 4-fold higher compared to the HIV Env-DNA vaccine. Moreover, this is similarly observed for all groups that received SHIV 89.6 VLPs in immunization (Groups 3, 4, 5, and 6) versus those that received HIV Env-DNA but no SHIV 89.6 VLPs in immunization (Groups 2 and 7), indicating that the IgG2a antibodies are primarily induced by immunization with SHIV 89.6 VLPs. The underlying mechanism for antibody subtype switch is still not clear and evidence suggests that a number of factors such as antigen forms as well as immunization routes may affect the levels of IgG2a antibodies [35, 36]. It has been reported that intramuscular immunization with plasmid DNAs expressing ovalbumin or hen egg lysozyme induced higher levels of IgG2a antibodies than immunization with purified soluble proteins [37]. In addition, it has also been shown that immunization with the secreted monomer hepatitis B virus e antigens induced predominantly IgG1 antibodies whereas immunization with the particulate hepatitis B virus core antigens elicited mostly IgG2a and IgG2b antibodies [38]. Moreover, it has been recently reported than VLPs can directly activate conventional B cells and promote B cell differentiation to IgG2a producing plasma cells [39]. Thus, it is possible that the presentation of multiple Env protein complexes on the surface of VLPs, which are particulate antigens, is more effective in eliciting IgG2a antibody responses. The induction of enhanced IgG2a antibody responses indicates a Th1-oriented immune response, which has been suggested to be more effective in complement activation for lysis of virus-infected cells [37, 40]. It will be interesting to investigate whether the induction of Th1 type antibody responses may provide additional benefit to the control of HIV infection through more effective complement fixation and antibody-dependent cell cytotoxicity (ADCC) to clear virus infected cells.

Of particular interest to vaccine development, we found that immunization with a mixture of the HIV Env-DNA with VLP vaccines significantly augmented induction of CD8 T cell responses compared to immunization with the HIV Env-DNA vaccine alone. Further, the enhancement in CD8 T cell responses was only observed in mice immunized with a mixture of the HIV Env-DNA vaccine with VLPs but not in mice immunized by separate injections. These results indicate that the VLPs in the vaccine mixture exert an adjuvant effect on the induction of CD8 T cell responses by the HIV Env-DNA vaccine. A number of studies have shown that using liposomes to absorb DNA vaccines enhances induction of immune responses by increasing uptake of DNA vaccines by host cells [41]. In this study, we found that the DNA plasmids bind to VLPs in the DNA and VLP mixture. Thus, the observed adjuvant activity of VLPs on DNA vaccines may result from increased uptake of DNA

vaccines in a mixture with VLPs by host cells, particularly by antigen-presenting cells (APCs) such as dendritic cells (DCs) that are more efficient in taking up particulate antigens [42, 43]. On the other hand, we also observed that SHIV 89.6 VLPs as well as their mixture with HIV Env-DNA potently stimulate cytokine secretion by DCs, and such stimulation is not observed for the HIV Env-DNA alone. Thus, it is possible that the VLPs may also augment induction of CD8 T cell responses by HIV Env-DNA through stimulating APCs for more effective antigen presentation. In contrast to CD8 T cell responses, immunization with the mixture of HIV Env-DNA and SHIV 89.6 VLPs induced similar levels of antibody responses to those induced by injecting HIV Env-DNA and SHIV 89.6 VLPs at separate sites. Further, mixing the HIV Env-DNA vaccine with SIV Gag VLPs actually reduced induction of antibody responses despite a significant enhancement in CD8 T cell responses. This discrepancy suggests that the underlying mechanisms for the induction of antibody and CD8 T cell responses by the HIV Env-DNA vaccine are likely to be different. It is possible that the VLPs in the mixture may target DNA molecules to specific cell populations that are more specialized in stimulating CD8 T cell responses. Alternatively, it is also possible that augmented CD8 T cell responses may kill DNA-transfected APCs through their cytotoxic activities and thus reduce antigen production for antibody induction. Nonetheless, the induction of strong antibody responses by the mixture of HIV Env-DNA and VLP vaccines indicates that the VLPs effectively compensate for the reduced ability of DNA vaccines to elicit antibody responses in such a setting.

In summary, comparison of immune responses induced by HIV Env-DNA and VLP vaccines showed that these two vaccine platforms exhibit different abilities in eliciting antibody and CD8 T cell responses. Further, immunization with a mixture of HIV Env-DNA and VLP vaccines is able to induce both strong antibody and CD8 T cell responses as compared with immunization with either vaccine alone, indicating that this approach combines the advantages of DNA and VLP vaccines with respect to their abilities to induced antibody and CD8 T cell responses. Moreover, VLPs also exert an adjuvant activity on induction of CD8 T cell responses by DNA vaccines when given as a mixture and such an adjuvant activity is also observed for irrelevant control VLPs, indicating that this approach may also apply other DNA and VLP vaccines for obtaining similar results. The induction of both strong antibody and CD8 T cell responses by immunization with a mixture of DNA and VLP vaccines may be applied to the development of more effective vaccine strategies against HIV or other viruses for which an effective control will require both antibody and CD8 T cell responses.

Acknowledgments

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Research Article

Autocrine, Not Paracrine, Interferon-Gamma Gene Delivery Enhances Ex Vivo Antigen-Specific Cytotoxic T Lymphocyte Stimulation and Killing

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The adoptive transfer of antigen-specific cytotoxic T lymphocytes (CTL) shows promise in the treatment of cancer and infectious diseases. We utilize adeno-associated virus-(AAV-) based antigen gene-loaded dendritic cells (DCs) to stimulate such antigen-specific CTL. Yet further improvements in CTL stimulation and killing may result by gene delivery of various Th1-response interferons/cytokines, such as interferon γ (IFN-γ), as the delivered gene can continuously produce that interferon. However which immune cell type should optimally express IFN-γ is unclear as the phenotypes of both DC and T cells are enhanced by it. Here, we used AAV to compare and contrast IFN-γ gene delivery into DC or T cells, and versus the addition of exogenous IFN-γ, for stimulating carcinoembryonic antigen-(CEA-) specific CTL. It was found that AAV/IFN-γ delivery into T cells (autocrine) resulted in T cell populations with the highest CD8(+)/CD4(+) ratio, highest IFN-γ(+)/IL-4(+) ratio, highest CD69(+),CD8(+) levels, and lowest CD4(+)/CD25(+) levels, all consistent with the strongest Th1 response. Most importantly, AAV/IFN-γ transduction of T cells resulted in antigen-specific T cell populations with the highest killing capabilities, 49% above other treatments. These data strongly suggest that AAV/IFN-γ autocrine gene delivery into T cells is worthy of further study towards maximizing the generation of antigen-specific anticancer CTL killers.

1. Introduction

Adoptive immunotherapy, particularly adoptive transfer of antigen-specific cytotoxic T lymphocytes, has shown some success in clinical trials for treating both cancer and viral infections [1–5]. However, due to resident tolerance within the tumor environment the most robust CTL must be stimulated for highest efficacy. Delivery of antigen genes into dendritic cells (DCs) or precursor monocytes (Mo) allows for the stimulation of robust antigen-specific CTL [6]. However, there are a variety of improvements that might be made to further enhance CTL stimulation. One obvious course is the delivery of Th1-response interferons/cytokine genes into immune cells for their continuous expression.

Interferon gamma (IFN-γ) is an important Th1 response interferon/cytokine involved in CTL generation and function and might be used to enhance CTL stimulation. IFN-γ likely has multiple mechanisms of action [7–10]. It is believed to inhibit expression of the IL-4 receptor. This is important as IL-4 correlates with low and ineffective CTL activity. In addition, IFN-γ may have other non-T cell-associated attributes as it is believed to stimulate expression of HLA Class I and II molecules. However, overall IFN-γ is strongly linked to the induction of Th1 response, the generation of

CTL. Of all the Th1 response interferons/cytokines IFN-γ correlates most strongly with the Th1 response [9–11] as, in fact, many laboratories study T cell expression of IFN-γ as a substitute for carrying out CTL killing/chromium release assays [12, 13]. Moreover, IFN-γ plus IL-12 appears to act cooperatively in the generation of a very strong Th1 response [14, 15]. IFN-γ may also partially overcome low activation and expansion rates of low-avidity CTL [16, 17]. As with most of the Th1-response-associated chemokines, IFN-γ is associated with regulation of perforin/Granzyme B [18]. Thus, gene delivery of IFN-γ will likely be very useful in generating a robust Th1 CTL response.

While IFN-γ gene delivery would seem to be beneficial for generating robust CTL, it is unclear which cells should specifically express this cytokine for maximum CTL stimulation. While activated T cells naturally express IFN-γ, it has been shown that IFN-γ is able to significantly affect DC maturity and function as well as DC precursor Mo and macrophage (MΦ) phenotypes [19–21]. Thus it is unclear which immune cell type should express this cytokine during the initial stimulation of the CD8+ CTL. Two general approaches are available, transducing the DC (paracrine delivery) which stimulate the responder T cells, or the T cell themselves (autocrine delivery). Here we demonstrate that IFN-γ autocrine gene delivery resulted in significantly higher CEA-specific CTL killing compared with paracrine delivery or exogenous IFN-γ.

2. Materials and Methods

2.1. Cells. The SW480 colorectal adenocarcinoma cell line was obtained from The American Tissue Culture Collection (ATCC). A carcinoembryonic antigen- (CEA-) positive lymphoblastoid cell line (CEA+ LCL) was generated by transfecting an HLA-A2 positive LCL cell line with a CEA plus Neomycin resistance gene (Neo) expression plasmid and selection with 1 mg/mL G418 for two weeks. Peripheral blood mononuclear cells (PBMCs) from five HLA-A2 positive healthy donors were separated by routine Ficoll gradient method. All blood donors gave informed consent in writing, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by our Human Research Internal Review Board. The HLA haplotype of all donors was compatible with SW480 cells (HLA A2) and other cells used as controls.

2.2. Construction of Recombinant AAV Vectors. Human CEA and IFN-γ cDNAs were amplified by reverse-transcription polymerase chain reaction (RT-PCR). Trizol reagent (Invitrogen) was employed to isolate total RNA from SW480 cells and PHA-stimulated primary human T lymphocytes, respectively. Subsequently, the total mRNA was separate from the total RNA using Oligotex mRNA isolation kit (Qiagen). After the first-strand cDNA was generated, PCR amplification for each of the cDNA was carried out using the following primer pair: CEA: 5′-ACCATGGAGTCTCCC-TCG-3′ and 5′-CTATATCAGAGCAACCCC-3′ that amplify the sequence from nucleotides 112 to 2223 [22]; IFN-γ: 5′-TTCTCTCGGAAACGATG-3′ and 5′-GGCAGGACAACC-ATTAC-3′ that amplify the sequence from nt 94 to 622 [23]. All cDNAs were sequenced and determined to be identical to the published sequence. CEA and IFN-γ cDNA were inserted in the downstream of p5 promoter of an AAV vector, respectively, as described previously [24].

2.3. Transduction of DC by Recombinant AAV. The experimental scheme is shown in Figure 1. The rAAVs were generated, purified, and tittered as described previously [24, 25]. Titer is given as encapsidated genomes (eg) per mL. After freshly isolated PBMCs (5×10^6) were cultured for two hours in AIM-V medium, the nonadherent cells were removed. The remaining adherent Mos were infected immediately with 1×10^9 encapsidated genomes (eg)/mL of AAV/CEA virus or AAV/CEA plus AAV/IFN-γ virus. After four hours the medium/virus solution was removed and the cells were finally fed with the medium containing recombinant human GM-CSF (Immunex, 800 IU/mL). At day 2, to induce the maturation of Mo into DC, recombinant human IL-4 and TNF-α (R & D SYSTEMS.) were added to the medium at 1000 IU/mL and 20 ng/mL, respectively. The medium and cytokines were replaced every two days. Finally, at day 6 the DCs were mixed with CD3+ T cells.

2.4. Transduction of CD3+ T Cells by Recombinant rAAV. T cells were transduced as described previously [25]. Briefly, Pan T Cell Isolation Kit II (Miltenyi Biotec) was employed to isolate CD3+ T cells from the nonadherent cells from the PBMC according to the kit instruction. At day 5 the CD3+ T cells (1×10^6) were infected with 1×10^9 eg/mL of AAV/IFN-γ virus and cultured with 20 ng/mL of IL-2.

2.5. Analysis of rAAV Chromosomal Integration. The total DNAs were isolated from the rAAV-infected or uninfected DC or T cells using DNAzol reagent (Invitrogen) according to supplier's protocol. Chromosomal integration of the AAV/CEA genome was studied by vector-chromosome junction PCR amplification and southern blot analysis, as previously described [24].

2.6. RT-PCR Expression Analysis of Transduced DC or T Cells for CEA and IFN-γ Expression. At day 4 of Mo/DC culture and DC-T cell culture, isolation and amplification of the mRNA was done as described above, respectively. Subsequently, the CEA and IFN-γ cDNAs were amplified as described above. TFIIB was amplified as a housekeeping control at the same time. PCR amplification for the TFIIB cDNA was performed using the primer pair: 5′-TGTCTG-TTGTGTCTTGTTGC-3′ and 5′-TAGGCTATGTACAAC-AGGC-3′ that amplify the sequence from nt 319 to 1310 [26].

2.7. Analysis of Transduction and Expression of CEA Antigen and Cytokines by Intracellular Staining. At day 6 of Mo/DC culture and day 4 of rAAV infection of CD3+ T cells, intracellular staining assay was employed, similar to Pala et al. [27]. Briefly, cells were incubated with the FITC- or

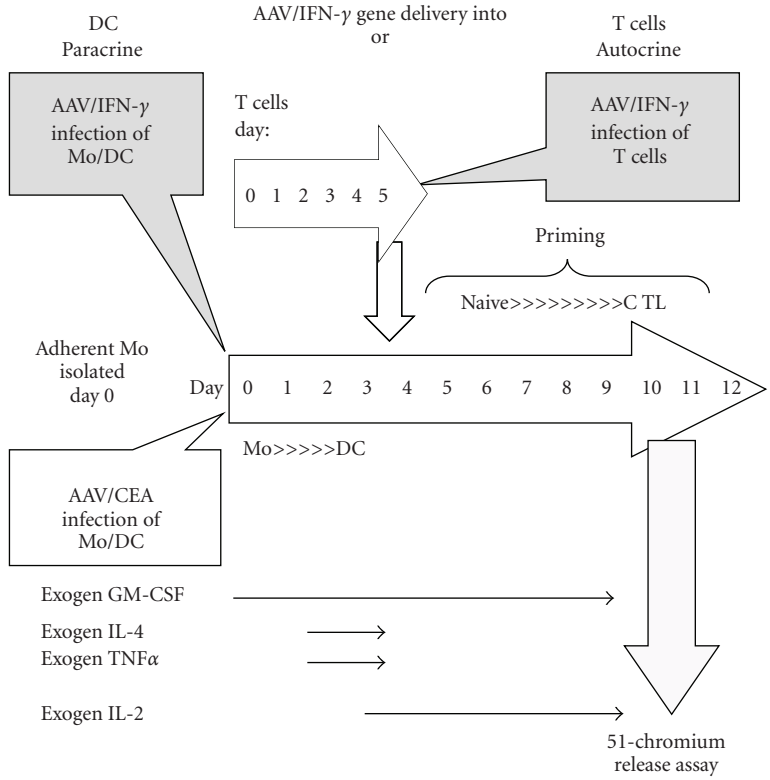

Figure 1: Structure of the cell treatment protocol. Shown is the temporal treatment of the Mo/DC and T cells and is self-descriptive. However, note that AAV/IFN-γ is used to infect Mo/DC at day zero, or naive T cells just prior to coincubation with AAV/antigen-loaded DC on day 5.

PE-labeled monoclonal antibodies recognizing the following antigens, respectively: CEA, IFN-γ, and IL-2 (BD Pharmingen). Control irrelevant isotype-matched FITC- or PE-conjugated monoclonal antibodies were also obtained from BD Pharmingen. A FACSCalibur flow cytometer (Becton-Dickinson) was used for data acquisitions. At least ten thousand events were counted for each sample.

2.8. Stimulation of Cytotoxic T Lymphocytes (CTLs) by Treated DC. At day 5-6 of DC culture the mature DCs were harvested and mixed with CD3+ T lymphocytes (ratios from 20 : 1, T : DC) in AIM-V medium, respectively. Some T cells were untransduced while others were previously infected with AAV/IFN-γ. Mixtures were cultured in AIM-V containing IL-2 (20 IU/mL) and IL-7 (20 ng/mL). The medium and cytokines were replaced every two days. Exogenous IFN-γ was used at 100 U/mL. At 7-8 days postaddition to DC the cells were harvested and analyzed further.

2.9. FACS Analysis of DC and T Cells. After 6 days the nonadherent DCs were harvested (>95% viable as assessed by Trypan blue exclusion) and the cells counted and distributed. For the analysis of DC a panel of FITC- or PE-labeled monoclonal antibodies recognizing the following antigens was used: CD14, CD40 (Chemicon International), HLA-DR, CD80, CD8, CD86, and isotype-matched antibodies (BD Pharmingen). Stained cells were assayed by fluorescence activated cell sorting (FACS) for these CD markers according to the routine method [25]. For the analysis of activated T cells, at day 8 of the mixed cell culture the stimulated T cell populations were analyzed for their surface markers with immunofluorescence staining by flow cytometry. A panel of FITC- or PE-labeled monoclonal antibodies recognizing the following antigens was used: CD4, CD8, CD25, and CD69 (BD Pharmingen). At day 8 postpriming T cells were harvested for analysis. Intracellular staining assay was performed to analyze the expression of IFN-γ in the T cells according to the method described above. FITC-labeled anti-IFN-γ monoclonal antibody (BD Pharmingen) was used.

2.10. Analysis of T Cell Proliferation Stimulated by rAAV-Infected DC. After the CD3+ T cells were mixed with the DC on day 6, each group of mixed cells was inoculated into wells of a 96-well cell culture plate. There were 5×10^5 cells (200 μl) in each well. After the mixed cells were cultured for 8 hours in 37°C, 5% CO_2, ^{3}H-TDR incorporation test was carried out according to the routine method [25].

2.11. Analysis of CTL Killing Activity. CTLs were generated from 3 donors. At day 14 (day 8 of T cell: DC coincubation), chromium-51 (^{51}Cr) release assay was used to analyze the killing activity of CTL elicited by AAV/CEA-transduced and control DC against the target cells as previously described [25]. Briefly, the CTL cells and ^{51}Cr-labeled target cells

Table 1: Surface expression of CD molecules on DC, as percentage.

DC treatment	CD14	CD40	CD80	CD83	CD86	HLA-DR
Ctrl (mock)	20.7	21.6	29.8	22.2	63.7	90.1
AAV/CEA	14.5	41.3	62.1	42.6	88.5	94.5
AAV/CEA + exo IFN-γ	16.3	40.0	60.4	39.9	86.2	90.3
AAV/CEA + AAV/IFN-γ	12.5	42.4	65.1	43.1	89.5	96.2

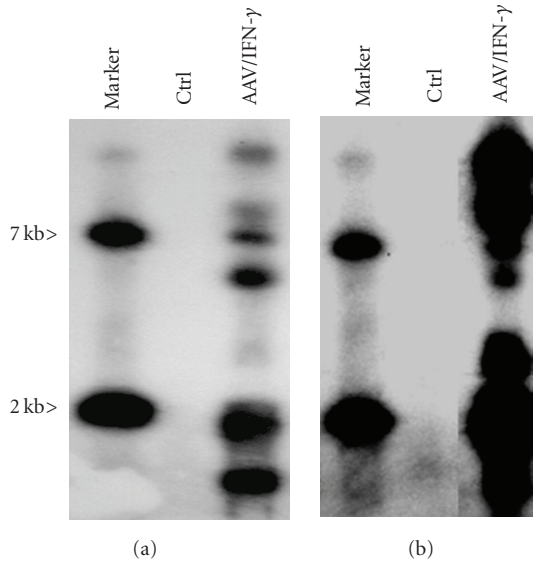

Figure 2: Chromosomal integration of AAV/IFN-γ vector DNA into DC and T cells. Cells were infected as described in the Materials and Methods section and then analyzed for proviral integration by, first, PCR amplification of the vector-chromosomal junctions, followed by agarose gel electrophoresis of the PCR products, Southern blotting and probing with ^{32}P labeled vector DNA. Each band represents a separate AAV integrant within the cell population. (a) shows the results from DC. (b) shows the results from T cells.

were mixed (20 : 1) and incubated for 6 hours at 37°C with 5% CO_2. To determine MHC/HLA Class I restriction of CTL killing anti-MHC Class I monoclonal antibodies were used to block cytotoxicity. The ^{51}Cr-labeled targets were preincubated with mouse antihuman MHC class I antibody (Serotec) for 1 hour before the ^{51}Cr release assay was performed. The mouse antihuman MHC class II antibody (Serotec) was also used as a control.

3. Results

3.1. Cloning of CEA, IFNγ, and Delivery into DC and T Cells. The CEA gene, a common adenocarcinoma tumor marker, was cloned from colorectal adenocarcinoma cell line SW480 and ligated down-stream of the AAV p5 promoter within a fully gutted AAV2-based vector (dl3-97). IFN-γ was also successfully cloned into AAV in a similar manner. rAAVs were generated as described previously [24, 25] and used to transduce DC and T cells at efficiencies above 85%. Our approach for transducing DC has been to infect freshly adherent peripheral blood monocytes with rAAV, to treat these cells with GM-CSF alone for one day, and then add IL-4 to induce their differentiation into DC [24, 25, 28]. This technique has proven to be very effective in generating specific antigen-presenting DC and cytokine-expressing DC [24, 25, 28].

3.2. rAAV Proviral Chromosomal Integration and Expression of CEA and IFNγ. One issue in the field of AAV-based gene therapy is the form of latency of the AAV proviral DNA within the transduced primary cells. In vitro tissue culture transduced cell lines often display a chromosomally integrated provirus, while in vivo transduced cells often show the latent rAAV DNA as an episomal element. To address this issue chromosomal DNA from the transduced T cells and DC were analyzed for integrated AAV/IFN-γ provirus by PCR amplification of vector-chromosome junctions by using one primer directed towards the vector and another directed towards the *Alu* I repetitive chromosomal element. Products were then agarose gel electrophoresed, Southern blotted, and ^{32}P-DNA probed for vector sequences. As shown in Figures 2(a) and 2(b) this technique clearly demonstrates some level of chromosomal latency in both DC and CD3+ T cells. We have previously shown AAV/CEA chromosomal integration in DC [25].

It is important that rAAV proviruses express their transgenes. Figures 3(a) and 3(b) show that the resulting rAAV/IFN-γ provirus does express by RT-PCR analysis, in both DC and T cells. To observe both the transduction efficiency and protein expression of IFN-γ we carried out an intracellular staining analysis of transduced and untransduced DC and T cells. The transduction efficiency of DC by AAV/IFN-γ, as shown in Figure 4(a) (DC) and Figure 4(b) (T cells), approached 90%. This agrees with our earlier studies with other transgenes, but it must be noted that T cells which were not transduced with AAV/IFN-γ displayed a high background expression of 48%. In any case, transduction efficiency using AAV 2 was high for both the DC and T cells.

3.3. Characterization of Transduced DC. The structure of the experimental scheme is shown in Figure 1. We infect DC with either the AAV/cytokine vector at day 0 or the T cells on day 5 just before their addition to the CEA antigen loaded DC. DCs (Mo) were always loaded by infection with AAV/CEA on day 0. We examined the DC on day 6, as shown in Table 1, for surface expression of CD14, CD40, CD80, CD83, and CD86 by FACS and found that CD80, CD86, and CD83 were upregulated by rAAV infection as shown previously [29]. The addition of exogenous IFN-γ further upregulated these

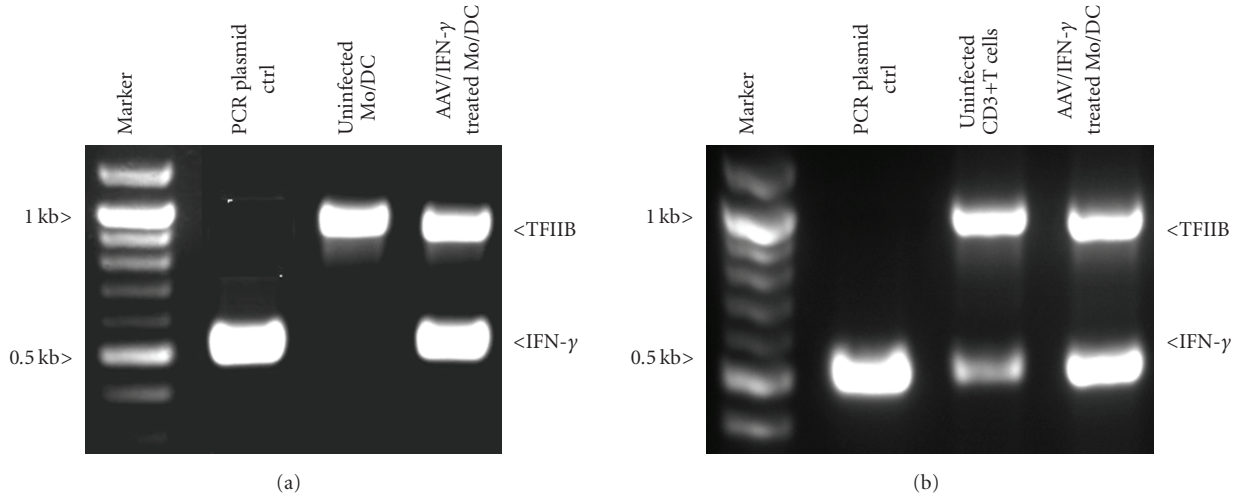

Figure 3: Analysis of IFN-γ expression in transduced DC and T cells by RT-PCR. Cells were infected, mRNA isolated and analyzed by RT-PCR as described in Section 2. (a) shows the results from DC. (b) shows the results from T cells. Note that both AAV/IFN-γ transduced DC and T cells demonstrate IFN-γ expression. Also note that untransduced T cells expressed some IFN-γ, but that AAV/IFN-γ transduced T cells demonstrate higher IFN-γ expression. This is confirmed in Figure 4.

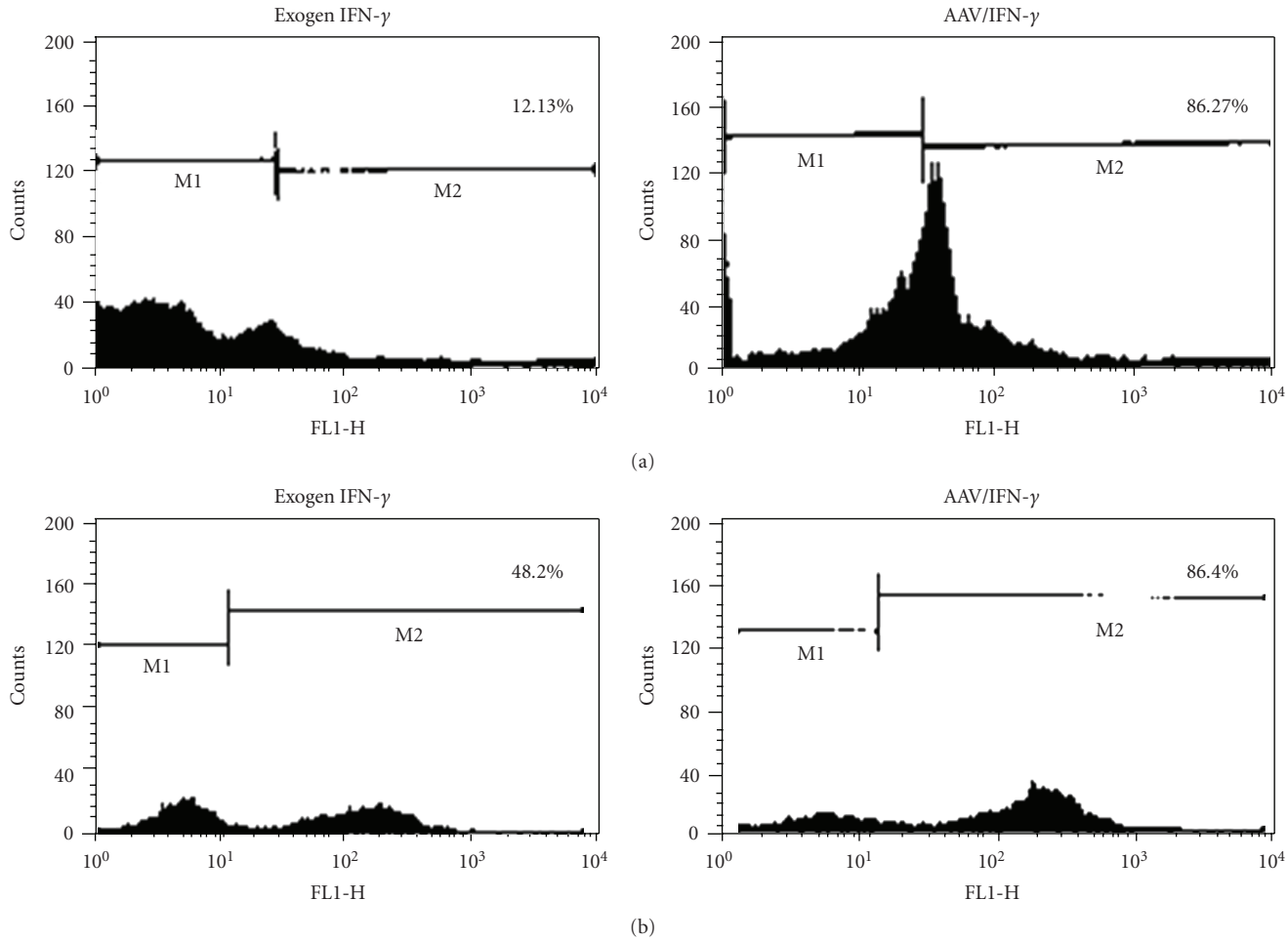

Figure 4: Transduction efficiency of AAV delivery into DC and T cells by intracellular staining. Cells were infected as described in Section 2 and analyzed by intracellular staining for IFN-γ. (a) shows the results from DC infection. (b) shows the results from T cell infection. Note that both AAV/IFN-γ transduced DC and T cells demonstrate high levels of IFN-γ expression, but that T cells exhibit a signifiucant basal level of expression.

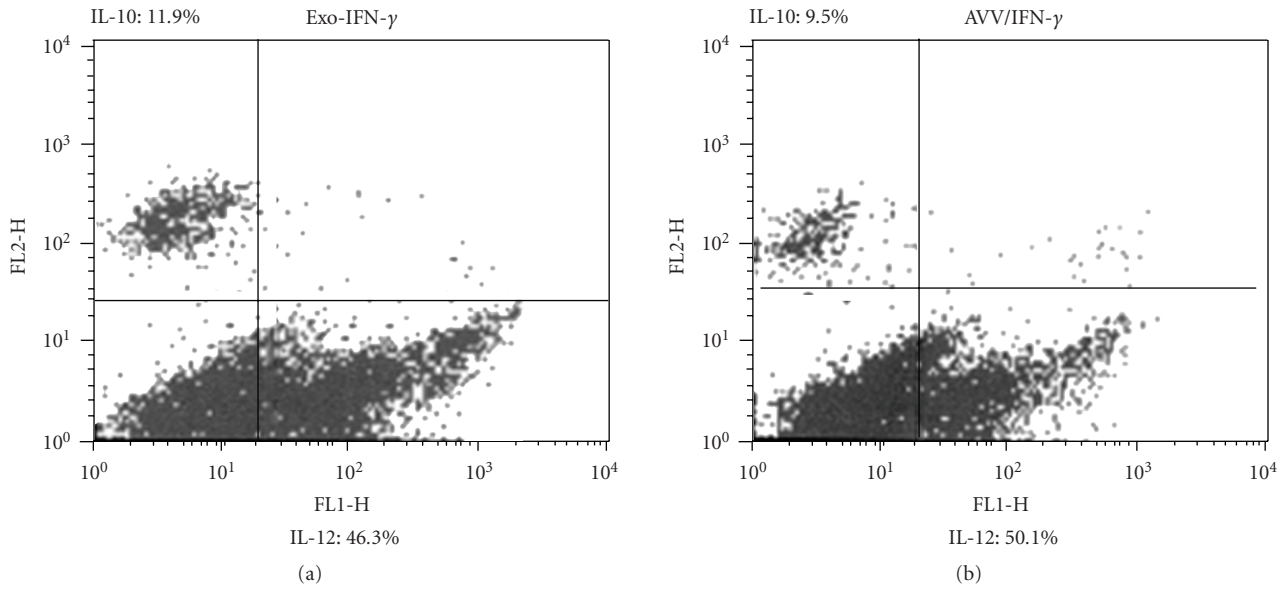

Figure 5: IL-12 and IL-10 expression in DC Analysis of IL-12 and IL-10 expression in DC under treatment with exogenous IFN-γ treatment versus AAV/IFN-γ transduction. Note that the IL-12/IL-10 ratio was slightly enhanced by AAV/IFN-γ transduction.

markers, but the use of AAV/IFN-γ had a more profound effect.

We further observed the resulting expression level of IL-12 and IL-10 in DC by these various treatments, as shown in Figure 5. DCs were treated with exogenous IFN-γ or AAV/IFN-γ. A high IL-12/IL-10 ratio reflects the likelihood that these DCs would stimulate a more robust Th1 CTL response; however both treatments gave a similar ratio. Secretion of IFN-γ from DC (and T cells) was also measured by ELISA. IFN-γ secretion from AAV/IFN-γ transduced DC, with or without AAV/CEA transduction, shown in Figure 6, was similar. Expression rose from 68 to 80 hours postinfection and then remained stable out to 92 hours. However, it is noteworthy that AAV/IFN-γ-infected T cells secrete more IFN-γ than AAV/IFN-γ-infected DC.

3.4. Characterization of Transduced and Stimulated T Cells. A robust MHC/HLA Class I-restricted Th1 CTL response is most consistent with a high CD8 : CD4 ratio. The resulting cell population stimulated by the various DC treatments was analyzed by FACS and the results listed in Table 2 upper panel. The CD8/CD4 ratio in T cell populations was higher when these cells were stimulated by AAV-transduced DC than with mock-treated DC (Table 2). However T cells derived from AAV/IFN-γ-treated DC had a slightly higher CD8/CD4 ratio, consistent these cells having slightly higher CD80, CD83, and CD86 expression (Table 1), and slightly lower IL-10 expression (Figure 5). However, the direct treatment of T cells by AAV/IFN-γ, stimulated by AAV/CEA-loaded DC, resulted in T cell populations with the highest CD8/CD4 ratio (7.9) (Table 2 lower panel).

A robust Th1 CTL response is also consistent with a high IFN-γ/IL-4 expression ratio. Again, the resulting T cells generated from any AAV-transduced DC had a higher IFN-γ/IL-4 expression ratio than by mock treated DC (Table 2 upper panel). However, the direct treatment of T cells with AAV/IFN-γ delivery, stimulated by AAV/CEA loaded DC, resulted in T cell populations with the highest IFN-γ/IL-4 ratio (18.3) (Table 2 lower panel). Moreover, a robust Th1 CTL response is also consistent with a high proportion of CD69+/CD8+ T cells. Yet again, the direct treatment of T cells by AAV/IFN-γ, stimulated by AAV/CEA loaded DC, resulted in T cell populations with the highest percent of CD69+/CD8+ T cells (91.3%) (Table 2 lower panel). Finally, a robust Th1 CTL would also be consistent with low CD25+/CD4+ Treg cell numbers. Again, the direct treatment of T cells by AAV/IFNγ, stimulated by AAV/CEA loaded DC, resulted in T cell populations with the lowest percent of CD25+/CD4+ Treg cells (17.1%) (Table 2 lower panel). Taken together these data suggest that AAV/IFNγ autocrine-delivery into T cells offers the most powerful Th1-T cell population as measured by the CD8/CD4 ratio, IFN-γ/IL-4 ratio, highest percentage CD69+, CD8+ cells, and lowest percent CD25+, CD4+ cells.

The generation of responder CTL involves both the proliferation of CD4+ helper T cells as well as proliferation of the CD8+ T cells themselves. To test the level of T cell proliferation we carried out the standard protocol for the generation of antigen-specific CTL. However, in addition to loading the DC with the antigen (AAV/CEA) we also added the delivery of AAV/IFN-γ into DC or T cells. Proliferation of CD3+ T cells was measured by the incorporation of 3H-TdR, and the results are shown in Figure 7. It appears that the use of IFN-γ in any form offered no advantage to

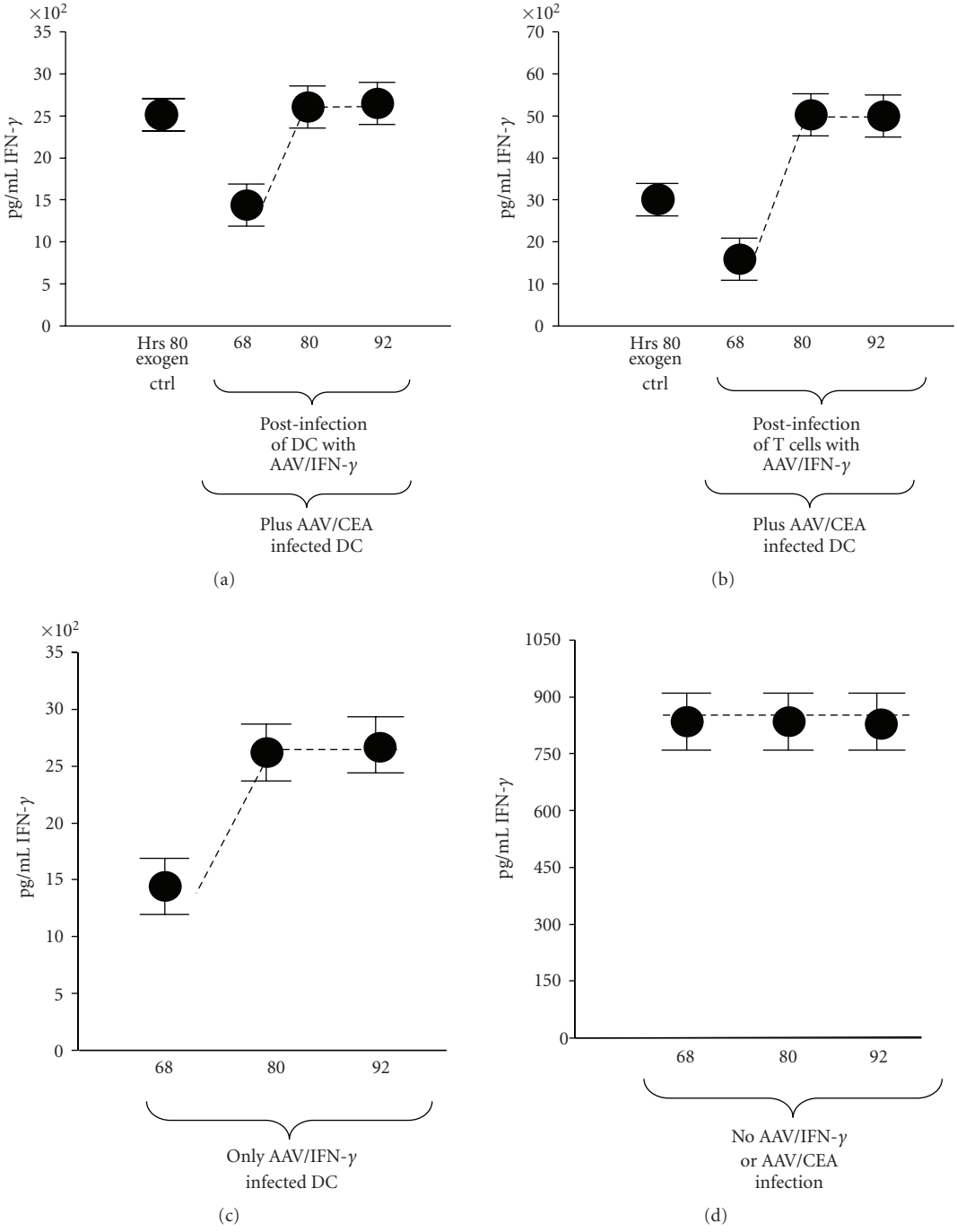

Figure 6: Secretion of IFN-γ from AAV-transduced cells over time. Cells were treated as indicated and secretion of IFN-γ was measured in the conditioned medium at the indicated time by ELISA. The conditions include AAV/IFN-γ delivery into DC (with concurrent AAV/CEA delivery), (a); AAV/IFN-γ delivery into T cells, (b); AAV/IFN-γ only delivery into DC, (c); cells with no treatment, (d).

that of the stimulation of proliferation by AAV/CEA-loaded DC. Overall T cell proliferation is relatively equal among all treatments (Figure 7), while the CD8/CD4 ratio increases (Table 2) when T cells are AAV/IFN-γ-infected. This suggests that CD8+ T cells are preferentially stimulated to proliferate.

3.5. Autocrine IFN-γ Gene Delivery Enhances CTL Killing. Having characterized the AAV/IFN-γ transduced DC and T cells we then assayed the resulting CTL for their ability to kill a genetically altered CEA-positive lymphoblastoid cell line (LCL) which was HLA A2-matched with blood donors. To do this we carried out the experiment depicted in Figure 1 and tested for SW480 cell target killing using the standard ^{51}Cr release assay, and the results are shown in Figure 8(a). As can be seen the highest level of CEA-directed killing results from AAV/IFN-γ autocrine delivery into T cells, a 49%

Table 2: Characterization T cell subsets and cytokine expression as percentages, from AAV-infected DC (top panel) and rAAV-infected T cells (bottom panel).

DC treatment	CD8+/CD4	IFN-γ/IL4	CD69+, CD8+	CD25+, CD4+
Ctrl (mock)	21.6/51.3	21.85/10.75	27.4	55.6
AAV/CEA	50.3/22.1	26.53/2.73	61.5	16.2
AAV/CEA + exogen IFN-γ	52.1/23.6	18.84/2.46	60.8	15.6
AAV/CEA + AAV/ IFN-γ	56.5/22.5	27.71/2.90	64.7	14.3
T cell treatment	CD8+/CD4+	IFN-γ/IL4	CD69+, CD8+	CD25+, CD4+
AAV/IFN-γ	84.6/10.7	43.4/2.37	91.3	17.1
exogen IFN-γ	ND	24.28/1.96	ND	ND
Untreated T cells	14.1/69.8	26.53/2.73	20.5	63.5

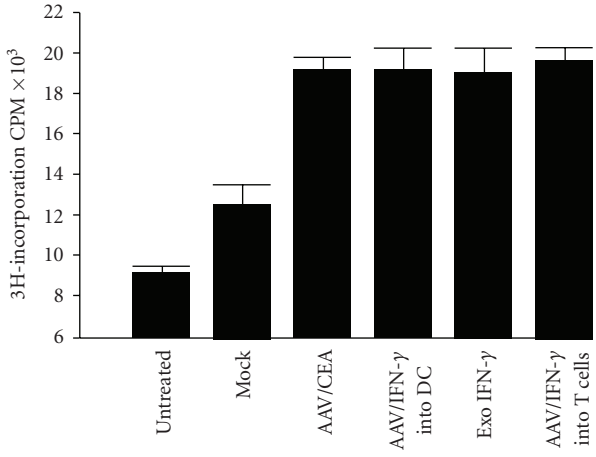

Figure 7: Proliferation of T cells. Tritium incorporation by T cells is shown under various treatments.

increase over AAV/CEA-only control. Killing was blocked by polymorphic anticlass I antibody, but not by anticlass II. The high killing that results from AAV/IFN-γ autocrine treatment is fully consistent with the highest CD8/CD4 ratio, highest IFN-γ/IL-4 ratio, highest percentage CD69+, CD8+ cells, and lowest percent CD25+, CD4+ cells. The higher killing may be attributed to the higher CD8+ T cell number due to higher CD8/CD4 ratio of T cells in AAV/IFN-γ-treated bulk T cell cultures versus the lower ratio generated from AAV/IFN-γ-treated DC, 84.6%/10.7% versus 56.5%/22.5%, respectively (Table 2). We also utilized CEA+ LCL cells as a target and, again, AAV/IFN-γ autocrine delivery into T cells resulted in CTL with the highest killing of CEA+ LCL, shown in Figure 8(b).

4. Discussion

While IFN-γ has multiple actions which promote Th1 response [7–10], it was unclear which immune cell type should best express this cytokine. This study demonstrates that only a specific approach of IFN-γ gene delivery, autocrine delivery, results in a CD3+ T cell population with higher killing ability. This was not fully anticipated as IFN-γ affects the phenotype of both DC and T cells. For DC IFN stimulates significantly higher MHC class II, CD86, and slightly higher CD80 levels [30–32]. IFN-γ also stimulates higher IL-12 expression and DC maturation [30–32]. Comparing DC from IFN knockout mice and normal mice, IFN-γ expression by DC also appears needed for the effective CD8+ T cell stimulation [33]. Normally IFN-γ expression in DC is dependent upon T-bet [34], but in this study we have circumvented this need by expressing it from the CMV promoter within the AAV backbone (AA). Moreover, NK and NKT cells are believed to be the major sources of IFN-γ for induction of macrophage maturation [35]. This pathway has also been circumvented by AAV/IFN-γ delivery. Paracrine IFN-γ expression by DC is believed to stimulate T cells to autocrine express their own IFN-γ. However, IFN-γ also has pronounced effects on T cells as well. While IFN-γ inhibits the formation of Th2 cells [36], its expression directly correlates with the formation of Th1 response and CTL killing, as mentioned earlier, so much so that IFN-γ secretion is used as a substitute for antigen specific killing assays [12, 13]. Yet IFN-γ appears needed for the activity of unusual CD8(+)LAP(+)foxp3(+) Treg cells. Thus, there is some evidence that IFN-γ may ultimately turn off the Th1 response that produces it [37].

Our purpose for these studies was to further analyze the mechanism of action of IFN-γ and to optimize ex vivo generated anticancer CTL for adoptive therapies in cancer. While the adoptive transfer of ex vivo manipulated dendritic cells (DCs) appears mostly ineffective in anticancer therapies [38], the adoptive transfer of ex vivo generated anti-cancer CD8+ MHC Class I-restricted cytotoxic T lymphocytes (CTLs) seem to be a more promising treatment therapy [39, 40]. While this study demonstrates that IFN-γ is best delivered and expressed via the autocrine route in the generation of antigen-specific CD8(+) CTL, it should be pointed out that the basal levels of natural IFN-γ expression also remain present and we are simply augmenting the level of IFN-γ. Thus, our interpretation of this data is that most likely the maximum effective ceiling of IFN-γ expression and effect in DC had been reached, while the effective ceiling of IFN-γ expression and effect in T cells had not been.

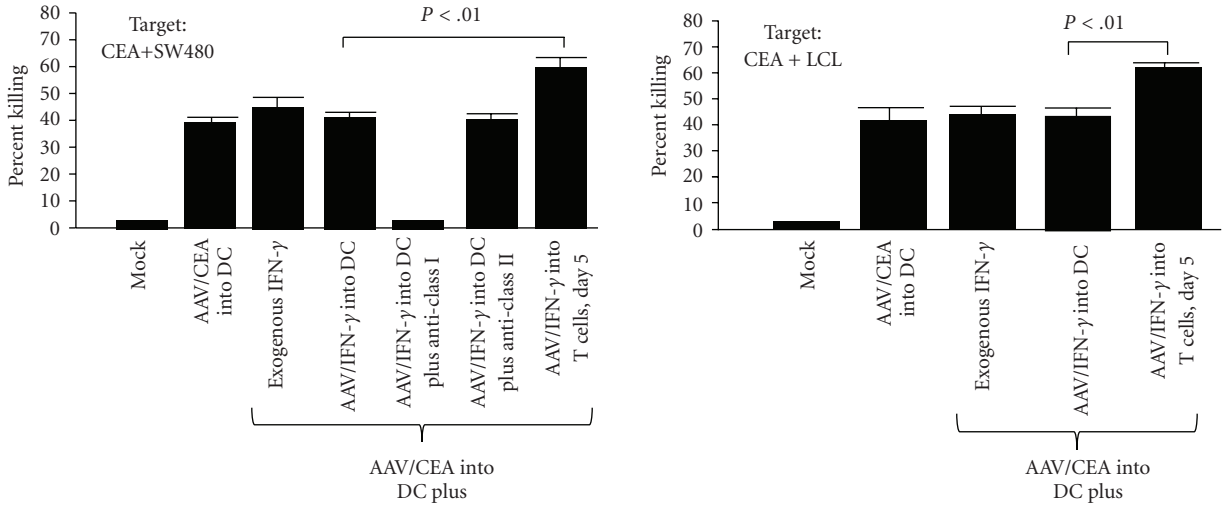

Figure 8: Enhanced CTL killing by IFN-γ autocrine delivery. (a) shows CTL killing after the indicated treatment, of CEA-positive targets, SW480 cells, by standard ^{51}Cr release assay. All assays were done at a effector: target ratio of 20 : 1. Note that AAV/IFN-γ autocrine delivery resulted in CTL with the highest killing ability. (b) shows a similar CTL killing assay using CEA+ LCL cells as the target. Note that, again, AAV/IFN-γ autocrine delivery resulted in CTL with the highest killing ability.

Another advantage of IFN-γ overexpression in CTL would be its known ability to upregulate MHC class I molecules, allowing for enhanced recognition of antigen expressing tumor cells [41]. Yet the practicality of the autocrine approach for certain Th1 response cytokines is questionable as the endogenous expression of certain cytokines in Th1 T cells may be problematic. The transgenic expression of IL-15 is linked with malignancy [42]. The most similar study involving IFN-γ was by Young et al., who generated transgenic mice which overexpressed IFN-γ in the thymus and spleen [43]. While bone-related granulomatous lesions and degeneration of cartilage was observed, there were no serious hyperproliferative cellular elements seen in the bone marrow, spleen, or lymph nodes. Thus, the adoptive transfer of IFN-γ-over expressing T cells in clinical trials may be further considered. In fact we observed no advantageous proliferation by the T cells (Figure 7). Moreover, if further work suggests that there are serious side effects of IFN-γ overexpression in T cells, then the inclusion of a suicide gene within the vector, such as herpes thymidine kinase gene, would allow for the elimination of these cells when needed.

This head-to-head comparison of IFN-γ overexpression in DC versus T cells was done with the purpose of enhancing Th1 CD8(+) CTL response. However, these data also give us information as to the primary mechanism of action of IFN-γ on Th1 immune response may be through endogenous T cell expression. Yet further analysis is needed to determine whether it is the CD4(+) or CD8(+) T cell which is the primary vehicle through which IFN-γ acts. Cell ratio studies (Table 2) suggest that it is the CD8(+) T cell. Ultimately DNA microarray analysis of the T cell's transcriptome and proteomics analysis are warranted to fully understand how high IFN-γ expression affects the cell's phenotype.

Abbreviations

AAV: Adeno-associated virus
CEA: Carcinoembryonic antigen
CD: Cluster of differentiation
DC: Dendritic cell
Foxp3: Forkhead box P3
HLA: Human leukocyte antigen
IFN: Interferon
IL: Interleukin
LCL: Lymphoblastoid cell line
MΦ: Macrophage
MHC: Major histocompatibility complex
Mo: Monocytes.

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Research Article

Increasing a Robust Antigen-Specific Cytotoxic T Lymphocyte Response by FMDV DNA Vaccination with IL-9 Expressing Construct

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Various chemokines and cytokines as adjuvants can be used to improve efficacy of DNA vaccination. In this study, we sought to investigate if a DNA construct expressing IL-9 (designed as proV-IL9) as a molecular adjuvant enhance antigen specific immune responses elicited by the pcD-VP1 DNA vaccination. Mice immunized with pcD-VP1 combined with proV-IL9 developed a strong humoral response. In addition, the coinoculation induced significant higher level of antigen-specific cell proliferation and cytotoxic response. This agreed well with higher expression level of IFN-γ and perforin in $CD8^+$ T cells, but not with IL-17 in these T cells. The results indicate that IL-9 induces the development of IFN-γ-producing $CD8^+$ T cells (Tc1), but not the IL-17-producing $CD8^+$ T cells (Tc17). Up-regulated expressions of BCL-2 and BCL-XL were exhibited in these Tc1 cells, suggesting that IL-9 may trigger antiapoptosis mechanism in these cells. Together, these results demonstrated that IL-9 used as molecular adjuvant could enhance the immunogenicity of DNA vaccination, in augmenting humoral and cellular responses and particularly promoting Tc1 activations. Thus, the IL-9 may be utilized as a potent Tc1 adjuvant for DNA vaccines.

1. Introduction

Foot and mouth disease virus (FMDV) is a member of genus *Aphthovirus* in the family *Picornaviridae* and causes a great economical loss for farm animals [1]. Since the current available inactivated FMDV vaccine is still considered as a potential outbreak, alternative vaccination methods should be developed [2]. DNA vaccination offers one of such alternatives, which is a relatively novel and powerful method of immunization, capable of humoral as well as cellular immunity [3–6]. To date, various approaches have been developed to enhance the immunogenicity of plasmid DNA vaccines, such as the use of plasmid expressing cytokine as a molecular adjuvant [7, 8].

IL-9, a cytokine produced by T cells, mast cells, eosinophils, and neutrophils, stimulates cell proliferation and prevents apoptosis [9]. In addition, IL-9 supports the growth of T cells and also increases the production of IL-6 in B lymphocytes in a phenomenon culminating in an enhancement of IgE or IgG1 synthesis [10, 11]. IL-9 producing-DC can be used to induce protective immune response against intestinal nematodes [12].

In spite of its immune effects, IL-9 or its expressing construct has not been examined to determine if it can be used as adjuvant, directly affecting elicited immune responses of vaccines. In this study, we demonstrated that coinoculation of the IL-9 expressing construct with VP1 DNA vaccine, encoding VP1 capsid protein of foot-and-mouth disease virus, induced strong humoral and cellular immune responses, including the antigen-specific CD8 Tc1 activation.

2. Materials and Methods

2.1. Reagents and Animals. FMDV VP1 peptide representing the T-cell epitope (aa133–147, SSKYGDTSTNNVRGD) was

Table 1: Immunization groups.

Groups	DNA vaccine	Adjuvant
1	Naïve	
2	100 μg pcD	100 μg proV
3	100 μg pcD	100 μg proV-IL9
4	100 μg pcD-VP1	100 μg proV
5	100 μg pcD-VP1	100 μg proV-IL9

Note: pcD and proV are empty vectors.

synthesized by GL Biochem Co., Ltd. (Shanghai, China). Conjugated Abs used for flow cytometry analysis were purchased from BD Pharmingen (San Diego, USA). Adult female C57BL/6 mice at 8–10 weeks of age were purchased from Animal Institute of Chinese Medical Academy (Beijing, China) and feed with pathogen-free food and water in a 12-h light-cycle.

2.2. Plasmid Construction and Preparations. The pcD-VP1 was constructed into pcDNA3.0 as described previously [13]. The coding sequence for mouse IL-9 was obtained from mRNA isolated from spleens by RT-PCR method and subcloned into the proVAX vector [14] to designate as proV-IL9. The plasmids were maxi-prepared by the alkaline method, subsequently purified by Qiagen Maxi prep kit (Qiagen Inc., Duesseldorf, Germany), and diluted in saline solution.

2.3. Transfection of the BHK Cell Lines. The purified plasmids proV-IL9 were transfected into BHK cells with Lipofectamine according to the manufacturer's instructions (Invitrogen, CA, USA). The transfected cells were harvested after 48 h and blocked with Fc-Block (BD Phamingen) in PBS for 30 min at 4°C before fixed with 4% paraformaldehyde and permeabilized with saponin. Cells were intracellularly stained with anti-IL-9-PE (BD Phamingen) for 1 hour at 4°C and analyzed with as FACScalibur using the Cell Quest Pro Software (BD Bioscience).

2.4. Immunization. The C57BL/6 mice were randomly divided into five groups (6 per group), and immunized intramuscularly on days 0, 14, and 28 listed in Table 1.

2.5. Detection of Anti-VP1-Specific Antibodies. The detection of anti-VP1-specific antibodies in the sera was carried out by quantitative ELISA assays as previously described [15]. The sera from immunized mice were tested individually on day 7 after the third immunization, and the concentrations calculated were the means of three independent assays.

2.6. Cell Proliferation. Single lymphocyte suspensions were obtained from spleens of the mice on day 7 after the third immunization. Cells in RPMI-1640 medium (Gibco, Eggenstein, Germany)/10% FBS were used to perform the cell proliferation by MTT method after the GST-VP1 stimulation in vitro for 48 h. This method was according to the previously described protocols [15]. The OD values were read at 490 nm by a plate reader (Magellan, Tecan Austria GmbH). Data were expressed as stimulation index (SI), calculated as the mean reading of triplicate wells stimulated with an antigen, divided by the mean reading of triplicate wells stimulated with the medium.

2.7. In Vivo Cytotoxic Assay. In vivo cytotoxic assay was performed as described previously [14] with the use of splenocytes from naïve C57BL/6 mice pulsed with 10^{-6} M VP1 peptide and labeled with a high concentration of CFSE (15 μM, $CFSE^{high}$ cells) as target cells. A portion of the same splenocytes was labeled with a low concentration of CFSE (0.5 μM, $CFSE^{low}$ cells) without peptide pulse as a nontarget control. The target and control cells were mixed in a 1 : 1 ratio and injected into immunized mice at 2×10^7 total cells per mouse via the tail vein on day 7 after the third immunization. Four hours later, lymphnodes and the spleens of injected mice were removed and the target and control cells were analyzed by their differential CFSE fluorescent intensities using an FACSCalibur (BD Biosciences, USA). Specific lysis was calculated using the following formula: ratio = percentage $CFSE^{low}$ /percentage $CFSE^{high}$. Percentage specific lysis = [1−(ratio unprimed/ratio primed) ×100].

2.8. Flow Cytometric Analysis. Splenic T cells were isolated on day 7 after the third immunization. T cells or $CD8^+$ T cells from splenocytes of C57/B6 mice isolated by MACS sorting (R&D Systems, Inc., Huntingdon Valley, PA, USA) at 0.5×10^6 cells/20 μL were stimulated in 96-well plates with VP1 peptide (5 μg/mL) and anti-CD28 (5 μg/mL) mAb for 6 h at 37°C and 5% CO_2. Monensin (2 μg/mL) was added for the last 4 h and the cells were washed three times with PBS/10%FCS. Cells were blocked with Fc-Block (BD Phamingen, San Diego, USA) in PBS for 30 min at 4°C before fixed with 4% paraformaldehyde and permeabilized with saponin, immunostained with isotype controls, or double stained with anti-CD8-FITC and anti-IFN-γ-PE, or anti-CD8-FITC and anti-IL-17-PE, or anti-CD8-FITC and anti-perforin-PE for 1 h at 4°C. The cells were washed and analyzed with an FACScalibur using the Cell Quest Pro Software (BD Bioscience).

2.9. RT-PCR. Total RNA was extracted from total splenocytes or from sorted $CD8^+$ T cells and then was reverse-transcribed. Seven days after the third immunization, $CD8^+$ T cells from splenocytes of C57/B6 mice were isolated by MACS sorting (R&D Systems,Inc). The sequences of the

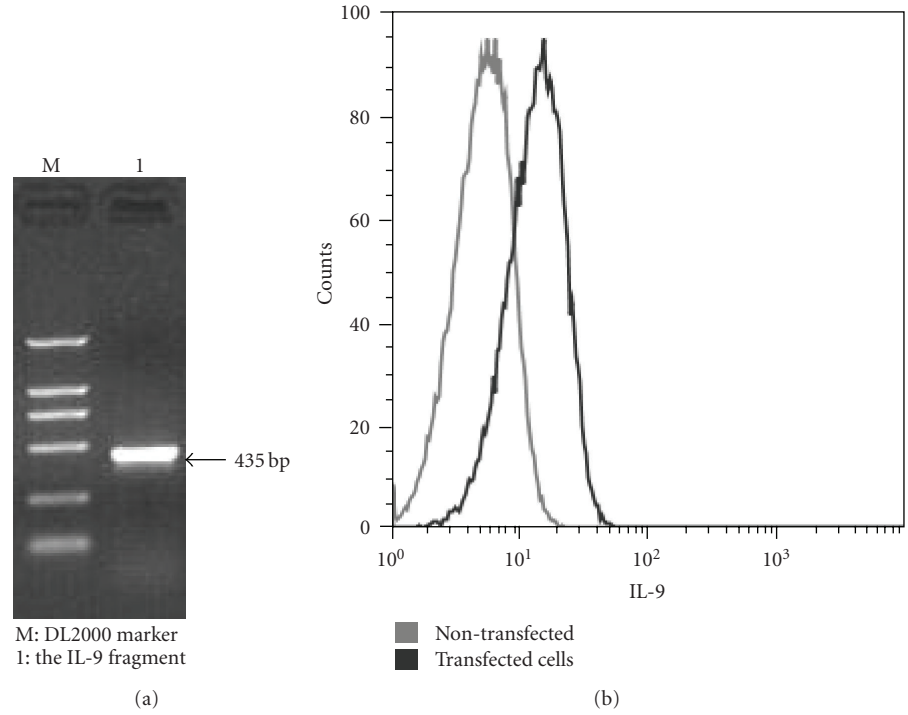

Figure 1: Cloning and expression of IL-9. (a) The coding sequence for mouse IL-9 was obtained from mRNA isolated from spleens. (b) BHK cells 48 h after the transfections with proV-IL9 and untransfected were collected, fixed, and intracellularly stained with anti-IL-9-PE. The results were analyzed by the FACScalibur.

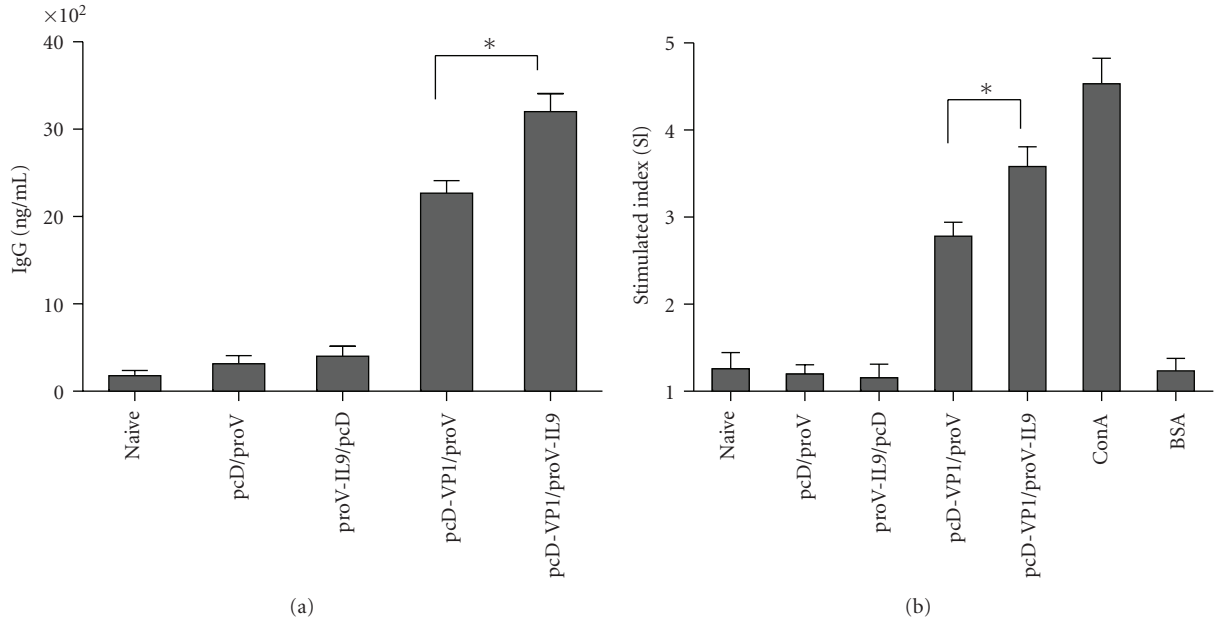

Figure 2: Effect of IL-9 as adjuvant on humoral and cell proliferative responses. (a) Serum samples from six mice per immunized group were analyzed for specific antibodies against VP1 by ELISA. Mouse IgG with a known concentration was serially diluted and a standard curve was obtained for concentration of specific binding. (b) T cells were isolated from mice (6 per group) of all groups on day 7 after the third immunization and stimulated with VP1 recombinant protein. A cell proliferation response was analyzed using MTT and expressed as stimulation index. Data showed are representatives from three independent experiments. $^*P < .05$ compared with pcD-VP1 group.

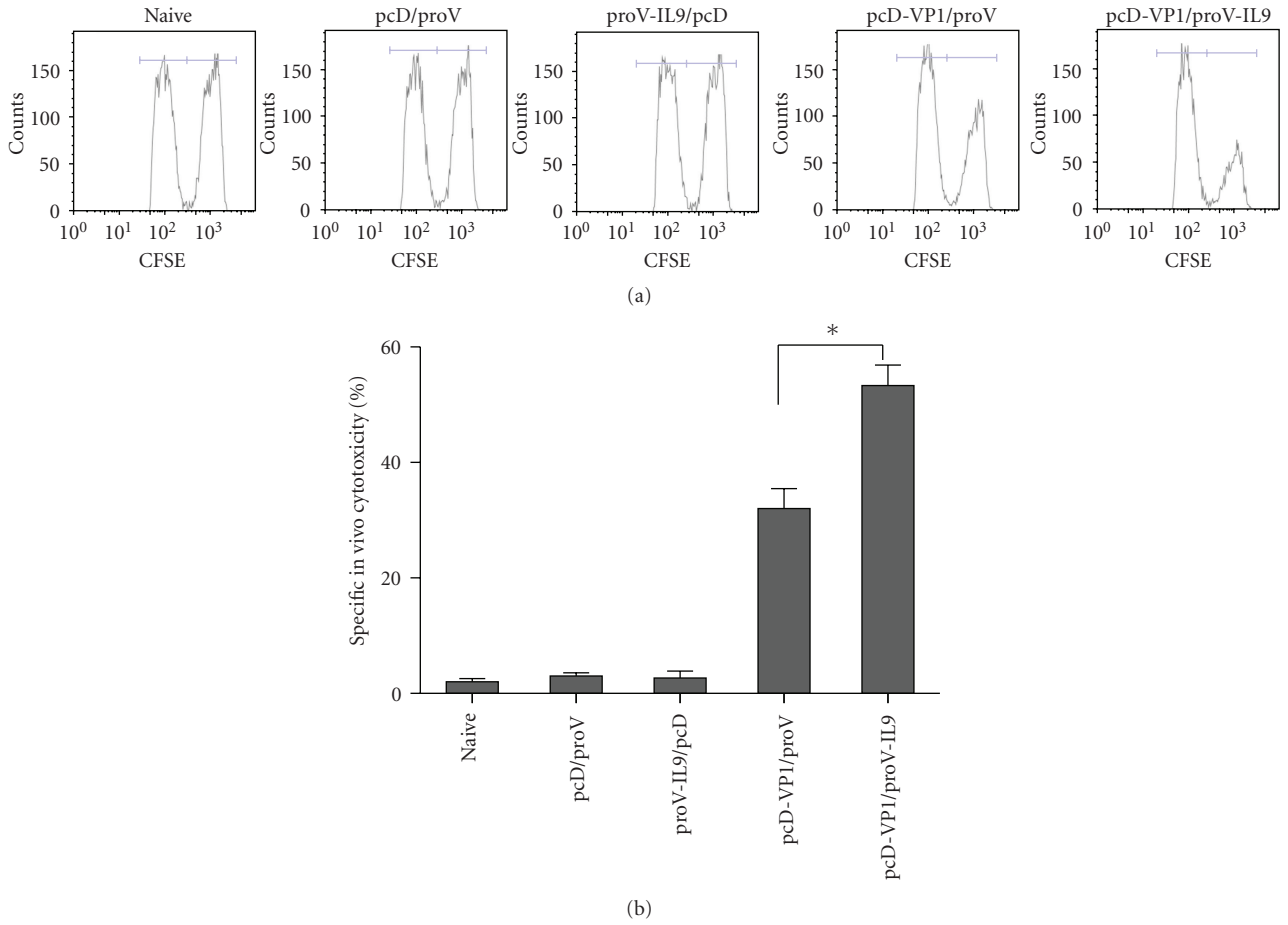

Figure 3: Effects of IL-9 on in vivo cytotoxic responses. (a) To analyze effect of IL-9 on VP1-specific cytotoxicity, the in vivo cytotoxic assay was performed by comparing the ratio of $CSFE^{high}$ of target cells versus $CSFE^{low}$ of controls after in vivo transfer into immunized mice by FACS. (b) The percentage of specific lysis is summarized in the means of the three independent experiments. **indicates $P < .01$ between groups.

primers are listed in Table 2. Samples were run by 1.5% agarose gel and visualized by staining with EtBr.

2.10. Statistical Analysis. Results are presented as means ± S.E.M. Student's *t*-test analysis was used for data analysis. A value of $P < .05$ was considered to be statistically significant.

3. Results

3.1. Cloning for IL-9 and Expression in BHK Cells. To construct the IL-9 expression plasmid proV-IL9, the entire mouse IL-9 cDNA (435 bp) was cloned, verified by sequencing, and subcloned into the proVAX vector for eukaryotic expression (Figure 1(a)). In order to confirm proV-IL9 protein expressing, transfected cells were used in intracellular staining analysis by the use of anti-IL-9-PE after 48 h of the transfection. Its eukaryotic expression was observed and showed in Figure 1(b).

3.2. IL-9 as a Molecular Adjuvant Enhances Humoral and Cell Proliferative Responses. To examine the effect of IL-9 on the humoral response in mice, serum total IgG antibodies against VP1 were determined by quantitative ELISA on day 7 after the third immunization. Compared to the group immunized with pcD-VP1 plus empty vector proV, a significantly enhanced level of the production of total IgG was found in the groups immunized with pcD-VP1 plus proV-IL9 (Figure 2(a)). To determine whether IL-9 influences T cell-mediated immunity, lymphocytes isolated from the mice on day 7 after the third immunization were stimulated with GST-VP1 protein as the specific antigen, ConA as a positive control, BSA as a nonspecific control, and medium as a negative control. The highest level of cell proliferation responses was induced in the groups immunized with pcD-VP1 plus proV-IL9 (Figure 2(b)). These data suggest that IL-9 as molecular adjuvant increased humoral and cell proliferative responses.

3.3. The Adjuvant Effect of IL-9 on Cytotoxicity. To examine whether proV-IL-9 could enhance antigen-specific cytotoxic response, in vivo cytotoxic assay was performed on day 7

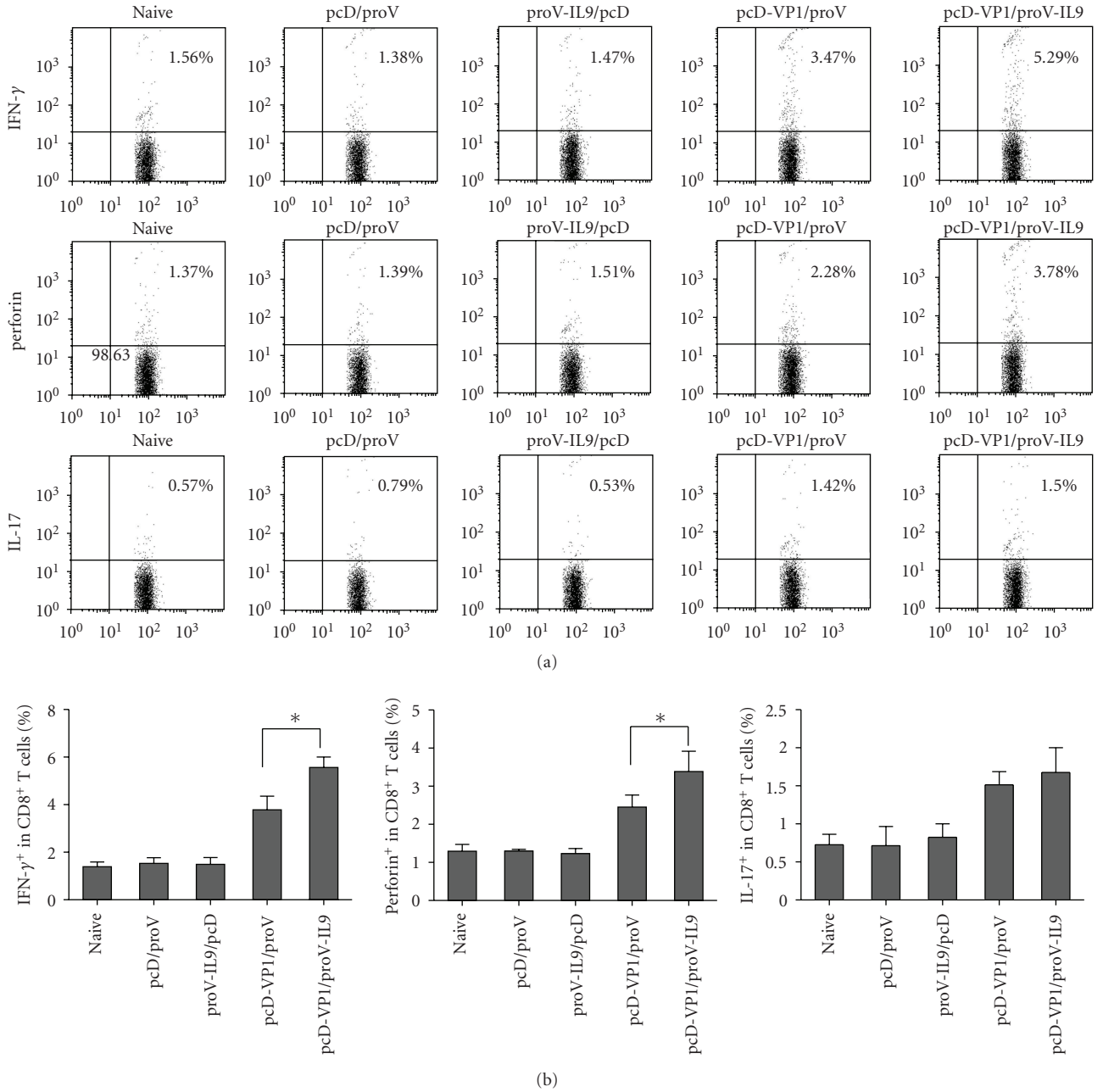

Figure 4: Analysis of antigen-specific cytokine productions in $CD8^+$ T cells by FACS. (a) $CD8^+$ T cells isolated from the spleen of C57BL/6 mice on day 7 after the final boost were stimulated with VP1 peptide for 4 h in culture. Intracellular staining for IFN-γ, perforin, and IL-17 in $CD8^+$ T cells was performed. (b) The summaries of percentage were shown in the means of the three independent experiments.

after the third immunization. As shown in Figure 3, the highest percentage of antigen-specific lysis was approximately 56.3%, from the mice immunized with pcD-VP1 plus proV-IL9, whereas it was 38.2% from the mice immunized with pcD-VP1 plus proV.

3.4. Effect of IL-9 on Cytokine Expression in $CD8^+$ T Cells. Since both IFN-γ−producing $CD8^+$ T cells (Tc1) and IL-17−producing $CD8^+$ T cells (Tc17) could enhance the cytotoxic responses [16, 17], we sought to determine which $CD8^+$ T cells were affected by the IL-9. $CD8^+$ T cells were purified before used to perform the intracellular stainings against IFN-γ, perforin, or IL-17 on day 7 after the third immunization. As a representative result shown in Figure 4(a)-4(b), the expression of antigen-induced IFN-γ and perforin in $CD8^+$ T cells were significantly higher in the mice immunized with pcD-VP1 plus proV-IL9 than the other groups, whereas the percentage of IL-17 in $CD8^+$ T cells of mice immunized with pcD-VP1 plus proV-IL9

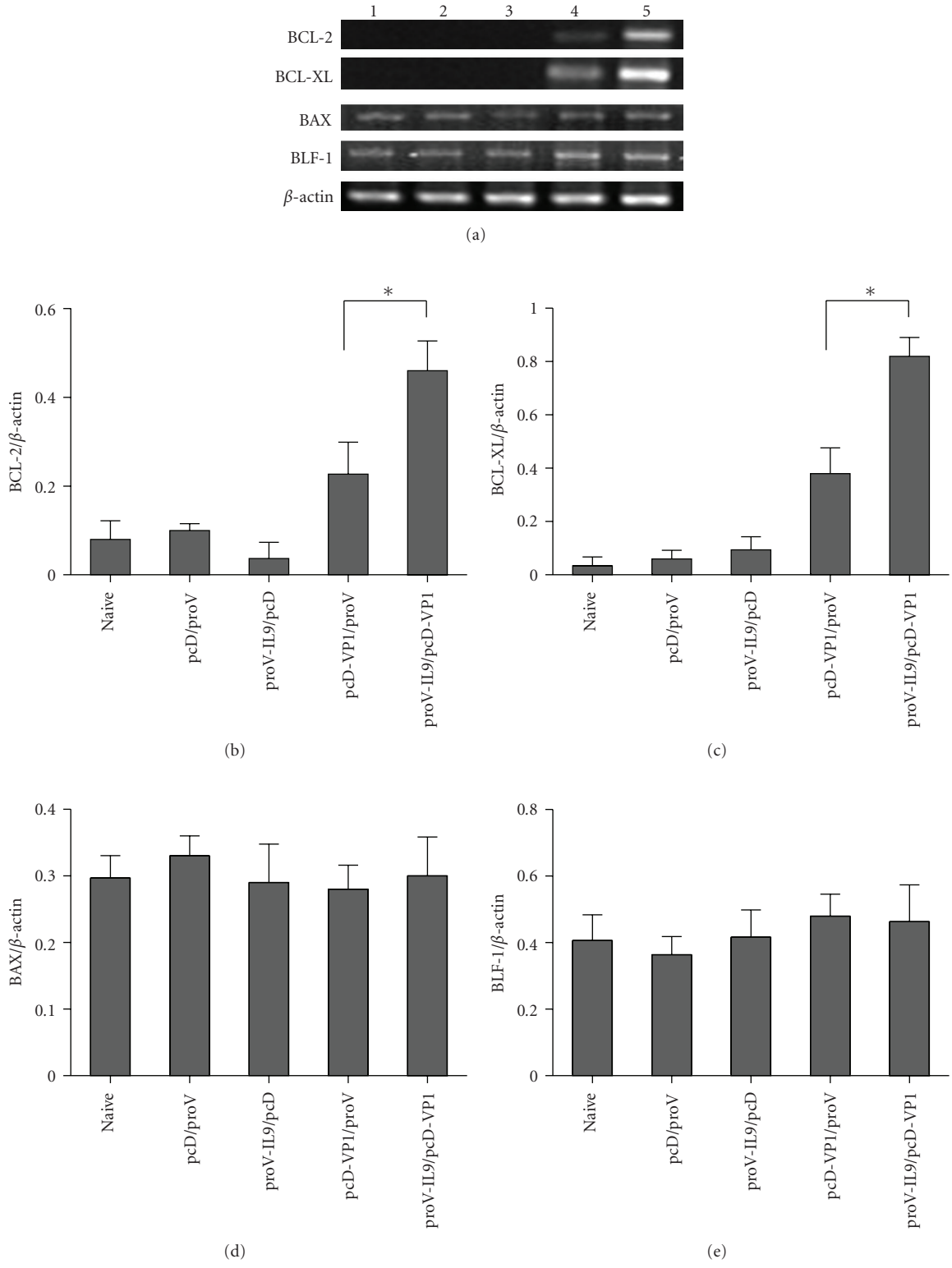

Figure 5: Expression of apoptotic or proapoptotic genes in $CD8^+$ T cells by RT-PCR. Total RNA was isolated from the splenic $CD8^+$ T cells of immunized on day 7 after final boost. (a) The expression levels of apoptotic or proapoptotic genes were semiquantitatively measured by RT-PCR through the normalization of the tested cDNA concentration to the amount of β-actin. (b), (c), (d), (e) the density of each band as determined using Alpha image software and expressed as a relative intensity against the β-actin band. From left to right, 1, naïve; 2, pcD/proV; 3, proV-IL9/pcD; 4, pcD-VP1/proV; 5, pcD-VP1/proV-IL9.

Table 2: Target gene primers.

Target gene	Primers
β-actin	5′-TGACGGGGTCACCCACACTGTGCCCATCTA
	3′-CTAGAAGCATTTGCGGTGGACGATGGAGGG
BCL-2	5′-GGCTACGAGTGGGATGCT
	3′-GGGTCATGTGTGTGGAGAG
BCL-XL	5′-CGATGAGTTTGAACTGCG
	3′-CACCTAGAGCCTTGGATCC
BAX	5′-CTGCAGAGGATGATTGCTGA
	3′-CCAACATTGCATGGTGCTAC
BLF-1	5′-CAGGGAAGATGGCTGAGTCT
	3′- TTCTGCCGTATCCATTCTCC

was at the similar level with that of pcD-VP1 plus proV. It may indicate that IL-9 favorably induces the development of IFN-γ−producing CD8$^+$ T cells (Tc1), not IL-17−producing CD8$^+$ T cells (Tc17).

3.5. IL-9 Enhanced Antiapoptotic Gene Expression in CD8$^+$ T Cells. Since IL-9 could affect on apoptosis [18], we examined the effect of IL-9 as adjuvant on Antiapoptotic and proapoptotic gene expressions in CD8$^+$ T cells on day 7 after the third immunization. As depicted in Figure 5, the levels of mRNAs for BCL-2 and BCL-XL induced by pcD-VP1 plus proV-IL9 were higher, whereas the levels of mRNAs for BLF-1 and BAX were not affected among the groups, indicating that the IL-9 may enhance survival of activated CD8$^+$ T cells via apoptotic mechanism.

4. Discussion

Our recent studies demonstrated that IL-9 as molecular adjuvant can induce strong humoral and cellular immune responses. Most importantly, a significant higher level of cytotoxic responses was observed in the mice immunized with pcD-VP1 plus proV-IL9. In addition, IFN-γ and perforin were up-regulated in CD8$^+$ T cells, suggesting that IFN-γ-producing CD8$^+$ T cells (Tc1) were mostly affected in the cytotoxic responses. This may be owned to high expression levels of anti-apoptosis genes of BCL-2 and BCL-XL induced in CD8$^+$ T cells by such adjuvant.

Immunization with DNA vaccine encoding an immunogenic antigen represents a novel and promising method in vaccine research and development. Many studies have demonstrated that the expressed antigen is naturally processed and presented to T cells, inducing a broad range of immune responses including antibody production and the activation of T cells [19–22]. However, plasmid DNA immunogenicity is relatively low compared to viral vectors; various strategies have been proposed to enhance it, such as molecular adjuvants.

IL-9 is a 14 kDa cytokine and involved in immune responses to helminthes as well as allergy [23, 24], it is generally attributed to T_H2 cells. To date, IL-9 seemed to be associated with the Treg and T_H17 cells [25–27]. Two studies suggested that IL-9 expression in T_H9 cells is distinct from other CD4$^+$ T cell subsets [28, 29]. Importantly, IL-9 supports the growth of T cells and also increases the production of IgG1 and IgE in B lymphocytes. However, IL-9 or its expressing construct has not been tested directly to determine what particular immune responses could be affected if it is used as a molecular adjuvant. From this study, we observed that IL-9 as the molecular adjuvant could increase humoral and cell proliferative responses.

Antigen-specific CTL response plays a key role in the protection against viruses or other intracellular pathogens. IFN-γ−producing CD8$^+$ T cells (Tc1) and IL-17−producing CD8$^+$ T cells (Tc17) can enhance cytotoxic responses. Tc17 cells, a unique subset of CD8$^+$ T cells, were found in the lung following primary challenge with influenza A and protected against lethal influenza challenge [30, 31]. Cytotoxicity can be induced by two distinct molecular pathways: up-regulation of perforin, or up-regulation of FasL (CD95L) [32, 33]. In our study, we found that high level expressions of IFN-γ and perforin were observed to associate with CD8$^+$ T cells, not the IL-17. This indicates that the IL-9-induced Tc1, but not the Tc17, enhances cytotoxic responses through up-regulation of perforin.

In sum, our results show for the first time that FMDV DNA vaccine combined with IL-9 expressing plasmid can induce strong immune responses and enhanced Tc1 mediated cytotoxic responses. This IL-9 may be served as a promising molecular adjuvant for DNA vaccinations.

Acknowledgments

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Review Article

Therapeutic Cancer Vaccines in Combination with Conventional Therapy

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The clinical efficacy of most therapeutic vaccines against cancer has not yet met its promise. Data are emerging that strongly support the notion that combining immunotherapy with conventional therapies, for example, radiation and chemotherapy may improve efficacy. In particular combination with chemotherapy may lead to improved clinical efficacy by clearing suppressor cells, reboot of the immune system, by rendering tumor cells more susceptible to immune mediated killing, or by activation of cells of the immune system. In addition, a range of tumor antigens have been characterized to allow targeting of proteins coupled to intrinsic properties of cancer cells. For example, proteins associated with drug resistance can be targeted, and form ideal target structures for use in combination with chemotherapy for killing of surviving drug resistant cancer cells. Proteins associated with the malignant phenotype can be targeted to specifically target cancer cells, but proteins targeted by immunotherapy may also simultaneously target cancer cells as well as suppressive cells in the tumor stroma.

1. Introduction

Traditional cancer treatment modalities include surgery, radiation therapy, chemotherapy, and for some cancer types, hormone therapy. Although these treatment modalities are life extending for many patients, they are rarely curative for disseminated cancers. The use of cancer vaccines to induce a therapeutic antitumor immune response in the patient has huge potential to complement traditional cancer therapies in a nonoverlapping way. Therapeutic cancer vaccines are designed to recalibrate the existing host-tumor interaction, tipping the balance from tumor acceptance towards tumor control to the benefit of the cancer patient. Additionally, the highly specific character of the host immune response minimizes the risk for unattractive adverse events associated with most other cancer therapies in use today. Such vaccinations have been ongoing since early 1990s [1] based on the long awaited characterization of human tumor antigens recognized by patient T cells [2]. Encouraged by sporadic successes, mainly in small phase I trials [3, 4], a high number of trials have been ongoing worldwide, however so far the clinical efficacy as demonstrated in large phase III trials has in most cases be absent or marginal. Recently, however, the US Food and Drug Administration (FDA) approved an autologous cellular vaccine (Provenge®) for the treatment of prostate cancer. The approval was based a clinical phase III trial including 512 patients with asymptomatic or minimally symptomatic, metastatic prostate cancer and was shown to increase overall survival (OS) by 4 months compared to placebo.

Initial attempts to improve vaccination induced biological and clinical responses focussed on the use of biological modifiers or adjuvants with the aim of increasing the magnitude of the response. To this end, immune stimulatory molecules such as interleukin-2 (IL-2) and granulocyte-macrophage colony-stimulating factor (GM-CSF) have been

used as adjuvants in many animal studies and vaccination trials. Over the past years it has been realized that the immune response is in fact comprised not only by responder cells and molecules, but also cells and molecules responsible for a counter-response [5, 6]. In turn this has led to intensified research into the cells and molecules involved with suppressing immune responses, and ways to inhibit or delete the counter response [7]. In this regard, it has been realized that chemotherapy could be used to specifically target suppressor cells of the immune system [8] or to "reboot" the immune system for induction of anti-cancer responses [9]. Moreover, chemotherapy could lead to immunogenic death of tumor cells [10], depending on the drug, dose, and administration.

Concerning the characterization of target epitopes recognized by T cells, the past decades has been characterized by an increased focus on proteins that functionally link to the malignant phenotype, for example, to metastasize and avoid apoptosis [11–16]. Importantly, some of the tumor associated antigens that comprise antigenic peptides are functionally related to resistance to conventional therapy, setting the stage for specifically targeting of cells that escape conventional therapy [17–21]. Last but not least, the tumor supportive role of cells in the stroma have encouraged the characterization of antigens which are expressed not alone by cancer cells but also or exclusively by cells in the tumor stroma [21–25].

Therapeutic strategies based on the above findings are likely to improve the clinical efficacy of therapeutic vaccinations against cancer. Nonetheless, combinations of chemotherapy—may be even with several drugs in combination—with immunological strategies, are complex to evaluate, and demand for careful design of clinical trials as well as development of new ways to evaluate dose and schedule combinations rationally [26]. The perspective, however, is future treatments that combine immunological treatment with conventional therapy leading to increased clinical efficacy to the benefit of the patient.

2. Combination with Chemotherapy Immunological Reboot, and Immunological Cell Death

Even few years back, the idea of combining chemotherapy with active immune therapy was unheard of. The dogma stated that chemotherapy would jeopardize any immune responses, in part due to the fact that dividing immune cells are vulnerable to chemotherapeutic drugs, and as a consequence concurrent active immune therapy with chemotherapy would be pointless. Nonetheless, the concept of combining immune therapy with sequential or even concurrent chemotherapy has lately gained much interest, for several reasons. First, a number of surprising findings suggested that combining immuno- and chemotherapy could lead to better responses in advanced cancer patients [27]. To this end, data from several studies suggest that clinical response to chemotherapy is improved if preceded by immunotherapy [28–31]. A possible explanation for this phenomenon is that cancer cell death by chemotherapy will lead to presentation of antigens that may further activate a treatment-induced T cell response leading to increased killing of cancer cells by T cells.

Obviously, not only cancer cells are inflicted by systemic chemotherapy but also cells of the tumor stroma and the immune system are influenced and as a consequence any improved reactivity may rely on effects imposed on the stromal cells, immune system, or on cancer cells—and probably all may play a role. In general terms, the influence of chemotherapy may have an impact on normal cells of the host; for example, cells of the immune system as well as an impact on cancer cells. These issues are discussed in some more detail below.

2.1. Chemotherapy in Relation to Normal Cells; Depletion of Suppressor Cells. Previously, there has been tremendous focus on the use of molecules or cells that would work in direction of a more powerful activation of the immune system in any given immunotherapy, however, the recent acknowledgement that counter-active cells are at play during immune responses has led to a focus on suppressor mechanisms. There seem to be at least two cell types involved as key players in cancer, myeloid derived suppressor cells (MDSC) [6], and regulatory T cells (Treg) [32]. MDSC are suppressor cells of the innate immune system capable of inhibiting both innate and adaptive immune responses [33]. Similarly, regulatory T cells of the adaptive immune system are capable of suppressing immune responses [5, 34]. Both cell types may be found in high frequencies in cancer patients—in blood but also at the tumor site, evidence is now emerging that both cell types are clinically relevant and predictive for patient survival [6, 35, 36]. Obviously, these suppressive cells play important roles in controlling and adjusting immune responses in general and are not in any way restricted to cancer patients. Hence, the existence of these populations of suppressive cells underscores the self-limiting nature of the immune system, characterized by dual actions, that is, the capacity to kill and destroy upon antigen recognition as well as the capacity to promote repair after antigen clearance, removal of dead cells and microbes, and the subsequent construction of new vessels and down expression of danger signals. This delicate balance is not only governed by presence or absence of antigen, but by a variety of cellular interactions and soluble factors. Thus, any immune response is composed by active as well as counter-active cells, and the mechanisms that control the immune response from initiation to full completion and repair are poorly understood.

It is now generally accepted that there is a link between inflammation and cancer—in turn leading to the notion that a tumor represents a group of cells selected for the capacity to induce immunological repair instead of clearance. Moreover, the intimate relation between inflammation and cancer underscores that the interplay between cells of the immune system and cancer cells are ongoing from initiation of oncogenesis and thus that suppressive or repair mechanisms are an intrinsic phenomenon of cancer [37]. Although data accumulating over the past few years have revealed much

new insight into the counter-active cells of the immune system, we may just have seen the tip of the iceberg. We recently identified regulatory CD8 T cells present in high frequencies in cancer patients, and capable of suppressing cytokine production, proliferation, and cytotoxic activity of other T cells with a hitherto unheard efficacy [38]. Reaching frequencies of 0.2% among peripheral blood lymphocytes (PBL) these CD8 T cells are specific for a single peptide from the protein heme oxygenase 1 (HO-1). Interestingly, this protein is expressed by tumor cells as well as during wound healing [39] and is a key molecule in local immune suppression [40], thus coupling the action of the novel CD8 T-cell population with the overall function of the protein recognized. The characterization of these antigen-specific suppressive CD8 T cells in cancer patients underscores that there are pros and cons of the immune system in relation to the interactions between cells of the immune system and cancer cells.

Thus, Treg, be it CD4 or CD8 cells seem to play a role for suppression of anticancer immune responses, but other cells in the tumor stroma as well as cancer cells themselves may also possess suppressive functions. Normal cells in the stroma and a fraction of cancer cells may express indoleamine 2,3-dioxygenase (IDO) [41, 42]. IDO may suppress T-cell responses directly by tryptophan deprivation in the microenvironment, and dendritic cells (DCs) may also express IDO leading to induction of Treg [43]. In turn, Treg may in fact induce IDO expression in DC further linking the suppressive network [44]. The drug 1-MT that inhibit IDO activity is in clinical testing [45], and may represent a suitable conditioning or combination partner to immunotherapy. Interestingly, we recently revealed that IDO is a target for specific T cells in cancer patients, and that such specific cells are capable of killing IDO-expressing tumor cells and also DC provided they express IDO [24]. Thus, the induction of a response against IDO could diminish the suppressive effect of IDO and thereby "unleash" a powerful response against the full spectra of tumor antigens expressed by the cancer cells. Strikingly, we have used 1-MT added to peptide stimulation cultures and shown that in some cultures there is a marked increase in INF-γ secretion measured by EliSpot, suggesting that PBMC cultures that comprise high frequencies of IDO-positive cells can be suppressive to T cells—a suppression that can be unleashed by 1-MT [46].

Steps to clear Treg cells prior to vaccination by a single administration of chemotherapy, for example, cyclophosphamide (CTX) have been attempted and have been shown to increase induced immune responses in murine [47] as well as human studies [48, 49]. However, a single clearance does not appreciate the continuous development of Treg [50], and other suppressive cells. Continued administration of low dose CTX was recently shown to be able to selectively clear Treg and would therefore be far more appealing [8]. However, we recently conducted a clinical vaccination trial in melanoma patients using the described low dose CTX without being able to induce Treg clearance or even decrease in Treg frequency (manuscript in preparation). Also taxanes which are widely used in the clinic have been studied for impact on cells of the immune system in particular Treg [51, 52]. Data from such studies suggest that both paclitaxel and docetaxel may impair the frequency of Treg either alone [51] or in combination with carboplatin [53] or CTX [54]. It could be speculated that the impact of chemotherapy on Treg depends on the subtype of the regulatory cell. To this end, it has been shown that induced Treg are rapidly dividing [55], and such cells could be more vulnerable to chemotherapy.

Concerning manipulations with MDSC this has been tested in murine models, where Gemcitabine were shown to selectively eliminate GR1 positive MDSC [56]. Similarly, it has been found that unresponsive tumor infiltrating lymphocytes (TIL) of prostate cancer can gain responsiveness by blockage of arginase 1 (ARG) known to be expressed by MDSC [57]. Cyclooxgenase 2 (COX-2) seems to play a role for induction of MDSC, and administration of COX-2 inhibitors have been shown to decrease MDSC development [58, 59]. We are currently running a clinical trial in melanoma patients in which DC vaccination is combined with concurrent administration of the COX-2 inhibitor Celebra (http://www.clinicaltrial.gov/). Whereas most attempts to manipulate suppressive cells like MDSC focus on lowering the frequency of the cells, a recent report demonstrated blockage of the suppressive function of MDSC by synthetic triterpenoid whereas the frequency of the cells was unchanged [60]. A potential difficulty concerning selective clearing or blockage of function of MDSC using chemotherapy is that these cells seem to possess different phenotypes in different cancers [6], and have different ways of actions [61, 62] which could be coupled to highly different properties of the cells in response to chemotherapeutic drugs.

Whether single individual drugs may synergistically work with, for example, therapeutic vaccination by selective clearing or decreasing the activity of cells with suppressive function remains so far elusive, and certainly we are by no means close to having defined optimal dosage, schedule, and so forth, for such combinations. Moreover, many cellular approaches are based on the use of allogeneic tumor cell lines transfected with vectors encoding immune stimulatory molecules, which obviously leads to a next level of complexity since the beneficial combination with chemotherapy may depend on the added stimulatory molecule. To this end, two recent studies in which allogeneic, GM-CSF-secreting tumor vaccines were administered to cancer patients in combination with CTX (and doxorubicin) suggested higher efficacy in the CTX group [26, 63]. These highly complex combinations will benefit from designs that are geared to identify the most clinically relevant combination of the interacting drugs [26].

2.2. Chemotherapy in Relation to Normal Cells; Conditioning prior to Adoptive Cell Transfer (ACT). As mentioned, it is still questionable whether selective clearing of immune cells with suppressive function is possible using chemotherapy; however, chemotherapy could also be beneficially used to "reboot" the immune system prior to initiation of immune therapy against cancer. For example, the efficacy of ACT using in vitro expanded TIL in melanoma patients seems to depend on prior conditioning using chemotherapy

and/or whole body irradiation therapy [64]. The biological background for the requirement of conditioning remains unknown but is probably related to creating space thereby enabling homeostatic cell division of transferred cells, and clearing of suppressive cells in the patient [9]. Detailed insight into the mechanisms that influence the success of TIL in ACT could potentially open the avenue for development of an ACT regimen that are associated with fewer and less serious side effect.

The induction of clinically relevant responses by ACT using lympho depleting procedures and TIL transfer could also in part be related to the broad spectra of reactivity comprised in these TIL cultures. In accordance, we have studied such TIL cultures from melanoma and head and neck cancer patients for reactivity against a panel of tumor antigens using EliSpot and tetramer analyses, and in general many of these cultures comprise a large number of T-cell specificities (paper in preparation). T-cell survival and expansion within the host depends on the availability of growth-promoting cytokines and regular encounters with cognate antigen. Under ideal conditions small numbers of infused T cells can undergo massive expansion *in vivo* [65]. However, this level of *in viv*o expansion may not occur following the adoptive transfer of tumor antigen-specific T cells owing to the poor immunogenicity of the tumor. Hence, an alternative approach is to combine adoptive transfer of T cells with vaccination to facilitate expansion and maintenance of T cells. The combination of ACT of either *in vitro* expanded specific T cells or gene-modified T cells [66] with vaccination [67] might provide synergism between the two treatment regimens.

2.3. Chemotherapy in Relation to Normal Cells; Activation of Cells of the Immune System. Chemotherapy may also influence cells of the immune system to more potent activity. To this end, it has been shown that gemcitabine treatment lead to increased efficacy of immune therapy in the absence of any direct effect on cancer cells [68]. As mentioned above CTX may have a role in the clearing of Treg and is used together with fludarabin as conditioning prior to ACT. Using CTX alone, it was recently shown in a murine model that the myelosuppressive action may lead to subsequent rebound DC generation with increased capacity to secrete IL-12 [69]. With a more direct action on DC, vinblastine has been shown to induce DC maturation [70], and functionally these DCs were superior to untreated DC in inducing CD8 T-cell responses [71]. Using non-cytotoxic concentrations, it was recently shown that several clinically relevant drugs (paclitaxel, doxorubicin, mitomycin C, and methotrexate) increase antigen presentation in an autocrine IL-12-dependent manner [72]. Thus, these data suggest that provided careful examination of the dose and schedule, it might be possible to harness the dual actions of chemotherapy to kill some cells and activate others.

2.4. Chemotherapy; Impact on Cancer Cells. Chemotherapy may also induce an antitumor immune response by direct influence on the tumor cells. To this end, a panel of chemotherapeutic agents was screened for inducing immunological cell death, each drug was studied functionally for the ability of chemotherapy-killed tumor cells to induce protective immunity upon immunization [73]. The data demonstrated that anthracyclin-treated tumor cells are particularly effective in eliciting an anticancer immune response, and further that the mechanism of the immunogenicity of antracyclin-induced cell death was the rapid preapoptotic translocation of calreticulin to the cell surface [73]. This surface exposure of calreticulin endows cancer cells with an "eat me" signal to dendritic cells, in turn leading to immunogenic uptake of tumor antigens and activation of tumor-specific T-cell responses [73]. Thus, to successfully combine chemotherapy with immune therapy careful selection of the chemotherapeutic agent is required since not all agents induce immunological death [74].

Another key denominator of immunogenic cell death is represented by high-mobility group box 1 (HMGB-1). Release of HMGB-1 from dying tumor cells leads to activation of toll-like receptors (TLRs) (2 and 4) and subsequent immune activation [75]. In this respect, HMGB-1 localization in the cytosol is associated with autophagy and cellular escape from apoptosis, in turn conferring resistance to several therapies including immunotherapy [74]. Also heat shock proteins (HSPs) which are upregulated upon specific stress may act as danger signals and be expressed on the cell surface as "eat me" signals to DC [76]. Interestingly, it has been shown that treatment of myeloma cells with the proteasome inhibitor bortezomib leads to surface expression of HSP90 on the cell surface [77]. A vaccination trial in which bortezomib is combined with peptide vaccination targeting the regulators of apoptosis proteins (RAPs) Bcl-2, Mcl-1, and Bcl-Xl [21] was recently initiated at our institution (www.clinicaltrial.gov).

Beyond inducing immunogenic tumor cell death, chemotherapy may render cancer cells more susceptible to killing by CTL. Thus, 5-fluorouracil (5-FU), CPT-11, or cisplatin (CDDP) were all shown to increase the sensibility of the SW480 colon cancer cell line to killing by T cells [78], and similar data have been found for renal cell carcinoma cells treated with adriamycin [79]. In both cases the enhanced lytic sensitivity was at least in part due to upregulation of costimulatory molecules on cancer cells, for example, LFA-3 and ICAM-1. More recently, Ramakrishnan and colleagues showed in a murine model that paclitaxel, cisplatin, and doxorubicin all sensitize tumor cells to more efficient killing by CTL [80]. In this system, however, the responsible mechanism on murine as well and human cancer cells went via upregulation of the mannose-6-phosphate (M6P) receptor on cancer cells which is required for granzyme B associated killing [81]. Importantly, CTL established upon administration of chemotherapy was capable of off-target killing of neighboring cells at the tumor site even in the absence of antigen expression.

The concept of using chemotherapy as an immune adjuvant is rapidly expanding. Obviously, future testing in clinical trials will benefit from basic research into the mechanism(s) of action; how do the cells die and which pathways are activated for immune activation [82, 83]. This

will set the stage for initiation of hypothesis driven clinical trials combining chemotherapy and immune therapy and form the basis for more rational biological monitoring.

3. Chemotherapy Resistance and Immune Targeting

Chemotherapeutic agents can induce a series of cellular responses that impact on tumor cell proliferation and survival. Perhaps the best studied of these cellular responses is apoptosis, a physiological cell death program that controls normal cell numbers during development and disease. A large number of various drugs in clinical use kill tumor cells by activation of common apoptotic pathways. Hence, most cytotoxic anticancer drugs, for example, microtubule binding drugs, DNA-damaging agents, and nucleosides induce apoptosis of malignant cells.

Many drugs are capable of inducing clinical response in the patients with metastatic cancer, however, in most cases the therapy is not curative due to selection of drug-resistant cancer cells. A frustrating property of such acquired resistance of cancer cells is that chemotherapy resistance may lead to cross-resistance to other drugs with different mechanisms of action [84]. Drug resistance is a major limiting factor for the effectiveness of chemotherapy in the treatment of disseminated cancer [85]. Cancer-associated defects in apoptosis play a vital role in resistance to chemotherapy and radiotherapy [86]. An important reason for this impaired apoptosis is overexpression of RAP [87], that is, the T-cell antigens survivin and proteins of the Bcl-2 family. Other mechanisms of drug resistance are exemplified by the tumor antigen CYP1B1 which may inactivate cytotoxic or cytostatic drugs thereby influencing the clinical outcome of therapy [88], and ATP transporters which act by transporting drugs out of the cell [89].

The mechanisms of drug resistance mentioned above are associated with expression of proteins that have been shown to be targets for T cell responses [17–21, 90–95]. Consequently, the combination of immunotherapy targeting these antigens with conventional chemotherapy appears to be particularly appealing. In such a setting, conventional therapy would kill the majority of the cancer cells, leaving only cells that express high levels of target antigens. Such high-expressers would be particularly vulnerable to killing by vaccination-induced T cells. Thus, the synergy of these measures could potentially give a more effective treatment than the added effect of either regimen alone, thereby strengthening the already described synergistic effect of anticancer vaccines and chemotherapy. Furthermore, it should be noted that chemotherapy only has an effect on dividing cells, whereas, for example, surviving-specific T cells in addition are able to kill resting tumor cells.

4. Radiation Therapy in Combination with Vaccination

Irradiation leads to immunogenic death [73]. Moreover, also sublethal irradiation may render cancer cells more vulnerable to killing by T cells, implying that even surviving irradiated cancer cells may still facilitate more efficient responses. Total body irradiation (TBI) is already being used by Dudley and colleagues as part of a conditioning regimen prior to ACT [9], indicating additive with more intensified TBI. Interestingly, a well-known phenomenon related to local radiation therapy is a bystander effect extending to distant untreated metastatic sites—an effect potentially mediated by the immune system [96]. Probably radiation could be pursued more conceptually in combination with vaccination as well.

5. Specific or Concurrent Targeting of Stroma Cells

One of the inherent properties of cancer cells is genetic instability which in turn allows cancer cells to "escape" during therapy. Obviously, the "active-imposing" term "escape" is in fact a selection process in which cells carrying an advantageous genetic, epi-genetic, and miRNA signature are given a survival and/or growth advantage. As already mentioned, this is a crucial problem concerning chemotherapy, but also for the success of immune therapy this poses a problem. In this regard, antigen loss [97] or HLA loss [98] has been described during immune therapy, and the background for this lies in the heterogeneity of cancer cells in turn enabling the presence of cells with escape properties. Conversely, stroma cells are genetically stable and possess limited proliferative capacity compared to cancer cells, implying that the risk of HLA-loss, antigen-loss, or antigen processing-loss is exceedingly low. Some antigens are expressed not only by tumor cells but also by cells in tumor stroma. This applies to survivin, and several other RAP.

Angiogenesis represents an important step in tumor development [99], and since antiangiogenic therapy targets the tumor vasculature and prevents tumor growth beyond micro-metastases, combination of antiangiogenic therapy and tumor-specific immunotherapy could lead to a synergistic effect. Active immunotherapy targeting endothelial products like vascular endothelial growth factor receptor (VEGFR)-2 protein can delay tumor progression [22], and it has been shown that vaccination with peptides derived from VEGFR-1 inhibits tumor growth in mice. This was associated with suppression of tumor angiogenesis in the absence of adverse effects [100]. In addition, similar to survivin, Bcl-2 and Mcl-1 are highly expressed in endothelial cells during tumor angiogenesis [101]. Thus, the targeting of survivin, Bcl-2, or Mcl-1 in a vaccination setting would beyond tumor cells also target endothelial cells and thus tumor-angiogenesis. Indeed this was shown in a mouse model; vaccination-induced survivin-specific T cells mediated the eradication of lung tumor metastases and the concurrent suppression of angiogenesis at the tumor site [102]. The efficacy of treatment did not inflict on wound healing or fertility of the mice.

A primary concern of immunizing against angiogenesis-associated proteins is a potential risk of interference with normal angiogenesis, especially if the effect is sustained. So far no vaccination associated toxicity was observed when late

stage melanoma patients were vaccinated with survivin in a compassionate use setting despite the fact that strong CTL responses were introduced in all patients [103]. Nevertheless, it is clear that even extensive phase I/II trials are not geared for analyses of potential side effects presenting several years after termination of the trial, and these issues demand further attention.

Certainly several targets are available for targeting of tumor stroma in particular for concurrent targeting of tumor cells and stroma cells. As already mentioned, IDO are expressed by fractions of cancer cells in vivo [42], but DCs, MDSC, and other immune cells in the tumor stroma may also be IDO positive, and characterized by the same targeting advantages as, for example, endothelial cells. To this end, it has been shown that regulatory T cells can be targeted by immune responses to peptides derived from Foxp3 [25].

6. Multiepitope Strategies and Additional Immunotherapy

Many current immunological strategies to combat cancer are already focusing on combining different cells and molecules to increase responses, for example, vaccination combined with the addition of cytokines or other immune modulating agents. However, so far most peptide-based vaccination trials have targeted only a single antigen and with the aim to increase efficacy an exciting strategy would be to cotarget biologically connected proteins, for example, RAP, in a multiepitope setting [104]. A number of different RAP have been described as T-cell antigens in a variety of cancers [21, 90, 105, 106]. Since coexpression of Bcl-2 family proteins is a frequent event [87], simultaneous targeting of Bcl-2 proteins may be more efficient than targeting one molecule alone.

Importantly, drug resistance is casually linked to over expression of RAP, for example, survivin and Bcl-2 [107, 108]. For combination therapies, vaccination against these molecules seems to be ideally suited for targeting of chemoresistant cancer cells upon conventional therapy, and thereby possibly prevents relapse of disease [109]. Underscoring this notion, the coexpression of survivin and Bcl-2 is associated with poor prognosis in breast cancer [110], implying that expression of more than one RAP is functionally significant at least in some indications. To this end, targeting both the Bcl-2 family proteins and survivin would be particularly attractive since they act in different apoptosis pathways, and the targeting of both to kill resistant cells upon chemotherapy would supposedly increase the chances of success. Combined with adjuvant chemotherapy after primary surgery, adjuvant vaccination in high risk cancer patients would be suited for a vaccination that specifically targets not only apoptosis pathways but also proteins associated with metastatic behavior, for example, RhoC [16, 111] or heparanase [15].

7. Conclusions

The immune system has a unique capacity to specifically recognize and kill cancer cells while leaving normal cells unharmed. As a consequence, to harness the immune system in therapeutic vaccinations against cancer is a very promising approach for the therapy of disseminated cancer. So far, however, the clinical impact of vaccination has been limited. Over the past few years, New insight has been achieved concerning the main suppressive mechanisms that hamper induction of more powerful immune responses, and also revealed new knowledge as to how suppressive cells and molecules could potentially be cleared or inhibited, for example, in combination with chemotherapy. Similarly, the immunogenicity of cancer cells has been shown to depend on the death process induced by the specific drug, thus some drugs are more prone to act synergistically with vaccination than others. Added to the above, cancer cells treated with chemotherapy may be rendered more vulnerable to killing by T cells, only adding to the potential of improving the efficacy of vaccination when combined with chemotherapy. Moreover, antigens may be selected and combined to target various traits of cancer cells and/or target stroma cells in the suppressive tumor environment. The available experimental data should form the basis for initiation of carefully planned hypothesis driven clinical trials that coupled with stringent and robust biological and clinical monitoring will be able to firmly demonstrate the most effective combinations.

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Review Article

TAA Polyepitope DNA-Based Vaccines: A Potential Tool for Cancer Therapy

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DNA-based cancer vaccines represent an attractive strategy for inducing immunity to tumor associated antigens (TAAs) in cancer patients. The demonstration that the delivery of a recombinant plasmid encoding epitopes can lead to epitope production, processing, and presentation to CD8+ T-lymphocytes, and the advantage of using a single DNA construct encoding multiple epitopes of one or more TAAs to elicit a broad spectrum of cytotoxic T-lymphocytes has encouraged the development of a variety of strategies aimed at increasing immunogenicity of TAA polyepitope DNA-based vaccines. The polyepitope DNA-based cancer vaccine approach can (a) circumvent the variability of peptide presentation by tumor cells, (b) allow the introduction in the plasmid construct of multiple immunogenic epitopes including heteroclitic epitope versions, and (c) permit to enroll patients with different major histocompatibility complex (MHC) haplotypes. This review will discuss the rationale for using the TAA polyepitope DNA-based vaccination strategy and recent results corroborating the usefulness of DNA encoding polyepitope vaccines as a potential tool for cancer therapy.

1. Introduction

The discovery that the abnormal expression of a given self tumor-associated antigen (TAA), resulting from overexpression, conformational changes, or posttranslational modifications, can overcome self-tolerance and induce immune responses to cancer cells has grown the idea that the immune system can fight against cancer [1–5]. The identification and molecular characterization of TAAs provided the basis for the development of cancer vaccines targeting TAAs [6]. Full-length recombinant forms of TAAs have been administered to animal models and then to cancer patients, with the aim of inducing induce a systemic immune response to the TAA that may result in the killing of cancer cells in the recipient [7].

The use of epitope-based cancer vaccines as the most specific method to trigger T cells against cancer cells has been first investigated by employing peptide-based vaccination. This approach was further supported by the knowledge that: (a) within a given antigen, a T cell recognizes only a single epitope (peptide) bound to major histocompatibility complex (MHC) proteins; (b) MHC class I and class II molecules bind to short peptides of 8–10 and 13–20 amino acids, respectively; (c) CD4+ and CD8+ T cells are activated by MHC class I- and class II-presented peptides, respectively; (d) TAA peptides are exposed on cancer cells [8–10]. Peptide-based cancer vaccines display unique features: they are relatively easy to prepare for clinical use, have minimal toxicity, and can include multiple peptides in order to bypass tumor heterogeneity and immune escape. Further, the induced T cell-response can be easily analyzed. On the other hand, the use of peptide-based cancer vaccines might display several drawbacks: (a) T-cell epitope restriction to a particular MHC haplotype, (b) inadequate activation of the innate immune system, (c) the need of adjuvants to trigger immune responses to subdominant or weakly immunogenic peptides, and (d) possible immunoselection of epitope-loss variants after single peptide vaccinations [8–10].

2. The Choice of the TAA Epitope/s: A Lesson from Peptide-Based Clinical Trials in Cancer Patients

2.1. Promise and Limits of Peptide-Based Single Epitope Vaccination. Most of the knowledge on the employment of TAA single epitope-based cancer vaccines was obtained from single peptide-based vaccination trials in cancer patients. Recent studies are reported below. These studies revealed fundamental concepts on the in vivo immunogenicity of TAA peptides in cancer patients.

2.1.1. Single Epitope Peptide-Vaccination. Many single epitope peptide-based cancer vaccines have been developed in preclinical as well as in clinical settings [8–16]. Peptides from melanoma antigens were the first to be employed as immunogens in phase I and II clinical trials for the treatment of melanoma patients [11]. It should be pointed out that the first clinical trials were conducted in melanoma patients since melanoma is the human (nonvirally induced) most immunogenic cancer. Then, similar trials have been conducted for less immunogenic cancers. Rosenberg et al., by investigating the reactivity of PBMCs from 8 of the 9 melanoma patients immunized with the native g209–217 form of the gp100 melanoma-associated antigen, showed that only 2 of 8 patients had reproducible evidence of immunization to the native g209–217 peptide and only one patient experienced an objective cancer regression that lasted 4 months [11]. It was also demonstrated that the in vivo administration of the MART-1(27–35) peptide from the melanoma-associated antigen could safely augment Cytotoxic T Lymphocytes (CTL) reactivity against epitopes commonly expressed by melanoma cells in melanoma patients. However, despite the enhancement of CTL reactivity, tumor regression was not achieved [13]. Conversely, it was found that among the 25 patients who received 3 subcutaneous injections of the MAGE-3.A1 peptide, 7 displayed significant tumor regression [14]. Intradermal vaccination of patients with metastatic NY-ESO-1-expressing cancers with 3 HLA-A2-binding NY-ESO-1 peptides was shown to elicit immune responses as well [15]. Remarkably, 10 of 22 patients with high-risk, resected, stages IIB, III, and IV melanoma, immunized with an immunodominant 9-amino acid peptide derived from the MART-1 tumor antigen (AAGIGILTV), developed an immune response that was associated with a prolonged time to relapse [16].

Based on these clinical trials, peptides derived from other TAAs were employed to immunize patients affected by different types of cancer [17–22]. For instance, early-staged breast cancer patients provided evidence for the induction of intra- and interantigenic epitope spreading after minimal essential HER-2/neu epitope (E75) vaccination [17]. Disis et al. showed that the majority of patients with HER-2/neu-overexpressing breast, ovarian, or nonsmall cell lung cancers developed immunity to both HER-2/neu peptides and protein when immunized with subdominant peptide epitopes derived from HER-2/neu [18]. Patients with advanced cancer showing an immune response to the mutant ras 17-mer peptides used as vaccines had prolonged survival from the start of treatment compared to nonresponders [19]. Yamamoto's group demonstrated that after vaccination with a 100-mer MUC1 peptide consisting of the extracellular tandem repeat domain, 7 out of 8 patients with advanced pancreatic and bile duct cancers had progressive disease while 1 out of 8 had stable disease with a tendency for increased circulating anti-MUC1 IgG antibodies [20]. Vaccination of pancreatic cancer patients with a 100-amino acid peptide corresponding to five 20-amino acid long repeats of the same antigen and SB-AS2 adjuvant resulted in an increased percentage of CD8+ T cells in the peripheral blood. In addition, 2 of 15 resected pancreatic cancer patients were alive and disease-free at follow-up of 32 and 61 months [21]. Furthermore, phase I/II studies demonstrated that the combined administration of irinotecan/high-dose 5-FU/leucovorin with CAP-1, that is, the immunodominant MHC class I HLA-A2-restricted nonamer epitope of the carcinoembryonic antigen (CEA), increases CAP-1-specific T cells in 47% of colorectal cancer patients after vaccination [22].

Accordingly, in spite of the solid preclinical rationale, single peptide-based cancer vaccines have generated comparatively poor objective clinical responses in cancer patients. Still, the development of novel engineered peptides and the knowledge of the molecular mechanisms regulating immunity to cancer cells have offered new motivations for the use of single epitope peptide-based vaccination in cancer patients.

2.1.2. Improvement of Single Epitope Peptide Immunogenicity. Although the discovery of immunodominant epitopes was essential for vaccine development, the knowledge of unconventional epitopes involved in antigen immune recognition could provide useful indications to increase immunity to TAAs, thus leading to the development of novel cancer vaccine strategies. A starting point in this respect was the demonstration that although self TAAs can elicit immune responses in cancer patients, the induced T cells response is weak and ineffective [23–26]. One explanation for this event is that thymic negative selection leads to the deletion of T cell progenitors which are able to recognize with high avidity self-peptides bound to MHC molecules displayed on the surface of antigen presenting cells (APCs). Yet, an individual tolerant to immunodominant self-epitopes can recognize unconventional self-epitopes generated from nontraditional sources of peptides/proteins and/or mechanisms of translation, including cryptic epitopes and atypical epitopes originating from incompletely spliced messages, mutation of a normally noncoding intronic sequence, exon extension, ribosomal frameshifting, initiation codon scanthrough, initiation from non-AUG codons, and doublet decoding [23–26].

Different approaches were investigated in order to develop novel peptide constructs able to increase peptide immunogenicity or to increase the TCR repertoire available for immune recognition of tumors. To improve epitope immunogenicity, two general strategies have been employed. The first aims at increasing peptide affinity to MHC, essentially class I molecules. Indeed, an enhanced affinity results in a longer complex half-life on cell surfaces increasing

the chance of CTL stimulation. The second strategy aims at improving the processing/production of the epitopes. Recent examples of epitope modifications are described below. One methodology was based on the identification of heteroclitic peptides which represent altered peptide ligands (APLs) with enhanced functional activity relative to the parental peptide. Most studies of APLs of tumor antigens employed amino acid exchanges at anchor residue positions of the peptide to enhance its binding to class I MHC molecules [10]. Notably, the lateral chain of the major anchor residues being buried, the introduction of the most favorable residues at these positions should not alter epitope antigenicity. Other studies of APLs of tumor antigens employed the introduction of aromatic amino acids at P1, P4, and P5 position, modification of individual amino acid residues, modification of cysteine residues, or modification of TCR-interacting amino acid residues [10]. Other modifications have been empirically discovered with the same affinity effect. These modifications would not apply to all allelic forms of MHC class I molecules. For those modifications that have been found to increase immunogenicity of epitopes without increasing affinity, increased interaction with TCR remains an hypothesis.

Among different modifications, Zaremba et al. introduced a single amino acid substitutions to the CAP-1 peptide (YLSGANLNL). Whereas CAP-1 failed to generate CTLs from normal PBMCs, the agonist peptide was able to generate CD8+ CTL lines that recognized both the agonist and the native CAP-1 sequences [27]. In addition, the modification of position 6 of the CAP-1 peptide from asparagine to aspartic acid dramatically shifted the dose of peptide resulting in maximal production of inflammatory cytokines [28]. Loftus et al. identified several partial agonists or antagonists of MART1(27–35)-reactive CTL clones derived from tumor infiltrating lymphocytes (TILs) [29]. It was also demonstrated that a singly substituted peptide derived from the epitope MART1(27–35), containing a leucine in position 1, acted as a superagonist by inducing specific T cells with enhanced immunological functions in vitro [30]. The introduction of a single leucine to alanine substitution at position 2 of the Melan-A26–35 decapeptide sequence into a recombinant vaccinia virus encoding the Melan-A26–35 minigene resulted in a strongly increased immunogenicity in vitro and in vivo [31]. Two more immunogenic variants of the wt p53(264–272) peptide were identified by amino acid exchanges at positions 6 and 7 of the peptide. These variants were capable of inducing T cells that recognized the parental peptide from PBMCs of nonresponsive donors [32]. The HER-2.369 V2V9 variant, generated by introducing in the wild type peptide a valine at position 2 and a valine at the C terminus, was shown to be a more potent immunogen than the wild-type epitope, and T cell responses activated by this analogue were able to recognize the naturally processed epitope on tumor target cells. The increased immunogenicity was associated with only a marginal increase in HLA class I binding of the variant epitope [33].

A novel approach is based on the knowledge that self-tolerance to immunodominant TAA epitopes might be circumvented through the recruitment of high avidity T cell repertoires specific for subdominant or cryptic TAA epitopes. On this regard, many groups have aimed at enhancing immunogenicity of nonimmunogenic low-affinity HLA-A2.1-binding peptides, by introducing a tyrosine in the first position of the amino acid sequence (P1Y). Such strategy was described for the first time by Tourdot et al. The P1Y substitution was able to increase peptide affinity of HLA-A2.1-binding peptides without altering their antigenic specificity [34]. On this line, Scardino et al. used the heteroclitic peptide approach to identify six low affinity, cryptic HER-2/neu, and hTERT epitopes presented by HLA-A*0201. These epitopes, in their P1Y heteroclitic form, stimulated CTLs that specifically lysed HER-2/neu- or hTERT-expressing tumor cells of various histological origins [35].

MHC class II-restricted immune responses can be increased by targeting the peptide through the invariant (Ii) chain-MHC class II biosynthesis pathway. The Ii chain protein is proteolyzed leaving a peptide called CLIP inside the peptide-binding groove of MHC class II [36]. CLIP appears to have superagonistic properties interacting with the T cell receptor and the MHC class II molecule at or near the binding site for the bacterial superantigen Staphylococcal enterotoxin B, and thus it can affect MHC class II stabilization, peptide antigen exchange, and antigen presentation [37]. Hess et al. investigated whether the N-terminal flanking region of the Ii peptide could augment the immunogenicity of cryptic "self" TAAs. Indeed, by introducing the flanking region of the Ii peptide, one expects a very efficient cut by the cathepsins since the amino acids of these regions are well conserved across species, insuring a very efficient final fragmentation of the Ii before MHC class II peptide loading. The authors demonstrated that immunogenicity of an MHC class II-binding peptide (p1171–1185) from the rat (c-neu) HER-2/neu oncogene was augmented by the addition of the N-terminal segment of CLIP [37]. Similarly, Voutsas et al. demonstrated that Ii-Key/HER-2/neu(776–790) hybrid peptides primed higher frequencies of CD4+ T cells compared to the native peptide following active vaccination in DR4-IE Tg mice. The hybrid peptide-induced CD4+ T cells in turn stimulated higher HER-2/neu peptide-specific CTL responses and resulted in tumor regression in SCID mice xenografted with an HER-2/neu+ tumor cell line [38]. The Ii-Key/HER-2/neu(776–790) hybrid peptides were also shown to induce more effective immunological responses over the native peptide in lymphocyte cultures from patients with HER-2/neu+ tumors [39]. In addition, an Ii-Key hybrid of HER-2/neu peptide 776 to 790, when injected in node-negative breast cancer patients expressing varying levels of HER-2/neu, induced a potent immune response in the absence of an immunoadjuvant [40]. Indeed, the Ii-Key/MHC class II epitope hybrid acts on MHC class II molecules to facilitate replacement of antigenic peptides with the epitope tethered to the Ii-Key motif. In this way, it greatly increases Th1 or Th2 responses to MHC class II epitopes [41].

A novel approach to enhance immunity against a self-HER-2/neu CTL epitope by vaccinating with xenogeneic, altered peptide ligands was proposed by Gritzapis et al. [42]. Two HER-2/neu-derived E75 APLs that demonstrated

increased affinities for the HLA-A*0201 allele compared with wild-type E75 peptide were also developed [43]. Kobayashi described a strategy to identify helper T-cell epitopes for HER-2/neu that were able to trigger human T cell responses in individuals expressing diverse HLA-DR alleles (promiscuous epitopes) [44].

2.1.3. Polyepitope Delivery by Multipeptides-Based Vaccination. The rationale for the first use of TAA polyepitope cancer vaccines was based upon the evidence that: (a) as compared to a single epitope-based cancer vaccine, the injection of multiple epitopes can overcome the potential loss of expression of a given TAA-single epitope in cancer cells; (b) the ability to target more than one TAA can counteract the heterogeneous expression of TAAs by different cancer cells within a tumor; (c) the vaccination with multiple epitopes would allow to enroll patients with different HLAs [9, 10]. Afterwards, multiepitopes-based cancer vaccines were considered not only for their potential to circumvent the variability of immunodominant peptides presentation by tumor cells, but also because, by using engineered peptides, they can induce the recruitment of diverse high avidity TCR repertoires specific for self TAA peptides in vivo. Results from recent clinical trials of polyepitope peptide-based vaccines are reported below. A phase I/II trial was conducted to assess the feasibility and tolerability of vaccination with a cocktail of 11 synthetic peptides derived from several TAAs, including prostate specific and membrane antigens, in 19 HLA-A2 positive patients with hormone-sensitive prostate carcinoma. The result of this trial demonstrated that the multipeptide vaccine stabilized or slowed down prostate specific antigen (PSA) progress in 4 of 19 cases, with long-term stability delaying androgen deprivation up to 31 months [45]. Kirkwood et al. reported the results of a randomized, multi-institutional trial of multi-epitope peptide vaccination for patients who failed prior therapy for metastatic unresectable melanoma. Three HLA class I-restricted lineage antigen epitopes were administered alone (arm A), or combined with granulocyte-monocyte colony-stimulating factor (GM-CSF) (arm B), or with IFN-α2b (arm C), or with both IFN-α2b and GM-CSF (arm D). The authors showed that immune responses to at least one melanoma antigen were observed in 35% of patients and that 6 out of 115 patients had objective clinical responses. Neither IFN-α2b nor GM-CSF significantly improved immune responses [46]. Thirty-three of 38 patients with high-risk, resected, stage III or IV melanoma showed an immune response after vaccination with two tumor antigen epitope peptides derived from gp100 (209–217) and tyrosinase (368–376) emulsified with incomplete Freund's adjuvant administered alone or in combination with IL-12 [47]. In the clinical trial performed by Slingluff et al., 120 eligible patients with resected, stage IIB to IV melanoma were vaccinated with 12 MHC class I-restricted melanoma peptides to stimulate CD8+ T cells, plus an HLA-DR-restricted tetanus helper peptide to stimulate CD4+ T cells, emulsified in incomplete Freund's adjuvant, with or without GM-CSF. The authors reported that high immune response rates were achieved using this multipeptides vaccine, but that CD8+ and CD4+ T-cell responses were lower when the vaccine was administered with GM-CSF [48]. On the other hand, it was also demonstrated that by mixing the highly immunogenic gp100 : 209–217 peptide and the less immunogenic tyrosinase: 368–376 peptide in the same emulsion, the elicited immune response was altered as compared with that obtained with the same peptides injected separately [49]. Five class I MHC-restricted synthetic peptides derived from multiple ovarian cancer-associated proteins plus a class II MHC-restricted synthetic helper peptide derived from tetanus toxoid protein were used to immunize 9 patients with epithelial ovarian, fallopian tube, or primary peritoneal carcinoma, who were HLA-A1-, HLA-A2-, or HLA-A3-positive. In this clinical trial, CD8+ T-cell responses were detected in 1 participant ex vivo and in 8 of 9 participants (89%) after in vitro stimulation [50]. In patients with stage IIIB or IV nonsmall-cell lung cancer the clinical efficacy, safety, and immunogenicity of a multiepitopes vaccine consisting of 9 HLA-A2 supertype-binding epitopes (2 native and 7 analog epitopes modified for optimal HLA binding or T-cell receptor stimulation) covering five TAAs including CEA, HER-2, MAGE3, and the universal helper pan-DR epitope, formulated as a stable emulsion with incomplete Freund's adjuvant was evaluated. One complete and one partial response and longer survival in patients demonstrating an immune response to epitope peptides were observed [51].

3. Polyepitope DNA-Based Cancer Vaccines: Parameters Affecting the Immune Response

Multiepitopes-based vaccines may represent promising tools for inducing antitumor immune responses in cancer patients. The presence of immunodominant and unconventional epitopes simultaneously delivered by a polyepitope DNA-based vaccine can induce a multiple response by generating different self-reactive T cell repertoires. The demonstration that the in vivo injection of RNA and DNA mammalian expression vectors containing foreign genes into mouse skeletal muscle was able to induce foreign protein expression in muscle cells opened up the discovery of a novel type of immunization [52]. Indeed, shortly after, Ulmer et al. demonstrated that a plasmid DNA encoding influenza A nucleoprotein injected into the quadriceps of BALB/c mice resulted in the generation of nucleoprotein-specific CTLs and protection from a subsequent challenge with a heterologous strain of influenza A virus [53]. After this study, a consistent number of reports demonstrated the ability of DNA vaccines encoding tumor antigens to induce protective immunity against cancer cell growth [52]. DNA cancer vaccines are a type of genetic vaccines which depend on delivery of plasmid DNA molecules encoding a whole protein or epitope of a given TAA [54]. The usefulness of DNA-based cancer vaccines has been demonstrated most effectively by targeting TAAs in mouse experimental models [55–57]. In general, plasmid DNA encoding TAA epitope/s, due to its content of unmethylated CG sequences, furnishes a potent danger signal, which represents a natural adjuvant stimulating the innate immunity. In addition, the expression

of a given DNA-encoded TAA epitope will be delivered at the site where resident APCs can be activated resulting in inflammation and enhanced antigen processation and presentation. The in vivo synthesis permits appropriate folding and posttranslational modification of the encoded protein, its prolonged expression, and its presentation with MHC class I. Furthermore, DNA vaccines allow the delivery of multiple epitopes and yet more than one full-length antigen in conjunction with immunostimulatory molecules. A supplementary feature supporting the development of plasmid DNA-based vaccines takes account of the easy preparation of the vaccine [55–57].

Intramuscular or skin injection is usually employed to delivery DNA vaccines. Injected myocytes or keratinocytes express the delivered antigen cDNA and serve as a target for immune effector cells. They can also increase the expression of MHC class I and costimulatory molecules [58]. However, it is believed that the obtained immune response upon recombinant plasmid DNA vaccination is due to indirect antigen transfer to APCs (cross-presentation) [58] (Figure 1). Skin APCs include immature Langherans cells in the epidermis and mature dendritic cells in the dermis. Although the cross-presentation process appears to support CD4+ T-cell responses, recent evidences demonstrated that endoplasmic reticulum-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells [59].

One of the advantages of using DNA fusion gene vaccines is the possibility to incorporate in the same DNA construct genes which encode for potentially adjuvant proteins which are able to increase the innate immune response against the given TAA. Accordingly, recombinant DNA plasmids were generated by fusing genes encoding cytokines, chemokines, Fc receptors, complement components, or antibodies targeting APCs, with the aim of increasing antigen uptake and presentation [60].

Yet, one crucial feature in the construction of the DNA vaccine is the ability to improve TAA presentation and processing. It is known that those antigens which are targeted to the endoplasmic reticulum will be able to induce the production of antibodies, whereas the antigens targeted to the cytosol will be delivered for proteasomal degradation for induction of peptide-specific CD8+ T cells [58, 60]. Accordingly, a supplementary gene encoding ubiquitin can be incorporated in the construct for induction of CTLs [59]. In a similar way, CD4+ T cell responses can be enhanced by targeting TAA expression to the endosomal or lysosomal compartment [61].

Different parameters can affect the immunological properties of a polyepitope vaccine: (a) the presence of a T helper (Th) epitope, (b) the addition of spacers between the epitopes for optimal cleavage of the epitopes by the proteasome, (c) the design of new junctional epitopes, and finally (d) the affinity of the selected epitopes for MHC molecules and transporters (TAPs). However, all the results available from the literature do not allow to establish a consensus on the precise role of each one of these parameters. If exceptions have been reported regarding the advantage conferred by the addition of T helper epitopes or alanine flanking sequences, at least these two actions have not been reported to have adverse effects.

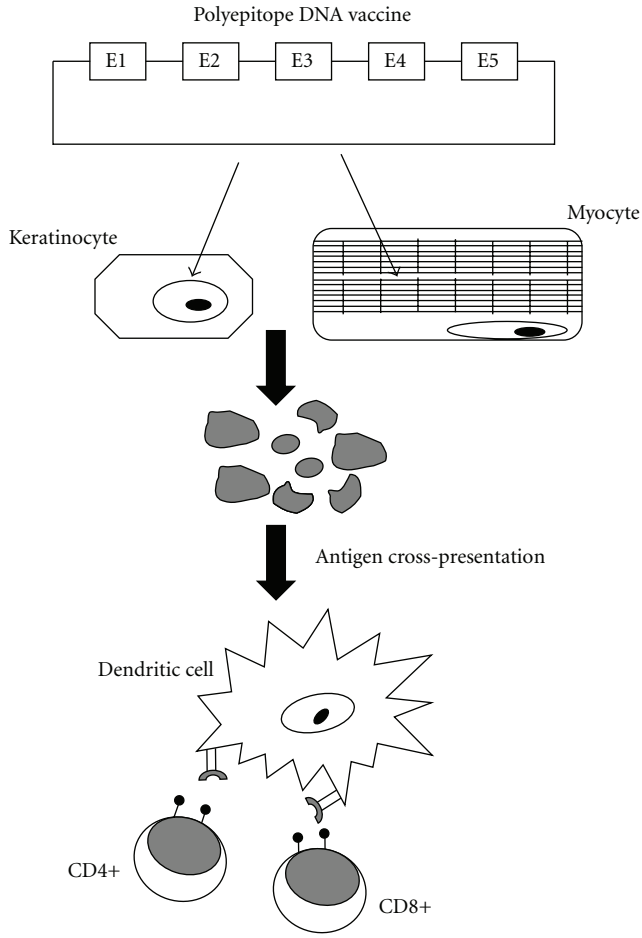

Figure 1: *Polyepitope DNA-based cancer vaccines and cross-antigen presentation.* After injection of the recombinant polyepitope DNA vaccine into myocyte or keratinocytes, antigenic materials is transferred to dendritic cells (antigen cross-presentation).

Mateo et al. suggested that the immunogenicity of peptides included in a melanoma polyepitope recombinant vaccine was conditioned by their affinity for the MHC [62]. Conversely, Ishioka et al., by employing an HIV polyepitope DNA vaccine, showed that the affinity of the epitopes for MHC does not interfere with their capacity to induce a CTL response [63]. On the other hand, Palmowski et al. demonstrated that the differential MHC affinity of peptides included within a polyepitope DNA vaccine was crucial for skewing of immune response and CTL immunodominance [64]. Concerning the need for a Th epitope, Ishioka et al. have also demonstrated the utility of including the pan-DR universal Th cell epitope (PADRE) for improving vaccination efficacy [63]. Conversely, Velders et al. did not observe differences in inducing antitumor immunity between wild type and CD4−/− mice vaccinated with a polyepitope DNA vaccine containing the tetanus-toxoid Th epitope [65]. A consensus, however, appears to be recognized for the function of polyepitope organization (epitope rearrangement,

addition of spacers): it should allow an appropriate cleavage of all epitopes and should prevent the creation of new junctional epitopes with high affinity for MHC molecules [63, 64]. Therefore, the design of a DNA polyepitope vaccine requires that the arrangement of the epitopes allows the processing of each peptide at its C-terminal position and does not create new junctional peptides with high affinity for MHC. Further, such design should include the selection of native epitopes or of heteroclytic variants of the epitopes with high MHC affinity, which should also display high immunogenicity in vivo and in vitro in preclinical settings. The definition of the C-terminal processing position can be evaluated by using predictive models of proteasome cleavage [66].

Initial concerns on the use of polyepitope DNA-based vaccines arose on the possibility that the processing of multiple epitopes could fail to incorporate them in their natural context of flanking amino acids residues. It has been demonstrated that the multicatalytic proteasome complex and its low molecular weight protein subunits are responsible for the production of peptides with hydrophobic or basic C-terminal residues which are preferentially associated with MHC I molecules [67]. To fit in the grove of most MHC class I molecules, peptides should possess a length of 8–10 residues [68]. However, how the final peptide length is attained is not well understood due to conflicting evidence on the production and transport of antigen-derived peptides. The proteasome degrades proteins to peptide fragments of 2–25 residues. While the COOH-terminal residues of peptides presented to MHC-class I are determined by cleavages within the proteasome, the NH_2-termini are often generated by aminopeptidases which trim longer N-extended proteasome products to mature peptides [68]. Some authors have suggested that peptides of suboptimal length in respect to MHC-binding could be produced when they are more efficiently translocated into the endoplasmic reticulum (ER) by the TAP molecules. These peptides are then trimmed in the ER before association with class I molecules [69]. Indeed, endoplasmatic reticulum aminopeptidase 1 (ERAP1), an IFN-γ-induced aminopeptidase, trims long precursors to the mature peptides presented on MHC class I [68]. ERAP1 prefers peptides of 9–16 residues and seems to lose activity when are generated epitopes 8 or 9 residues long [68]. The activity of ERAP1 depends on the COOH-termini of the substrate, the enzyme having a high affinity for large hydrophobic COOH-terminal residues. This preferential affinity has immunological implications: since human TAP shows affinity for basic as well as hydrophobic COOH-terminal residues, epitopes with basic COOH-terminal residues are most likely produced independently of ERAP1 [68]. Indeed, different authors report that TAP molecules themselves select peptides of optimal length for MHC association, which are produced solely by cytosolic endopeptidases [70]. The molecular determinants of peptide cleavage indicate that different stages of the antigen processing pathway are likely to be important in the successful presentation of polyepitope. For example, if a particular epitope requires a cleavage at a specific point, a number of residues upstream of its N-terminus, which are necessary for efficient translocation, will need to be removed. However, this process could have a deleterious effect on the processing of immediately adjacent epitopes which can be internally cleaved. For this reason, several constructs have been made by inserting alanine spacers between residues to provide peptidase cleavage points outside the epitope sequence [71].

Del Val et al. demonstrated that not only the sequence of the presented peptide but also that of its flanking residues determine the efficiency of processing and presentation and that a low yield of antigenic peptide due to an unfavorable integration site could be overcome by flanking the insert with oligo-alanine to space it from disruptive neighboring sequences [72]. This result indicated the need to include particular flanking residues in the construct in order to obtain a correct peptide cleavage and processing. Conversely, Thompson et al. found that a construct incorporating directly-linked Epstein-Barr virus epitopes with no flanking sequences was capable as well of inducing CTL recognition of all its epitopes when delivered to cells by vaccinia virus [73].

4. Polyepitope DNA-Based Cancer Vaccines: Results of Preclinical In Vivo and In Vitro Studies

Polyepitope DNA-based cancer vaccines have demonstrated promising results in preclinical studies.

Results of these studies are described below. Qin et al. selected and ligated together several DNA fragments encoding multiple CTL and Th cell epitopes from human prostate-specific membrane antigen (hPSM), mouse prostatic acid phosphatase (mPAP), and human prostate-specific antigen (hPSA). It was thus formed a novel fusion gene, termed 3P gene, which was inserted into a plasmid DNA along with a gene encoding for the human IgG Fc to construct a DNA vaccine. Vaccination with this polyepitope DNA induced a strong antitumor response in a mouse tumor model, significantly inhibited tumor growth and prolonged survival time of the tumor-bearing mice. CTLs were also induced which could specifically kill hPSM-, hPAP-, or hPSA-expressing tumor cells [74]. The authors then formulated a novel chemotactic antigen DNA vaccine encoding chemokines and multiepitopes of prostate-TAA. This vaccine enhanced antitumor immunity. In addition, when human lymphocytes were stimulated by autologous PBMCs transfected with the plasmid DNA vaccine, CTLs were induced which could kill hPSM-, hPAP-, or hPSA-expressing tumor cells [75]. The utility to insert a chemokine in the polyepitope DNA vaccine was also demonstrated by Sun et al., who showed that the immunization of mice with a fused-gene DNA vaccine containing the N-terminus of both MHC class I-restricted and class II-restricted T-cell epitopes from HER-2/neu and p53 linked to the sequence encoding for human secondary lymphoid-tissue chemokine, and the C-terminus linked to a cell-binding domain of IgG, reduced the size of established tumours, prolonged the lifespan of tumour-bearing mice, and enhanced the antigen-specific cellular and humoral immune responses [76].

The usefulness to insert a Th epitope or an adjuvant mimic gene was reproducibly reported. Zhang et al. linked the full-length human prostate stem-cell antigen (PSCA) gene to the N- or C-terminus of human heat shock protein HSP70 as adjuvant and investigated whether HSP70 could enhance the potency of the DNA vaccines. Mice vaccinated with PSCA-HSP plasmids generated a strong PSCA-specific CD8+ T-cell immune response [77]. Similarly, it was demonstrated that a DNApoly1 vaccine encoding a protein containing an ER (Ig jchain) signal sequence, the PADRE Th epitope, and a number of HPV CTL epitopes elicited powerful effector CTL responses and long-lived memory CTL responses in immunized mice [78]. In addition, it was proven that the combination of the gene which encodes a protein, called the class II MHC transactivator, (CIITA) with calreticulin/E6 and Ii-PADRE (pan HLA-DR-reactive epitope) DNA vaccines represents a potentially effective cancer vaccine, because of the ability of the vaccine to increase the levels of MHC class I/II molecules and lead to enhanced presentation of the antigen via the MHC class I and II processing pathways [79]. On the other hand, the advantage of removing the second domain of fragment C (FrC) of tetanus toxin Th was demonstrated, because of potentially containing competitive epitopes which were able to depress induction of CEA-specific CTLs [80]. The injection of DNA encoding HPV E6 or E7 antigen with DNA encoding Ii-PADRE led to significantly stronger E6- or E7-specific CD8+ T-cell immune responses and more potent protective and therapeutic antitumor effects against an E6/E7-expressing tumor model in mice as compared to the administration of E6 or E7 DNA with Ii DNA, thus indicating that administration of DNA vaccines with Ii-PADRE DNA is an effective tool to elicit stronger antigen-specific CD8+ T-cell immune responses [81]. Immunogenicity of cryptic epitopes delivered by a multiepitopes DNA-based vaccine was also demonstrated. In this respect, Scardino et al. designed a cDNA vaccine encoding 12 different HER-2/ErbB-2-derived, including HLA-A*0201-restricted, dominant and high-affinity heteroclitic cryptic epitopes. Vaccination of HLA-A*0201 transgenic HHD mice with this ErbB2 multiepitopes vaccine triggered multiple ErbB-2-specific CTL responses in vitro and significantly delayed the growth of challenged ErbB-2-expressing tumors in vivo. In addition, a T cells multiple response from HLA-A*0201 healthy donors was obtained in vitro upon stimulation of dendritic cells with the multiepitopes vaccine [82].

However, a biased T cells response upon a multiepitopes DNA-based vaccine was also showed. This event might be due to the presence of an immunodominant epitope within subdominant epitopes in the vaccine. Indeed, it was proven that a single immunization with a DNA plasmid minigene construct encoding four human leucocyte antigen (HLA)-A2-restricted epitopes belonging to tumour antigens CEA, MAGE2 and MAGE3, as well as the universal PADRE epitope recognized by Th lymphocytes, induced a monospecific immune response only against the immunodominant CEA epitope [83]. Polyepitope design and constructions should take into account the respective position of subdominant and dominant epitopes, in order to trigger an efficient and broad T cell epitope response. Yet, results obtained after HDD mice vaccination with an HER-2 multiepitopic DNA construct containing heteroclytic cryptic epitopes within native and immunodominant epitopes did not show any prevalent immunodominance among different epitopes, although CTL activity on target cells bearing certain HER-2 peptides was variable. In addition, stimulation of human PBMCs from different donors with the polyepitope DNA vaccine showed evidence of CTL response variability among individuals, demonstrating the possibility to mobilize human T cell repertoires specific for multiple HER-2 epitopes including cryptic epitopes. The multiple epitopes-induced CTLs were capable to kill MCF-7 tumor cells in vitro [82]. Conversely, Smith et al. provided in vitro evidence that a melanoma polyepitope cDNA vaccine was able stimulate lymphocytes from normal human donors to simultaneously generate multiple antigen-specific responses [84]. Interestingly, the use of multiepitopes DNA vaccines delivered by dendritic cells (DC) was also investigated. DCs transfected with human telomerase reverse transcriptase (hTERT)-IL18 gene were capable of eliciting a stronger hTERT-specific CTL response in vitro than that stimulated with the hTERT construct only [85]. In addition, an efficient induction of tumor antigen-specific immune response in vitro by DCs pulsed with a recombinant fusion protein of Hsp70 and CEA(576–669), a fragment of the carcinoembryonic antigen (CEA) containing CAP-1, was reported [86].

5. Polyepitope DNA-Based Cancer Vaccine: Results of Clinical Trials and Future Perspectives

The use of DNA-based cancer vaccines appears a novel tool for efficient activation of diverse T cell immune responses against cancer. However, the promising data obtained in preclinical models were not immediately followed by encouraging results in cancer patients. In fact, early clinical trials employing DNA-based vaccines showed a limited immunity to the delivered TAA. For example, Rosenberg et al. were unable to demonstrate significant clinical or immunological responses to the administration of a plasmid DNA encoding the "self-" nonmutated gp100 tumor antigen in 22 patients with metastatic melanoma. Only one patient exhibited a partial response of several subcentimeter cutaneous nodules [87]. Similarly, Triozzi et al. demonstrated that although the injection of a plasmid containing MART-1 was safe when administered to 12 patients with resected melanoma at risk for relapse, no patient manifested increased MART-1-specific lymphoproliferative responses [88].

Conversely, evidence of both immune and clinical responses in the same patients was observed in prostate cancer patients administered with a DNA vaccine encoding prostate-specific antigen [89, 90]. A multiepitopes-DNA-based vaccine was employed by Tagawa et al. who vaccinated stage IV melanoma patients with a recombinant plasmid DNA vaccine encoding two peptides derived from human tyrosinase, that is tyrosinase 207–216 and tyrosinase 1–17. The authors reported that immune responses were

detected in 11 of 26 patients. No clinical responses were seen. Still, survival of the heavily pretreated patients on this trial was unexpectedly long, with 16 of 26 patients alive at a median follow-up of 12 months [91]. Klencke et al. observed that 10 of 12 eligible subjects with HPV-16 anal infection and a HLA-A2 haplotype responded to the vaccination with a plasmid DNA encoding for multiple HLA-A2-restricted epitopes derived from the HPV-16 E7 protein. In addition, 3 patients obtained a partial histological response [92]. Conversely, Smith et al. produced no direct evidence that 2 injections of a plasmid DNA encoding 7 melanoma tumor antigen CTL epitopes were capable of priming an immune response against the recombinant gene products when administered in HLA-A*0201-positive patients with surgically treated melanoma [93]. According to these trials it appears that the immune responses elicited by TAA polyepitope DNA vaccines in cancer patients are weaker than those induced by other forms of recombinant vaccines.

TAA polyepitope DNA vaccines aim at inducing TAA specific CTLs. However, accumulating evidence shows that tumor cells can escape immune destruction [94]. In this regard the tumor microenvironment plays a pivotal role in determining the fate of the emerging anti-cancer immune response [3, 94, 95]. Solid tumors are composed of cancer cells embedded in a variety of non-malignant cells (macrophages, lymphocytes, vascular cells, fibroblasts) which form the tumor stroma [94, 95]. Beside the alteration in antigen processation and presentation, cancer cells and tumor stromal cells can produce local immunosuppressive factors [94]. Cancer cells can also become resistant to CTL-mediated cytotoxic pathways, for instance acquiring resistance to perforins and granzyme B or through the alteration of death receptors expression or signaling [94]. In addition, cancer cell genetic instability can lead to the alteration of pro-apoptotic regulators, one of the most important being p53. It has been demonstrated that the p53 status of cancer cells has a key role in determining the fate of the anti-tumor CTL response since it regulates Fas receptor expression, cellular FLICE/caspase-8 inhibitory protein (cFLIP) short protein degradation, and CD95-mediated apoptosis [94]. Cancer cell cytoskeleton disorganization can confer resistance to CTLs as well [94].

The limited anti-tumor and clinical responses obtained with TAA polyepitope DNA-based vaccines might be due also to the poor immunogenicity of plasmid DNA itself, in particular when the plasmid DNA is used alone and delivered through needle injection [56]. Indeed, one issue related to the low immunogenicity of DNA vaccine is represented by inefficient plasmid DNA uptake following either skin or muscle injection [54]. In addition, the effectiveness of in vivo DNA transfection attained in murine models might not correctly be converted into proper dosage for humans [56]. Different techniques can be employed to enhance DNA uptake and immunogenicity, including gene gun delivery and electroporation. Besides, CpG neutralizing sequences present within the plasmid DNA backbone can inhibit the activation of DNA uptake by APCs [54]. In this respect, it would be of help to choose CpG sequences which allow the maturation of selected immune cell types, while replacing CpG inhibitory motifs [56].

Due to the low immunogenicity of plasmid DNA, efficacy in priming the immune response is often below detection levels at the end of the immunization protocol, but it emerges after the host is boosted with a different form of vaccine delivering the same antigen [96]. It is clear that antigen cross-presentation from skin or muscle cells to antigen-presenting cells (APCs) is the major route for immune response priming [60]. However, a long lasting immune response is obtained through a two step-process, that is, priming and boost. While priming of immune cells requires a low dose of antigen, the boost needs a larger amount of antigen [96]. The immunogenicity of DNA vaccines could be enhanced by increasing the administered dose, although the cost of using high amounts of plasmid DNA will make this approach economically unaffordable [96]. Thus, it is likely that DNA vaccines could efficiently induce cell-mediated immunity mainly via priming of dendritic cells in vivo. The adjuvant nature of bacterial DNA plasmids used as delivery vectors also furnishes a potent immunostimulatory effect for the Th1 phenotype and can promote CTL-induction even in the absence of specific helper.

Finally, polyepitope DNA constructs should be optimized before clinical usage. All modifications and every combination of epitopes require previous validations by experimental prediction (motif sampler, weight matrix, and artificial neural network) [97] and in vitro preclinical studies. In fact, future studies should be focused on the development of strategies to enhance epitope expression, to improve immune recruitment, and to select the best combination of epitopes which can drive a strong T cell response toward a TAA/s. Future studies should be focused on the development of strategies to enhance epitope expression, to improve immune recruitment and to select the best combination of epitopes which can drive a strong T cell response toward a TAA/s. In addition, a better understanding of the proteasome/TAP/ER-mediated processing of polyepitope proteins will allow the design of DNA constructs optimized for efficient presentation of all incorporated epitopes. Undoubtedly, a key feature for the use of polyepitope DNA-based cancer vaccines is their extraordinary feasibility, which in the future may allow to design specific DNA vaccines tailored on the MHC/TAA epitopes profile peculiar for each patient.

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Review Article

RNA Vaccines in Cancer Treatment

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The Cancer Report from the World Health Organization states that in the year 2000 12% of all death cases worldwide were caused by cancer. In the western world, the cancer death rates are often devastating, being at about 25%. This fact stresses the urgency to find effective cures against malignant diseases. New approaches in the treatment of cancer focus on the development of immunotherapies to fight the disease. Besides other methods, the usage of tumor-specific RNA as part of vaccines is investigated lately. RNA, administered alone or used for transfection of dendritic cells, shows several advantages as a vaccine including feasibility, applicability, safeness, and effectiveness when it comes to the generation of immune responses. This review concentrates on results from in vitro experiments and recent trials using RNA vaccines to present an overview about this specific strategy.

1. Introduction

The diverse causes for cancer malignancies suggest that the therapy of patients should be individualized to offer an effective treatment for each person. Additionally, it is the goal to intervene at the molecular level and manipulate cells and ongoing processes to fight disease. For quite some time, the idea of using the own body's immune system for the treatment of cancer has been fascinating for its apparent simplicity and probable effectiveness. Due to this, studies on the topic have been going on since the 1970s [1–3]. At this time point first ideas to employ RNA for the generation of immune responses occurred and the term immunotherapy emerged. So-called immune RNA was extracted from lymphoid tissues of animals immunized with tumor cells and injected into patients as adjuvants [1, 2].

As easy as the idea sounds, however, researchers have been challenged with the complexity of the immune system and the generation of specific and effective immune responses against desired targets. One issue, that makes things both easier and more difficult, is the origin of cancer, which is altered self. It is easier, because tumor cells exhibit distinct differences in comparison to nonaltered body cells. Difficult, because, for one, these cells are still self cells and, second, they apply diverse mechanisms to evade recognition by the immune system.

Nevertheless, diverse approaches have been taken to use the differences between normal and tumor cells to teach the immune system to recognize malignancies and eliminate them. The goal in the generation of immunotherapeutic vaccines is the induction and perpetuation of a tumor specific immune response. As a consequence the body should be cleared from tumor cells, and additionally, the immune system should prevent the recurrence of the tumor. Therefore it is necessary to generate a complete immune response and to activate several leukocyte populations like antigen-presenting cells, CD4+ and CD8+ T cells and B cells. This will lead to the generation of memory cells, which have a protective function after the clearance of tumor cells.

One focus of current research is the usage of messenger RNA (mRNA) in diverse forms as a vaccine. In these approaches mRNA encoding for tumor-associated antigens or whole tumor cell RNA is applied to induce specific CTL responses [4, 5]. Proceedings in experimental research and recent trials are the topic of this review.

2. Tumor-Associated Antigens (TAAs)

An important step for vaccine development has been and still is the identification of tumor-associated antigens (TAAs) [6]. As immune responses shall be induced, it is necessary

to find immunogenic molecules, which are upregulated or best exclusively expressed in cancerous tissues but not or only lightly in healthy adult tissues.

Many TAAs are shared by tumor and normal tissues but are indeed overexpressed in tumor cells. The use of these broadly distributed antigens should be evaluated carefully, as they induce tolerance due to the negative selection processes during T cell development. If tolerance can be overcome, the generation of immune responses against these antigens might lead to the induction of autoimmune diseases. A subgroup of shared TAAs are differentiation antigens. They are not ubiquitously expressed, but specific for certain tumors and the tissues these tumors derived from. An example for differentiation antigens is Melan-A/MART-1 [7], which is almost solely expressed in melanoma cells and melanocytes. Although the distribution of differentiation antigens is restricted to certain tissues, the induction of tolerance towards these antigens still poses a problem. And again, it might be possible to create autoimmune effects when interfering with these TAAs.

A safer way to utilize the immune system would be the induction of immune responses against antigens that are uniquely expressed in tumor tissues. Two types of TAAs are indeed specific for tumor cells. Cancer/testis (CT) antigens [8] are expressed in several tumor tissues and in the germline but cannot be found in somatic tissues. CT antigens are not presented in the germline as MHC class I and class II molecules get not expressed at site. This means that the antigens will only be recognized in the tumor. In opposition to shared tumor antigens, the generation of immune responses will not lead to autoimmunity. An example for CT antigens is NY-ESO-1 [9], which already has been used as a target in immunotherapeutic approaches. Mutation antigens are also exclusively expressed in tumor tissues. Cancer cells often acquire several mutations, which lead to the translation of altered proteins. If mutations occur in oncogenes or tumor suppressor genes, this will induce the invasiveness of the malign tumor. Mutation antigens are suited perfectly for immunotherapy as these TAAs are not considered as self by the immune system and no tolerance has been induced towards them. Examples for mutation antigens are CDK4 [10], Kras [11], caspase8 [12], or TGF-β receptor [13–15]. Another group of tumor-specific antigens are TAAs of viral origin. Several viruses, like HTLV-1, HIV, and EBV, induce the generation of tumor cells. Particularly, the development of non-Hodgkin lymphomas (NHL) and multiple myelomas (MM) is associated with former viral infection. The altered cells present viral antigens on their surface, which can be the starting point for vaccine generation. In NHL and MM disease, the idiotype of the immunoglobulin can be targeted as well.

So far, depending on the type of disease, several useful TAAs have been identified. For melanoma patients, MART-1/Melan A [7], gp100 [16], and tyrosinase [17, 18] seem to be promising targets. The TAAs Survivin [19–21] and MUC-1 [22, 23] are expressed in a broad variety of epithelial and haematological malignancies. CEA [24] is associated with pancreatic, colorectal, or gastric cancer, while MAGE-A [6] and NY-ESO-1 [9] are cancer testis antigens. Adipophilin [25, 26], MMP-7 [27], RGS1 [28], and RGS5 [25, 29] have recently been identified as renal cell carcinoma TAAs. Examples for leukemia-associated antigens are Wilms' tumor protein (WT1) [30], proteinase 3 [31, 32], receptor for hyaluronan acid-mediated motility (RHAMM) [33], and Bax inhibitor-1 [34].

3. Vaccine Delivery

Having found specific differences between tumor and healthy tissue, the next challenge is to teach the immune system to perceive them. This is obviously done by administering the TAAs to the body. The question is still: How is it done in the best way?

Important issues to consider are the type of antigen, which should be applied, and the way the antigens should be delivered. When it comes to the type of antigen, diverse approaches have been taken. Peptides from TAAs or whole proteins have been used, whole cell tumor lysates or irradiated, apoptotic tumor cell bodies have been applied, and TAA-encoding plasmids or TAA/ whole tumor cell RNA were to try out. Besides the choice of the antigen the strategy of antigen delivery must be optimized. The above stated tumor antigens could be used as vaccines alone or together with additives like GM-CSF, IFN-α, or IL-12. Instead of using the antigens as direct vaccines, they can also be brought into the body by dendritic cells. This review will focus on the use of RNA either alone or in combination with dendritic cells as a cancer vaccine. An overview over both methods is given in Figure 1.

4. Dendritic Cells

In the development of vaccines against cancer, many research groups focus on the usage of dendritic cells (DCs). DCs are, besides B cells and macrophages, antigen presenting cells (APCs) and are seen as the most potent population executing this function.

In their immature state, DCs mostly reside in tissues and in part travel through the blood and lymph streams. They express major histocompatibility complex (MHC) class II molecules, but only few costimulatory molecules like CD80 (B7.1) or CD86 (B7.2). While migrating through the body's periphery, DCs eventually encounter antigens. The cells incorporate these antigens through a variety of processes, namely, receptor-mediated endocytosis, phagocytosis, or macropinocytosis. After antigen ingestion, DCs migrate towards afferent lymphoid organs and differentiate into mature cells. During the maturation process DCs undergo several phenotypic and functional changes. They upregulate the expression of MHC class I and II molecules [35], the costimulatory molecules CD80, CD86 [36], and CD40 [37], as well as the adhesion molecules CD54 (ICAM-1), CD102 (ICAM-2), CD11a (LFA-1), CD58 (LFA-3), and CD209 (DC-SIGN) [38–40]. As another prominent change, mature DCs increase the secretion of cytokines (IL-6, IL-12, IL-23, TNF-α) and chemokines like CCL18 (DC-CK-1), which draws naïve T cells to the location.

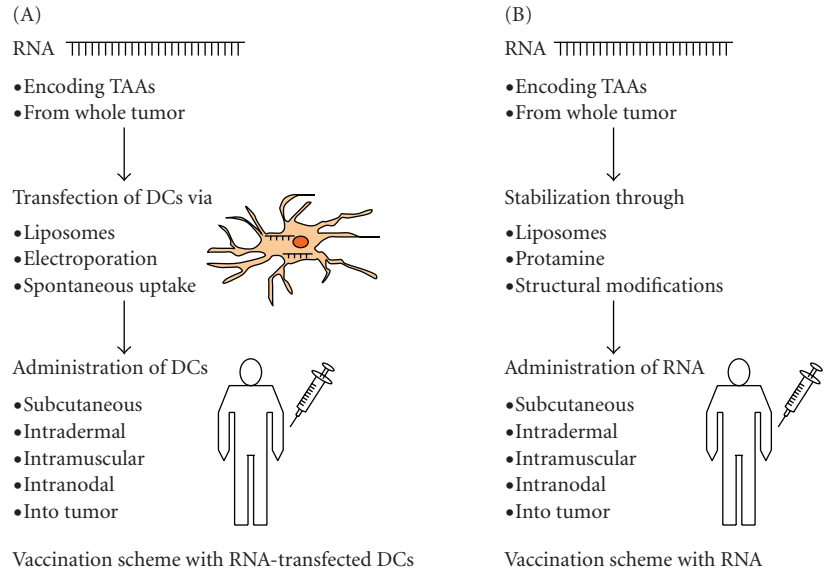

Figure 1: Overview over RNA-vaccination schemes using DCs (A) or pure/stabilized RNA.

Through their matching adhesion molecules, DCs and residing T cells interact with each other, forming immunological synapses. As a consequence, DCs present the processed antigens to T cells, these get activated, and antigen-specific responses are generated.

Depending on the nature of the antigen, either MHC class I or class II molecules are involved in their presentation. Like almost all cells of the body, DCs have the ability to present antigens via MHC class I molecules. This route is taken when antigens are of endogenous origin. After proteasomal degradation in the cytosol, generated peptides are transported into the ER, loaded onto MHC class I molecules, and brought to the cell's surface. There, the MHC class I molecules interact with T cell receptors from CD8 positive cytotoxic T lymphocytes (CTLs).

DCs can present exogenous antigens over MHC class II molecules. After incorporation, the antigens are degraded in endosomal-lysosomal compartments by cathepsins. Generated peptides are loaded onto MHC class II molecules, which interact with CD4 positive T cells.

Besides following the "classical" pathways of antigen processing and presentation, DCs are able to present exogenous antigens through MHC class I molecules. Engulfed antigens are transferred from lysosomal compartments into the cytosol and then follow the MHC class I pathway of processing and presentation. Through this so-called cross-priming, effective CTL responses can be generated [41–43].

5. In Vitro Generation of Dendritic Cells

The employment of DCs in vaccination strategies requires large amounts of this cell type. Until now, several protocols have been established for the generation of high quantities in vitro. DCs can be generated from CD34 positive precursors from bone marrow or blood, when the cells are incubated with cytokine cocktails containing granulocyte-macrophage colony stimulating factor (GM-CSF), IL-4, or TNF-α [44–46]. A different approach uses CD14 positive monocytes from blood and the cytokines GM-CSF and IL-4 or CD40 ligand to generate myeloid DCs [47–49]. These cells are immature and express few costimulatory molecules on their surface but can be matured with different inflammatory stimuli like Toll-like receptor ligands, TNF-α, or a mixture of compounds containing PGE_2, TNF-α, IL-1β, and IL-6 [50].

6. Usage of RNA

RNAs, ribonucleic acids, are single-stranded polymers in their primary structure, containing the four nucleotides adenosine, guanosine, cytidine, and uridine monophosphate. These are linked through the 3′ and 5′ phosphate residues of the ribose sugars. In immunotherapy, messenger RNA (mRNA) is used, which contains the genetic information for proteins. The structure of processed and spliced mRNA has five structural features: A Cap structure, which is a 7-methylguanosine triphosphate, the 5′-untranslated region (UTR), the open reading frame (ORF) beginning with the start codon at the 5′ end and ending with the stop codon at the 3′ end, the 3′-UTR, and a poly A-tail at the 3′ end containing 100–250 residues.

In the first vaccination approaches, mRNA was extracted from autologous tumor tissues from patients. This procedure had the disadvantage that sufficient amounts of tumor cells had to be available. Nowadays it is possible to prepare high amounts of RNA in vitro. From few tumor cells one can extract RNA and generate complete tumor cDNA libraries to obtain the necessary amounts of RNA encoding multiple patient specific TAAs in the needed quality [51, 52]. Besides the easy access to large quantities, the application of mRNA provides several advantages as a vaccine. RNA molecules are considered to be safe vaccines, as they are easily degraded. They are cleared quickly out of the organism and cannot,

as opposed to plasmid DNA, integrate into the genome and influence the cell's gene expression in an uncontrollable manner [53]. Due to the stated facts, it is also unlikely that RNA vaccination causes severe side effects like the generation of autoimmune disease or anti-DNA antibodies. As a plus, the application of mRNA is, unlike peptide-based vaccinations, not MHC-restricted. An immune response against a broad variety of antigens can be generated and CD8 positive CTLs as well as CD4 positive T helper cells can get induced. A polyclonal immune response makes tumor escape less likely. Although the expression of genes cannot be regulated when RNA is used, this is eventually not necessary due to the molecule's instability. Unlike DNA, which has to be transfected into the nucleus, the transfection with RNA requires only insertion into the cell's cytoplasm, which is easier to achieve. All these issues speak for the utilization of RNA-based vaccines.

7. RNA-Pulsed DCs as Vaccines

One basic approach in RNA vaccination is the use of in vitro generated DCs as tumor antigen-presenting cells. Generated DCs can be transfected with RNA encoding single or multiple TAAs or whole tumor cell RNA. After transfection, the genetic information introduced into the DCs is translated into protein. These proteins underlie intracellular degradation processes and the generated peptides are presented on MHC class I and class II molecules.

Several in vitro experiments showed that DCs pulsed with whole tumor RNA or RNA encoding specific TAAs induce the generation of specific CTLs. This procedure was first described by the group of Gilboa and formed the basis for ongoing experiments [54]. Milazzo et al. electroporated monocyte-derived DCs with whole RNA from LP-1 and U266 cell lines and induced specific CTLs that lysed LP-1 and U266 myeloma cells [55]. In different experiments, scientists used RNA from tumor-bearing patients instead of cell lines to generate CTL responses. Heiser et al. transfected DCs with RNA isolated from renal tumor cells [56]. The group showed the generation of polyclonal CTL responses and subsequent lysis of primary and metastatic tumor cells. Apparently, the polyclonal T cell activities were more potent than the CTL responses generated against a single antigen. However, in other settings the potency of immune responses induced by single TAAs was investigated. Nair et al. transfected DCs with CEA mRNA and stimulated specific CTL responses in vitro [24]. Grunebach et al. analyzed the influence of cotransfection of two different TAAs and electroporated DCs with Her-2/neu and 4-1BBL RNA. They found that costimulatory molecules were upregulated and immune responses were increased in comparison to single TAA transfection. Both CD8 and CD4 T cell responses were induced [57].

The transfection of DCs with RNA already leads to the initiation of the maturation process. It is still questioned if these stimuli are enough or if additional signals lead to more capable cells. Several additional maturation stimuli have been analyzed on the effect on DC phenotype and effectiveness on T cell proliferation and thus the generation of specific immune responses. Onaitis et al. transfected DCs with mRNA encoding Mart-1 and partly matured the cells with CD40 ligand [58]. An enhanced T-cell proliferation was found in the preparations with CD40L stimuli, which was due to the increased IL-12 secretion by the treated DCs. The same group discovered that the sequential stimulation with a cytokine cocktail (TNF-α, IL-1β, IL-6, and PGE_2) followed by incubation with CD40L enhances the generation of Mart-1-specific T cells, an effect again mediated by increased IL-12 production [59]. They also described the effective maturation of DCs by diverse Toll-like receptor ligands [60, 61]. In mouse experiments the generation and further enhancement of T cell responses has been analyzed. Boczkowski et al. induced OVA-specific CTLs and protection from OVA-expressing tumors in mice [54]. Naka et al. cotransfected mouse DCs with tumor RNA as well as GM-CSF RNA and used the cells as a vaccine [62]. The animals had already received immunotherapeutic treatment in a previous experiment and the re-growing of the tumor should be inhibited. The co-transfected DCs successfully induced augmented CTL responses and suppressed tumor growth. Kim et al. investigated whether it is possible to enhance immune responses against the antigen CEA when the TAA is modified. The group generated a fusion gene containing a truncated CEA ($\triangle$CEA) devoid of its signal sequences, calreticulin (CRT), and the HIV TAT protein transduction domain (TAT-PTD) and pulsed DCs with the according RNA. The vaccine enhanced CEA-specific CD4 and CD8 responses and inhibited tumor growth and led to a prolonged survival of treated mice [63].

The promising results from in vitro experiments and studies in mice led to several human trials where patients with different malignancies were vaccinated with RNA transfected DCs (see Table 1).

Heiser et al. conducted a study on metastatic prostate cancer [64]. Sixteen patients were initially enrolled in the phase I trial and 13 followed the immunization schedule with PSA RNA-transfected DCs. The patients received 3 intravenous vaccination cycles with escalating doses of DCs, namely 1×10^7 cells (low dose), 3×10^7 cells (medium dose), and 5×10^7 cells (high dose), together with, 1×10^7 DCs intradermally administered at weeks 2, 4, and 6. The evaluation of specific T cell responses was carried out via ELISPOT and [^{51}Cr]-chromium-release assays. All nine patients that were analyzed showed PSA-specific CTL responses. Additionally, a decrease in the log slope PSA was detected in six of seven patients and three patients transiently showed clearance of circulating tumor cells. No severe adverse effects were observed after the vaccinations, stating the safety of this type of vaccine.

CEA is an important tumor marker expressed in colorectal, pancreatic, and gastric carcinomas as well as in the majority of breast and nonsmall cell lung cancers. The TAA has been object of recent phase I/II studies to evaluate its use in vaccination therapy. Immunizations with CEA mRNA-transfected DCs were administered to three patients with resected pancreatic adenocarcinoma [65]. The injections were given monthly for a time period of six months and the treated patients showed no recurrence of disease in the more

Table 1: Recent clinical trials using RNA-transfected DCs.

Cancer type	RNA source/target	Vaccination schedule	Number of study subjects	Immunological response	Clinical response	Reference
Colorectal cancer	Total autologous tumor	10^6 DCs pulsed with 25 μg RNA and KLH intravenously 4 times on monthly intervals.	15	NA	NA	[73]
Adenocarcinoma (lung)	Total autologous tumor	3×10^7 DCs transfected with 300 μg RNA intravenously, followed by 10^6 DCs transfected with 10 μg RNA intradermally 4 times on monthly intervals.	1	1/1	NA	[93]
Pancreatic cancer	CEA	10^7 DCs transfected with 20 μg RNA intradermally 6 times on monthly intervals.	3	NA	NA	[65]
Prostate	PSA	10^7–5×10^7 DCs transfected with 1.5 μg RNA/10^6 DCs intravenously, 3 times biweekly with escalating dose and 10^7 DCs intradermally.	16	9/9	NA	[64]
CEA-expressing cancers	CEA	10^7-10^8 DCs transfected with 2 μg RNA/10^6 DCs intravenously and 0–10^6 trasnfected DCs intradermally 4 times biweekly.	42	NA	NA	[94]
RCC	Total autologous tumor	10^7–5×10^7 DCs transfected with 50 μg RNA/10^7 DCs intravenously, 3 times biweekly with escalating dose and 10^7 DCs intradermally.	15	6/7	NA	[72]
Brain cancer	Total autologous tumor	0.5–5×10^7 DCs/m^2 transfected with 5 μg RNA/ 10^6 DCs intravenously and 0.5×10^7 DCs/m^2 intradermally, 3 times biweekly with escalating dose, 3 times at 3-month intervals.	9	NA	2/7 SD	[95]
Neuroblastoma	Total autologous tumor	0.5–5×10^7 DCs/m^2 transfected with 5 μg RNA/10^6 DCs intravenously and 0.5×10^7 DCs/m^2 intradermally, 3 times biweekly with excalating dose, 3 times at 3-month intervals.	11	NA	1/7 SD	[96]
RCC, OVA	Total tumor from clear cell carcinoma tissue	Arm 1: 10^7 DCs electroporated with 5 μg RNA/10^6 DCs intradermally, 3 times biweekly, 18 μg/kg DAB_{389} IL-2 prior to vaccination. Arm 2: 10^7 DCs electroporated with 5 μg RNA/10^6 DCs intradermally, 3 times biweekly.	11 Arm 1: 7 Arm 2: 4	10/11 Arm 1: 7/7 Arm 2: 3/4	NA	[83]
Prostate	hTERT, LAMP hTERT	Arm 1: 10^7 DCs electroporated with 1 μg hTERT RNA/106 DCs intradermally, 3 (6 patients) or 6 (5 patients) times weekly. Arm 2: 10^7 DCs electroporated with 1 μg LAMP hTERT RNA/106 DCs intradermally, 3 (6 patients) or 6 (3 patients) times weekly.	20 Arm 1: 11 Arm 2: 9	17/18	NA	[97]
Prostate	Total tumor from prostate cancer cell lines DU145, LNCaP, PC-3	2×10^7 electroporated DCs intranodally or intradermally, at least 4 times weekly.	19	12/19	11/19 SD	[98]
Melanoma	Total autologous tumor	Arm 1: 2×10^7 electroporated DCs intradermally 4 times weekly. Arm 2: 2×10^7 electroporated DCs intranodally 4 times weekly.	22 Arm 1: 10 Arm 2: 12	9/19	2/20	[68]
Melanoma	Mage-A3, Mage-C2, tyrosinase, gp100	1.25×10^7 electroporated TriMix DCs intradermally, 4 times biweekly.	3	2/2	NA	[71]

NA: Not applicable; SD: Stable disease.

than 2.5 years. In a parallel study, DCs loaded with CEA mRNA were administered to patients with CEA-expressing tumors (phase I study) or with resected hepatic metastases of colon cancer (phase II study) in escalating doses. While 24 patients were enrolled in the phase I trial, only one showed a complete response, 2 patients exhibited minor responses, and 3 patients showed a stable disease. The clinical response in the phase II study was similarly poor. Nine of 13 patients showed recurrence of disease.

These examples can stand for several clinical phase I/II studies that have been performed and that show an analog outcome. The induction of specific CTLs after vaccination works well and strong immune responses can be observed in vitro, but only little effects are seen when looking at the clinical responses. The majority of the treated patients showed no benefit from the vaccinations; that is, no tumor regression or protection from recurring disease was noticeable. The difference between in vitro analyses and in vivo outcome could be due to the large tumor burden of the patients, the occurrence of regulatory T cells or myeloid suppressor cells, and induction of tolerance or consequences of medical pretreatment.

Still, scientists have thought of multiple ways to make vaccinations with RNA-transfected DCs more efficient. There are many variables when it comes to the generation of DC vaccines and many issues must be considered. One item is the question of the vaccine dose. This entails in detail the number of administered DCs and if an escalating dose should be used or not, the number of injections, and the injection interval. Another point is the route of administration. Different injection sites are possible for the administration of DC-based vaccines. The cells can be injected intradermally, subcutaneously, intravenously, intranodally, and directly into the tumor. As DCs migrate to lymphatic tissues after their administration, it is subject of investigation which route leads to the most effective immune responses. Intranodal or intradermal close to lymph nodes seemed to be promising, more than i.v. in one trial using peptide-pulsed DCs [66, 67]. When comparing intradermal versus intranodal injections of RNA-transfected DCs, the intranodal injection showed no signs of superiority [68, 69]. The different results might be due to the complexity of accurate DC vaccination. Intranodal injections lead to the accumulation of DCs in the lymph nodes. However, the accurate administration is more difficult than i.d. or i.v. injections and vaccines can be accidently delivered into perinodal fat [70]. As the route of administration affects the migration of DCs significantly, the optimal way has to be found for the treatment of different malignancies.

Another important issue is the antigen that has to be transfected into DCs. It is possible to pulse the cells with one or several TAAs or whole cell tumor RNA. Theoretically the application of the latter allows the generation of immune responses against a broad variety of antigens. It is still a matter of investigation if this approach is more effective than the use of several specific TAAs. If enough tumor material is available, whole RNA can be extracted from tumor tissues and used for vaccine preparation. If this is not the case, cDNA libraries can be created followed by cloning, amplification in E. coli, and finally in vitro transcription of RNA [51]. What actually is an advantage when it comes to the number of patients that can be treated following this approach also has a negative impact on the composition of the RNA mixtures, as in vitro transcription leads to the occurrence of shorter fragments [51].

The actual delivery of RNA into dendritic cells can be achieved in different ways. The transfection can be performed using liposomes and cationic lipids. These bind automatically to RNA due to their charge and form complexes that interact with the target cells. The cationic lipids DOTAP [54] or DMRIE [24] have been used for successful DC transfection. One disadvantage of lipid-mediated transfection is the toxicity of the mixture. Therefore, the ratio between RNA and lipids and the used concentration must be optimized. Another successful approach is the electroporation [71] of dendritic cells in the presence of RNA. The application of an electrical pulse causes the cells to take up enough RNA for antigen presentation. Finally, RNA can be introduced into DCs by passive transfection [72]. Immature DCs absorb particles and media as part of their function without external stimuli. This fact suggests that the cells will take up RNA present in the surroundings of the DCs. The passive RNA pulsing without the use of additives was shown to work successfully as well.

As in vitro experiments showed, the add-on of further stimuli can lead to enhanced immune responses as well. Rains et al. conducted a study on patients suffering from colorectal cancer [73]. They pulsed DCs with tumor RNA and keyhole limpet hemocyanin (KLH) for vaccine preparation. KLH is an immunogenic protein supposed to enhance the efficiency of the DC vaccination. In the study, eleven of 13 patients developed responses against KLH and seven patients showed a decrease in CEA levels.

A complex in vivo study was performed by Bonehill et al. in which the group generated so-called TriMix DCs [71]. Dendritic cells were electroporated with mRNA encoding CD40L, CD70, and a constitutively active TLR4 as enhancing elements. The cells were additionally electroporated with either Mage-A3, Mage-C2, tyrosinase, or gp100 mRNA. Two melanoma patients received four biweekly intradermal injections at four different sites of 1.25×10^7 TriMix DCs per antigen. While antigen-specific CD8 T cell responses were detected in both patients after finished treatment, no data about clinical responses were published on this trial.

The discussed approaches have analyzed several possibilities in vaccine preparation and administration. Even more and different settings have to be investigated in the future to find the optimized conditions for this type of treatment.

8. RNA Vaccines

Besides using in vitro preparations of dendritic cells for antigen presentation, another approach directly uses RNA vaccines. It has been shown that RNA can be transfected into DCs without the help of additives like liposomes or electroporation. Therefore, an obvious approach is the vaccination with naked RNA.

To deliver mRNA into patients in an effective manner, several issues have already been considered and tried out. RNA molecules themselves are thought to be relatively instable and easily degradable. For this reason there used to be much doubt, if the injection of the naked RNA molecules makes sense. However, Wolff et al. injected naked RNA into the skeletal muscle of mice and showed that the encoded protein was expressed in situ [74]. Furthermore, it could be shown that even the application of unprotected RNA could induce specific CTL responses in patients. Still, some thought has been given to stabilize RNA molecules and make delivery more efficient.

One possibility for RNA administration is to code the nucleic acid on gold particles and subsequent "gene-gun delivery" [75]. The particles are used as shuttles to carry the RNA molecules through skin. After incorporation by DCs, the encoded proteins are expressed and presented to T cells.

Another approach, which has been analyzed, is the packaging of RNA into liposomes. Liposomes contain cationic lipids which interact electrostatically with negatively charged nucleic acid molecules and form stable complexes [76]. Liposomes not only stabilize RNA, but already activate immune cells by themselves, and thus are supporting as adjuvants [77, 78].

Another way to stabilize RNA is its condensation to protamine [52], an arginine-rich protein essential for DNA condensation in spermatogenesis. Protamine forms spontaneous complexes with RNA in vitro, which are immunostimulating for several hours. Besides protection of RNA, protamine functions as a danger signal and activates in a MyD88-dependent way human DCs as well as monocytes, B cells, NK cells, and granulocytes.

Other ways to modify RNA to make it more resistant against degradation and more efficient for translation are the elongation of the poly-A tail at the 3′-end of the molecule and the manipulation of the Cap structure at the 5′-end. If the original 7-methylguanosine triphosphate is replaced by an Antireverse Cap Analog (ARCA), the efficiency of transcription is strongly enhanced.

To provide the immune system with even more potent signals, RNA has been modified to generate sequences with a phosphorothioate backbone [79]. Phosphothioate RNA serves as a danger signal and activates mouse DCs through MyD88 [79].

As discussed in connection with RNA-transfected DCs, the optimal injection site for immunostimulatory RNA has been a matter of research. RNA alone can be injected using the following routes: subcutaneous, intradermal, intramuscular, and intranodal. Injections close or into the lymph nodes seem to be preferable, as they induce the generation of strong immune responses.

By now, several in vivo studies have been carried out to analyze the use of RNA in tumor vaccines. Carralot et al. injected β-globin UTR-stabilized RNA encoding β-galactosidase intradermally into BALB/c mice [80]. The model antigen was translated in vivo, which was shown by specific staining. Interestingly, IgG1 antibodies against β-galactosidase were found after the vaccination, which are characteristic for a TH2 immune response. This result seemed to be contrary to other studies using DNA vaccines, where the generation of IgG2 antibodies was induced. The latter is typical for a TH1 response, which is preferred in anti-tumor immunotherapy. The study showed the importance of the type of antigen used for the vaccine in means of DNA or RNA, as it is obviously more important than the delivery route.

Until now, only few phase I/II trials have been carried out using RNA as a vaccine (see Table 2). Schmidt et al. vaccinated 30 RCC patients with naked tumor RNA coding for the TAAs MUC1, CEA, Her-2/neu, telomerase, surviving, and MAGE-1. The patients received intradermal injections on days 1, 14, 28, and 42 (group A), while group B received vaccinations on days 0–3, 7–10, 28, and 42 following monthly injections. The generation of antigen-specific immune responses was analyzed in vitro by ELISPOT assays. Both CD4+ and CD8+ T cell responses were induced for diverse antigens. Seven patients additionally showed a clinical response. No severe adverse effects occurred demonstrating the safety of the approach [81].

Weide et al. carried out two distinct trials on metastatic melanoma [5, 82]. In the first phase I/II study patients with stages III and IV received intradermal injections of in vitro transcribed naked whole tumor RNA. The patients were vaccinated with four times every two weeks followed by monthly injections for six months. 24 hours after each vaccination, the patients were injected subcutaneously with GM-CSF as an adjuvant. After treatment, melanoma cell line-specific antibodies were detected in four of 15 patients and specific T cell responses were probably induced in five patients. Looking at the clinical response, two patients showed a mixed response and five patients a favourable course of disease, but no clinical regression was seen. The vaccine itself proved to be safe, as only mild and reversible side effects occurred.

In the second phase I/II study, not whole tumor RNA was used for vaccination, but six defined RNAs encoding the TAAs Melan-A, tyrosinase, gp100, Mage-A1, Mage-A3, and survivin. The metastatic melanoma patients received escalating doses of RNA, a higher number of injections in comparison to the first study, and instead of using naked RNA the nucleic acids were stabilized with protamine. Additionally, KLH was used as a helper antigen and added to the vaccine of half of the patients. Again, GM-CSF was injected 24 hours after vaccination. The intense vaccination schedule contained 12 vaccinations administered in 19 weeks. The analysis of induced T cell responses showed no consistency between different patients. Out of four patients, one showed an increase in CD8 and CD4 T cells, a second patient showed first an increase for both T cell populations, but then a decline and no difference was seen in two other patients. The few number of patients and the individual differences make the outcome uncertain. In addition, the numbers of immunosuppressive cells were monitored. While the KLH+ arm exhibited a decrease of regulatory T cells (Tregs), the KLH-arm showed a decrease in myeloid suppressor cells. A clinical response was seen in one of seven stage IV patients. Again, the study results showed only few achievements by the administered immunotherapy. The group pointed in their

Table 2: Recent clinical trials using RNA.

Cancer type	RNA	Vaccination schedule	Number of study subjects	Immunological response	Clinical response	Reference
Melanoma	Total tumor	200 μg naked RNA intradermally, biweekly for 8 weeks, followed by monthly injections for 6 months. 150 μg GM-CSF subcutaneously 24 h after RNA injection.	15	NA	2/13 MR 3/13 NED	[5]
Melanoma	Melan-A, tyrosinase, gp100, Mage-A1, Mage-A3, survivin	Arm 1: 3.2–80 μg RNA per antigen + 128 μg protamine intradermally on days 1, 3, 5, weeks 2, 3, 4, 5, 6, 7, 11, 15, 19. 200 μg GM-CSF subcutaneously 24 h after RNA injection. Arm 2: 3.2–80 μg RNA per antigen + 128 μg protamine + 4 mg KLH intradermally on days 1, 3, 5, weeks 2, 3, 4, 5, 6, 7, 11, 15, 19. 200 μg GM-CSF subcutaneously 24 h after RNA injection.	21 Arm 1: 11 Arm 2: 10	Vaccine directed T cells: 2/4	Arm 1: 1/11 CR 4/11 NED Arm 2: 1/10 NED	[99]
RCC	MUC1, CEA, Her-2/neu, telomerase, surviving, MAGE-1	Arm 1: 20 μg naked RNA per antigen intradermally on days 0, 14, 28, 42, followed by monthly injections. 100 μg/m^2 GM-CSF subcutaneously 24 h after RNA injection. Arm 2: 50 μg naked RNA per antigen intradermally on days 0–3, 7–10, 28, 42, followed by monthly injections. 250 μg/m^2 GM-CSF subcutaneously 24 h after RNA injection.	30, Arm 1: 14 Arm 2: 16	CD4+ ELISpot: 3/7 CD8+ ELISpot: 8/9 CD8+ Cr-Release Assay: 7/11	Arm 1: 1/14 PR 6/14 SD Arm 2: 9/16 SD	[81]

CR: Complete response; MR: Mixed response; NA: Not applicable; NED: No evidence of disease; PR: Partial response; SD: Stable disease.

report to the possible role of immunosuppressive cells for the treatment. This issue is eventually one reason why there is a huge discrepancy in clinical trials when comparing the generation of specific antitumor immune responses in vitro and the clinical response of patients.

9. Optimization of Immunotherapy

CD4+CD25+ regulatory T cells are important for self-tolerance and have suppressor functions in the immune system. They control immune responses and reduce the risk of T cell responses being harmful to the body. Elevated numbers of Tregs are linked to a reduced survival in tumor patients. Thus, the depletion of Tregs could prolong the life of patients and strengthen the induced immune responses.

Dannull et al. vaccinated RCC patients with RNA-transfected DCs and additionally reduced the number of regulatory T cells [83]. For the depletion of Tregs they used the recombinant IL-2 diphtheria toxin conjugate DAB_{389}IL-2 (ONTAK), which selectively eliminates CD25-positive regulatory T cells. A significant increase of tumor-specific CD8 and CD4 T cell responses was observed for the combinational therapy opposed to injections with DC vaccines alone.

Another approach to eliminate inhibitory immune mechanisms is the targeting of CTLA-4. Cytotoxic T lymphocyte antigen (CTLA)-4 is homologous to the costimulatory B7 molecules and a negative regulator for T cell proliferation. Like B7, CTLA-4 binds to CD28. However, the affinity of CTLA-4 to CD28 is higher in comparison to B7 and the interaction leads to the inhibition of T cell proliferation. After T cell activation, CTLA-4 expression is increased, making the interaction with CD28 more and more likely and giving a negative feedback mechanism. The importance of CTLA-4 is pointed out by certain CTLA-4 polymorphisms which lead to autoimmune diseases [84]. Another evidence for the importance in controlling T cell responses is found in mice with lympho-proliferatory diseases not expressing CTLA-4 [85, 86].

The inhibition of CTLA-4 could lead to the generation of enhanced T cell responses. Tremelimumab and Ipilimumab are both humanized anti-CTLA-4 inhibitory antibodies. Their application in melanoma trials led to better clinical responses in treated patients [87]. Along with these promising results went severe side effects like diarrhea, panhypopituitarism, and autoimmune thyroiditis. These immune-related adverse events (IRAEs) probably go along with positive clinical responses. Further investigation should stress on the safety issues of CTLA-4 inhibition, as long-time treatment could lead to autoimmune phenomena. In ongoing studies the utilization of several immune-modulatory mechanisms together with CTLA-4 inhibition is investigated [88].

Besides the inhibition of undesirable immune effects, it is possible to further stimulate mechanisms that activate the immune system. Compounds with enhancing qualities are, for instance, nonmethylated cytosine-guanine dinucleotides

(CpGs) [89, 90]. These synthetic oligodeoxynucleotides are homologues to bacterial or viral DNA and stimulate TLR9. TLR9 is a pattern-recognition receptor belonging to the TLR family. It is located in the endosomal compartments of dendritic cells and macrophages and activates APCs at the occurrence of PAMPS. The synthetic oligodeoxynucleotide PF-3512676, for instance, enhances the induced immune response and prevents induction of immune tolerance [91].

Another possibility to enhance antitumor effects is the combination of RNA-vaccination with the administration of tyrosine kinase inhibitors (TKIs). The TKIs sorafenib and sunitinib inhibit intracellular signaling pathways leading to proliferation and angiogenesis. Sorafenib is applied in the therapy of renal cell carcinoma (RCC) and hepatocellular carcinoma (HCC). Sunitinib is administered in RCC and gastrointestinal tumor (GIST) treatment. Recent mouse experiments showed that pretreatment with sorafenib reduced the induction of antigen-specific T cells, while sunitinib had no such effect [92]. In human monocyte-derived DCs, sunitinib had no influence on DC phenotype and T cell proliferation, but sorafenib inhibited maturation processes in DCs and the stimulation of T cells. These findings indicate that sunitinib might be a good choice for combinational therapy with RNA vaccinations.

10. Summary

In the last 20 years many research groups have focused on the development of immunotherapies to fight malignant diseases. The main idea behind this approach is the utilization of apparent differences between the tumor and the normal healthy tissue it originated from. An important discovery supporting ongoing experiments was the finding of tumor-associated antigens, which demonstrate the difference of altered tissues on the molecular level. TAAs have been the starting point for several in vitro experiments and in vivo studies to generate specific immune responses against tumor cells.

One feasible and apparently safe approach for vaccination is the usage of mRNA encoding TAAs or the use of whole tumor RNA altogether. RNA can be used to transfect dendritic cells, which present TAAs, in their function as antigen-presenting cells, to T cells and generate a specific immune response. In more recent studies, the use of naked RNA plus possible enhancers has been analyzed in vaccination trials as well. For both methods exist a broad variety of variables that can be modified at vaccine administration. Due to this diversity, ongoing trials basically never follow the same approach twice, but instead single items are systematically changed for each new study. Many more trials might be needed before the best vaccination schemes will be found. However, the use of RNA vaccination might be the key for the cure of diverse types of malignant diseases.

Until now, in vivo trials have in common that the generation of specific T cells is induced after the vaccination of patients, but clinical outcomes are observed rarely. Often, the progression or recurrence of disease is observed instead of tumor clearance. Thus, latest studies deal with the enhancement of the effectiveness of vaccines. One possibility is the combination of RNA vaccination and the further stimulation of the immune system by cytokines and TLR ligands together with the inhibition of cell populations that suppress immune responses. When the most effective vaccination mechanisms will be found, patients will receive a specific treatment against their individual disease. The stimulation of the patient's immune system will lead to the generation and maintenance of an effective immune response. Hopefully, the vaccination with RNA will enable the control of malignant disease.

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Review Article

Recent Advancements in Cytotoxic T Lymphocyte Generation Methods Using Carbohydrate-Coated Liposomes

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Both tumor-specific $CD4^+$ and $CD8^+$ T cells have been identified, and the latter is known as a major effector of adaptive antitumor immune responses. Optimal antitumor immune responses are considered to require the concomitant activation of both $CD8^+$ and $CD4^+$ T cells and the additional selective activation of $CD4^+$ T cells with helper, but not regulatory function. As optimal antitumor immune responses are generated by the concomitant activation of both T cell types, it is necessary for vaccine methods involving cytotoxic T-lymphocytes (CTLs) generation to possess a mechanism whereby antigen presenting cells can present administrated exogenous antigens on not only Major histocompatibility complex (MHC) class II, but also MHC class I molecules.

1. Introduction

We have previously reported the development of a new drug delivery system (DDS) based on the carbohydrate recognition properties of phagocytic cells to control metastatic cancer in extranodal lymphoid tissue of the abdominal cavity [1]. Further, we demonstrated that our DDS could be used for the induction of CTLs through the presentation of exogenous antigens on MHC class I molecules of phagocytic cells [2].

In accordance with findings from many attempts, including ours, to generate antigen-specific CTLs, this paper provides an overview of current trials of liposome-based vaccines. Furthermore, we discuss the feasibility concerning our vaccination technique by summarizing accumulated knowledge regarding receptor candidates.

2. Overview

In order to reject invading pathogens and cancer cells, expansion of T cells is known to be activated by small peptides on Major histocompatibility complex (MHC) class I or MHC class II molecules on the cell surface of antigen-presenting cells (APCs) such as dendritic cells (DCs) and macrophages. We will mainly introduce recent progression of vaccine methods to generate $CD8^+$ cytotoxic T lymphocytes (CTLs) in this paper, while first mentioning the indispensable roles of $CD4^+$ helper T cells that support the expansion and persistence of CTLs [3–5]. Indeed, optimal antitumor immune responses are generated by the concomitant activation of both $CD8^+$ and $CD4^+$ T cells because of the selective activation of $CD4^+$ T cells with helper, but not regulatory functions [6]. Generally, exogenous antigens presented by MHC class II molecules are intended for $CD4^+$ T cells, whereas internal antigens from the cell itself, components of virus infected cells, and cancer antigens are presented on MHC class I molecules for activation and expansion of $CD8^+$ T cells. Consequently, it is necessary for vaccine development methods involving CTL generation to possess a mechanism whereby administrated exogenous antigens can be presented not only on MHC class II, but also class I molecules of APCs [6–8].

For vaccine development methods whereby exogenous antigens are exhibited both on MHC class I and class II molecules to induce antigen-specific $CD8^+$ and $CD4^+$ T cells,

our novel drug delivery system (DDS) using oligomannose-coated liposomes (OMLs) [1] that target phagocytic cells can be tailored for this purpose [2]. Indeed, a novel OML-based vaccine could reject transplanted tumor cells, prevent progression of encephalitis and vertical transmission, and reduce offspring mortality of *Neospora caninum* as shown in a feasibility study for its clinical use [2, 9–11].

OML-based vaccines produce strong adjuvanticity for CTLs. As liposomes coated by oligomannose are exclusively taken up by $F4/80^+$ intraperitoneal mononuclear cells and gathered at extranodal lymphoid tissues, the so-called "milky spots in abdominal cavity" [1], the underlining mechanisms of OML-based vaccine appear to be accompanied by an immune surveillance system for detecting pathogens invading the abdominal cavity in either a mannose dependant or mannose independent manner. Important roles for macrophages and complement systems are well known in the clearance of foreign materials, invading bacteria, and tumor cells from the abdominal cavity. Moreover, it is the milky spots that are the exact locus of this clearance process [12–15]. Taken together, a line of clearance process for OMLs may associate with strong adjuvanticity to induce CTLs.

Some diseases such as hepatitis C virus infection and malignancies still remain to have vaccine methods developed for them using disease-specific CTLs by elucidating their basic roles [5, 16, 17]. Many attempts to generate antigen-specific CTLs have been conducted, based on new experimental evidence. In accordance with these efforts, this paper will provide an overview of current trials concerning liposome-based vaccine delivery, and we discuss the feasibility of an OML-based vaccine based on recently accumulated knowledge of the carbohydrate recognition system as a target for OML-based vaccine delivery systems.

2.1. Liposome-Based Vaccine Delivery to Generate CTLs. Based on results from materials investigated for immunization, many types of liposomes have been tested for use in attempts to increase the effect of CTL generation against delivered vaccine antigens. New materials used in liposome preparation have been investigated to see whether they could effectively generate CTLs while monitoring the following three effects [18–20]: (1) an increase in the fusion efficiency between the cell membrane and liposomes, (2) the stabilization of liposomes in blood circulation, and (3) efficient delivery of vaccine antigens to APCs.

The approach tried first was to increase the fusion efficiency between the cell membrane and liposomes, because external antigens spilling from endosomes into the cytosol is considered to be the most important step for entry into the class I processing pathway for CTL generation [18, 21, 22]. To this purpose, use of peptide sequences referred to as antennapedia homeodomain [23, 24], and the hemolytic protein of *Listeria monocytogenes*, listeriolysin O [25], succeeded in enhancing the introduction of CTL epitopes into the class I processing pathway, resulting in the increased generation of CTLs. Furthermore, fusogenic liposomes prepared by fusing simple liposomes with Sendai virus particles can deliver encased antigens into the cytosol to generate CTLs [26].

Retaining liposomes in blood circulation is another way to increase the efficiency of CTL generation. Increasing retention time has been achieved by reducing surface-macromolecule interaction, which provides less opportunity for liposomes uptake by phagocytic cells and hepatocytes [27, 28]. The approach is highly effective for induction of CTLs against antigens encased in liposomes [29, 30]. To this purpose, polyethylene glycol (PEG)-modified lipids have become universally used in the preparation of liposomes (PEG-liposome) [29–31], which can more greatly enhance the generation of a $CD8^+$ T cell response than when given in soluble form or in conventional or positively charged liposomes [32]. Moreover, new lipids isolated from Archaea have also been used in the preparation of liposomes because of their stabilizing effect on liposomes in a manner similar to that for PEG-modified lipids. Archaea liposomes showed higher stability against extreme pH, oxidation, elevated temperatures, and action of lipases than conventional liposomes [33–35].

While higher stabilization in blood circulation increases CTL generation as discussed above, enhanced uptake by phagocytic cells has been indicated to elicit strong adjuvanticity to induce antigen-specific CTL responses [19]. The relevant examples of specific delivery of liposomes to phagocytic cells are cationic liposomes and OMLs. Positive charge on a liposome surface enhances uptake by APCs more than neutral membranes, and more robust immune responses for CTL generation and antibody production were seen in mice immunized using positively charged liposomes than with neutral liposomes [23, 28, 36–38]. To add positive charge to the surface of liposomes in earlier studies, cationic cholesterol derivatives such as 3β[N-(N′, N′-dimethylaminoethane)-carbamyl] cholesterol hydrochloride were frequently used.

Coating with ligands for pattern recognition receptors such as a mannose receptor (MR) on APCs is expected to have the analogous effect of adding positive charge to the liposome surface using cationic cholesterol derivatives, because ligand binding triggers endocytosis of liposomes by APCs such as DCs and macrophages [39, 40]. Related to this concept, Chikh et al. have indicated a line of phagocytic receptors for a variety of ligands, FCγ RI [41, 42], mannose [39, 40], $\alpha M\beta 2$ integrin (CD11b CD18) [43, 44], CD36, and $\alpha v\beta 5$ integrin [45], for forced uptake by APCs [19].

2.2. Carbohydrate Coatings on Liposomes to Deliver Vaccine Antigen to APCs. To induce robust immune responses using carbohydrates recognition by phagocytic cells, either mannose residues coupling on antigens or coating on an antigen encased liposomes appears to show promise. Using these methods, antigens were able to efficiently deliver phagocytic cells such as APCs, due to the uptake by mannose recognition receptors such as macrophage mannose receptor (MMR, CD206) and DC-specific intercellular adhesion molecule (ICAM)-3-grabbing nonintegrin (DC-SIGN) preferentially expressed on them, resulting in effective induction of CTLs [1, 46–50]. It may be associated with a nature of mannose residues, which significantly enhances immunogenicity of antigens and strongly promotes DC maturation through

TLR4 function [51]. Concerning carbohydrate coupling on antigen, not only the high-mannose oligosaccharide [47] and O-linked short mannose (2-3 mannoses) from *P. pastoris* [48–50], but also fucosylated oligosaccharides such as Lewis X or Lewis B [50], could be used to specifically deliver to dendritic cell by the other preferential binding specificity of DC-SIGN. Indeed, either mannosylated or fucosylated antigens could enhance CTLs responses depending on antigen presentation via class I molecules. [47, 49, 50].

Concerning mannose residues coating on liposome surfaces, we used mannotriose-dipalmitoylphosphatidylethanolamine (Man3-DPPE) [52] in the preparation of liposomes with dipalmitoyl phosphatidylcholine and cholesterol, at a molar ratio of 1 : 10 : 10 [52, 53]. Man3-DPPE, a neoglycolipid that is composed of Man3 and DPPE, is synthesized by reductive amination between an aldehyde group of oligosaccharides and the amino group of DPPE [52]. Because of the hydrophobic lipid moiety of the neoglycolipid, Man3-DPPE can easily be incorporated into the lipid bilayer of liposomes. The liposomes contain Man3-DPPE, and we have named them OMLs.

As mentioned in our previous studies, OMLs injected into the abdominal cavity are taken up by $CD11b^+$ phagocytic cells that deliver material to milky spots [1, 46]. Indeed, when 5-fluorouracil (5-FU) was encapsulated in the OMLs, more than 60% of the administered 5-FU accumulated in the omentum where milky spots gathered [1]. In other words, the OML-based DDS targets $CD11b^+$ phagocytic cells that act as cellular vehicles for material delivery. Recent use of hematopoietic or mesenchymal stem cells as cellular vehicles has led to significant progress in gene delivery techniques, while Burke has indicated the advantages of using phagocytic cells as natural cellular vehicles [54]. Phagocytic cells such as macrophages in the abdominal cavity take up large amounts of particles and accumulate them at not only lymphoid tissue, but also various pathological sites such as cancer lesions, wounds, atherosclerotic plaques, and arthritic joints [54]. Consequently, for delivery of materials, OMLs are a valuable tool for exploiting the nature of phagocytic cells.

We recently demonstrated the usefulness of OMLs as carriers for the delivery of vaccine antigen to generate and expand CTLs by employing ovalbumin (OVA) as a model cancer antigen [2]. Indeed, APC came to express OVA-derived peptides obtained by OML-based delivery in the context of both MHC class I and II molecules, which were evaluated by the detection of interferon gamma (IFNγ) production in the coculture with OML-delivered APCs and either $CD8^+$ or $CD4^+$ T cells from the spleens of T cell receptor transgenic mice OT-I (specific for H-2Kb/$OVA_{257–264}$) [55, 56] or OT-II (H-2Ab/$OVA_{323–339}$) [57], respectively [2]. Moreover, only the spleen cells from mice immunized with OML-OVA, but not bare liposomes without coating-encased OVA, showed cytotoxicity against E.G7-OVA, and only the mice preimmunized with subcutaneous challenge by OML-OVA rejected E.G7-OVA, but not EL4. These results together indicate that the OMLs can be used as an effective antigen delivery system for immunotherapy activating both CTL and Th subsets. OMLs are very useful not only for the promotion of nonglycosylated protein uptake by APCs, but also for the enhancement of antigen processing of encased antigens. This advantage of OML-mediated immunization will hopefully facilitate the simultaneous activation of tumor antigen-specific $CD4^+$ and $CD8^+$ T cells [2], and have the potential for use in cancer immune therapy [9].

It is well known that carbohydrates binding protein on APCs and complement lectin pathways recognize conserved motifs of glycans on pathogens. Carbohydrates binding proteins on phagocytic cells participate in the capture of materials to internalize, while the complement lectin pathway actively generates peptide fragments from C3, facilitating opsonophagocytosis by phagocytic cells through the complement receptors (CRs). Inhibition of complement component C3 and complement receptor type 3 (CR3, CD11b/CD18) could block the uptake process of OMLs by phagocytic cells [58, 59]. These observations support the hypothesis that carbohydrates binding receptors promote the uptake of liposomes in accordance with the activated lectin pathway, acting as an essential step in robust CTL responses against antigens encased in liposomes [59–61].

3. Possible Target Receptors on APCs Using Liposomes with Carbohydrate Coatings

In this section, we focus on receptor candidates on APCs for uptake of liposomes with carbohydrate coatings. Based on recent advancements in technologies to investigate structure–function relationships of glycans, knowledge about the properties of carbohydrate-binding proteins has dramatically increased, and offers their possible use as targets for the delivery of vaccine antigens. Here, we introduce DC-SIGN (CD209), MMR (CD206), and CRs to illustrate the possible mechanism for OML delivery to induce CTLs.

DC-SIGN (CD209) is a type II membrane protein, which is now established as a mannose-binding protein [62], and which appears to be a major receptor for OMLs [63]. It exhibits higher avidity to mannose through multimer formation, while there is not a one-to-one correspondence between the mannose and carbohydrate recognition domains (CRD) of DC-SIGN because of the binding specificity for *N*-acetylglucosamine (GlcNAc) and fructose (fuc) [62]. DC-SIGN was initially reported as having specific binding for HIV gp120 [64], and further, ICAM-2 and ICAM-3 on T cells were identified as ligands [65]. Because the interaction between DC-SIGN and ICAM-3 is inhibited by added free mannose, mannose residues of ICAM-3 act as ligands for CRD of DC-SIGN [65]. Though the ligand binding has been considered to enhance T cell activation by MHC class II (and possibly MHC class I) molecules in a restricted manner [66, 67], the exact role of how DC-SIGN associates to induce and activate CTLs remains to be elucidated.

The initial immunological role of MMR/CD206 has been considered to be for the surveillance of invading pathogens such as *Candida albicans* and *Pneumocystis carinii* [68, 69]. MMR has eight CRD/C-type lectin-like carbohydrate recognition domains, one fibronectin type II repeat domain (Fn-II D), five CRDs (CRD4–8), which bind with mannose, but also fuc and GlcNAc [70, 71]. Targeted delivery using a specific antibody to MMR increases the uptake of delivered

Table 1: Lectin-like receptors, complement receptors (CR), and ligands.

Receptors	Ligands
DC-SIGN (CD209)	Fucose, mannose, N-acetylglucosamine
DEC-205(CD205)	Unknown
Mannose receptor (CD206)	Fucose, mannose, N-acetylglucosamine
CR1 (CD35)	C3b, iC3b
CR2 (CD21)	C3d
CR3 (CD11b/CD18, Mac-1)	iC3b
CR4 (CD11 *c/CD18)*	iC3b
CR5 (CRlg, VSIG4)	iC3b

antigens by phagocytic cells, resulting in the concomitant activation of both $CD8^+$ and $CD4^+$ T cells through antigen presentation on MHC class I and class II molecules [39, 72]. This observation suggests a possible role of MMR in the induction of CTLs when OML is used for immunization.

Though DEC-205/CD205 has 10 CRDs and is highly homologous with MMR, its avidity to any glycans has not been detected. It may be caused by the limited number of glycan structures to perform binding analysis [73–75]. DEC-205 expresses exclusively on mature DCs, but for macrophage and immature DCs, targeting DEC-205 to deliver liposome-containing vaccine antigen has the potential to improve the efficiency of CTL generation because of possible adjuvanticity [76].

To date, five members of CRs (CR1-5) have been identified, and all of which are associated with opsonophagocytosis through the activation of the complement system [77]. Expression of all CRs has been exclusively detected on monocyte/macrophage lineage cells, while the distinct presence of CR1 and CR2 on erythrocytes and B cells is known [78]. Table 1 shows the differential binding specificity of each CR for C3 fragments [79–81].

CRs appear generally to have other functions besides facilitating opsonophagocytosis, known as their classical role. CRIg/VSIG4 is a recently identified fifth member (CR5) of the CR family, the long form of which is identical to Z39Ig reported in earlier studies [78, 81]. CR5, CRIg/VSIG4/Z39Ig, on monocytic cells can bind C3b and iC3b to internalize opsonized materials in the same way as other CRs, while it appears to play other roles, such as B7 family molecules, to suppress the activation and proliferation of CTLs [82, 83]. In addition, CR2/CD21 on B cells was demonstrated to transduce positive signals for antibody production upon complex formation with CD19 and CD81 [84]. Taking into account that CRs have another function besides internalization, CR3 (CD11b/CD18, Mac-1) [85] and CR4 (CD11b) may play some role in generating CTLs upon taking up OML [84, 85].

4. Possible Infectious Disease Targets for a Vaccine Strategy Using OMLs

In order to prevent manifestation of some diseases whose main effectors are CTLs, administration of recombinant antigen as a vaccine requires particular adjuvants to induce CTLs sufficiently to reject causative pathogens. To date, live vaccines are exclusively applied for universal prophylaxis of domestic animals, because they appear to have higher efficacy for illness given infection than either recombinant or inactivated vaccine containing adjuvant because of the advantage of induction efficiency of CTLs. Because vaccination with OMLs is expected to induce concomitant activation of both CTLs and Th1 cells, it has the potential to alter general approaches for infection control in domestic animals from live vaccines to recombinant antigen.

Based on increasing knowledge regarding OML-based vaccines, we have attempted to use them for control of some infectious diseases. Promising results using OMLs could be obtained for infection by *N. caninum* [10] and *Leishmania major* [53] using animal models. By administration of *Neospora* antigen NcGRA7 encased in OMLs to mice, NcGRA7-specific Th1 cells were generated, preventing the transition of the infection to the brain and transplacental vertical transmission [10]. Moreover, administration of apical membrane antigen 1 of *Neospora* using OMLs succeeded to reduce offspring mortality [11]. Whether the induced CTLs eliminated *N. caninum* in infected mice, it remained to determine increased numbers of the infection-specific CTLs by ELISpot assay, and estimate disease activity using genetically engineered protozoans expressing OVA as surrogate antigens. For the *L. major* infection, as earlier models with genetically engineered protozoans showed important roles of CTLs in the elimination of *L. major* [86] similar to the intracellular protozoan parasite *Toxoplasma gondii* [87], both antigen specific CTLs and Th1 cells induced by OML vaccination would show efficacy in preventing development of the illness.

We consider that CTL generation by OML-based vaccines can also be applied to *Theileriosis*. *Theileriosis* is a serious infection in cattle caused by tick-borne parasites, and is classified as a lymphoproliferative or hemoproliferative disease, depending on the principal pathogenic feature. *T. parva* and *T. annulata* are agents of lymphoproliferative *Theileriosis* [88, 89], and CTLs against *T. annulata* and *T. parva* are known to prevent disease progression [90, 91]. To date, live vaccine is known to be efficacious as a prophylactic agent. Administration of either *T. annulata* attenuated by long time in vitro culture or the infection-and-treatment method for *T. parva* [92, 93] are known as effective vaccination methods preventing the development of the lymphoproliferative disease. These results suggest that

it is possible to use OML vaccines to prevent the onset of lymphoproliferative *Theileriosis*.

5. Conclusion

In accordance with the findings from many attempts to generate antigen-specific CTLs, this paper presents an overview of current trials concerning liposome-based vaccine delivery. We discuss the feasibility of using OML-based vaccine based on recently accumulated knowledge regarding carbohydrate recognition systems as targets for OML-based vaccine delivery systems. In order to practically use OML-based vaccine to introduce CTLs for prophylaxis of some infectious diseases, general use of ELISpot assays may be needed to monitor the efficacy of CTL generation. Taking into account the recent trend of surveying for latent infection of *Mycobacterium tuberculosis* [94] using this method, universal use of OML-based vaccine to induce CTLs is not limited by this issue. Further studies characterizing the type of immune response induced by OML-based vaccine delivery in cattle are planned and should provide additional insights for the optimal development of OML-based vaccine to generate CTLs.

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Review Article

Combination of Intensive Chemotherapy and Anticancer Vaccines in the Treatment of Human Malignancies: The Hematological Experience

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In vitro studies have demonstrated that cancer-specific T cell cytotoxicity can be induced both ex vivo and in vivo, but this therapeutic strategy should probably be used as an integrated part of a cancer treatment regimen. Initial chemotherapy should be administered to reduce the cancer cell burden and disease-induced immune defects. This could be followed by autologous stem cell transplantation that is a safe procedure including both high-dose disease-directed chemotherapy and the possibility for ex vivo enrichment of the immunocompetent graft cells. The most intensive conventional chemotherapy and stem cell transplantation are used especially in the treatment of aggressive hematologic malignancies; both strategies induce T cell defects that may last for several months but cancer-specific T cell reactivity is maintained after both procedures. Enhancement of anticancer T cell cytotoxicity is possible but posttransplant vaccination therapy should probably be combined with optimalisation of immunoregulatory networks. Such combinatory regimens should be suitable for patients with aggressive hematological malignancies and probably also for other cancer patients.

1. Introduction

During the last two decades, effects of immunotherapy and autologous stem cell transplantation have been extensively studied in the treatment of human cancer. Immunotherapy often includes cancer vaccines, but vaccine-induced anticancer reactivity is often not associated with significant clinical responses [1–3]. Similarly, high-dose chemotherapy combined with autotransplantation has become a part of routine clinical practice only for a minority of cancer patients due to limited clinical benefits [4, 5].

Anticancer immune reactivity is probably important in autotransplantation, because early lymphoid reconstitution is associated with prolonged progression- or disease-free survival in many malignancies [6, 7]. This has been described in patients with B-cell malignancies, acute myeloid leukemia (AML), and solid tumors, suggesting that early reconstitution represents a general anticancer effect [7–10]. Even though cancer patients often have both disease-associated and treatment-induced immune defects that may persist for several months [11], the combined use of autotransplantation and anticancer vaccines should be considered to try to increase anticancer effects.

In the present paper we review the experience with intensive chemotherapy and immunotherapy for patients receiving intensive chemotherapy for aggressive hematological malignancies. We focus on acute myeloid leukemia (AML), one of the most aggressive human malignancies that is usually treated with very intensive therapy eventually in combination with stem cell transplantation. The experience from these patients is that anticancer immune reactivity is maintained and can be induced after the intensive treatment. It seems likely that similar therapeutic strategies should be possible also in other patients receiving less intensive chemotherapy for less aggressive malignancies.

2. Effects of Conventional Intensive Chemotherapy on T Cells

2.1. Early Effects of Chemotherapy on the T Cell System. Patients with acute myeloid leukemia receive intensive chemotherapy followed by a period of severe leukopenia, but even these patients have a functional T cell system, and rapid lymphoid reconstitution is associated with a decreased risk of AML relapse [11, 12]. T cell functions during cytopenia are characterised by the following.

(i) Circulating T cells are mainly T cell receptor (TCR)$\alpha\beta^+$ with only a minority of TCR$\gamma\delta^+$ cells. The CD4/CD8 ratio varies considerably between patients [13].

(ii) Chemotherapy-induced lymphopenia is not a random process and these patients have a decreased percentage of circulating clonogenic T cells. Their most important growth factors are IL-2 and IL-15, but several other cytokines can also cause detectable T cell proliferation [14]. T cell proliferation can be induced through the TCR-CD3 complex even in the presence of AML accessory cells [14], and responses are increased by CD28 mediated costimulation [15].

(iii) Activated T cells release several cytokines; high levels are detected for IFNγ, IL-6 and GM-CSF and detectable release of IL-2, IL-3, IL-4, IL-10, IL-13, and TNFα is often seen [16]. These responses can be enhanced by the protein kinase C agonist PEP005 [16].

(iv) Local T cell recruitment to the cancer cell compartment is essential for antileukemic T cell reactivity, and the chemotactic gradients are then determined by the systemic serum levels and the local levels in the cancer cell compartment. The systemic levels vary between patients and can be influenced by several factors, including the type of chemotherapy, patient age, and complicating febrile neutropenia [17]. Constitutive release of T cell chemotactic chemokines by the cancer cells may be an important determinant of the tissue chemokine levels [18].

Altogether, these observations clearly demonstrate that a functional T cell system remains during the severe post-treatment leukopenia even for patients receiving the most intensive conventional chemotherapy, and T cell targeting therapy may be possible even during this period.

2.2. Late Effects of Chemotherapy on Circulating T Cells. Studies of immunological reconstitution after conventional intensive chemotherapy has been carried out for patients with nonHodgkin's lymphoma, sarcomas and brain tumors, but these patients generally received less intensive chemotherapy than patients with AML [19–23]. These studies have described a decrease in circulating $CD4^+$ cells that may last for several months, and there seems to be a predominance of memory-type ($CD4^+CD45RO^+CD45RA^{+/-}$) cells. This defect seems to be at least partly age-dependent and is less pronounced in children [23]. The number of $CD3^-CD16^+CD56^+$ NK lymphocytes is usually normalised within 6 weeks [24]. It seems likely that similar defects are also seen after conventional chemotherapy for other malignancies, including the most intensive AML therapy. Certain drugs seem to cause more severe $CD4^+$ T cell defects; this is especially true for fludarabine that can be used as a part of conventional chemotherapy and in the reduced intensity conditioning before allogeneic stem cell transplantation [25]. Finally, the effect of chemotherapy on Treg cells seem to differ between cytotoxic drugs as reviewed recently [26], myeloid suppressor cells can be transiently increased early after recovery from chemotherapy and the heterogeneity within the dendritic cell population varies during the recovery phase after chemotherapy [27]. The status of all these three cell types may influence the response to anticancer vaccines.

3. Immunocompetent Cells in Autologous Stem Cell Grafts

3.1. Sources of Hematopoietic Stem Cells: Bone Marrow versus Peripheral Blood Harvesting. Hematopoietic stem cells (HSC) are now preferably harvested from peripheral blood after growth factor mobilisation [28, 29], and this method is associated with earlier engraftment than HSC aspired from bone marrow [30]. Neutrophil engraftment with peripheral blood neutrophils $>0.1 \times 10^9$/L is usually seen within 10–12 days and platelet engraftment with thrombocytes $>20 \times 10^9$/L within 12–14 days [30, 31]. The conclusion from the overall results is therefore very clear: (i) mobilisation of peripheral blood stem cells is safe and effective; and (ii) the short time until hematopoietic reconstitution when using mobilised cells has increased the safety of the procedure [32].

3.2. Preparation of Peripheral Blood Stem Cell Grafts. G-CSF is the most widely used drug for stem cell mobilisation [33] and is thought to stimulate immature stem cells to produce progenitors of all classes [34]. In addition G-CSF indirectly stimulates the production of progenitor cells by increasing the production of hematopoietic growth factors [35] and it promotes the release of progenitors into the general circulation [35]. When administered early after chemotherapy, G-CSF acts synergistically with the natural increase in HSC that is seen during the recovery phase [36]. The morbidity during and after G-CSF mobilisation is very low [37], and the most frequent side effects are bone pain, fever, and nausea. Successful mobilisation can also be achieved with longer-acting G-CSF (pegylated G-CSF) [38] its main advantage is less frequent administration [33].

Several strategies have been tried to increase the mobilisation of $CD34^+$ cells. A combination of G-CSF plus stem cell factor (SCF) will often more than double the yield of $CD34^+$ cells compared to G-CSF alone [39, 40]. The CXCR4 antagonist AMD3100 can also be combined with G-CSF to improve HSC mobilisation [41–43]. Finally, even though mobilisation regimens that include chemotherapy have more side effects than G-CSF alone [37], the use of chemotherapy is appealing because insufficient mobilisation of $CD34^+$ cells

is more common in cancer patients with G-CSF alone [44], and the disease-specific chemotherapy in such regimens can have additional anticancer effects.

3.3. Cryopreservation of Peripheral Blood Stem Cell Grafts: Effects on Immunocompetent Cells. Autologous stem cell grafts are usually cryopreserved, and the protocols are generally based on the use of the cryoprotectant dimethyl sulphoxide (DMSO) in the freezing medium [45–47]. After harvesting, the final product is often diluted with autologous plasma if the nucleated cell concentration is higher than 200 $\times$ 10^6 cells/mL to improve cell viability [48]. Most centers will use a controlled programmed freezer and storage in nitrogen at -160°C.

The grafts are usually prepared by apheresis procedures alone without further enrichment of $CD34^+$ stem cells, and large numbers of immunocompetent cells are therefore reinfused together with the stem cells. Early posttransplant lymphocyte reconstitution after both auto- and allotransplantation is associated with prolonged relapse-free survival in several malignancies [7–10]. For allotransplanted patients reconstitution of $CD4^+$ T cells seems particularly important, and infusion of a high number of $CD4^+$ T cells and NKT cells seems to be associated with a better prognosis [49]. This may also be true for autografted patients, and these observations suggest that immunological events early after reinfusion are important for the risk of later relapse/progression. The amount and quality of reinfused lymphocytes may therefore be essential.

We investigated the viability of total lymphocytes and the distribution of various T cell subsets in peripheral blood stem cell autografts after long-term storage with 2%, 4%, 5% and 10% DMSO [50]. The viability of the total lymphocyte population was significantly higher for cells preserved in 4% and 5% DMSO, but the DMSO effect differed between T cell subsets (Table 1). First, NKT cell viability was dependent on the DMSO concentration used. Second, naive and central memory T cells usually express CD62L, late effector T cells show intermediate/low expression, while effector memory T cells do not express CD62L [51]. The effect of DMSO differed between these CD62L-defined T cell subsets. Third, the CCR7 chemokine receptor is expressed by naive and central memory T cells and directs migration to lymph nodes; this homing process is important for initiation of immune responses [52]. Again the DMSO effect differed between CCR7-defined subsets and also between CCR2/CCR4 defined subsets. Finally, Foxp3 positive T cells are referred to as thymus-derived natural T regulatory (Treg) cells [53]. The percentage of $CD4^+CD25^+Foxp3^+$ cells among $CD4^+$ T cells was significantly lower after cryopreservation with 10% DMSO.

Several NK cell subsets have now been characterised, including immunoregulatory ($CD3^-CD56^{bright}CD16^{low/neg}$) and cytotoxic cells ($CD3^-CD56^{dim}CD16^{bright}$) [54–56]. Cryopreservation with various DMSO concentrations did not alter the percentages among viable graft lymphocytes of total NK cells ($CD3^-CD56^+$) or the various NK cell subsets [50].

Taken together these results suggest that the procedures used for cryopreservation affect lymphocyte viability; this is not a nonspecific effect but rather an effect that differs between various T cell subsets and it may thereby affect post-transplant T cell reconstitution and immunocompetence. The mobilisation and harvesting procedures would also be expected to influence the graft lymphocyte content but to the best of our knowledge this has not been investigated in clinical studies.

3.4. Enhancement of Antileukemic Reactivity in Autologous Stem Cell Grafts. The studies described above demonstrate that cytotoxic T cells are preserved and reinfused in autologous stem cell grafts. Furthermore, recent studies have demonstrated that T cells specific for leukemia-associated antigens remain in the circulation after intensive AML chemotherapy (see Section 5), and for this reason one would expect such cells also to be present in the autografts. At present it is not known whether reinfusion of such cells as a part of the transplantation procedure will have any clinical impact, or whether the immunocompetent cells in the graft will differ between various mobilisation procedures.

It is possible to separate graft cells into $CD34^+$ and $CD34^-$ cells before freezing [57], and the $CD34^-$ subset could then be used for ex vivo enrichment of cancer-reactive T cells before reinfusion together with the $CD34^+$ cells. Although techniques for enrichment of leukemia-reactive T cells are available, they have not been used in a large-scale clinical setting in combination with stem cell transplantation.

3.5. The Possible Use of Ex Vivo Expanded Cancer-Reactive T Cells. As stated above, in vitro expanded autologous T cells can be used in the treatment of cancer. This strategy has not been widely used in the aggressive hematological malignancies, but the experiences from solid tumors (e.g., malignant melanoma) suggest that such cells can induce clinically relevant antitumor activity [27, 58]. In a recent study a comparable strategy was also used as a posttransplant treatment in allotransplanted patient; donor-derived leukemia-reactive T cells were then reinfused in patients with leukemia relapse after allotransplantation [59]. Taken together these studies suggest that infusion of ex vivo generated cancer-reactive T cells can be combined with high-dose chemotherapy and possible also with vaccination strategies.

4. T Cell Reconstitution after Autotransplantation

T cell functions during the early posttransplant period with severe treatment-induced leukopenia seem to be very similar to AML patients receiving intensive conventional chemotherapy, and the later reconstitution also shows many similarities [60] (Table 2). Time until complete immunological reconstitution in adults may take years [61–63]. However, after transplantation the absolute number of circulating $CD3^+$ cells usually remains decreased for three to five months

Table 1: Cryopreservation of autologous peripheral blood stem cell grafts in cancer patients; a summary of the effects on immunocompetent cells when grafts were prepared with 2%, 4%, 5%, and 10% DMSO.

T cell population	Functional characteristics	Effects of cryopreservation with DMSO at various concentrations
Major T cell subsets		
$CD3^+CD4^+$	T helper	No effect of different DMSO levels
$CD3^+CD8^+$	Cytotoxic	No effect of different DMSO levels
$CD3^+CD56^+CD16^+$	NK T cells	Highest viability with 2% DMSO
$CD3^+CD56^+CD16^-$	NK T cells	Highest viability with 2% DMSO
CD62L-defined subsets		
$CD4^+CD62^+$, $CD8^+CD62^+$	Naive and central memory	Highest viability when using DMSO 5%
$CD4^+CD62^{low}$, $CD8^+CD62^{low}$	Late effector T cells	$CD4^+$ cells show highest viability with DMSO 2% and 4%
$CD4^+CD62^-$, $CD8^+CD62^-$	Effector memory	No effect of different DMSO levels
Subsets defined by chemokine receptor expression		
$CD4^+CCR7^+$, $CD8^+CCR7^+$	Naive and central memory T cells	The $CD4^+$ cells showed decreased viability when using 10% DMSO
$CD3^+CD45^+CCR4^-CCR6^-$		Decreased viability when using 2% DMSO
Other CCR2, CCR4, CCR7 defined T cell subsets		No effect of different DMSO levels
$CD4^+CD25^+FoxP3^+$ T cells	Natural regulatory T cells	Decreased viability when using 10% DMSO

Autografts were prepared for cancer patients after mobilisation with chemotherapy plus G-CSF. After the aphereses cell concentrations were adjusted and cells stored in nitrogen for 5-6 years as described in the text [50].

[64]. Low levels of circulating $CD4^+$ cells have been reported for 12–18 months [65], whereas $CD8^+$ T cells usually recover within 3–12 months [64]. Defective proliferation of both $CD4^+$ and $CD8^+$ T cells in response to antiCD3 and antiCD2 persists for at least for 2–4 months, and this seems to be caused by defective IL-2 responsiveness [64, 66]. Furthermore, the specific cytotoxic T cell response against Epstein-Barr virus is significantly impaired for 2–5 months [67], whereas the frequency of circulating cytokine-secreting T helper cells and IL-2 responding T cells can be decreased for up to 5 years posttransplant [68].

Peripheral blood mobilized stem cells (PBMSC) are now used for most cancer patients treated with autologous stem cell transplantation. The immunological reconstitution differs between patients receiving peripheral blood and bone marrow autografts [64], and the following data on T cell subset reconstitution refers to patients transplanted with mobilized stem cells. PBMSC autografted patients show early recovery of $CD14^+$ monocytes and $CD56^+$ NK cells during the first month after autotransplantation [64, 69, 70]. The homeostasis of total circulating dendritic cells is usually achieved relatively early after transplantation, although differences in dendritic cell subset composition may be detected for several months [70]. However, as pointed out by these authors the kinetics of dendritic cell reconstitution may differ between patients and also depend on the chemotherapy regimen [70]. In contrast, a long-lasting T cell defect similar to chemotherapy-treated patients is also observed after autotransplantation [69, 70]. This defect is detected after 6 months for most patients, and for a minority the defect will last for more than a year [69].

Table 2: T cell reconstitution after autologous stem cell transplantation.

Immunocompetent cell	Time until reconstitution
Number of circulating $CD4^+$ T cells	1–5 years
Number of circulating $CD8^+$ T cells	3–12 months
T cell proliferation	3 months–5 years
T cell cytokine production	6 months–5 years
T cell response to exogenous IL2	7 months–5 years
Cytotoxic T cells	2 months–5 years

Adapted from article by Porrata et al. [60]. In general there is considerable variability in the data that have been found for T cell reconstitution, both in-between patients and studies. Thus, the timeframes indicate when the majority of patients can expect to reach normal values.

The total levels of $CD8^+$ T cells seem to normalize within a few months, whereas total $CD4^+$ T cell counts remain decreased for several months. The CD4 defect is mainly due to a reduction of naive $CD3^+CD4^+CD45RA^+$ T cells, and there seems to be a reduction even of $CD8^+$ naive T cells [69, 70].

As discussed by Dreger age may influence the T cell reconstitution after autotransplantation [69]. Immunogenetic factors may also be important for posttransplant T cell functions; single nucleotide polimorphisms in immunoregulatory chemokine/cytokine genes seem to influence the risk of infections, graft versus host disease and leukemia relapse after allogeneic stem cell transplantation. (for a detailed discussion and additional references see

Loeffler [71]). Such immunogenetic influences may also be important for the response to posttransplant vaccination therapy after conventional chemotherapy or stem cell transplantation.

The number of mature B cells is markedly decreased during the first 3 months posttransplant, but will thereafter gradually increase although complete normalisation may take up to 18 months [64]. Both T cell-dependent and independent B cell response are decreased for 12–18 months [72], and the IgM production will normalise earlier (often within 6 months) than the corresponding IgG response [72].

NK cells usually recover within 1 month after transplantation [73]. The NK cell number is often increased on day 15 posttransplant compared with normal individuals, but by day 75 NK cell activity has usually returned to a normal level. NK cells are important for rejection of malignant cells [74], and pharmacological agents that increase cancer cell susceptibility to NK cell mediated lysis are now being developed [75].

As described in a recent review the immunological reconstitution after allogeneic stem cell transplantation differ between patients receiving myeloablative and reduced intensity conditioning therapy [25]. Briefly, allotransplantation is also associated with a similar quantitative defect in $CD4^+$ T cells, this defect may last for several months but early normalization seems to be more common for patients receiving reduced intensity conditioning (for detailed discussion and additional references see [25].

5. Induction of Anticancer Reactivity by Vaccination Is Possible in Heavily Pre-Treated Patients

A major question is whether it is possible to induce anticancer T cell reactivity and especially cytotoxicity in patients who have recently received intensive chemotherapy. T cells specific for cancer-associated antigens can be detected in healthy individuals and also in untreated cancer patients despite their disease-induced immunosuppression, and many of these cells seem to be $CD8^+$ cytotoxic effector memory cells [76]. Many cancer patients develop additional therapy-induced immune defects. However, if it is possible to induce anticancer T cell reactivity by vaccination in AML patients who have a very aggressive disease and receive the most intensive chemotherapy, one would expect immunotherapy to be effective also in other cancer patients. Several strategies for immunotherapy in AML have been tried (Table 3). In this paper we will review the results from vaccination studies.

5.1. AML-Associated Peptide Antigens Used for Vaccination. AML patients are very heterogeneous with regard to genetic abnormalities encoding leukemia-specific antigens [77]. To find a common vaccination strategy the available studies have therefore focused on the use of either whole AML cells (cell lysates or modulated AML cells) or peptides derived from AML-associated proteins.

WT1 is a zinc finger transcription factor that is expressed in normal $CD34^+$ hematopoietic cells, myoepithelal progenitors, renal podocytes, and some cells in testis and ovary [78]. This protein is overexpressed in several hematological malignancies and solid tumors [78]. Antibodies against this molecule have been detected in cancer patients and several immunogenic peptides have been identified [78]. Briefly, both CD4 and CD8 T cell epitopes have been identified, and HLA-A0201 and HLA-A24-restricted $CD8^+$ T cell cytotoxicity against WT1 expressing cancer cells have been detected. The CD4 peptides bind to different HLA-class II molecules and induce $CD4^+$ T cell responses that enhance cytotoxic T cell reactivity either through induction of $CD4^+$ cytotoxic T cells or through induction of $CD4^+$ Th1 helper cells. Some peptides include epitopes recognised both by $CD4^+$ and $CD8^+$ T cells. Finally, a recent study demonstrated that WT1-specific cytotoxic T cells remain even after remission-inducing intensive AML chemotherapy [79].

Proteinase 3 is a differentiation antigen that is also overexpressed in leukemic blasts [78]. A proteinase 3-derived peptide named PR1 has been identified by screening for binding avidity to HLA-A0201 [78]. In vitro studies suggest that PR1-specific T cells can kill leukemic cells, including the more immature clonogenic subsets, but not normal hematopoietic stem cells [78, 80, 81]. However, it should be emphasized that these data are mainly based on studies of healthy individuals and cancer patients receiving low-toxicity chemotherapy. Untreated AML patients have also been investigated and circulating PR1 specific T cells could not be detected then [78]. This may be due to apoptosis of high-avidity T cells induced by exposure to high peptide concentrations or leukemic cells overexpressing the proteinase 3 [78]. However, PR1 specific T cells can be detected later after remission-inducing AML chemotherapy [79].

The receptor for hyaluronic-acid-mediated motility (RHAMM) is overexpressed in leukemic blasts from AML and CML patients but not in normal $CD34^+$ hematopoietic cells [78]. Greiner et al. identified a RHAMM-derived peptide (referred to as the RHAMM-R3 peptide) that could be presented by HLA-A2 and recognised by $CD8^+$ T cells [78, 83]. This peptide was a naturally processed T cell epitope, specific T cells were detected in AML patients even following intensive chemotherapy, and in vitro primed T cells could lyse human AML blasts.

Taken together these studies clearly demonstrate that cancer-specific, HLA-restricted T cell reactivity, including specific cytotoxicity, is maintained even after the most intensive chemotherapy.

5.2. Peptide Vaccination in AML. Oka et al. investigated the effect of WT1 peptide vaccination in 26 cancer patients, including 13 patients with *de novo* AML [84]. They used intradermal injection of a modified 9-mer WT1 peptide emulsified in Montanide ISA51 adjuvant; 18 of the 26 patients completed the vaccination protocol with 3 or more injections every second week and most patients were

Table 3: Immunotherapy in AML; the advantages and disadvantages of the various approaches that have been investigated in clinical trials [82].

Strategy	Advantage	Disadvantage
Peptide vaccination	Easy to produce and administer	Selection of patients with certain HLA types and high antigen expression in the malignant cells
Normal dendritic cells loaded with AML-associated peptides	Presentation of several leukemia-specific and leukemia-associated antigens	Work-consuming in vitro procedures for preparation and antigenic loading (lysates, mRNA).
Whole tumor cell vaccines with irradiated AML cells:		
(i) Leukemic cells plus systemic administration of immunostimulatory cytokines	Relatively easy to prepare, several antigens presented	Clinical side effects
(ii) Modified leukemic cells expressing GM-CSF or CD80+IL2	Several antigens presented	Complicated and work-consuming ex vivo handling
Leukemic dendritic cells	Presentation of several leukemia-specific and leukemia-associated antigens	Heterogeneity between patients with regard to efficiency; work-consuming in vitro procedures for preparation and antigenic loading (lysates, mRNA).
IL-2 therapy	Easy to administer, induces innate and specific immunity	Serious side effects

vaccinated with a modified peptide that gave stronger cytotoxic T cell responses than the natural peptide. All patients were HLA-A2402 positive and their malignant cells showed high WT1 expression. Tetramer flow cytometry of circulating cells showed an increase in specific T cells during vaccination for 9 of the 13 AML patients. An increase in antigen-specific induction of IFNγ expression was also observed for 6 of the patients. Only 10 of these patients could be evaluated with regard to clinical responses: (i) 2 patients showed decreased residual AML; (ii) stable disease was seen for 2 patients; (iii) bone marrow expression of WT1 was used as a surrogate marker of residual disease for those patients without detectable AML blasts, and for 5 of these patients decreased expression was detected following vaccination; (iv) 1 patient showed progressive disease. There was a statically significant correlation between clinical and immunological responsiveness. These observations suggest that WT1 vaccination can induce a specific T cell response; these T cells can locate to the bone marrow compartment and they mediate WT1-specific antileukemic effects.

Another study examined a regimen with GM-CSF therapy on days 1–4 and vaccination with antigenic peptide + keyhole limpet hemocyanine on day 3 [1]. All patients were HLA-A2 positive and had high expression of WT1 in their leukemia cells, 17 out of the 19 included patients had received previous chemotherapy and all patients had detectable AML with increased bone marrow blasts. The vaccines induced immunological responses judged from tetramer analyses of peripheral blood, and a significant increase of these cells was also seen in the bone marrow. Importantly, responses were recorded especially in patients that had received previous chemotherapy and showed relatively low levels of bone marrow blasts (<50%). Reduction of bone marrow WT1 levels was observed for a subset of patients following vaccination. Thus, previous intensive chemotherapy does not eradicate leukemia-reactive T cells; the chemotherapy-induced reduction of the AML cell burden rather seems to reduce disease-induced immune defects and thereby increase the efficiency of the vaccination.

PR1 responses have been investigated in a study that combined vaccination with WT1 and PR1 peptides [81]. This vaccine was also based on concomitant GM-CSF administration and subcutaneous administration of peptides in Montanide adjuvant. Five AML patients in complete remission after previous intensive chemotherapy were included. Responses were evaluated by tetramer staining, and immunological responses were detected for one or both peptides in most patients. Increased T cell responses were also detected by IFNγ expression after specific stimulation. Bone marrow expression of WT1 was used as a surrogate marker for residual disease, and these levels decreased when immunological responses became detectable. Thus, even though in vitro exposure of PR1-specific T cells to AML cells with high antigen levels causes apoptosis of these cells (see Section 5.1), detectable PR1 T cell responses could be induced early after induction chemotherapy.

The RHAMM-R3 peptide identified in previous in vitro studies has been tried for vaccination in HLA-A2$^+$ patients with hematological malignancies [85]. This study included only 10 patients with AML, MDS, or multiple myeloma; all 3 AML patients had received intensive chemotherapy before vaccination and the 4 myeloma patients had received autologous stem cell transplantation. The vaccine consisted of injection of 300 μg peptide in incomplete Freund adjuvant

subcutaneously on day 3, GM-CSF was administered on days 1 and 5, and this cycle was repeated 4 times with 2-weeks intervals. Immunological responses were evaluated by tetramer flowcytometry and ELISpot analysis for IFNγ and Granzyme B. An immunological response was detected by at least one of these methods for 9 patients, only 1 AML patient in relapse did not respond. Vaccination-induced T cell cytotoxicity towards autologous AML cells or HLA-A2$^+$RHAMM$^+$ target cells could also be detected. A clinical response with further reduction of bone marrow blasts was observed for 1 AML and 2 MDS patients. This study illustrates that T cell reactivity against leukemia-associated antigenic epitopes is maintained after intensive conventional chemotherapy as well as autologous stem cell transplantation, and this reactivity can be enhanced by peptide vaccination.

The clinical toxicity of vaccination was generally low, the most common side effect being grade 1-2 reactions with pain and erythema at the injection site. One study observed progressing leukopenia in two patients with MDS and MDS-AML, respectively; this may be caused by immunological reactivity against normal stem cells in patients with disease-induced reduction of normal hematopoiesis [3]. Oka et al. also described a patient with a febrile reaction during the first injection [3].

5.3. Cell Vaccines. A recent article described two patients vaccinated with ex vivo generated monocytic dendritic cells that had been incubated with leukemic cell lysates and keyhole limpet hemocyanine [86]. Subcutaneous injection of the pulsed dendritic cells was well tolerated. Another study also investigated preparation of monocyte-derived dendritic cells in AML; these authors combined ex vivo generation of the cells followed by cryopreservation before transfection of WT1 mRNA by electroporation [87]. The procedure was successful for all patients and injections were well tolerated. It is difficult to see from these articles whether immunological responses were induced by the vaccination. An additional advantage with this approach could also be activation of NK cells and not only specific T cells and thereby induction of an additional anticancer effect [88].

Primary human AML cells can be induced to differentiate in the direction of a dendritic cell phenotype by exposure to various cytokines or cytokine combinations. These cells show dendritic morphology, increased expression of T cell costimulatory molecule, increased antigen-presenting capacity and a constitutive chemokine release profile consistent with a dendritic cell phenotype [89, 90]. One study has reported the efficiency and toxicity when using a vaccine based on subcutaneous injection of dendritic AML cells [91]. Five patients treated in a palliative setting were included, and the authors observed increased immune responses towards a peptide derived from the leukemia-associated antigen PRAME. Thus, this methodological approach also seems to be effective and feasible. However, care should be taken when injecting ex vivo expanded cells, and possibly the cells should be irradiated before injection [89].

Even though the experience with dendritic cell vaccines in aggressive hematological malignancies is limited, the experience from other malignancies is promising [92]. The dendritic cells orchestrate a repertoire of immune responses, but various dendritic cell subsets differ in their immunoregulatory characteristics [93]. Dendritic cell vaccines can thereby be used for cross-presentation of cancer-associated antigens; a possible approach then being to load the cells with autologous cancer cell lysates [94]. This therapeutic strategy seems safe and effective. As an example, a recent study in patients with lung cell cancer showed no serious side effects and increased T cell responsiveness to cancer-associated antigens for more than half of the patients vaccinated with antigen-loaded autologous dendritic cells [94]. Another strategy is viral transfection of cancer-associated antigens alone or antigens together with various immunostimulatory molecules [95, 96]. The experiences from other cancers suggest that such therapeutic strategies should be further investigated also in hematological malignancies, and combination of chemotherapy and dendritic cell vaccination should then be possible. However, the optimal procedures for antigenic loading and dendritic cell preparation remain to be established.

6. Future Directions

6.1. Design of the Chemotherapy in Combination Regimen. The general intensity of the chemotherapy has to be decided based on a clinical evaluation of the patients, and one has to take into consideration that there is evidence from clinical studies that a low cancer cell burden is associated with increased anticancer T cell reactivity [1, 97]. Several additional points also have to be considered. Firstly, if possible one should use a regimen that induces immunogenic cancer cell apoptosis that will enhance anticancer immune reactivity; this has been described especially for the anthracyclines [98] (Figure 1). Second, if possible the chemotherapy should reduce the levels of regulatory T cells [99] (see Section 6.3). Finally, the vaccine studies described above started at least 4 weeks after chemotherapy, but even patients with severe chemotherapy-induced cytopenia have an operative T cell system and immunotherapy can probably start even earlier after chemotherapy [100].

6.2. The Advantage of Including Autologous Stem Cell Transplantation. There are several possible advantages if autotransplantation is combined with vaccination therapy. Firstly, additional disease-directed chemotherapy can be administered both for mobilisation and as high-dose intensive chemotherapy before transplantation; this is safe and may further reduce the cancer cell burden [101]. Secondly, graft preparation offers the possibility to manipulate the immunocompetent graft cells and thereby combine chemotherapy and immunotherapy. CD34 enrichment is now possible as a part of routine therapy before cryopreservation [102], and by using similar methodological approaches as previously used in experimental studies, one

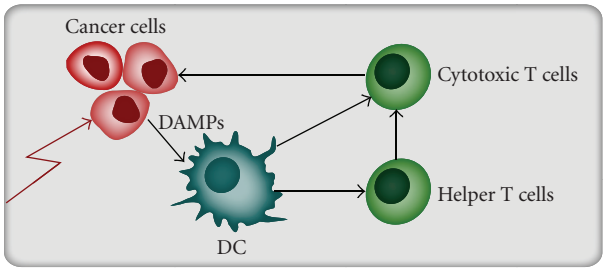

Figure 1: Induction of immunogenic apoptosis. Certain chemotherapeutics agents (e.g., the anthracyclines) will induce immunogenic apoptosis in cancer cell through induction of Danger-Associated Molecular Patterns (DAMPs). Examples of DAMPs are various Heat shock proteins and ecto-calreticulin exposure on the cancer cell surface. This pattern will induce dendritic cell (DC) maturation with development of specific T helper cell responses and enhancement of cancer-specific T cell cytotoxicity.

could enrich anticancer cytotoxic T cells and reinfuse them as a part of the transplantation procedure. Finally, even though both chemotherapy and autotransplantation seem to induce a general quantitative $CD4^+$ T cell defect, intensive chemotherapy does not eradicate cancer-specific T cells and enhancement of this reactivity in graft immunocompetent cells or posttransplant cells should therefore be possible.

6.3. The Vaccination Procedure. The administration route of vaccines will probably be important. For example, animal experiments have shown that dendritic cells administered subcutaneously will localise to the draining lymph nodes, whereas intravenous administration will cause localisation to the spleen [103]. Several techniques are now available for preparation of the vaccines; these strategies have been reviewed recently and are exemplified by the results summarized in Figure 2 and Tables 3 and 4 [82, 104].

Manipulation of immunocompetent cells through stimulation of Toll-like receptors (TLR) seems to be of particular interest [108, 109]. TLR9 is expressed by dendritic cells and B cells, and CpG oligonucleotides can be used as TLR9 agonists. These oligonucleotides will increase anticancer immune reactivity through several mechanisms, including increased presentation of cancer-associated antigens by dendritic cells [105–107, 110] (Figure 2). These agents have been used alone, but they can also be used as adjuvants in cancer vaccines and be combined with the Montanide adjuvant used in the peptide vaccines (see Section 5.2). However, the overall results from experimental and clinical studies with regard to effects of adjuvants on T regulator cells (Treg) are conflicting and require further studies.

Other adjuvants should also be tried in vaccination therapy, especially chemokines that are now considered as possible therapeutic targets in proinflammatory conditions [111]. These mediators can be released by malignant cells and have several biological effects including chemotactic, growth-stimulatory, immunomodulatory, and angioregulatory effects [26, 43, 112]. Other cytokines than GM-CSF are now investigated as vaccine adjuvants, including Flts-ligand as well as the chemokine CCL5 and CXCL9 [113–115]. These experiences from animal cancer models suggest that such approaches should be tried also in the treatment of human cancers, although the possible roles of chemokine decoy receptors have to be explored [116].

An alternative to in vivo expansion of antigen-specific T cells through vaccination would be ex vivo enrichment of specific cells followed by infusion of these cells. Based on experimental observations various strategies may be possible, including (i) stimulation and thereby in vitro prokiferation/expansion of antigen-specific T cells; or (ii) ex vivo generation of antigen-specific T cell reactivity through viral transduction of specific T cell receptor genes. First, antigen-loaded dendritic cells can be used to stimulate proliferation of cancer-specific cells, and this expansion can be increased by subsequent costimulation through CD28 [117, 118]. Costimulation through the CD40/CD40-ligand system may also be possible [119]. Second, T cell receptor gene-modified lymphocytes can be generated, such cells persist in patients after infusion and reduction of tumor cell burden has been described [120, 121]. Whether in vivo (vaccination) and ex vivo expansion (in vitro culture) of cancer-reactive T cells can be combined in cancer patients has not been clarified, and future studies also have to clarify whether these therapeutic strategies will be effective in hematologic malignancies.

6.4. Targeting of Immunoregulatory Cells Together with Leukemia-Specific Cytotoxicity: The Importance of Th17 Cells and Treg Cells. IL-17 is a family of T cell derived cytokines that triggers the production of proinflammatory cytokines and chemokines by a wide range of cells, including epithelial cells, endothelial cells and macrophages [122, 123]. In healthy individuals circulating Th17 cells constitute less than 1.0–1.5% of total circulating T cells [124], increased levels are observed in patients with advanced cancers, and tumor infiltrating Th17 cells have been detected in ovarian, pancreatic and renal cell cancer [125]. Several experimental observations suggest that Th17 cells can increase specific antitumor immune activity [126] as well as anticancer NK cell reactivity [127]. These animal studies suggest that the role of Th17 cells in human cancer should be further investigated, including the cryopreservation of Th17 cells in autologous stem cell grafts and the possibility to enhance anticancer reactivity through ex vivo enrichment of Th17 cells in the autografts before reinfusion.

Immunosuppressive Treg cells comprise 5–15% of peripheral $CD4^+$ T cells [128, 129]. In animal models these cells prevent autoimmune diseases, graft rejection and anticancer reactivity [128, 130]. Several studies suggest that cytotoxic agents can alter the levels of Treg cells. Fludarabine therapy in patients with chronic lymphocytic leukemia often causes a decrease or abrogation of the activity of Treg cells [131]. Suppression of Treg cells by cyclophosphamide may allow immunotherapy of established tumors to be curative in animal models [132], but no effect is observed after a single cyclophosphamide infusion combined with nonspecific

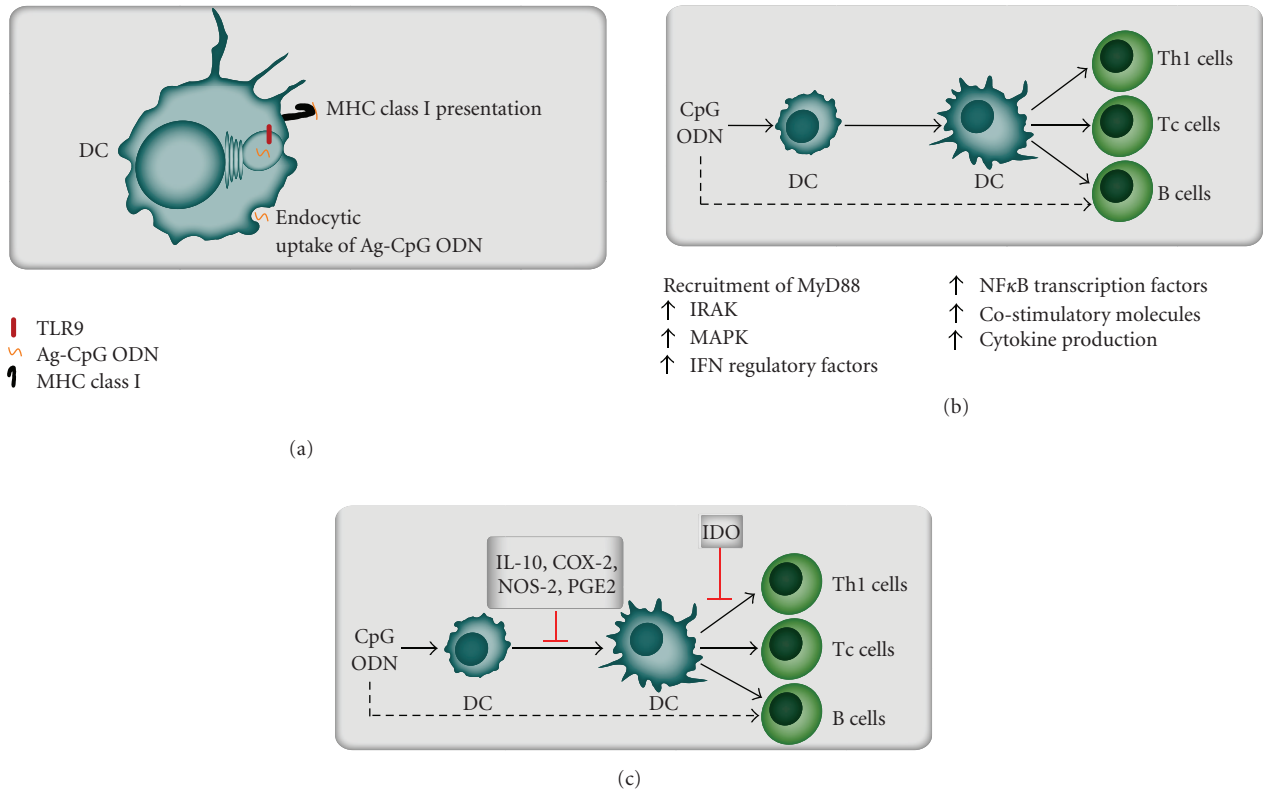

Figure 2: The immunostimulatory effect of TLR9 ligation by CpG oligonucleotides. (a) TLR9 is normally activated by nonmethylated CpG dinucleotides (DNA motifs). In vaccination therapy TLR9 can be activated by synthetic oligodeoxynucleotides (ODN) containing CpG motifs (CPG ODN), these molecules can be linked to antigenic peptides (Ag CpG ODN). This complex is endocytosed by dendritic cells (DC); the antigen is then presented and CpG ODN enhances the accessory cell function of the dendritic cells [105–107]. (b) Binding of CpG ODN by TLR9$^+$ dendritic cells initiates signal transduction through members of the IL-1 receptor-associated kinase (IRAK) family, mitogen activated kinases (MAPK) or Interferon (IFN) regulatory factors. These events lead to activation of nuclear factor kappa B (NFκB) transcription factors with increased cytokine release and expression of costimulatory molecules [108]. (c) Inhibitory control mechanisms of CpG-mediated immune activation seem to include induction of IL-10, cyclooxygenase-2 (COX-2), NO synthase 2 (NOS-2) and prostaglandin E2 (PGE2). Intravenous administration of CpG ODN to mice induce splenic expression of the enzyme indoleamine 2,3-dioxygenase (IDO) that is an enzyme associated generation of regulatory T cells (Treg) and thereby inhibition of Th1 cells, cytotoxic T cells (Tc cells) and B cells [108].

Table 4: Preparation of vaccines for lymphomas, an overview of possible methodological approaches [104].

Procedure	Advantages
Improving antigen delivery	
DNA vaccines	Skin or muscle injection of cDNA encoding the antigen. Protein is endogenously produced, and the epitopes can be combined with sequences from the carrier proteins or adjuvant proteins that increase immunogenicity
Liposomal vaccines	Antigens are supposed to be delivered both for endosomal (CD4$^+$ responses) and cytosolic processing (CD8$^+$ responses), combination with adjuvant is possible and custom-made vaccines can rapidly be produced.
Increasing antigen presentation	
Normal dendritic cell vaccines	Dendritic cells are regarded as the most powerful antigen-presenting cells; the cells can be pulsed by either cell lysates, heat shock proteins with bound client proteins or apoptotic cell organelles.
CpG vaccines	Dendritic cells are activated via toll-like receptors; these antigen-presenting cells will take up cancer-derived peptides and this approach thereby bypasses the step of custom-made vaccines. One approach is pre-vaccination local therapy that induces apoptosis, and local CpG-injection will then enhance the uptake and presentation of peptides derived from malignant cells
Malignant dendritic cells	Can be prepared for various hematological malignancies; these cells will present several tumor-specific as well as tumor-associated antigens.

immunotherapy in patients with metastatic carcinoma [133]. The mTOR antagonist rapamycin that is used in anticancer therapy, increases the number of Treg cells [134], whereas docetaxel does not seem to have any effect [135]. Thus, the effects of anticancer therapy on Treg levels seem to differ between therapeutic agents.

Previous studies in AML have demonstrated that increased Treg cells are detected in patients with newly diagnosed AML, and these high levels persist even after intensive chemotherapy and induction of disease control with hematological remission [136]. Thus, the overall intensity of the chemotherapy is not decisive for elimination of these cells; rather the design and use of specific drugs seem to be essential. Experimental studies have demonstrated that suppressive Treg cells can be stimulated to develop into proinflammatory Th17 cells [137]. The possibility to use this approach instead of chemotherapy for elimination of Treg cells and enhancement of immunoreactivity should be explored both with regard to ex vivo manipulation of stem cell grafts and in vivo immunomodulation.

6.5. The Roles of Immunogenetics. Many of the vaccination studies reviewed above included only patients with certain HLA-types known to bind and present the vaccine peptides. Future studies have to consider how vaccination strategies should be designed to include all patients and not only selected subsets. Other immunogenetic factors also need to be considered then, for example, genetic polymorphisms in the chemokine system [71] or in T cell regulatory molecules [110].

6.6. Final Comment. Available studies have demonstrated that cancer cell vaccines can induce anticancer immune reactivity. This is possible even for patients with the most aggressive hematological malignancies that are treated with very intensive chemotherapy, and it should therefore be possible also in other malignancies. The future challenge will now be to design optimal combinations of conventional disease-reducing therapy (chemotherapy, surgery, and irradiation), induction of antigen-specific immunity through vaccination and antigen-nonspecific immunomodulation (e.g., targeting of Treg and Th17 cells as well as NK cells) to enhance anticancer reactivity.

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Research Article

Cell Density Plays a Critical Role in Ex Vivo Expansion of T Cells for Adoptive Immunotherapy

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The successful ex vivo expansion of a large numbers of T cells is a prerequisite for adoptive immunotherapy. In this study, we found that cell density had important effects on the process of expansion of T cells in vitro. Resting T cells were activated to expand at high cell density but failed to be activated at low cell density. Activated T cells (ATCs) expanded rapidly at high cell density but underwent apoptosis at low cell density. Our studies indicated that low-cell-density related ATC death is mediated by oxidative stress. Antioxidants N-acetylcysteine, catalase, and albumin suppressed elevated reactive oxygen species (ROS) levels in low-density cultures and protected ATCs from apoptosis. The viability of ATCs at low density was preserved by conditioned medium from high-density cultures of ATCs in which the autocrine survival factor was identified as catalase. We also found that costimulatory signal CD28 increases T cell activation at lower cell density, paralleled by an increase in catalase secretion. Our findings highlight the importance of cell density in T cell activation, proliferation, survival and apoptosis and support the importance of maintaining T cells at high density for their successful expansion in vitro.

1. Introduction

T cells are a critical component of the cellular immune response. In the past two decades, adoptive transfer of tumor reactive T cells into cancer patients has been created as an immunotherapy method to combat cancer [1]. This includes the early studies with lymphokine-activated killer (LAK) cells derived from ex vivo amplification of autologous lymphocytes with interleukin-2 (IL-2), late studies with tumor infiltrating lymphocytes (TILs) isolated from tumor specimens, and recent studies with genetically modified tumor reactive T cells [2]. The prerequisite for the success of adoptive immunotherapy relies on the successful ex vivo expansion of a large amount of T cells (up to 10^{11}).

The ex vivo expansion of T cells for adoptive immunotherapy usually involves two phases. The first phase is T cell activation, in which resting T cells are activated with anti-CD3 antibody or plus anti-CD28 antibody supplemented with IL-2. The second phase is activated T cell (ATC) proliferation. After activation, resting T cells become ATCs and undergo vigorous cell proliferation for about two to three weeks and the ATCs lose their proliferation capacity in about four weeks. Maintaining high cell density has been considered important among investigators performing ex vivo T cell expansions for clinical therapeutic applications. This report addresses formally the basis for this observation.

Cell density has been reported to be an important factor in maintaining certain T and B cells in vitro. Resting T cells die rapidly by apoptosis when cultured under diluted conditions but survive for extended periods when cultured at high cell density [3]. This effect was found to be mediated by soluble factors and independent of integrin-mediated signals. An acute T-lymphocytic leukemia cell line, CCRF-CEM, was reported to display a cell density-dependent growth characteristic [4]. CEM cells grow well at cell density $>2 \times 10^5$ cells per mL, but at low cell densities the

cultures rapidly undergo apoptosis. The viability of low-density CEM cells could be preserved by supplementing with "conditioned" medium from high-density CEM cultures. Catalase was identified as the active component in the conditioned medium. B cell chronic lymphocytic leukemia (CLL) was reported to be dependent on cell density for surviving in cultures [5]. CLL cells survival was strongly enhanced at high cell density. Conditioned medium from high cell density CLL cells produced a marked increase in the viability of low cell density autologous cells. Again, autocrine catalase was identified as the survival factor in the high cell density cultures.

Reactive oxygen species (ROS) have been shown to contribute to the death of CEM cells and CLL cells at low cell density [4, 5]. ROS are highly reactive metabolites that are generated during normal cell metabolism. Intracellular ROS derive mainly from leakage of electrons from mitochondrial electron transport chains that reduce molecular oxygen to superoxide ions. Cells possess antioxidant systems to control their redox state, to reduce oxidative stress and to maintain cell survival [6]. Superoxide ions are converted to hydrogen peroxide (H_2O_2) by the action of Cu^{2+}/Zn^{2+}-dependent or Mn^{2+}-dependent superoxide dismutases, and H_2O_2 is then detoxified by catalase or glutathione peroxidase. H_2O_2 can also react in vivo to generate the highly damaging hydroxyl radical by the Fe^{2+}-dependent Fenton reaction or the Fe^{2+}-catalyzed Haber-Weiss reaction [6, 7]. At subtoxic levels, ROS may play an essential signaling role in cell growth and differentiation [8–11]. At elevated levels, however, intracellular ROS are sufficient to trigger cell death [12–16]. Antioxidants that limit ROS-induced cell damage can suppress apoptosis in many systems. For example, N-acetylcysteine (NAC), which elevates intracellular glutathione levels, delays activation-induced cell death of a T cell hybridoma [17]. NAC or the iron chelator pyrrolidine dithiocarbamate (PDTC) or enforced expression of Mn^{2+}-dependent superoxide dismutase inhibits apoptosis induced by TNF-α which can stimulate ROS production [18–21]. Similarly, cell death through oxidative mechanisms has been shown to be opposed by protein albumin at physiologic concentrations directly by scavenging for free oxygen radicals through the free cysteinyl sulfhydryl moiety and indirectly by maintaining the reduced state of cellular proteins [22–24].

ROS have also been shown to be the decisive contributors to the death of activated T cells (ATCs) [25–28]. First, the ATCs have increased levels of ROS [25, 26, 29–31]. Second, ATC death is prevented by manganese (III) tetrakis (5, 10, 15, 20-benzoic acid) porphyrin (MnTBAP), an antioxidant that has been shown to inhibit ROS-induced death in different types of cells [25]. Evidence shows that ROS lead to ATC death by at least two pathways, one mediated by caspase activation and subsequent proteolytic cellular disintegration and the other driven by ROS themselves [25].

While cell density has been found to be important for the survival of resting T cell and certain but not all leukemic T and B lines, it remains unclear if normal T cells behave in a cell density-dependent manner during T cell expansion. In this study, we confirm a critical role of cell density in resting T cell activation and ATC expansion. We found that resting T cells need to be kept at high cell density for optimized activation and ATCs need to be kept at high cell density for optimized expansion. We show that the cell density-related ATC apoptosis is triggered by oxidative stress that is in turn opposed by the secretion of an autocrine survival factor, mainly catalase: at high cell density, more catalase accumulates in the medium and opposes the oxidative apoptosis. Further, we confirm the antioxidant activity of added NAC or of serum albumin at high concentrations that protects ATCs from apoptosis when cultured at low cell density.

2. Materials and Methods

2.1. Reagents. N-acetylcysteine (NAC), catalase, and 3′-amino-1,2,4-triazole (ATZ) were purchased from Sigma Chemical (St Louis, MO, USA). Human serum albumin was from Bayer Corporation (Elkhart, IN, USA).

2.2. Cell Purification, Activation, and Culture. Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood from healthy adults by centrifugation over Histopaque-1083 (Sigma). For resting T cells activation study, PBMCs were cultured in 6-well plates in different cell densities and were activated in serum-free AIM V media (Gibco, Gaithersburg, MD, USA) supplemented with 100 U/ml IL-2, 100 ng/mL mouse antihuman CD3 antibody OKT3 (Ortho Biotech, Raritan, NJ, USA) only or with the addition of 100 ng/mL mouse antihuman CD28 antibody mAb9.3 (gift from Dr. Carl H. June, University of Pennsylvania, Philadelphia, PA, USA). For preparation of ATCs, PBMCs were activated at 1×10^6/mL cells in serum-free AIM V media supplemented with 100 U/ml IL-2, 100 ng/mL OKT3 in 75 cm^2 flasks for 3 days. ATCs were then washed and cultured in the AIM V medium supplemented with 100 U/mL IL-2 for an additional 7 days before use in experiments.

2.3. Monitor of T Cell Activation and Cell Division. Accompanied with T cell activation, there is a significant cell size enlargement from resting T cells to ATCs, with cytokines such as IL-2 and IFN-γ production and surface molecules such as CD69 and CD25 expression. ATCs can be distinguished from resting T cells and other types of PBMCs as the enlargement in forward-angle light scatter (FS) by flow cytometric analysis and it is identical with CD69 or CD25 staining. T cell activation was monitored by counting the percent of ATCs in the culture by flow cytometer and confirmed with microscope. To monitor cell division, ATCs were labeled with 1 μM carboxy-fluorescein diacetate succinimidyl diester (CFSE) (Molecular Probes, Eugene, OR, USA) [32] and cultured for 6 days. Cells were harvested and cell division was analyzed by the dilution of CFSE in the daughter cells by flow cytometer.

2.4. Detection of Apoptosis. ATC apoptosis was determined by cell shrinkage and DNA cleavage. Cell viability of ATCs was analyzed by flow cytometry [5, 33, 34]. Cell shrinkage

accompanying ATC apoptosis was detected as a reduction in forward-angle light scatter (FS) by flow cytometric analysis. Agarose gel electrophoresis was used to detect internucleosomal cleavage fragments of DNA following apoptosis [4, 35]. 2×10^6 cells were pelleted and resuspended in 500 μL of ice-cold lysis buffer (20 mM Tris·HCl, 10 mM EDTA, and 0.2% Triton X-100, pH 7.4). Proteinase K was added at 100 μg/mL and incubated at 50°C overnight, followed by further incubation at 37°C for 2 hours with the addition of RNase. DNA was extracted twice with phenol/chloroform at 1 : 1 and precipitated with isopropanol. The DNA was electrophoresed through a 1% agarose gel and stained with ethidium bromide.

2.5. Measurement of Intracellular Oxidative Stress. Cells were gently resuspended in 10 μM dihydrorhodamine 123 (DHR) (Molecular Probes) and incubated for 30 minutes and then analyzed by flow cytometry [5, 7]. The level of intracellular ROS was inferred from the mean fluorescence intensity (MFI) of DHR-stained cells. Dead cells and debris were excluded from analysis by electronic gating of forward and side scatter measurements.

2.6. Generation of Conditioned Medium. Cells were cultured at 1×10^6/mL (ATCs) or confluent (MIP101 cells, a human colon carcinoma cell line) in 75 cm^2 flasks. After 2 days in culture, the conditioned medium (CM) was removed from the culture flasks and centrifuged at 1500 rpm for 10 minutes. The CM was then passed through a sterile 0.45 μm filter.

2.7. Detection of Catalase. Detection of catalase was performed by western blot and dot blot. For western blot, control medium, ATCs CM, and cell lysates from ATCs were run on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and electrotransferred to nitrocellulose membranes. For dot blot, 100 μL culture medium from untreated, anti-CD3 activated and anti-CD3 plus anti-CD28 activated PBMCs were blotted on to nitrocellulose membrane by using a dot blot apparatus (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (20 mM Tris/500 mM NaCl, pH 7.5) for 1-2 hours. Membranes were washed with TTBS (0.05% Tween-20 in Tris-buffered saline), and mouse antihuman catalase mAb antibody (Sigma) was added at a dilution of 1 : 1000 for 1 hour, followed by incubation with HRP-conjugated goat antimouse IgG antibody at a dilution of 1 : 1000 for 1 hour, all in 1% non-fat dry milk in TTBS. The membrane was developed with enhanced chemiluminescence reagent (Amersham, England, UK) and exposed to X-ray film for 5–60 seconds.

2.8. Detection of Intracellular Albumin. Analysis of intracellular albumin was performed using immunofluorescence and flow cytometry. Cells were fixed and permeabilized with a Fix & Perm kit (Caltag Laboratories, Burlingame, CA, USA) and then stained with mouse antihuman albumin (Sigma), followed by staining with a secondary goat antimouse FITC-conjugated antibody (Caltag Laboratories).

3. Results

The objective of this study was to determine the role of cell density in ex vivo expansion of T cells. To mimic the actual situation in preparation for clinical applications, resting T cells were not further purified from the bulk PBMCs in the experiments. Typically, PBMC are composed of 50–70% T cells, and smaller numbers of B cells, NK cells, and monocytes.

3.1. Cell Density Determines the Fate of T Cells Activation. Efficient activation of resting T cells requires signal 1 (TCR, CD3) and signal 2 (CD28) costimulation [36]. Activation of T cells through soluble OKT3 (anti-CD3) antibody depends on crosslinking of the antibody through Fc receptor on monocytes present in PBMCs preparations that also provide costimulation through B7 to CD28 on the T cells [37]. Typically, PBMCs cell concentrations of at least $\mathbf{1 \times 10^6}$/mL are specified for this T cell activation. At lower cell densities, the opportunities for cell-cell contact are diminished and activation is incomplete or absent.

This is confirmed in our results. Following activation with OKT3 and IL-2 at 1×10^6 cells/mL for 6 days, 61% of cells in the culture showed a T blastic morphology by flow cytometry versus 23%, 9%, and 8% for the lower PBMCs densities of 1×10^5, 1×10^4 and 1×10^3/mL, respectively (Figure 1). At an intermediate T cell density of 1×10^5/mL, addition of an agonist anti-CD28 antibody that bypasses need for B7 on monocytes partially compensates for the reduced cell contacts, doubling the activated fraction from 23% to 47%. At the lowest cell densities, this maneuver was not effective and at the highest it was unnecessary, where the activated phenotype was maximal with OKT3 alone. At higher density, there are more chances of cell contacts and B7-CD28 interactions, and costimulation is maximal without added anti-CD28 antibody. Such maximally activated T cells (ATC) were applied throughout this study.

As analyzed by flow cytometry from one typical experiment, the PBMCs isolated from normal human blood were 57.2% CD3+, 38.8% CD4+, 21.6% CD8+, 0.37% CD4+CD8+, 39.2% CD4−CD8−, 49.8% CD28+, 53.6% CD2+, and 60.6% CD11a+. After 3 days activation with OKT3 and expansion for 7 days (total 10 days), the cell populations were 99.7% CD3+, 81.7% CD4+, 15.6% CD8+, 1.9% CD4+CD8+, 0.76% CD4−CD8−, 99.4% CD28+, 99.5% CD2+, and 100% CD11a+. Such 10-day cultures were used in the ATC studies that follow.

3.2. ATCs Proliferate at High Cell Density. To examine the relation between cell density and ATC proliferation, T cells were activated under high density conditions as in Section 3.1 and then reseeded at varying cell densities and monitored over time for viable cell numbers. There was a progressive increase in the number of viable cells when ATCs were cultured at 1×10^5/mL, but a progressive decline in viable cells when ATCs were cultured at 1×10^4/mL. After 6 days, the number of viable ATCs increased 340% when cultured at 1×10^5/mL but decreased by 75% when cultured at $1 \times \mathbf{10^4}$/mL

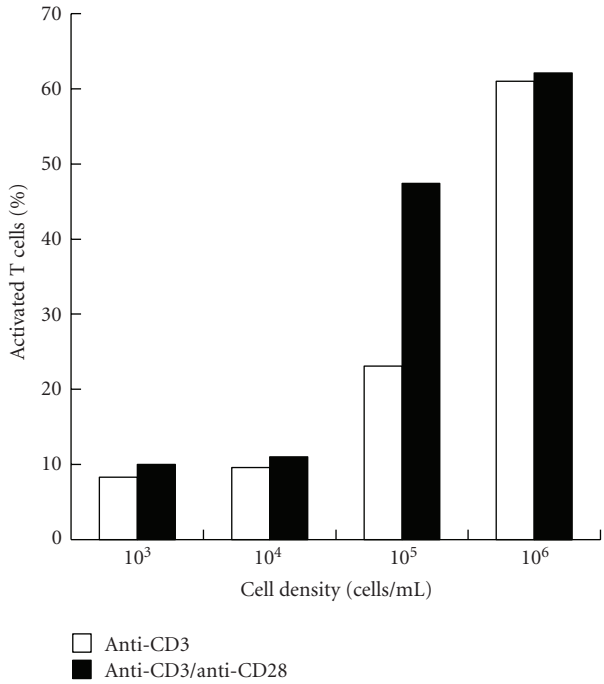

Figure 1: Cell density determines the fate of T cell activation. PBMCs cultured at different cell densities in 6-well plates were activated with anti-CD3 antibody alone or plus anti-CD28 antibody in the presence of IL-2. T cell activation was measured as percentage of activated T cells in culture after six days of activation by morphological criteria of T cell blasts on flow cytometer. Similar results were obtained in two independent experiments.

(Figure 2(a)). Furthermore, when measuring dividing cells by CFSE, 68% of ATCs underwent division when cultured at 1×10^5/mL, 35% at 1×10^4/mL, and only 15% at 1×10^3/mL (Figure 2(b)). These results establish a correlation between cell density and ATC expansion in vitro.

3.3. ATCs Undergo Apoptosis at Low Cell Density. To examine the relation between cell density and cell viability, ATCs cultured at different cell densities were evaluated after 24 hours. A condensed cell size by morphology on flow cytometry has been shown to correlate with apoptosis/cell death markers under experimental conditions close to our own [5, 33, 34] and was used to distinguish apoptotic from viable cells in our tests. At cell density of 1×10^5/mL, viable cells remained unchanged at 92% after 24 hours but declined to 62% and 34% when ATCs were cultured at 5×10^4/mL and 1×10^4/mL over the same time interval (Figure 3(a)). Increasing IL-2 100-fold from 100 IU/mL to 10,000 IU/mL did not rescue ATCs from death at low cell density (data not shown).

To determine by another measure whether ATCs died by apoptosis when cultured at low cell density, a DNA fragmentation assay was employed. Apoptosis is a distinctive form of cell death that occurs in a wide range of physiological and pathological situations [38]. It differs fundamentally from degenerative cell death or necrosis and was originally defined by the orderly sequence of ultrastructural changes that accompanies cell elimination during development [39]. Biochemically, it is best characterized by the presence of internucleosomal cleavage of DNA into 180–200 base-pair fragments [39, 40], which can be demonstrated by gel electrophoresis. As shown in Figure 3(b), DNA was fragmented when ATCs were cultured at low cell density (1×10^4/mL) but remained intact at high cell density (1×10^6/mL), confirming that apoptosis is the mechanism of ATC death at low cell density.

3.4. ROS Are the Mediators of ATC Apoptosis at Low Cell Density. Elevated intracellular ROS can trigger cell death [12–16]. We hypothesized that ROS were the mediators of ATC apoptosis at low cell density. To test this hypothesis, we measured the levels of intracellular ROS in ATCs cultured at different cell densities and tested if antioxidants can block ATC apoptosis at low cell density.

ROS include superoxide and hydroxyl-free radicals and H_2O_2. To measure intracellular ROS, we used the oxidation-sensitive fluorescent probe DHR [7]. DHR is nonfluorescent, uncharged, and accumulates within cells, whereas R123, the product of intracellular DHR oxidation, is fluorescent, positively charged, and trapped within cells [41]. ATCs cultured at high cell density (1×10^5/mL) and low cell density (1×10^4/mL) for 24 hours were incubated with DHR, and R123 fluorescence was measured by flow cytometry. The rate of DHR oxidation was significantly greater in ATCs cultured at low cell density than in ATCs cultured at high cell density (Figure 4(a)), confirming a correlation of intracellular ROS with apoptosis.

Antioxidants inhibit ROS-mediated apoptosis in many systems by limiting ROS-induced cell damage [16]. We therefore tested the effects of three antioxidants on ATC apoptosis at low cell density. NAC elevates intracellular glutathione [17] that is a substrate for glutathione peroxidase to catalyze the breakdown of H_2O_2; catalase detoxifies H_2O_2 produced by superoxide ions [7]; and albumin can directly scavenge reactive oxygen species through its free cysteinyl—SH [42]. ATCs were cultured at low cell density (1×10^4/mL) in the presence of antioxidants at different concentrations for 24 hours. Cell viability and intracellular ROS were measured by flow cytometry. All agents significantly protected cells from death in a dose-dependent manner. In general, the increased survival was paralleled by a reduction in intracellular ROS levels with higher levels of the antioxidants (Figure 4(b)). This further supports the hypothesis of ROS as a mediator of apoptosis in ATCs cultured at low cell density.

Albumin is the most abundant plasma protein and has been suggested to constitute an important extracellular antioxidant [43, 44]. Interestingly, the reduction in ROS with albumin appeared somewhat less than predicted versus the survival benefit observed (Figure 4(b)), suggesting that there may be additional, downstream means by which albumin may mitigate the harmful effects of ROS. A further feature of interest was the demonstration of increased albumin endocytosis in the low density ATCs versus those grown at high density (Figure 4(c)). Whether this was a generalized effect on endocytosis or selectively related to albumin was not

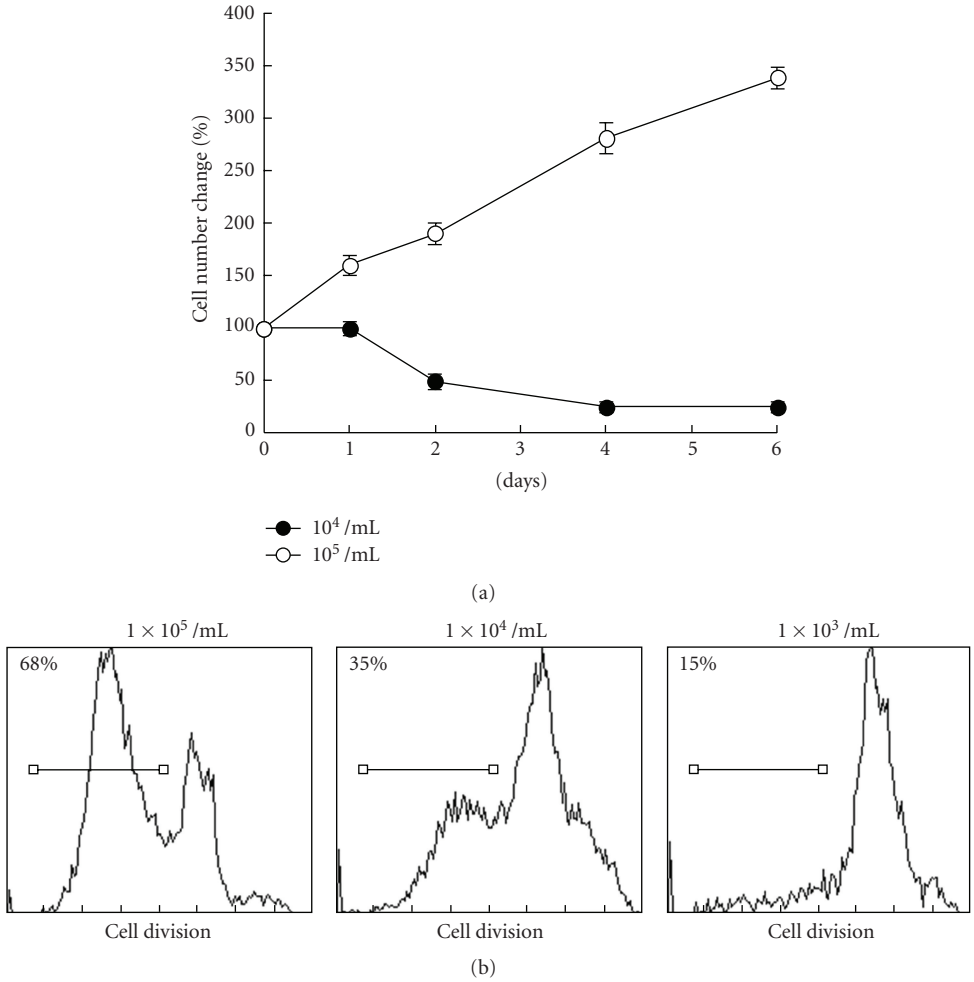

Figure 2: ATCs proliferate at high cell density. ATCs were activated at high density and then reseeded in fresh growth medium plus IL-2 at varying concentrations. (a) Numbers of ATCs at different cell densities. ATCs were cultured at high cell density (1×10^5/mL) or low cell density (1×10^4/mL) in 75 cm^2 flasks for 6 days. Cell number change rate (%) was monitored by direct cell counting. (b) Cell division of ATCs at different cell densities. After labeling with CFSE, ATCs were cultured at 1×10^5/mL, 1×10^4/mL, and 1×10^3/mL in 75 cm^2 flasks for 6 days. Cell division was monitored by CFSE dilution in the dividing cells as analyzed by flow cytometry.

examined. It was also not discriminated whether it was the internalized albumin or the external protein that mediated the survival benefits; its mechanism of action was not further investigated.

3.5. Soluble Factor Secreted by ATCs at High Cell Density Prevents Apoptosis of ATCs at Low Cell Density. The protective effect of high cell density indicated that either cell-to-cell contacts or soluble factor(s) produced by ATCs were inhibiting ATC apoptosis under this condition. To assess whether soluble factor(s) were involved, conditioned medium from ATCs cultured at high cell density (1×10^6/mL) was collected and added to ATCs cultured at low cell density (1×10^4/mL). With increased fractions of conditioned medium in the total medium, the intracellular ROS levels decreased in ATCs cultured at low cell density and their viability increased (Figure 5(a)). These data confirmed the secretion of one or more soluble factors at high cell density that functioned as antioxidant to protect ATCs from apoptosis.

A similar protective effect was observed with conditioned medium from a human colon carcinoma cell line, MIP101 (Figure 5(b)) and from a human T cell leukemia cell line (data not shown). These results indicate that the protective soluble factor(s) secreted by ATCs at high cell density are not ATC-specific. This result is compatible with the potential of diverse cell lines to function as feeder cells during T cell cloning procedures [40].

3.6. Autocrine Catalase Protects ATCs from Apoptosis. Based on prior studies of cytoprotective effects of catalase in cultures of lymphoid leukemia cells [5, 40], we hypothesized that the cell density effect on ATC survival was also mediated by secreted catalase. To determine whether catalase was one of the autocrine survival factors, western blot was performed (Figure 6(a)). The enzyme was clearly detected in cell lysates

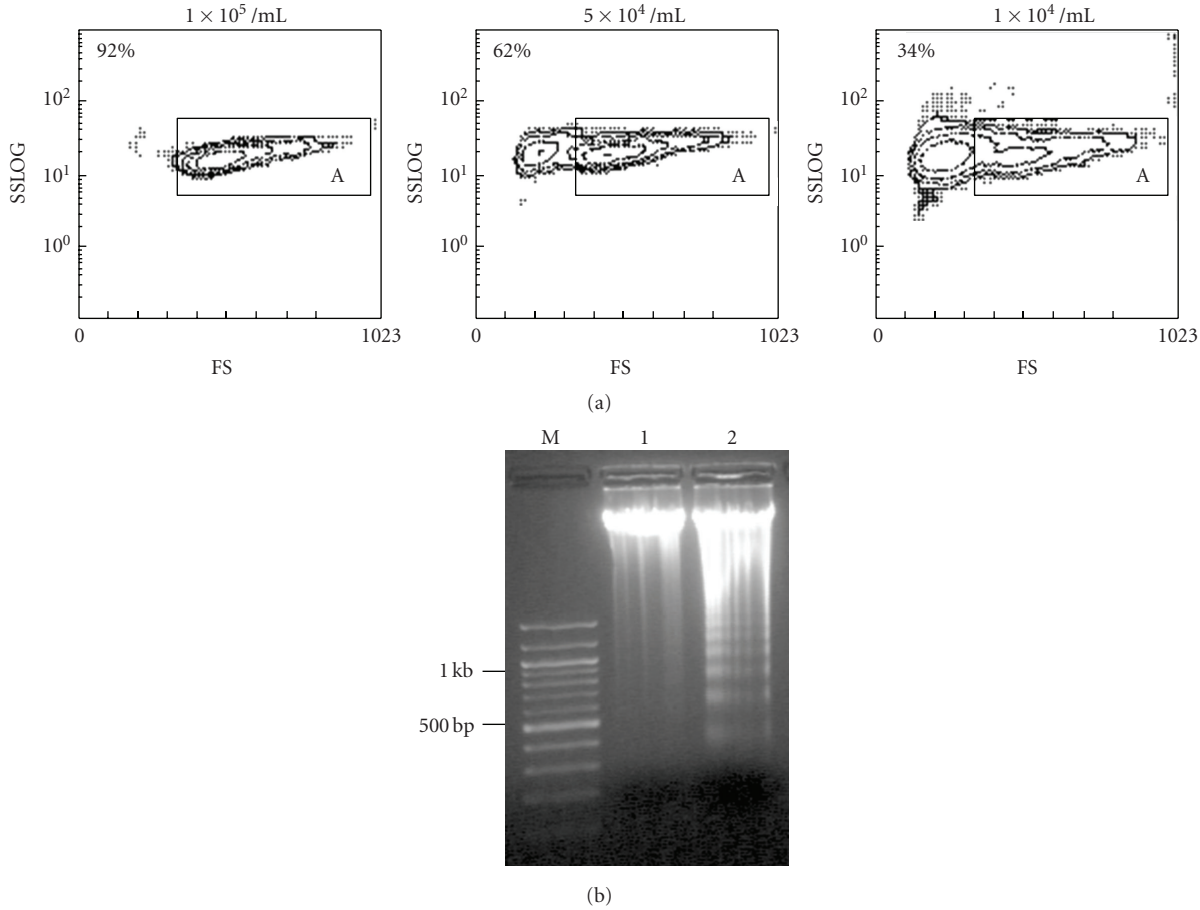

Figure 3: ATCs undergo apoptosis at low cell density. (a) Cell viability of ATCs at different cell densities. ATCs were cultured at different cell densities: 1×10^5, 5×10^4, and 1×10^4/mL in 75 cm^2 flasks for 24 hours. Cell viability was analyzed by flow cytometry. Viable cells are included in the rectangle and percentages of viable cells are indicated within the icon top left. Similar results were obtained in 3 independent experiments. (b) DNA fragmentation of ATCs at different cell densities. The DNA extracts of ATCs cultured at high (1×10^6/mL) (lane 1) or low (1×10^4/mL) (lane 2) cell density in 75 cm^2 flasks for 24 hours were electrophoresed through a 1% agarose gel and stained with ethidium bromide. 100 bp DNA ladder markers were included as markers (M).

and conditioned medium, but not in control medium. In two separate assays, catalase in CM (e.g., lane 2) was estimated at 2.5–5 μg/mL (6–12 units/mL) in comparison with control purified catalase. Estimates from cell lysate (lane 3) are 2 μg per 10^6 cells. This means that in 24 hours 10^6 cells secrete into 1 mL of medium, a quantity (2.5–5 μg) that equals or exceeds what is present in the cells themselves. Further, this concentration of catalase in CM corresponds closely with levels that give maximum benefit to ROS control and cell viability in Figure 4(b) (e.g., ~10 units/mL).

We next sought to establish whether catalase contributed to the survival-enhancing effect of the conditioned medium. To address this question, the effect of the selective catalase inhibitor, 3′-amino-1,2,4-triazole (ATZ) [5, 45–48] was examined. ATZ significantly blocked the survival-enhancing activity of the conditioned medium (Figure 6(b)), indicating that catalase plays the major role in this cytoprotective effect.

3.7. Elevated Autocrine Catalase Accumulation in CD28 Costimulated T Cell Activation. Having established the role of autocrine catalase as a cell survival factor in ATC proliferation, an interesting question arises: whether autocrine catalase also plays a role in CD28 costimulated T cell activation. An intermediate cell concentration was shown in Figure 1 to benefit from CD28 costimulation, PBMCs at 1×10^5/mL were activated with anti-CD3 antibody without or with anti-CD28 antibody in the presence of IL-2. Compared with anti-CD3 antibody activation alone, there was a significant more amount of autocrine catalase accumulation in the anti-CD28 antibody costimulated cell cultures (Figure 7). These results indicate that the improved T cell activation at lower cell density with CD28 is paralleled by an enhanced autocrine catalase secretion.

4. Discussion

Cell density has been reported to be important for cell survival in cultures of resting T cells [3] and certain leukemic T and B cell lines [4] but not reported in other leukemic T cell lines such as Jurkat and H9 T cells [5]. Although

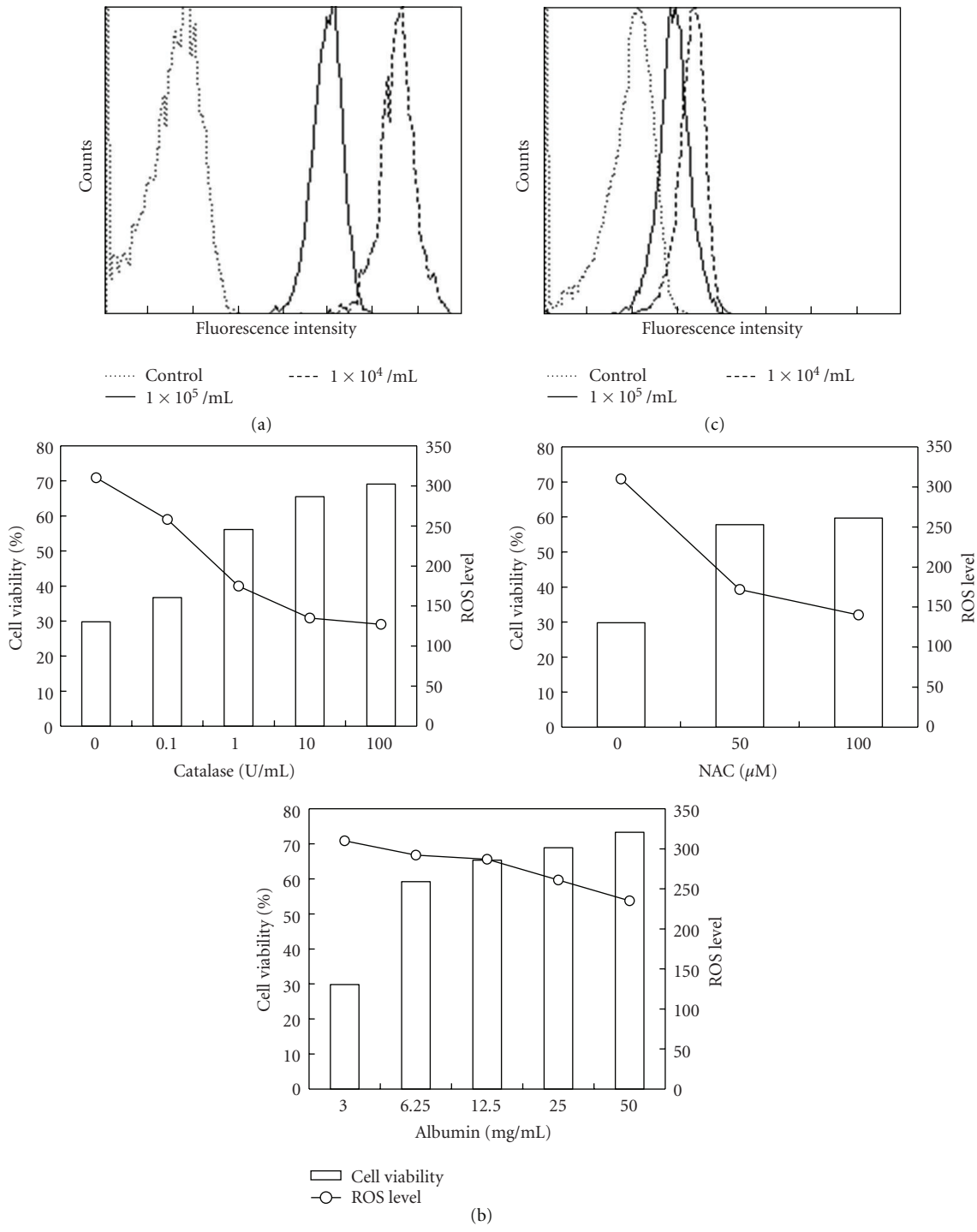

Figure 4: Reactive oxygen species (ROS) are the mediators of ATC apoptosis at low cell density. (a) Intracellular ROS levels in ATCs cultured at different cell densities. ATCs were cultured at high cell density (1×10^5/mL) or low cell density (1×10^4/mL) in 75 cm^2 flasks for 24 hours. After staining with DHR for 30 minutes, intracellular ROS levels of ATCs were analyzed by flow cytometry. Nonstaining ATCs were used as negative control. (b) Antioxidants protect activated T cells from apoptosis at low cell density. ATCs were cultured at 1×10^4/mL in 75 cm^2 flasks in the presence of catalase, NAC, and human serum albumin (HSA) in different concentrations. HSA in unsupplemented serum-free medium was measured at 3 mg/mL by Bradford assay. Both cell viability and intracellular ROS levels were analyzed by flow cytometry as in (a). ROS levels were indicated as mean fluorescence intensity (MFI) of DHR-stained cells. Similar results were obtained in 2 independent experiments. (c) Elevated intracellular albumin in ATCs cultured at low cell density. ATCs were cultured at high cell density (1×10^5/mL) or low cell density (1×10^4/mL) in 75 cm^2 flasks for 24 hours. Cells were fixed and permeabilized and then stained with mouse antihuman albumin antibody, followed by staining with a secondary goat antimouse FITC-conjugated antibody. Cells stained with only secondary antibodies were used as negative controls.

there are frequent communications between investigators performing ex vivo T cell expansions for clinical therapeutic applications that maintaining high cell density is an important consideration factor in T cell expansion, the relation between cell density and T cell expansion remains unclear. In this study, we systematically examined the relation between cell density and normal human T cell expansion in vitro, providing evidence for optimizing T cell expansion protocols for clinical applications. From our results, PBMCs have to be seeded at high cell density ($\geq 1 \times 10^6$/mL) for optimal T cell activation. The addition of CD28 costimulation helps resting T cells to be activated at lower cell density and coordinately yields elevated catalase secretion and accumulation in the cultures. Maintaining high cell density is also important for ATC proliferation. ATCs undergo apoptosis when cultured at cell density of 1×10^4/mL or less. Our mechanistic studies support the role of ROS and oxidative stress apoptosis in ATC death at low density. At high cell density, the extracellular accumulation of secreted catalase reduces intracellular ROS species and alleviates their toxic effects.

4.1. Cell Density Plays a Critical Role in Ex Vivo Expansion of T Cells. Current protocols for ex vivo expansion of T cells for clinical adoptive immunotherapy usually involve the activation of PBMCs with OKT3 alone or plus anti-CD28 antibody in the presence of IL-2. When expanding T cells in vitro, our results indicate that it is critical to maintain the cells at high cell density during both T cell activation and ATC expansion phases. It is reported that OKT3 has to be immobilized on plastic or crosslinked via accessory cells in PBMCs through Fc receptor binding for the activation of T cells [37]. At low cell density, where the cell-cell contact is poor, the OKT3 may not be efficiently crosslinked to activate resting T cells. We also found that the addition of costimulation through anti-CD28 antibody improves the activation of resting T cells at lower cell density. Unlike the OKT3, the soluble form of mAb9.3 can induce costimulation directly without the need for crosslinking [37]. Interestingly, there is a correlation between CD28 costimulation and elevated catalase accumulation in the cultures. At present, there is no basis on which to infer whether catalase is in the CD28 pathway or merely one of the many downstream components of T cell activation. Once being activated, the ATCs have to be kept at high cell density for survival and efficient proliferation.

When T cells are activated, T cells secrete abundant cytokines such as IL-2 and other growth factors for cell proliferation and survival. Maintaining high cell density in culture may enable the accumulation of such autocrine products to reach a relatively high concentration to support the proliferation and survival of ATCs. It is interesting to notice that under physiological conditions, T cell immune responses can only be exclusively induced in organized lymphoid tissues but not elsewhere in the body [49–53]. Naïve T cells traffic constitutively through secondary lymphoid organs where they encounter antigen-loaded dendritic cells and are activated to proliferate and differentiate into activated effector T cells. Following this, effector T cells then migrate to peripheral tissues to perform their function

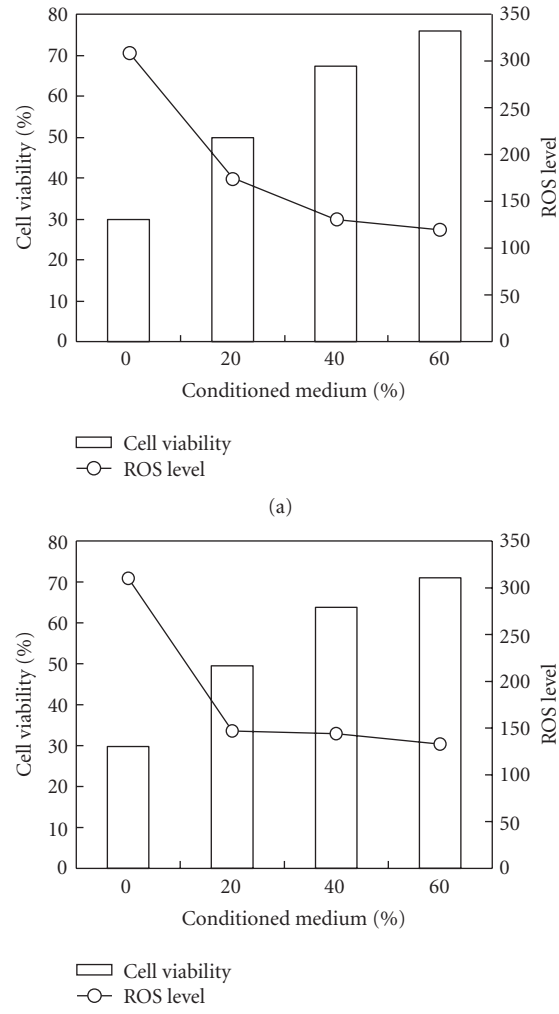

Figure 5: Soluble factors secreted by ATCs at high cell density prevent apoptosis of ATCs at low cell density. ATCs cultured at 1×10^4/mL in 75 cm^2 flasks were supplemented with conditioned medium from (a) ATCs at high cell density or (b) MIP101 cells in different concentrations for 24 hours. Cell viability and intracellular ROS levels were analyzed by flow cytometry. Similar results were obtained in two independent experiments.

[54]. Is it true that at locations such as the T cell areas of secondary lymphoid organs, high cell density enables the initiation of T cell responses with supported T cell survival and proliferation, whereas at sites of peripheral tissues, low cell density prevents T cell proliferation and minimizes immunopathology? The question remains to be answered.

4.2. ROS Are the Mediators of ATC Apoptosis at Low Cell Density. To understand why T cells have to be maintained at high cell density for optimal expansion, it is important to know why the T cells die at low cell density. ROS such as superoxide and hydroxyl radicals and H_2O_2 are continuously produced by cells, and their levels are regulated by a number of enzymes and physiological antioxidants. Excessive generation of ROS or failure to suppress elevated

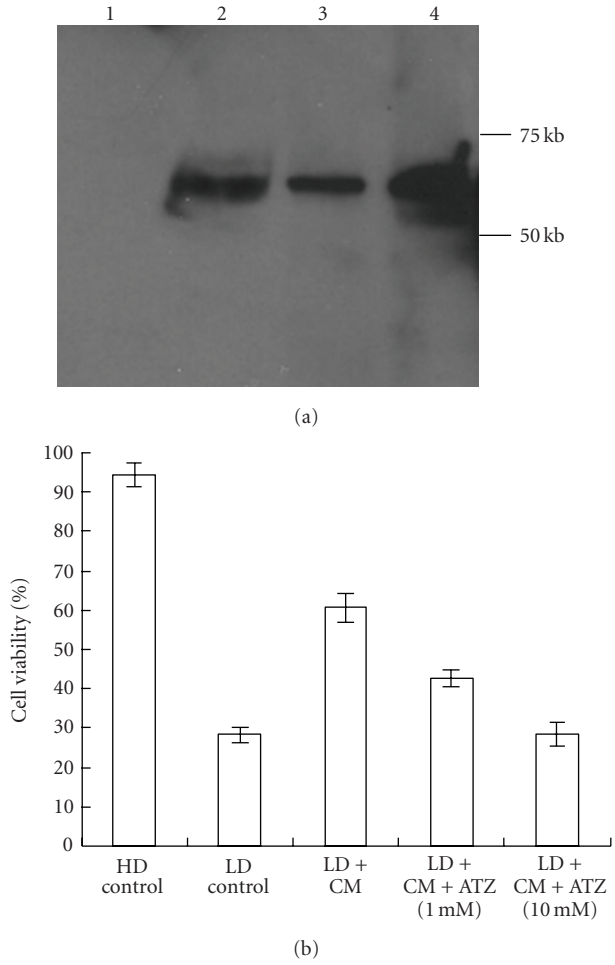

Figure 6: Autocrine catalase is the conditioned medium product that protects ATCs from apoptosis. (a) Expression and secretion of catalase by ATCs. Western blot of control medium (lane 1), conditioned medium from ATCs (lane 2), cell lysates of ATCs (lane 3), and catalase control (lane 4) probed with anticatalase antibody. (b) Catalase inhibitor ATZ abrogates the protective effect of conditioned medium from ATCs. ATCs were cultured at high cell density (1×10^5/mL) (HD) or low cell density (1×10^4/mL) (LD) in 75 cm^2 flasks in the presence or absence of 50% conditioned medium from ATCs (CM) and ATZ in 1 or 10 mM for 24 hours. Cell viability was analyzed by flow cytometry.

intracellular ROS by the cellular regulatory systems has been associated with cell death [12–15].

Differential effects of ROS on cell death are observed depending on the level of ROS within the cell [26, 55]. High levels of ROS lead to lipid peroxidation, damage to cellular membranes, inactivation of caspase enzymes, and necrotic cell death. Low levels of ROS can activate protein kinases and phosphatases, mobilize Ca^{2+} stores, activate or inactivate transcription factors, and lead to apoptotic cell death. ATCs have been shown to have increased levels of ROS [25, 26, 29–31] and ROS have been shown to be one of the decisive contributors to the death of ATCs [25–28].

ROS are intermediates in the induction of FasL after TCR engagement during activation induced cell death [25]. ROS-driven Bcl-2 downregulation is a necessary signal for activated T cell autonomous death [25]. Besides, ROS may affect many other molecules, such as membrane lipids, transcriptional factors, and signal transduction proteins that are involved in T cell apoptosis [25].

Our data demonstrate that ATCs cultured at low cell density have higher levels of ROS than ATCs cultured at high cell density and that reversal of high ROS in culture improves T cell proliferative response and survival, implying that ATC apoptosis at low cell density is triggered by ROS.

4.3. Antioxidants Promote ATC Survival at Low Cell Density. Glutathione (GSH) is the major intracellular redox buffer and plays an essential role in protecting cells against oxidative damage [56]. In addition, changes in the intracellular GSH levels modulate the expression of several genes involved in the control of cell growth and differentiation [57, 58]. In T lymphocytes, intracellular GSH is critical for the proliferative response to mitogens or antigens [59–62]. Our experiments demonstrate that by supplementing the GSH precursor, NAC, ATCs can be protected from apoptosis at low cell density, suggesting that the GSH peroxidase antioxidant system may play an important role in ATC survival.

Other interventions that reduce intracellular ROS were also effective in reversing the effect of low cell density to inhibit T cell proliferation and survival. These included the supplementation of cultures with purified catalase, which detoxifies H_2O_2, and addition of high concentrations of serum albumin, which contributes reducing cysteines that can scavenge oxygen radicals.

One of the striking features of human serum albumin is the presence of 34 cysteine residues forming 17 disulfide bonds, and one free thiol at the Cys-34 position [63]. One-third of the albumin molecules form mixed disulfides with either GSH or half-cystine. The remaining sulfhydryl group of the Cys-34 residue of albumin constitutes the major extracellular source of reactive free thiol [64]. In this context, it has been suggested that albumin constitutes an important extracellular antioxidant in plasma [43]. The role of albumin as an ROS scavenger has been confirmed in cell-free systems with a wide variety of oxidative species, including HOCl, H_2O_2, $^{\bullet}OH$, carbon radicals, and peroxynitrite [22, 65, 66], as well as in intact cell systems such as macrophages and renal tubular epithelium [42]. However, the mechanism by which albumin exerts its antioxidant effects is most likely multifactorial [42]. It is possible that the free sulfhydryl group of albumin enables it to act not only as an antioxidant but also as a reducing agent via modulation of cellular GSH levels [44]. GSH in turn affects a wide variety of cell proteins, the function of which is dependent on redox state, such as the N-methyl-D-aspartic acid receptor, the DNA binding protein activator protein-1, and NF-κB [67, 68].

Albumin has been an essential component of non-serum culture mediums for expansion of T cells for clinical applications. We explored the antioxidant role of albumin in cell density-related death of ATCs. Concentration-dependent reduction of intracellular ROS in ATCs cultured at low cell density suggests that albumin reacted as an antioxidant to

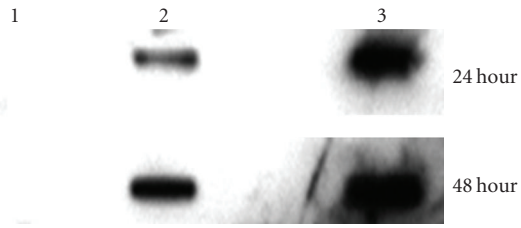

Figure 7: Improved activation with CD28 costimulation at low cell density is accompanied by higher autocrine catalase secretion. Slot blot of culture media from unactivated (lane 1), anti-CD3 antibody (lane 2) and anti-CD3 antibody plus anti-CD28 antibody (lane 3) activated PBMCs at 1×10^5/mL at 24 and 48 hours probed with anticatalase antibody.

scavenge ROS. Compared to antioxidants catalase and NAC, however, the effectiveness of albumin in reducing ROS is not as strong, compatible with its action via these other indirect mechanisms to rescue ATCs from apoptosis. The mechanism of albumin's action was not investigated further.

Interestingly, elevated intracellular albumin was detected in ATCs cultured at low cell density, implicating increased endocytosis under this condition, possibly in response to elevated intracellular ROS. Albumin is bound on the surface of lymphoid cells of all mammalian species tested [69], but its function is relatively unknown. ATCs express albumin binding proteins and the binding of serum albumin increased considerably upon blastic transformation [70]. Albumin is endocytosed and the internalized albumin is detected in peroxidase-conjugated form in lysosome-like bodies by ultrastructural cytochemistry. Pulse-chase experiments show that internalized albumin is finally released mainly in a degraded form from the cells [70]. Whether increased endocytosis of albumin under low-density conditions is an adaptive response to elevated intracellular ROS to import further reducing species into the cell is uncertain and was not investigated further.

It is likely that those nontoxic antioxidants such as sodium pyruvate [71], β-mercaptoethanol [72], DL-penicillamine and thiolactate that exhibit protective effects against ROS may have the same effect to protect ATCs from apoptosis at low cell density. Whether these agents are useful in the maintenance and growth of ATCs remains to be determined.

4.4. Autocrine Catalase Protects ATCs from Apoptosis. Among the naturally occurring variables examined, only one correlated with the reversal of high levels of ROS under conditions of high ATC cell density: the accumulation of secreted catalase that raised extracellular levels of this potent antioxidant enzyme. This extends studies of cultured leukemic T and B cells [4, 5] suggesting that autocrine catalase functions as a cytoprotective antioxidant in protecting cells at high cell density from apoptosis. Furthermore, our results and others' indicate that this factor is not cell-type restricted and is compatible with the observation that so-called "feeder" cells of various origins can function to support T cells under single-cell cloning conditions. We may infer that secretion of catalase to suppress intracellular oxidative stress is a key component of the supportive role of feeder cells.

How catalase is released from the cells is unclear. Catalase lacks a leader sequence and cannot therefore be secreted by the classic endoplasmic reticulum-Golgi secretory pathway [73]. It is suggested that, like some cytokines, catalase may be secreted via a leaderless secretory pathway [5]. It is unlikely that the appearance of catalase in the medium derives from dying cells. First, at high cell density, ATCs only begin to die at late stages of expansion (after 3-4 weeks). The CM we collected are from ATCs cultured at early stage of expansion (2 weeks) with good viability. Second, we have shown that CM from other cell lines that have very limited cell death can also prevent ATC apoptosis.

It was noted that the endogenous cellular catalase was similar in quantity to that secreted by the cells in 24 hours. Whether the intracellular enzyme is in a compartment that is functional or inactive is not addressed by these studies. However, it is clear that cellular catalase is not a substitute for the secreted component for maintaining cellular health; otherwise, the cell concentration effects would not be observed and CM would not rescue lower ATC densities from apoptosis.

Finally, the quantities of catalase in the CM (6–12 units/mL) (Figure 6(a)) corresponded closely to levels of purified catalase (~10 units/mL) that provide maximum control of ROS and high ATC viability (Figure 4(b)).

Regarding how extracellular catalase activity might regulate intracellular oxidative stress, it is suggested that the extracellular decomposition of H_2O_2 may create a concentration gradient favoring the diffusion of H_2O_2 out of the cells [5].

A final speculation is warranted on the relevance of these findings to the in vivo setting. The autocrine of catalase by T cells may be an important factor for their proliferation. At the time of activation, ATCs are at the highest level for ROS generation and most vulnerable to oxidative cell death [29]. At sites of high cellular density and low fluid efflux, such as lymph node paracortex, catalase could be expected to accumulate to high levels and support ATCs viability and proliferation. On the other hand, ATCs trafficking through the peripheral circulation will be dependent upon blood/tissue levels of ROS and tissue secretion of catalase, as well as serum albumin.

Another question remains to be answered is that the elevated ROS in ATCs and an increased rate of their death at low cell density may be a result of or partly related to excessive oxygen concentration in the growth medium cultivating in traditional incubators in comparison with oxygen concentration in the blood. Nevertheless, our studies clearly indicate that it is important to keep an antioxidant environment for optimized expansion of T cells in vitro.

In conclusion, the present study examined the role of cell density in T cell expansion in vitro. Several conclusions are drawn from this work. First, we confirm that cell density plays a critical role in T cell activation and ATC proliferation. Resting T cells were activated to expand at high cell density but failed to be activated at low cell density. ATCs grew

rapidly at high cell density but underwent apoptosis at low cell density in culture. Second, apoptosis of ATCs cultured at low cell density correlated with elevated intracellular ROS levels and was reversed by antioxidants NAC, catalase, and albumin, indicating that the apoptosis of ATCs at low cell density was mediated by ROS. Third, the increased survival of ATCs at high cell density was due to non-IL-2 factor(s) secreted by ATCs and non-ATCs alike. Fourth, autocrine catalase was demonstrated to be the key survival factor regulating ATC survival at high density by suppressing intracellular ROS. Fifth, CD28 costimulation that improves T cell activation at lower cell density is accompanied by enhanced autocrine catalase secretion. Our findings highlight the importance of cell density in T cell activation, proliferation, survival, and apoptosis and suggest that it is critical to maintain T cells at high cell density for the successful expansion of T cells ex vivo for adoptive immunotherapy.

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Review Article

Direct Microbicidal Activity of Cytotoxic T-Lymphocytes

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Cytotoxic T-lymphocytes (CTL) are famous for their ability to kill tumor, allogeneic and virus-infected cells. However, an emerging literature has now demonstrated that CTL also possess the ability to directly recognize and kill bacteria, parasites, and fungi. Here, we review past and recent findings demonstrating the direct microbicidal activity of both $CD4^+$ and $CD8^+$ CTL against various microbial pathogens. Further, this review will outline what is known regarding the mechanisms of direct killing and their underlying signalling pathways.

1. Introduction

The adverse consequences of the acquired immune deficiency syndrome (AIDS) or T cell immunodeficiency provide evidence of the vital role of cytotoxic T-lymphocytes (CTL) in the immune response. Indeed, CTL are well-known elements of the immune response to virus-infected, tumour and allogeneic cells [1–3]. More recently, the role of CTL was expanded significantly when the ability to mediate direct killing of microbial pathogens was identified. Research defining the precise mechanisms underlying CTL killing of microbes, however, is still in its infancy. Remarkably, granulysin (in contrast to granzymes) has emerged as a fundamental mediator of microbial killing. The mode of action of granulysin appears to be through the disruption of membrane permeability [4]. It follows that granulysin has been found to insert into the microbial membrane through ionic interactions between the positively charged amino acid residues and negatively charged phospholipids. Insertion of granulysin in turn disrupts membrane permeability resulting in the influx of fluid into the cytoplasm and death by osmotic lysis [4]. Other mechanisms identified in the killing of tumor cells may also play a role, including Ca^{2+} influx and K^+ efflux [5], and activation of a sphingomyelinase associated with the cell membrane to generate ceramide [6].

CTL killing of extracellular pathogens involves direct microbial recognition by the CTL (Figure 1(A)). In contrast to tumour and virus-infected cells, recognition of extracellular pathogens occurs through an apparent MHC-independent mechanism (as microbes have not been found to express MHC). Successful recognition induces the release of granulysin which may directly bind and kill the microbe. In the case of intracellular microbes, CTL must first bind to the infected host cell. These interactions, like recognition of tumor and virus-infected cells, are often MHC-restricted and antigen-specific. Binding triggers the release of granulysin, which must enter the infected host cell for microbial killing to occur (Figure 1(B)). This is thought to be mediated through perforin-generated pores in the host cell membrane. These pores facilitate the influx of extracellular Ca^{2+} which triggers the target cell to endocytose the damaged region of the membrane and internalize nearby granulysin [7–9]. Alternatively, CTL may mediate lysis of the infected host cells releasing microbes which may then be recognized and killed by nearby CTL (Figure 1(C)).

To acquire microbicidal activity, T cells must be primed. This priming event can occur in response to cytokines such as T cell growth factors, stimulation by a mitogen, or antigen-specific responses. The receptors and signalling pathways involved in priming may be the same, but also

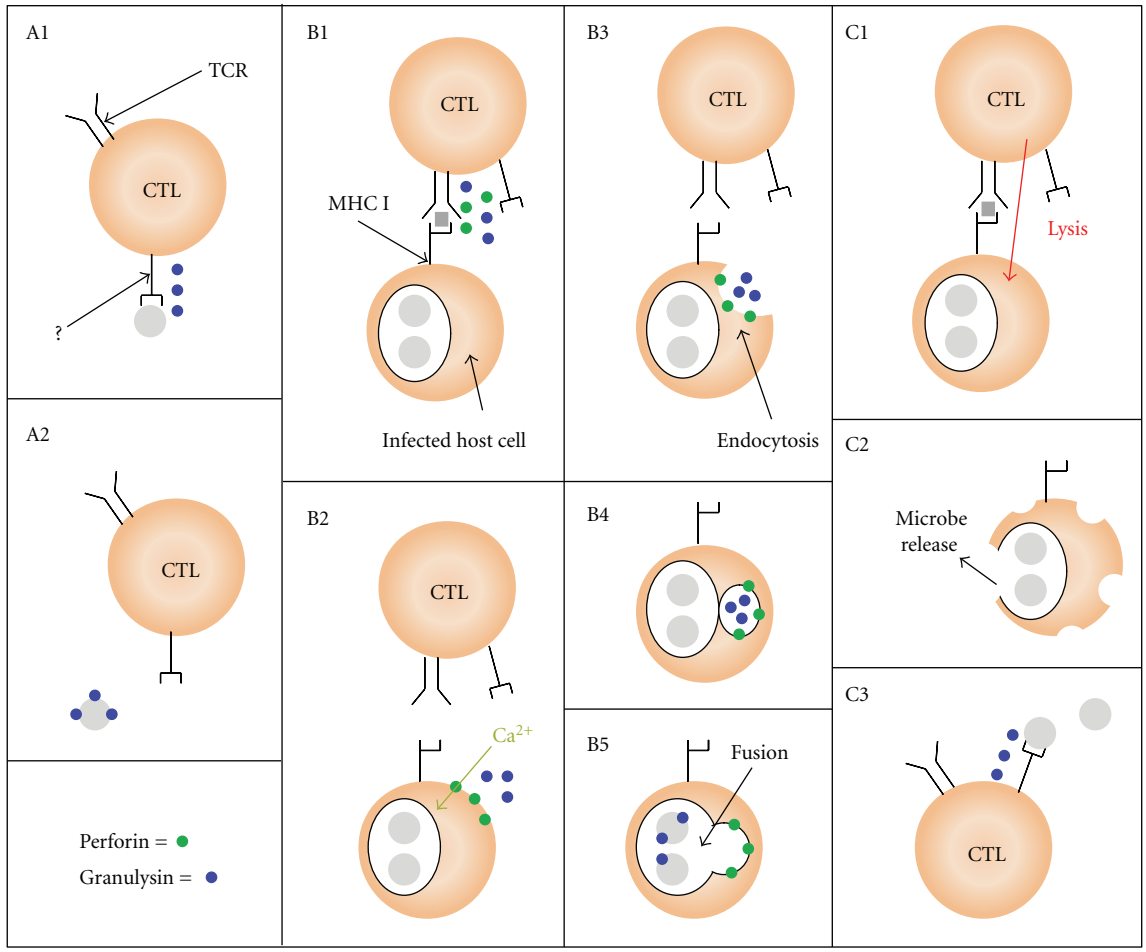


Figure 1: Killing of extracellular and intracellular microbes by CTL. (A) CTL bind to an extracellular microbe (grey) via an unknown receptor (?) independently of MHC (panel A1). Binding triggers CTL to release granulysin (blue) which binds to and kills the microbe (panel A2). By contrast, CTL killing of intracellular microbes involves MHC-restricted antigen-specific recognition of infected host cells. (B) CTL-host cell interactions induce the release of perforin (green) and granulysin (panel B1). Perforin-generated pores in the host cell membrane facilitate the influx of extracellular Ca^{2+} which induces endocytosis of the membrane region damaged by perforin, along with nearby granulysin (panels B2–B4). Granulysin-containing endosomes fuse with intracellular compartments containing the microbe allowing granulysin to bind to and kill the microbe (panel B5). (C) Alternatively, CTL induce lysis of an infected host cell causing the release of intracellular microbes which are then killed by other nearby CTL (panels C1–C3).

might be quite distinct from the receptors and signalling that lead to immediate killing. Following the priming event, the microbicidal CTL is then ready to receive the signal that triggers them to kill the pathogen.

2. Direct Killing of Bacteria by CTL

One of the earliest studies describing bactericidal CTL came from investigations with *Pseudomonas aeruginosa* [10]. T cells from mice immunized with *P. aeruginosa* polysaccharide were stimulated with macrophages from nonimmune mice with or without the addition of heat-killed bacteria. The T cells were then able to kill live *P. aeruginosa*. Macrophages had to be present during the priming, but surprisingly, heat-killed bacteria did not. This suggested that the bactericidal activity of these T cells was dependent on their interaction with macrophages but did not require presentation of *P. aeruginosa* antigens. Supernatants collected from immune T cells exposed to macrophages and *P. aeruginosa* were found to kill *P. aeruginosa* in addition to *Staphylococcus aureus* and *Escherichia coli* suggesting that these T cells were producing a soluble bactericidal product.

CTL have also been found to mediate killing of *Mycobacterium tuberculosis* through the release of bactericidal products [11–13]. *M. tuberculosis* growth was reduced as much as 74% in the presence of $CD4^+$ CTL and 84% in the presence of $CD8^+$ CTL [12]. When $CD8^+$ CTL were pretreated with strontium chloride (which depletes granule contents), it caused a clear reduction in the ability to kill *M. tuberculosis* that correlated with a marked decrease in granulysin content [13]. In vitro, purified granulysin was able to kill extracellular *M. tuberculosis* in a dose-dependent fashion. However, killing of intracellular *M. tuberculosis* required the addition of

perforin, which was not directly bactericidal, but was able to lyse *M. tuberculosis*-infected macrophages. Together this data suggests that perforin facilitates entry of granulysin into *M. tuberculosis*-infected cells where granulysin can access and kill the intracellular pathogen. In vivo studies have provided additional support for the conclusion that perforin is required for $CD8^+$-mediated clearance of *M. tuberculosis*. In these studies, irradiated mice infected with *M. tuberculosis* were found to have a significantly greater bacterial load after receiving adoptively transferred perforin-deficient $CD8^+$ T cells compared to wild-type $CD8^+$ T cells [14].

However, for other bacteria, killing can occur independently of perforin. Studies conducted with *Listeria innocua* support a perforin-independent mechanism in which granulysin is actively taken up by the infected cell [15]. Both healthy and *L. innocua*-infected dendritic cells (DC) were found to take up recombinant granulysin in a temperature-sensitive manner, indicative of active internalization. Furthermore, cholesterol depletion abrogated granulysin uptake and killing of infected DC, suggesting lipid raft involvement. This was further supported by data showing colocalization of granulysin and cholera toxin (used as a marker of lipid rafts) during and shortly following uptake. Immunofluorescent microscopy showed granulysin trafficking through the endocytic pathway following internalization. Ninety minutes after uptake, granulysin was found to colocalize with phagosomes containing *L. innocua* DNA. Indeed, granulysin was found to kill both extracellular *L. innocua* and *L. innocua*-infected DC in a dose-dependent manner. While granulysin may function independently, perforin might also help. Another study found that perforin treatment (whether simultaneous or sequential to granulysin treatment) augmented granulysin-dependent killing of intracellular *L. innocua* which was not due to the formation of stable pores in the DC membrane [16]. Rather, perforin treatment was found to stimulate a transient change in the plasma membrane permeability (assessed by Ca^{2+} influx) that enhanced fusion of granulysin-containing endosomes with phagosomes containing *L. innocua*.

CTL killing of *Mycobacterium leprae* has also been found to involve granulysin. The highest level of expression of granulysin occurred in patients afflicted with the localized tuberculoid form, rather than the disseminated lepromatous form of the disease [17]. This correlation suggests that granulysin release by CTL may limit the spread and severity of *M. leprae* infection. Remarkably, granulysin expression was mostly limited to $CD4^+$ T cells. $CD4^+$ T cell lines derived from tuberculoid leprosy lesions were found to lyse *M. leprae* infected macrophages and kill intracellular mycobacteria. Strontium treatment abrogated both the cytolytic and bactericidal activity of these $CD4^+$T cells, which suggested that killing was mediated through the granule-exocytosis pathway. Blocking Fas using anti-Fas antibody also partially inhibited the cytolytic activity of two of four $CD4^+$ T cell lines suggesting that the Fas-FasL pathway may also play a minor role in the lysis of *M. leprae*-infected cells. It follows that the extent of Fas-FasL mediated lysis may depend on the nature of the CTL [18] or the infected target cell [19].

3. Direct Killing of Parasites by CTL

Early studies with *Schistosoma mansoni* described the capacity of CTL to directly kill parasites [20]. T cells stimulated with phytohemagglutinin (PHA), a mitogenic lectin, as well as various oxidative mitogens, antigens, and alloantigens were found to kill schistosomula. Killing was found to correlate with increased binding to schistosomula suggesting a contact-dependent mechanism. In support of this, supernatants from PHA-stimulated T cells were ineffective at killing, although the concentration of parasiticidal products in the supernatants may not have been high enough to mediate an appreciable response. Moreover, it remains unclear how PHA induces CTL parasiticidal activity. Removal of PHA prior to incubation of T cells with schistosomula was found to impair killing suggesting that PHA may have an ulterior function in addition to stimulating T cell activation and proliferation. CTL killing of *Entameoba histolytica* closely resembled that of *Schistosoma mansoni* in many respects [21]. First, nonspecific activation of T cells with PHA was found to significantly enhance killing of *E. histolytica* trophozoites compared to unstimulated T cells. PHA-stimulated T cells killed as many as 92% of trophozoites whereas amoebic viability in the presence of unstimulated T cells remained relatively unchanged [21]. Second, binding correlated with killing suggesting a contact-dependent mechanism. Third, and surprisingly, killing required the continuous presence of PHA, again alluding to direct participation. One possibility is that PHA bridges the T cells and *E. histolytica*. However, bridging the T cells and *Entameoba* cannot be the only mechanism by which PHA participates in killing because at least 18 hours of preincubation was required to elicit killing. Similarly, in studies with *S. mansoni*, pretreatment of schistosomula with PHA was not sufficient to induce parasiticidal activity of unstimulated T cells [20]. Thus, PHA appears to act both by activating the T cells as well as possibly functioning as a bridge for binding.

Specific activation of T cells isolated from patients with an amoebic liver abscess with an *E. histolytica* lysate also induced killing [22]. In contrast to prior studies with PHA [20, 21], stimulation with antigens from *E. histolytica* was found to augment binding intensity of T cells to the trophozoites rather than the binding frequency [22]. Thus, the more important determinants in killing amoeba may be the binding characteristics of the CTL rather than the number of CTL bound. The mechanism underlying the parasiticidal activity of these T cells is poorly understood. Anti-TNF-α antibody blocked killing of immune T cells from mice immunized against *E. histolytica*, although large doses of TNF-α did not directly kill the amoeba [23]. These results demonstrate the complexity of the system and the possible role of additional mediators.

CTL have also been reported to directly kill the pathogenic parasite *Toxoplasma gondii*. $CD8^+$ T cells from mice immunized with P30 (a *T. gondii* membrane protein) and subsequently restimulated in vitro with P30 were found to kill extracellular *T. gondii* [24]. Unlike studies conducted with *S. mansoni* and *E. histolytica* [20, 21],

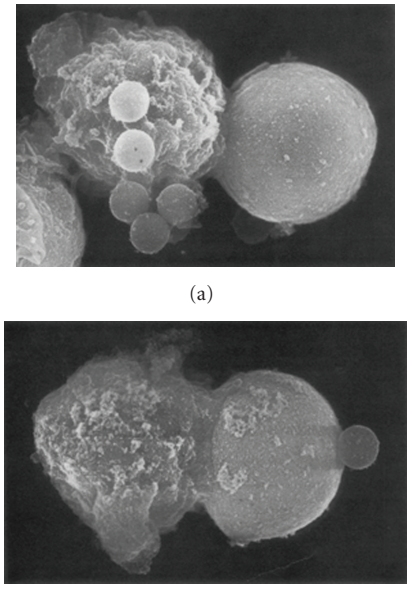
killing of *T. gondii* appeared to be antigen specific. P30-antigen-specific splenocytes killed significantly more *T. gondii* than splenocytes stimulated with the mitogenic lectin, concanavalin A (ConA), despite greater T cell proliferation in response to ConA [24]. Although antigen stimulation resulting in microbicidal priming was likely to have been MHC-restricted, killing was direct. Since there is no evidence for expression of MHC or MHC-like molecules by *T. gondii*, this suggests that direct cytotoxicity was MHC-independent. MHC-unrestricted T cell killing of extracellular *T. gondii* has also been reported by others [25]. However, the mechanism by which antigen-specificity is accomplished in the absence of MHC is not yet defined.

CTL responses against intracellular *T. gondii*, on the other hand, have been found to proceed through an MHC-restricted mechanism [26]. Splenocytes from mice immunized against *T. gondii* mediated killing of both infected macrophages and intracellular *T. gondii* [26, 27]. These activities were found to be mediated by $CD8^+$ T cells. Concanamycin, an inhibitor of the vacuolar ATPase that is required to maintain perforin in lytic granules, significantly impaired killing, implicating perforin in the response against *T. gondii* [27]. Indeed, perforin has been found to play a critical role in chronic toxoplasmosis. Perforin-deficient mice chronically infected with *T. gondii* were found to have a higher rate of mortality and a greater number of brain cysts compared to wild-type mice [28], although other studies came to disparate conclusions [29]. Moreover, the ability of $CD8^+$ T cells to kill intracellular *T. gondii* is controversial. In one study, $CD8^+$ T cells failed to kill *T. gondii*-infected B cells as assessed by quantitative PCR of the SAG-1 gene [30]. Further studies will be required to resolve the discrepancy between these studies.

Emerging from this uncertainly, *Plasmodium falciparum* is clearly killed directly by CTL. $\gamma\delta$ T cells were found to kill blood-stage *P. falciparum* through a contact-dependent mechanism [31–33]. Killing appeared to be stage-dependent with extracellular merozoites being the suggested targets [31, 32]. In vitro studies revealed a correlation between the parasiticidal activity of the $\gamma\delta$ T cells and expression of granulysin [33]. Treatment of the T cells with antigranulysin antibody abrogated this activity. Together, this data indicates that parasiticidal CTL mediate killing of *P. falciparum* via the release of granulysin.

4. Direct Killing of Fungi by CTL

Several studies have reported the ability of CTL to kill the opportunistic fungus *Candida albicans*. IL-2 stimulation of murine splenocytes was found to induce fungicidal activity against *C. albicans* as well as several other *Candida* species [34]. Anti-*Candida* activity required at least 3 days of priming with IL-2 and peaked at 7 days. The induction of fungicidal activity correlated with enhanced killing of an NK cell-resistant tumor cell line. Supernatants taken from IL-2 stimulated splenocytes had no effect on the growth of *C. albicans* suggesting a contact-dependent fungicidal mechanism. IL-2 stimulated human PBMC were also found to inhibit the growth of *C. albicans*. Moreover, another study found that the $CD8^+$ T cells, and not $NK1.1^+$ cells (NK cells), mediated fungicidal activity against *C. albicans* in response to IL-2 [35].



(a)

(b)

Figure 2: Scanning electron micrographs of conjugates formed between T cells (left) and *C. neoformans* (right). Magnification in both panels is $\times 16,370$. Cells in (a) were labelled with mouse monoclonal anti-CD3 antibody followed by goat antimouse IgG bound to latex beads. Latex beads are seen attached to the effector cell (a). Cells in (b) were labelled with UPC10 as a control IgG_{2a} followed by goat antimouse IgG conjugated to latex beads. One latex bead is seen associated with the cryptococcal cell; however, no beads attached to the effector cell (b).

The CTL response against the yeast-like pathogen *Cryptococcus neoformans* has been described extensively. In one of the earlier studies, lymphoid cells were isolated after mice had been immunized with heat-killed *C. neoformans* in complete Freund's adjuvant [36]. The anticryptococcal activity of these cells was measured by assessing colony forming units, and delayed-type hypersensitivity was assessed by footpad swelling following administration of *C. neoformans* antigen. Only lymphoid cells from immunized mice were found to inhibit the growth of *C. neoformans*. Growth inhibition reached as high as 60–80% as assessed by CFU [36]. Furthermore, the magnitude of the anticryptococcal activity correlated with the intensity of the delayed-type hypersensitivity response. T cells within the peripheral lymphoid compartment were found to be the mediators of the anticryptococcal activity [36]. In another study, T cells were observed to interact directly with *C. neoformans* in vitro suggesting a contact-dependent mechanism of killing (Figure 2) [37]. However, between 11 and 35% of T cells were found to bind to *C. neoformans* suggesting recognition by a receptor other than the T cell receptor. Other studies reported similar killing with human peripheral blood mononuclear cells (PBMC) stimulated with dead

C. neoformans [38] or cultured for 7 days with IL-2 and GM-CSF (but not with TNF, IFN-γ, or vitamin D3) [39]. In agreement with prior studies [36, 37], T cells (as well as NK cells) isolated from the PBMC were found to directly bind to, and possess fungicidal activity against *C. neoformans* after priming with IL-2 [40]. Another study found that freshly isolated T cells could mediate killing of *C. neoformans* without the need for IL-2 stimulation [41]. However, T cells cultured without IL-2 soon lost their ability to kill, suggesting that IL-2 is required for T cells to maintain their fungicidal activity. Treatment of T cells with the proteases trypsin and bromelain, which have been found to cleave several receptors on T cells [42], also impaired killing of *C. neoformans* suggesting that receptor-ligand interactions were involved in anticryptococcal activity [41].

Investigators have asked whether CTL kill *Cryptococcus* or exert their anticryptococcal activity by inhibiting the growth of the organism. Studies using the viability dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) showed that *C. neoformans* failed to demonstrate metabolic activity after incubation with T cells [43]. *C. neoformans* also becomes permeable and labels with propidium iodide after incubation with CTL providing further evidence for killing (unpublished observations, Anowara Islam). Furthermore, CTL can reduce the burden of organisms below the starting innocula [41, 44], and consistent with previous studies that suggested a contact-dependent mechanism of killing, separation of T cells and *C. neoformans* with a porous membrane during incubation was found to abrogate anticryptococcal activity [43]. Thus, the burden of evidence is that killing occurs.

4.1. Effector Mechanisms Used by CTL to Kill Fungi. Early studies investigating the mechanism of CTL anticryptococcal activity examined possible receptors and effector mechanisms. Antibody-mediated blockade of various T cell surface molecules such as LFA-1 and CD3 did not significantly inhibit killing [45]. Similar results were found using putative ligands to block mannose and hyaluronate receptors. Together this data suggests that CTL killing of *C. neoformans* may proceed through a novel receptor that is yet to be identified.

These early studies also examined the role of reactive hydroxyl radicals [45], which have been suggested to play a role in eosinophil-mediated killing [46]. Additionally, cyclooxygenase inhibitors and prostaglandin E2, which have been found to inhibit NK cell cytotoxicity [47, 48] and NF-κB activation [49], were assessed. Among the 3 hydroxyl radical scavengers used, only catechin was found to impair killing of *C. neoformans* [45]. Similarly, only 1 (salicylic acid) of the 3 cycloxygenase inhibitors abrogated killing, while prostaglandin E2 failed to have any appreciable effect. One study, however, described an effector mechanism by which IL-15 stimulated $CD8^+$ CTL kill *C. neoformans* [44]. In vitro studies have previously shown that IL-15 from *C. neoformans*-stimulated monocytes induced T cells to become anticryptococcal [50]. This led to studies showing that the anticryptococcal activity of IL-15-stimulated $CD8^+$ T cells correlated with the level of granulysin expression [44]. Furthermore, both strontium treatment and siRNA knockdown of granulysin abrogated anticryptococcal activity, directly implicating granulysin in the CTL response against *C. neoformans*. Perforin was not required, as neither concanamycin A nor EGTA treatment impaired anticryptococcal activity. These results are in contrast to NK cells, which depend on perforin, but not granulysin for killing [51].

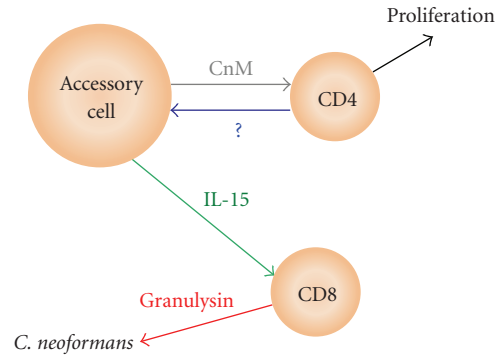


Figure 3: $CD8^+$ T cells are primed for microbicidal activity following stimulation with *C. neoformans* mitogen (CnM). Recognition of CnM (presented by accessory cells) by $CD4^+$T cells triggers their activation and proliferation. Meanwhile, a reciprocal signal from $CD4^+$T cells mediated through an unknown receptor-ligand interaction (?) induces accessory cells to express IL-15, which in turn primes $CD8^+$ T cells for granulysin expression and anticryptococcal activity.

These results were extended using $CD8^+$ T cells purified from PBMC that had been stimulated with *C. neoformans* mitogen (CnM) [44], a protein mitogen within the cryptococcal cell wall and membrane [52, 53]. Priming of anticryptococcal activity and granulysin expression by stimulation with CnM was dependent on $CD4^+$ T cells [44]. Stimulation of PBMC with CnM in the presence of anti-IL-15 abrogated anticryptococcal activity and granulysin expression suggesting that the dependence on $CD4^+$ T cells was mediated through IL-15. However, in the absence of accessory cells, $CD4^+$ T cells were not sufficient to induce $CD8^+$ T cell anticryptococcal activity and granulysin expression. Together this data suggests that the proliferating $CD4^+$ T cells provide a retrograde stimulus to accessory cells that results in production of IL-15, which then primes $CD8^+$ T cell for granulysin expression and anticryptococcal activity (Figure 3) [44]. Indeed, both $CD4^+$ and $CD8^+$ T cells in vivo have been found to be indispensable for an effective response to *C. neoformans* [54–58].

Aside from their role in licensing accessory cells to prime $CD8^+$ CTL anticryptococcal activity [44], $CD4^+$ T cells have also been demonstrated to directly kill *C. neoformans* [59]. Upon stimulation with IL-2, $CD4^+$ T cells increased the expression of granulysin (but not perforin) which correlated with increased fungicidal activity. Both strontium treatment and granulysin siRNA knockdown abrogated $CD4^+$ T cell killing of *C. neoformans*. Perforin involvement was excluded as neither concanamycin A nor perforin knockdown could

interfere with anticryptococcal activity of $CD4^+$ T cells stimulated with both IL-2 and anti-CD3 antibody (which stimulated both perforin and granulysin expression). Together this data suggests that $CD4^+$CTL (like $CD8^+$) directly kill *C. neoformans* through a granulysin-mediated mechanism.

The high prevalence of serious cryptococcal infection in HIV-infected patients [60, 61] may be at least partially due to a defect in this fungicidal activity. Indeed, $CD4^+$ T cells from these patients were found to exhibit defective killing of *C. neoformans* [59]. Neither IL-2 nor IL-2 plus anti-CD3 stimulation could induce granulysin expression in these patients' cells. Furthermore, stimulation with IL-2 alone was sufficient to induce perforin expression. These results suggest that profound dysregulation of perforin and granulysin expression may account for the failure of $CD4^+$ T cells from HIV-infected patients to kill *C. neoformans*.

4.2. Signalling Pathways Used by CTL to Kill Fungi. The signalling pathways regulating granulysin expression in $CD8^+$ and $CD4^+$ CTL are relatively unknown. In preparation for killing of fungi, $CD4^+$ T cells were stimulated with IL-2, which resulted in expression of granulysin [62]. Expression of granulysin correlated with short-term (0–60 min) and long-term (1–5 days) phosphorylation of extracellular signal-regulated kinases (ERK)1/2, p38 mitogen-activated protein (MAP) kinase, and p54 c-Jun N-terminal kinases (JNK) as well as the transcription factor signal transducers and activators of transcription (STAT)5. Meanwhile, no phosphorylation of the human oncogene Akt (signifying phosphoinositide 3-kinase (PI3K) activation) could be detected following continuous IL-2 stimulation. Akt phosphorylation could only be detected when $CD4^+$ T cells were stimulated with IL-2 for 3–5 days, rested (24 hours) and restimulated with IL-2 for 5 min, indicating that PI3K undergoes a transient activation after initial stimulation with IL-2. Continuous stimulation of $CD4^+$ T cells with IL-2 was also found to increase expression of the IL-2Rα and IL-2Rβ subunits of the IL-2 receptor (by day 3) followed by expression of granulysin (on day 5). Pharmacological inhibition of Janus kinase (JAK)3/STAT5 or PI3K abrogated both granulysin expression and anticryptococcal activity of IL-2 stimulated $CD4^+$ T cells, which correlated with decreased expression of the IL-2R subunits. Together this data suggests that granulysin expression requires acquisition of one or more of the IL-2R subunits. Indeed, blockade of IL-2Rβ expression using anti-IL-2Rβ antibodies or siRNA knockdown abrogated granulysin expression in IL-2 stimulated $CD4^+$ T cells. Thus, IL-2 (and perhaps other T cell growth factors) signals T cells to increase expression of IL-2Rβ, which is then available for signalling that is necessary for granulysin expression.

Previous studies have shown that $CD4^+$ T cells from HIV-infected patients exhibit dysregulated expression of perforin and granulysin [59]. Comparison of IL-2 signalling in $CD4^+$ T cells from healthy donors and patients infected with HIV revealed the underlying cause to be defective STAT5 and PI3K signalling [62]. IL-2 stimulation of $CD4^+$ T cells from HIV-infected patients failed to induce phosphorylation of STAT5 and failed to increase expression of IL-2Rβ. Furthermore, in contrast to $CD4^+$ T cells from healthy donors, IL-2 failed to induce phosphorylation of Akt. Together these results demonstrate a critical role for JAK/STAT and PI3K signalling in granulysin-mediated killing of *C. neoformans* by $CD4^+$ T cells.

The response in $CD8^+$ T cells is similar. In a recent study, JAK/STAT signalling was also found to be required for granulysin expression in $CD8^+$ T cells in response to IL-15 and IL-21 [63]. $CD8^+$ T cells stimulated with either IL-15 or IL-21 were found to increase granulysin expression, which correlated with increased phosphorylation of STAT3 and STAT5. IL-15 was found to induce phosphorylation of both STAT3 and STAT5, while IL-21 only induced phosphorylation of STAT3. Pharmacological inhibition and siRNA knockdown of JAK/STAT signalling was found to abrogate granulysin expression by $CD8^+$ T cells in response to IL-15 and IL-21. Together this data indicates that JAK/STAT signalling regulates granulysin expression by $CD8^+$ T cells in response to IL-15 and IL-21. Consistent with previous studies conducted with $CD4^+$ T cells [59, 62], HIV-infected $CD8^+$ T cells exhibited reduced phosphorylation of STAT3, STAT5, and granulysin expression in response to IL-15 and IL-21 as compared to mock-infected cells [63].

5. Summary

It is clear that both $CD4^+$ and $CD8^+$CTL mediate direct killing of a wide range of bacterial, parasitic, and fungal pathogens. Studies demonstrate several key features of the microbicidal CTL response. First, with rare exceptions, killing is contact-dependent. Several studies reported a correlation between the frequency [20, 21] or intensity [22] of binding and killing. Moreover, microscopy [37, 40, 41] revealed direct CTL-microbe interactions. Finally, T cells separated from *C. neoformans* by a porous membrane were unable to mediate killing [43]. Contact may induce release of cytotoxic products by the CTL or provide a microenvironment or concentration at which the cytotoxic product can be efficacious. The latter may explain why supernatants from activated T cells were often unable to mediate killing [20, 34, 40, 41]. Second, killing is mediated primarily through the granule-exocytosis pathway. In many experimental systems, pretreatment of CTL with strontium (which depletes granule contents) was found to abrogate killing [13, 17, 44, 59]. Furthermore, granulysin, a granule constituent [4], has been implicated in the killing of several microbes [13, 15–17, 33, 44, 59]. Third, killing (at least of extracellular microbes) is neither antigen-specific nor MHC-dependent. Nonspecific stimulation with T cell growth factors [34, 35, 39, 40, 44, 59] or mitogenic lectins [20, 21] was often sufficient to prime the effector cells for killing.

The effector mechanisms at work during microbial killing by CTL are gradually being unravelled. While granulysin appears to be highly involved in killing, the role of perforin and other effector molecules is not as clear. In the case of extracellular pathogens, perforin has not been found to play a role in killing [54, 59], although it plays a role in

direct killing by NK cells [51, 64, 65]. By contrast, killing of intracellular microbes requires perforin [13, 27], although some investigations have suggested a perforin-independent mechanism [15]. To be sure, this topic demands the attention of future studies.

Much less is known of the signalling pathways. JAK/STAT and PI3K signalling have been found to be essential in CTL killing of *C. neoformans* [62]. Defective JAK/STAT and PI3K signalling in CTL from HIV-infected patients [62, 63] may explain why these patients experience such a high incidence of severe cryptococcal infection.

During the process of evolution, CTL have developed the ability to specifically recognize altered self through complex TCR-MHC interactions. As a consequence, they have become the epitome of specific cell-mediated immunity. It is fascinating to now discover that during this process, CTL have, at the same time, preserved one of the most rudimentary immune functions of all, namely, the ability to directly recognize a microbe, bind, and without the help of antigen presenting cells or other effector cells kill the invading pathogen with competence and precision.

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Review Article

Vaccines against Human Carcinomas: Strategies to Improve Antitumor Immune Responses

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Multiple observations in preclinical and clinical studies support a role for the immune system in controlling tumor growth and progression. Various components of the innate and adaptive immune response are able to mediate tumor cell destruction; however, certain immune cell populations can also induce a protumor environment that favors tumor growth and the development of metastasis. Moreover, tumor cells themselves are equipped with various mechanisms that allow them to evade surveillance by the immune system. The goal of cancer vaccines is to induce a tumor-specific immune response that ultimately will reduce tumor burden by tipping the balance from a protumor to an antitumor immune environment. This review discusses common mechanisms that govern immune cell activation and tumor immune escape, and some of the current strategies employed in the field of cancer vaccines aimed at enhancing activation of tumor-specific T-cells with concurrent reduction of immunosuppression.

1. Introduction

The role of the immune system in limiting tumor growth, designated as cancer immunosurveillance [1], has been first elucidated in mouse models of immune deficiency characterized by a high incidence of spontaneous and chemically induced tumors [2]. Those studies have identified several components of the innate and adaptive immune response being responsible for tumor elimination, including $\alpha\beta$ and $\gamma\delta$ T-cells [3], and NK cells [4]. Reinforcing the role of cytotoxic T lymphocytes (CTLs) in the eradication of malignant cells, transgenic mice deficient in perforin, a component of the cytolytic granules of T and NK cells, are more susceptible to spontaneous and chemically induced tumors than their wild type counterparts [5]. In humans, evidence on the role of the immune system in limiting tumor growth and progression is linked to observations indicating a positive correlation between the presence of tumor infiltrating CD8+ T-cells and good prognosis in various types of cancer. In colorectal cancer, for example, significantly higher levels of early memory and effector memory CD8+ T-cell infiltrates positively correlate with good clinical outcome, defined as absence of metastatic invasion, less advanced pathological stage, and increased survival [6, 7]. Similarly, the presence of intraepithelial tumor infiltrating CD8+ T-cells has been associated with the lack of tumor metastases in the draining lymph nodes of cervical cancer patients [8]. In non-small cell lung carcinoma patients, increasing numbers of tumor infiltrating CD8+, CD20+, and CD4+ T lymphocytes have also been shown to significantly correlate with improved disease-specific survival [9]. Altogether, these observations support a role for the immune system in controlling tumor burden and form the rationale for the development of vaccine-based interventions against cancer that rely on the stimulation of an effective antitumor immune response in the host.

The immune system, however, has two paradoxical roles in cancer. While various components of the innate and adaptive immune response are able to mediate tumor cell destruction, specific types of immune cells can also induce a protumor environment that favors tumor growth and the development of metastasis [10]. Among the latter are, for example, regulatory T (Treg) cells [11, 12], tumor associated macrophages (TAM) [13, 14], and type 2 helper CD4+ (Th2)

T-cells [15, 16]. These various immune cells have been shown to accumulate at the site of the tumor, negatively impacting the establishment of antitumor T-cell responses, that is, creating an immunosuppressive tumor environment.

Cancer cells themselves are also equipped with mechanisms that allow them to evade recognition by the immune system or to negatively affect the functionality of effector T-cells. In order to avoid immune recognition, tumor cells have been shown to downregulate antigen expression, components of the antigen-processing and presentation machinery, and expression of major histocompatibility complex (MHC) molecules [17]. Decreased expression of costimulatory molecules of crucial importance to T-cell activation, and enhanced surface expression of molecules that negatively regulate activation of T-cells, such as PD-L1/B7-H1 and B7-H4, have also been demonstrated in various types of tumors [18–20]. Cancer cells can also restrain the function of the immune system by secreting a milieu of soluble factors that ultimately inhibit the activation, proliferation, and differentiation of the various components of the immune response. Among these molecules are TGF-β [21], IL-10 [22], IL-13 [23], and VEGF [24].

The goal of vaccine-based cancer immunotherapy approaches is to induce a tumor-specific immune response that ultimately will reduce tumor burden by tipping the balance from a protumor to an antitumor immune environment (Figure 1). This review discusses strategies employed in the field of cancer vaccines aimed at enhancing activation of tumor-specific T-cells with concurrent reduction of immunosuppression. Specifically, vaccine design, immune adjuvants, and multimodal approaches using vaccines in combination with other treatment modalities will be discussed here, with a particular emphasis on studies conducted at the National Cancer Institute, NIH.

Table 1: Vaccine-delivery systems.

Vaccine-delivery systems	
Immunization against multiple antigens*	Immunization against specified antigen(s)
Cell-based	**Cell-based**
Autologous whole-tumor cells	DCs pulsed with peptide
Allogeneic whole-tumor cells	Genetically-modified DCs
Genetically-modified tumor cells	
DCs-tumor fusion	**Protein/Peptide based**
DCs loaded with tumor lysate	Protein
DCs transfected with tumor-derived RNA	Peptide
	Agonist peptide
	Antiidiotype MAb
Protein/Peptide based	Mab fusion proteins
Tumor lysates	**Vector-based**
Heat shock proteins-tumor peptides	*Plasmid DNA*
	Bacterial vectors
	Listeria
	Salmonella
	Yeast vectors
	Viral vectors
	Adenovirus
	Vaccinia
	Avipox (fowlpox)
	MVA

*Vaccine formulation includes known and unknown antigens.

2. Vaccine Design: Choice of Vaccine-Delivery System(s)

Depending on the vaccine-delivery system of choice, cancer vaccines can elicit an immune response against an individual or multiple tumor antigens. A list of the various types of vaccine-delivery systems under investigation in the field, either at the preclinical or clinical stages, is presented in Table 1. Multiple studies have demonstrated that combinations of some of the strategies in the form of diversified prime/boost regimens may enhance the outcome of the intervention against the tumor [25]. Moreover, it has also been shown that concurrent vaccination with two distinct vaccine platforms targeting the same antigen can elicit a more diverse population of antigen-specific T-cells thus resulting in higher antitumor immunity [26].

Among the various types of vaccine delivery systems, there are strategies based on the use of whole tumor cells, dendritic cells-(DCs-) tumor cell fusions, or preparations of DCs loaded with tumor protein lysates or tumor-derived RNA (Table 1, left column). These vaccine platforms induce an immune response against multiple tumor targets, either known or unknown. Other vaccine modalities (Table 1, right column) are based on the previous characterization of tumor antigens to be used as "specified" targets in the vaccine formulation.

3. Tumor Antigens

Tumor antigens are molecules either exclusively expressed in the tumor cells, designated as "tumor-specific antigens", or molecules that are overexpressed in cancerous versus normal tissues, designated as "tumor-associated antigens". A comprehensive list of tumor antigens and their corresponding T-cell epitopes can be found at [27, 28]. Table 2 shows a brief list of selected examples for either type of antigen. Tumor-specific antigens appear *de novo* after cancer cells acquire mutations within the coding regions of certain genes, for example the oncogene *ras* [29] and the tumor suppressor *p53* [30], or novel fusion proteins are generated as in the case of the Bcr-Abl fusion in chronic myeloid leukemia [31]. Moreover, in tumors driven by infectious agents like human papillomavirus (HPV) or Epstein-Barr virus (EBV), virally-derived products become *de novo* targets of T-cell immune responses directed against the tumor. The majority of tumor-associated antigens so far identified have a certain level of expression in normal tissues and thus tolerance to these antigens often exists.

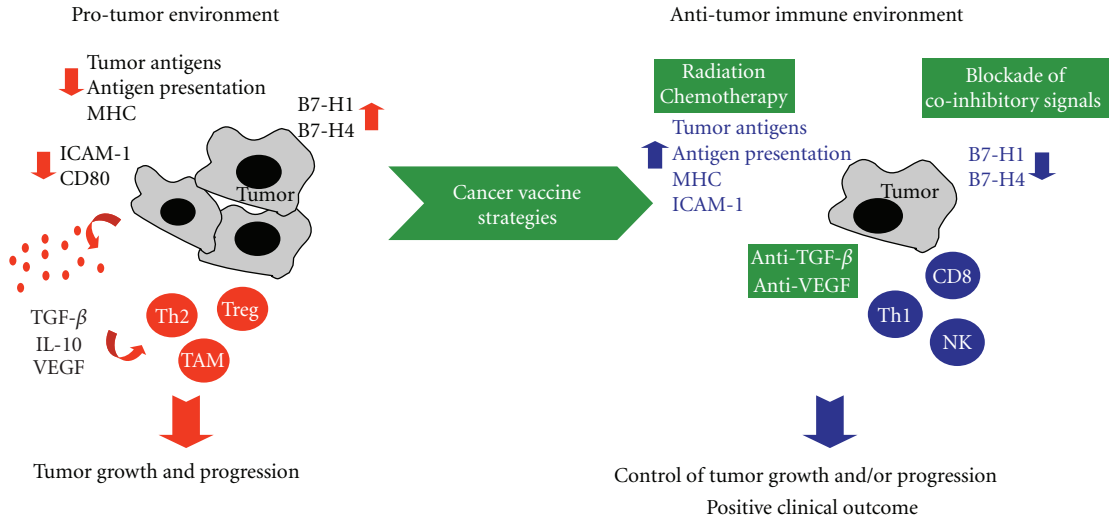

Figure 1: Cancer vaccine strategies aimed at shifting the immune environment of a tumor from protumorigenic to antitumorigenic.

A strategy used by tumors to escape immune recognition and destruction is the complete or partial loss of an antigen(s) [17]. Both in experimental animal models [32, 33], and in human cancer [34], it has been shown that "antigen-negative tumor variants", characterized by the loss of the targeted antigen, can emerge subsequently to an immune intervention. An approach to overcome this problem is the targeting of "functionally relevant antigens", which are proteins with an essential role during tumor initiation, growth, survival, or metastasis [35]. It can be hypothesized that an immune intervention directed against a functioning tumor antigen would greatly reduce the emergence of antigen-negative variants, since cells that lose the antigen will fail to grow, survive, or metastasize.

Table 2: Human carcinoma antigens.

Carcinoma antigens*	
Tumor-specific antigens	Tumor-associated antigens
Mutated molecules	Carcinoembryonic antigen (CEA)
K-RAS	Mucin 1 (MUC-1)
p53	Prostate-specific antigen (PSA)
Fusion molecules	Prostate acid phosphatase (PAP)
BCR-ABL	Prostate stem-cell antigen (PSCA)
	Brachyury
Virally-derived molecules	TERT
HPV-16 E6, E7	Wilm's tumor 1 (WT1)
EBNA1, LMP1 and LMP2	Her-2/neu
	Sox-2
	NY-ESO-1
	Cyclin D1
	Mesothelin
	Survivin

*Included is only a partial list of antigens for human carcinomas.

4. Targeting of Molecules that Control Metastatic Dissemination

In order to achieve the various steps along the metastatic cascade, epithelial tumor cells may need to undergo a phenotypic conversion into mesenchymal cells via a process designated as epithelial-to-mesenchymal transition (EMT) [36]. The EMT program involves the downregulation of epithelial proteins such as E-cadherin and cytokeratins, and the upregulation of mesenchymal proteins including Fibronectin, N-cadherin, and Vimentin. Various genes normally expressed in the early embryo have been implicated in the control of the EMT triggered during tumor progression, including *Twist*, *Snail*, *Slug*, *Goosecoid*, and *SIP1* [36, 37]. The transcription factors encoded by these genes can impart to tumor cells the traits of mesenchymal cells, including motility and the ability to invade the extracellular matrix (ECM). The expression of Twist, for example, has been found to be elevated in various types of cancer, including breast, prostate, and cervical cancer, with higher levels of Twist protein being detected in prostate cancer tissues of high Gleason score [38, 39]. Since the EMT process appears to be a necessary step for tumor cells to initiate the metastatic cascade [37, 40], interfering with EMT in early stages of the disease is likely to prevent tumor cell spreading and might also be effective in treating established metastatic lesions (Figure 2). An example of a tumor antigen with a functionally relevant role in the EMT program is the T-box transcription factor Brachyury, highly expressed in various human tumors of epithelial origin, but not in most human normal adult tissues [41]. It has been recently demonstrated that Brachyury overexpression in epithelial tumor cells induces an EMT, promoting the expression of mesenchymal markers and downregulation of epithelial markers, with concomitant increase in tumor cell migration and invasion [42]. Additionally, stable silencing of Brachyury expression in Brachyury-positive human carcinoma cells has been shown to downregulate mesenchymal markers

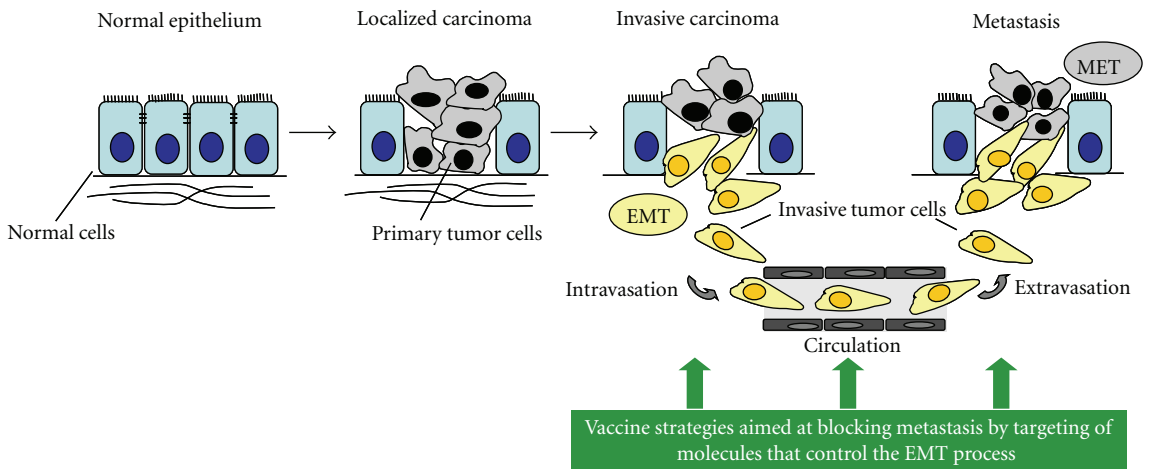

Figure 2: The epithelial-to-mesenchymal transition (EMT) in tumor progression: an opportunity to target metastatic tumor cells. The epithelial-to-mesenchymal transition (EMT) and its reverse process, designated as mesenchymal-to-epithelial transition (MET), are involved in the progression of epithelial tumors towards metastasis. Vaccine strategies targeting molecules that control the EMT process, for example, the transcription factor Brachyury, could be used to block tumor spreading.

and upregulate epithelial markers with simultaneous loss of cell migration and ECM invasion. In vivo, Brachyury-inhibited human tumor cells had a decreased ability to form experimental lung metastases after intravenous injection, as well as to disseminate from the primary, subcutaneous tumor to the site of metastases [42]. A CD8 T-cell epitope of Brachyury capable of expanding Brachyury-specific T-cells from the peripheral blood of cancer patients was recently identified [41]; Brachyury-specific T-cells have been used to efficiently lyse Brachyury-positive tumor cells in vitro. The successful expansion of T-cells directed against the transcription factor Brachyury exemplifies the ability of T-cell mediated immunotherapies to target (a) highly conserved tumor proteins, and (b) tumor proteins irrespective of their cellular localization. Because of its relevant role during tumor progression, Brachyury is an appealing tumor antigen for interventions aimed at interfering with the metastatic spreading of tumor cells (Figure 2). Additionally, the transcription factors Twist, Snail, and Slug, among others, which are critically involved in the control of EMT during tumor progression, could also be explored as novel tumor antigens for the targeting of metastatic disease.

5. Antigen Cascade

A phenomenon observed with cancer vaccines is the induction of immune responses to tumor antigens that are not present in the vaccine formulation. For example, it has been shown that CEA-transgenic mice cured of CEA-positive tumors by a CEA/TRICOM vaccine regimen were able to subsequently reject CEA-positive as well as CEA-negative tumors, and that this effect was mediated by the generation of specific T-cell immune responses directed against gp70, an antigen expressed by the tumor but not present in the vaccine [43]. The same phenomenon has also been reported in clinical studies [44, 45]. Altogether these studies showed that the immune response initiated against a tumor antigen included in the vaccine formulation is then followed by cross-priming and initiation of an "antigen cascade" that expands the immune response to additional antigens expressed on the tumor, thus potentiating antitumor immunity.

6. Enhancing Activation of Tumor-Specific T-Cells: The Use of Costimulation in the Vaccine Formulation

Optimal activation of T-cells is known to require at least two signals. The first signal is mediated by the interaction between the peptide/MHC complex on the surface of antigen presenting cells (APC) and the T-cell receptor (TCR) on the surface of T-cells. The second signal, also designated as costimulation, is mediated by the interaction between accessory molecules located on the surface of the APC and their corresponding ligand(s) on the T-cells [46]. Activation via the TCR in the presence of adequate costimulatory signals results in the clonal expansion, differentiation, and expression of effector functions by antigen-specific T-cells. Optimization of the mechanism of T-cell activation is critical to achieve a successful immune response to an antigen included in a vaccine formulation. A list of various strategies being explored in the field of cancer vaccines to achieve enhanced antitumor immune responses is presented in Table 3. One of them is the delivery of a single or multiple costimulatory molecules along with the tumor antigen as part of the vaccine formulation. One of the most studied T-cell costimulatory molecules is B7-1 [47, 48]. In preclinical studies it has been shown that mice immunized with an admixture of recombinant vaccinia (rV-) viruses encoding for a tumor antigen (rV-CEA or rV-MUC-1) and the costimulatory molecule B7-1 (rV-B7-1) generate effective antigen-specific T-cell immune responses that translate into successful antitumor immunity [49]. Furthermore, it was demonstrated that combinations of various costimulatory

molecules act synergistically to further enhance antigen-specific T-cell responses. Using recombinant vaccinia and fowlpox viruses encoding for the tumor antigen CEA and three different costimulatory molecules (B7-1, ICAM-1, LFA-3, designated as TRICOM), T-cell responses against the tumor antigen were further enhanced above the level observed when only one costimulatory molecule was used [50]. In preclinical studies with human T-cells in vitro, enhanced activation of antigen-specific T-cells was observed against DCs modified by infection with rF-TRICOM [51]. Moreover, the same vector was successfully used to enhance the antigen-presentation potency of freshly isolated B cells, resulting in enhanced activation of antigen-specific T-cell responses in vitro [52]. Results from a Phase II randomized clinical trial in 125 metastatic prostate cancer patients were recently reported [53]; patients were randomized to receive either a vaccine regimen consisting of two recombinant viral vectors each encoding for prostate specific antigen (PSA) and the TRICOM molecules (rV-PSA/TRICOM and rF-PSA/TRICOM), or control empty vector (control arm). The results from this trial demonstrated a 44% reduction in the death rate and an 8.5 month improvement in median overall survival (OS) in men in the vaccine compared to the control arm [53]. Additionally, NCI also reported the results from a randomized Phase II trial in 32 patients with metastatic castrate-resistant prostate cancer, who received a prime with rV-PSA/TRICOM and booster vaccinations with rF-PSA/TRICOM. Twelve of 32 patients showed declines in serum PSA post-vaccination; patients with greater PSA-specific T-cell responses showed a trend toward enhanced survival. In general, there was evidence of enhanced median overall survival, particularly among patients with more indolent type of disease [54]. Overall, cancer vaccine strategies incorporating costimulatory molecules have demonstrated the generation of antitumor immunity and evidence of clinical benefit in cancer patients.

Table 3: Strategies to enhance antitumor T-cell responses*.

Strategy
Use of costimulation in vaccine formulation
Cytokines
Effect on APC (GM-CSF)
Effect on T-cells (IL-2, IL-7, IL-15, IL-12)
Radiation
Chemotherapy
Small molecule targeted therapies
Inhibition of coinhibitory signals
At the tumor site (B7-H1, B7-H4)
Directly on T-cells (CTLA-4)
Depletion/inhibition of Treg cells
Inhibition of immunosuppressive cytokines
AntiTGF-β
AntiVEGF

* Only a partial list is included here.

7. The Use of Cytokines as Vaccine Adjuvants

7.1. Cytokines that Affect the APCs. Biological adjuvants are agents generally used for improving the immunogenicity of an antigen in a vaccine formulation. Several cytokines have the ability to enhance immune responses by either (a) promoting the differentiation, activation, or recruitment of APC, therefore enhancing antigen presentation and activation of antigen-specific T-cell responses, or (b) by directly acting on various subsets of T-cells. Within the first group, one of the most studied cytokines is the granulocyte-macrophage colony-stimulating factor (GM-CSF). It has been demonstrated in preclinical studies that subcutaneous injections of GM-CSF at the vaccination site can significantly increase the infiltration of DCs in regional lymph nodes that drain the site of vaccination [55, 56]. In several preclinical studies, tumor cells or DCs genetically engineered to secrete biologically active GM-CSF have been used to generate a systemic antitumor immune response [57, 58]. The use of GM-CSF at high doses, however, could be detrimental in the context of vaccines since it may result in immune suppression via the activation and expansion of myeloid-derived suppressor cells [59]. In the clinical setting, two placebo-controlled Phase III trials in patients with hormone-refractory prostate cancer, for example, have been performed with an autologous DC-based vaccine, designated Sipuleucel-T, which is genetically modified to express prostatic acid phosphatase (PAP) as a tumor antigen, and GM-CSF. Results from the initial trial with 127 patients [60] demonstrated a 4.5-month improvement in median survival in the vaccine versus placebo group, though without meeting the primary endpoint of time to progression. Results from a subsequent Phase III, placebo-controlled trial measuring overall survival as the primary endpoint have been recently reported [61], indicating a statistically significant survival advantage in patients in the vaccine versus placebo group.

7.2. Cytokines that Affect the T-Cell Compartment. The second group includes cytokines that directly affect the T-cell compartment by promoting T-cell proliferation, activation, and effector function. Among these, the cytokines IL-2 [62, 63], IL-7 [64], IL-15 [65, 66], and IL-12 [67] are currently under investigation to enhance antitumor immune responses elicited by a vaccine. The most used of these cytokines is IL-2, a T-cell growth factor which, as a single agent, has demonstrated clinical responses in patients with metastatic renal cell carcinoma [68] and metastatic melanoma [69]. A disadvantage, however, in the use of IL-2 therapy in vivo, particularly with high-dose IL-2, is the rate of associated toxicities [70]. Moreover, IL-2 mediates not only the proliferation of activated, effector T-cells and NK cells, but also the development and homeostasis of regulatory T-cells (Tregs), which constitutively express elevated levels of the IL-2R alpha (CD25) [71]. In vitro, IL-2 has also been used to expand tumor-specific T-cells to be used for adoptive immunotherapy [72].

The cytokines IL-15 and IL-7 are also T-cell growth factors; their function, however, is different to that of IL-2 in

vivo. IL-15 is necessary for the development and homeostasis of memory CD8 T-cells and NK cells [65]. It has also been demonstrated a role for IL-15 in the induction of long-lived, high avidity CD8 T-cells [73, 74] and, unlike IL-2, there is no role for IL-15 on the proliferation of Tregs. Up to date, studies with IL-15 as an adjuvant for cancer vaccine strategies have only been conducted in preclinical models, with encouraging results [67]. The cytokine IL-7, another T-cell growth factor, targets a different population of T-cells, promoting the expansion of naïve T-cells and thus increasing the diversity of T-cell repertoire after lymphopenia [64, 65]. IL-7 has also demonstrated positive results as an adjuvant cytokine for cancer vaccine interventions in preclinical studies [67].

Another cytokine that is under investigation as a vaccine adjuvant is IL-12, which promotes Th1 polarization, proliferation of activated T-cells and NK cells, and cell-mediated immunity. IL-12 has been shown to have potent antitumor effects in preclinical models [75]. In humans, however, the systemic delivery of IL-12 has resulted in elevated toxicities [76], hence leading towards the investigation of alternative modes for local delivery of IL-12. For example, in preclinical studies, a coformulation of IL-12 with chitosan intravesically delivered was well tolerated and very efficient at curing mice with superficial bladder cancer. A durable antitumor immune response was also generated in mice receiving IL-12/chitosan, providing them with complete protection from intravesical tumor rechallenge [77]. Overall, the use of cytokines as vaccine adjuvants to enhance the immune response to a tumor is a very promising and active field of investigation. Current research is focused on understanding the proper ways of delivery for each particular cytokine in order to maximize the immune adjuvant effects while reducing potential toxicities, when used in combination with various types of vaccine platforms.

8. Vaccine Plus Radiation

As it was mentioned above, a mechanism by which tumor cells escape immune recognition and attack is through the downregulation of tumor antigens, MHC expression, or various components of the antigen processing/presentation machinery. A strategy to overcome these obstacles is the use of radiation on tumor cells. Radiation is the standard of care for many types of cancer because of its direct cytotoxic effect on the tumor or its palliative effects on the patient. It has been recently reported that local irradiation of tumors with doses insufficient to induce tumor cell death could result in changes on the phenotype of the tumor cells that include the upregulation of MHC, Fas, ICAM-1, and various tumor associated antigens [78–80]. As a result of these changes, irradiated tumor cells are more susceptible to T-cell mediated immune attack. In preclinical studies with a murine colon carcinoma tumor model, sublethal, local tumor irradiation significantly improved the therapeutic efficacy of a recombinant rV-/rF-CEA/TRICOM vaccine regimen against CEA-positive tumors in CEA-transgenic mice [81], while radiation alone or vaccine alone had no effect on tumor growth. In the clinical setting, the approach has been investigated in a phase II clinical trial in patients with localized prostate cancer, randomized to receive a PSA-based poxviral vaccine plus radiotherapy versus radiotherapy alone [82]. The results from this trial indicated increases in PSA-specific T-cell responses of at least 3-fold in patients in the combination arm; the authors also reported evidence of de novo generation of T-cells to well-described prostate-associated antigens not found in the vaccine, providing indirect evidence of immune-mediated tumor killing. These studies thus demonstrated a new paradigm for the use of local tumor irradiation in combination with active vaccine therapy to elicit an effective antitumor immune response.

9. Vaccines Plus Cytotoxic Drugs

Because of the widespread use of chemotherapy for the treatment of most malignancies, it is rational to design combinatorial approaches using vaccines plus standard chemotherapeutic agents. Like radiotherapy, the use of various types of chemotherapy in combination with vaccines has resulted in enhanced antitumor immune responses. Although the mechanisms involved vary among the various types of cytotoxic drugs employed, in general, drugs can: (a) induce "immunogenic death" of tumor cells, leading to activation of DCs followed by antigen presentation to T-cells [83, 84], or (b) modulate the phenotype of the tumor cells making them more susceptible to immune-mediated killing. For example, it has been shown that treatment of human colon carcinoma cell lines with 5-fluorouracil or cisplatin enhances their lytic sensitivity to antigen-specific CD8+ cytotoxic T lymphocytes, by inducing expression of ICAM-1 and Fas [85]. Similarly, treatment of renal cell carcinoma cells with subtoxic concentrations of adriamycin has been shown to upregulate the expression of ICAM-1 and LFA-3, as well as to enhance T-cell mediated killing, Fas-mediated, and TRAIL-mediated killing of tumor cells [86]. Taxanes, on the other hand, are a widely used type of chemotherapeutic agents known to have various effects on the immune system, promoting macrophage activation and release of inflammatory cytokines at the tumor site, thus enhancing tumor lysis [87]. In preclinical studies with CEA-transgenic mice transplanted with CEA-positive tumor cells, enhanced antitumor effect was achieved by using a combination of a rV-/rF-CEA/TRICOM vaccine regimen plus docetaxel, compared to that of vaccine or docetaxel alone [88]. In preclinical models as well, cyclophosphamide, doxorubicin, and paclitaxel have all been shown to enhance the antitumor immune response elicited with a GM-CSF-secreting, HER2/neu-expressing whole-tumor cell vaccine in tumor bearing neu-transgenic mice [89]. The authors were able to demonstrate that the increased antitumor effects in the combination group (vaccine plus drugs) were due to enhanced vaccine efficacy rather than a direct cytolytic effect of the drugs on cancer cells [89].

Altogether, these studies demonstrated that, if used in appropriate schedule and at the correct doses, chemotherapeutic agents could enhance antitumor responses when used in combination with cancer vaccine modalities. Therefore,

further studies investigating optimum schedules and dosing for various chemotherapeutic agents are needed in order to optimize the use of different cytotoxic agents in combination with cancer vaccines.

10. Vaccine Plus Small Molecule Targeted Therapies

As the molecular pathways involved in the various steps of carcinogenesis and tumor progression are being elucidated with the advent of sophisticated genetic and molecular techniques, a novel group of therapeutic cancer drugs aimed at inhibiting specific molecular pathways is emerging, designated as small molecule targeted therapies. These drugs are also now being investigated for their immune-modulatory functions to be potentially used in combination with cancer vaccines. For example, the anticancer agent lenalidomide (Revlimid, Celgene Corp., NJ, USA), which is FDA approved for the treatment of patients with multiple myeloma, has been shown to have several immune-modulatory effects that include costimulatory effects on CD3-activated T-cells, augmentation of NK cell cytotoxicity, and suppression of Treg cells proliferation and function [90, 91]. Results from a study combining a small molecule BCL-2 inhibitor and a rV-/rF-CEA/TRICOM vaccine regimen were recently reported [92]. It has been shown that, when administered after the vaccine, the BCL-2 inhibitor GX15-070 was able to increase the intratumoral ratio of activated, CD8+ T effector to Treg cells, thus resulting in significant reduction of pulmonary tumor nodules in a mouse model of experimental lung tumors [92]. As with chemotherapeutic agents, the use of small molecule targeted therapies could also be associated with potential toxicities and, in particular, with negative effects on the immune compartment. Thus, further studies investigating optimum schedules and dosing for various small molecule targeted drugs are needed in order to optimize their use in combination with cancer vaccines.

11. Inhibition of Coinhibitory Signals

An alternative strategy to enhance the outcome of an antitumor immune approach is to eliminate negative signals imparted to T-cells by coinhibitory molecules such as B7-H1, B7-H4, and cytotoxic T-lymphocyte antigen-4 (CTLA-4), among others. B7-H1 is constitutively expressed in many types of human tumors and has been shown to promote evasion of tumor immunity by promoting apoptosis of activated effector T-cells [18] and tumor resistance to T-cell mediated lysis [93]. In preclinical studies, blockade of B7-H1 with a specific monoclonal antibody has resulted in enhanced antitumor immune responses [93, 94]. B7-H4 is another member of the B7 family that has been implicated as a negative regulator of T-cell immunity [95]. It has been demonstrated that B7-H4 can inhibit T-cell proliferation and IL-2 production, and that blockade of B7-H4 in preclinical animal models results in enhanced cytotoxic T-cell responses against an alloantigen [19]. The expression of B7-H4 has been observed in many types of human cancer, such as breast [96], ovary [97], and lung [98]. In renal cell carcinoma, its expression has been correlated with more aggressive tumors, particularly in those cases where both B7-H1 and B7-H4 are aberrantly overexpressed [99]. Therefore, blocking of molecules such as B7-H1 and B7-H4 expressed on tumor cells can reduce coinhibitory signals directly at the site of the tumor (Table 3), resulting in enhanced antitumor immune responses.

A different strategy involves blocking of inhibitory molecules directly expressed on T-cells. An example is CTLA-4, a negative regulator of T-cell activation, which is expressed on the T-cells and, like its homolog CD28, binds to B7 on the surface of the APC. Binding of CTLA-4 to B7 initiates a negative signal cascade that leads to downregulation of the T-cell response [100, 101]. The blockade of CTLA-4 with specific, monoclonal antibodies (mAb) has been explored as a monotherapy or in combination with vaccine therapy in preclinical and clinical studies. In preclinical studies, the use of antiCTLA-4 mAb as a monotherapy has shown antitumor activity with immunogenic tumors [100, 101], but not with poorly immunogenic tumors, such as MC38 [102]. In combination with vaccines, antiCTLA-4 mAb has been used to enhance antitumor T-cell responses elicited by the vaccine, thus resulting in effective antitumor effects [102]. In the clinic, antiCTLA-4 has been used also as a monotherapy or in combination with other immune-mediated anticancer modalities [103]. Results from a phase I clinical trial with antiCTLA-4 mAb (ipilimumab) in patients with metastatic melanoma or renal cell cancer recently reported a 14% response rate and multiple, and at times severe, immune-mediated toxicities such as nephritis, panhypophysitis, and enterocolitis, among others [104, 105], this last point constituting a potential disadvantage of the approach.

12. Depletion/Inhibition of Regulatory T-Cells

Naturally occurring regulatory T (Treg) cells, characterized by expression of IL-2R alpha, the transcription factor Foxp3, CTLA-4, and glucocorticoid-induced tumor necrosis factor (TNF) receptor (GITR), constitute 5% to 10% of peripheral $CD4^+$ T-cells [11, 106]. Treg cells represent an important mechanism of peripheral T-cell tolerance through their inhibition of self-reactive effector T-cells [107], and have also been implicated in the lack of effective antitumor immunity as the number of Treg cells are increased in the tumors and peripheral blood of cancer patients [108, 109]. Previous studies in several models of mouse tumors have demonstrated that deletion of Treg cells by using an antiCD25 mAb enhanced the development of antitumor immunity leading to tumor rejection [110, 111]. Furthermore, it has been previously shown that antigen-specific T-cell responses induced by poxviral vaccines can be augmented by simultaneous administration of antiCD25 mAb in mice [112]. Among alternative modalities that can be used in humans to delete Treg cells is denileukin diftitox, (DAB_{389}IL-2), a fusion protein of IL-2 and diphtheria toxin, previously shown in mouse preclinical studies to reduce Treg cells and to enhance antigen-specific immune responses induced by poxviral

vaccines [113]. A potential drawback of both approaches is that activated, effector T-cells also transiently upregulate the expression of CD25 on their cell surface, therefore being at risk of depletion by strategies that target CD25. In the clinic, denileukin diftitox was previously shown to reduce Treg cells and to lead to objective clinical responses in patients with ovarian cancer [12, 114]. Moreover, antitumor immunotherapy approaches combining denileukin diftitox-mediated deletion of Treg cells followed by vaccination with RNA-transfected DCs, tumor antigen peptides, or DCs modified by infection with rF-CEA(6D)/TRICOM, have been shown to improve tumor-specific T-cell responses in patients with renal cell cancer, melanoma, or CEA-positive malignancies, respectively, [115–117].

13. Inhibition of Immunosuppressive Cytokines

Tumors can also evade immunosurveillance by directly secreting a number of inhibitory cytokines or by inducing various types of immune cells to secrete cytokines associated with reduced immune responses. One of these cytokines is the transforming growth factor beta (TGF-β), which can be directly secreted by many types of tumor cells, including breast, prostate, colon, liver, lung, and melanomas, among others [118, 119]. TGF-β exerts its negative effects on T-cells, NK cells, macrophages and DCs [120]. Several preclinical studies have demonstrated that blockade of TGF-β can reverse the immunosuppressive effects of the tumor microenvironment. For example, a small molecule TGF-β inhibitor was used in a mouse tumor model to rescue the functionality of infiltrating CD8+ T-cells (TILs), which are usually hyporesponsive [121]. Recently, it was also reported a synergistic improvement of a peptide vaccine modality in combination with a monoclonal antibody against TGF-β, in a murine tumor model [122]. Although no adverse effects have been observed in studies so far conducted, the dual role of TGF-β on normal versus tumor cells, where it can function as a suppressor or promoter of tumor development, respectively, may constitute a potential problem for this approach and indicates the necessity for detailed studies aimed at optimizing the use of antiTGF-β reagents while minimizing the potential for adverse effects.

Another cytokine that has a negative impact on the development of antitumor T-cell responses is the vascular-endothelial growth factor (VEGF). A major role of VEGF is to induce the process designated as tumor angiogenesis, which involves the development of an adequate tumor vasculature that will support a blood supply to the growing tumor [123, 124]. Additionally, VEGF contributes to tumor immune escape by inducing the development of immune cell populations with immunosuppressive functions, like immature DCs [24] and the recruitment of tumor-associated macrophages (TAM) to the tumor stroma [13, 14, 125]. Strategies aimed at inhibiting VEGF or its receptors, therefore, will not only disrupt the tumor vasculature thus impairing tumor growth, but will also improve antitumor immunity by eliminating inhibitory cell populations, resulting in enhanced responses to cancer vaccines. In preclinical studies, for example, an antiVEGF antibody enhanced the efficacy of a peptide-pulsed DC-based vaccine that resulted in prolonged and pronounced antitumor effect [126]. Therefore, inhibition of VEGF may be a valuable adjuvant in the immunotherapy of cancer. A disadvantage of antiVEGF therapies in the clinic has been the emergence of toxicities that included wound healing complications as well as adverse vascular effects.

14. Conclusions

Progress in understanding the molecular mechanisms that govern immune activation as well as the mechanisms used by tumor cells to evade surveillance by the immune system are advancing the development of immune-mediated therapies that could be effectively used against a range of human cancers. The combination of cancer vaccines with other therapeutical modalities, in particular established therapies such as radiation and chemotherapy, as well as small molecule targeted therapies, provides an opportunity to further improve the outcome of vaccine interventions against cancer. Moreover, several studies also indicated that patients who receive a cancer vaccine have an enhanced outcome to subsequent therapies, thus providing another possible approach for the use of cancer vaccines prior to other cancer interventions. A prospective randomized trial is being initiated to substantiate these findings. Unlike other modalities, cancer vaccines have so far demonstrated no associated toxicities and therefore their use could not only result in improved patient survival but also in improvements in quality of life.

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Review Article

The Emerging Role of HLA-E-Restricted CD8$^+$ T Lymphocytes in the Adaptive Immune Response to Pathogens and Tumors

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Human leukocyte antigen (HLA)-E is a nonclassical major histocompatibility complex (MHC) class I molecule of limited sequence variability that is expressed by most tissues albeit at low levels. HLA-E has been first described as the ligand of CD94/NKG2 receptors expressed mainly by natural killer (NK) cells, thus confining its role to the regulation of NK-cell function. However, recent evidences obtained by our and other groups indicate that HLA-E complexed with peptides can interact with $\alpha\beta$ T-cell receptor (TCR) expressed on CD8$^+$ T cells. Although, HLA-E displays a selective preference for nonameric peptides, derived from the leader sequence of various HLA class I alleles, several reports indicate that it can present also "noncanonical" peptides derived from both stress-related and pathogen-associated proteins. Because HLA-E displays binding specificity for innate CD94/NKG2 receptors, as well as all the features of an antigen-presenting molecule, its role in both natural and acquired immune responses has recently been re-evaluated.

1. Introduction

Major histocompatibility complex (MHC) class I molecules may be subdivided into two families, MHC class Ia (classical) and MHC class Ib (nonclassical). Both classical and nonclassical MHC class I molecules are expressed at the cell surface in association with β2-microglobulin. MHC class Ia molecules (human leukocyte antigen (HLA)-A, -B, and -C, in humans) [1] play a central role in adaptive immunity. They interact directly with T-cell receptors (TCRs) and with the coreceptor molecule CD8 on cytotoxic T cells (CTLs). In humans, the MHC class Ib family members include HLA-E, -F, -G, and HFE (HLA-H) [2]. They are best known for their capability of regulating innate immune responses. Nevertheless, there is now accumulating evidence that, like the MHC class Ia molecules, certain class Ib molecules can play a role also in regulating acquired immune responses to bacteria and viruses [2, 3]. While, classical MHC class I molecules are extremely polymorphic, nonclassical MHC class I molecules are poorly polymorphic and, in most instances, exhibit a narrow tissue distribution. In addition, cell surface expression of MHC class Ib molecules is generally lower than that of MHC class Ia molecules. In humans, HLA-E is the least polymorphic of all the MHC class I molecules [4]. Several studies reported that only two alleles predominate in the Caucasian population. These two alleles HLA-E*0101 (HLA-E^{107R}) and HLA-E*0103 (HLA-E^{107G}) differ at only one amino acid position. Thus, an arginine at position 107 in HLA-E*0101 (HLA-E^{107R}) is replaced by a glycine in HLA-E*0103 (HLA-E^{107G}). In most cases the HLA-E^{107G} allele is expressed at significantly higher levels than the HLA-E^{107R} allele on normal cells [5]. The differences in surface expression of the two alleles do not reflect only the higher affinity of HLA-E^{107G} for available

peptides, but also the higher stability of surface HLA-E^{107G}/peptide complexes. HLA-E displays a relatively high degree of peptide binding ability [6]. Under physiological conditions, cell surface expression of HLA-E depends on binding of nonamer peptides derived from the signal sequence of MHC class I molecules [7]. The assembly of HLA-E with the signal peptide-derived ligands is strictly dependent on a functioning processing machinery (i.e., proteasome and the transporter associated with antigen processing, TAP) [7, 8]. Unlike the other MHC class Ib molecules, HLA-E is transcribed virtually in all human tissues and cell lines, although at lower levels than MHC class Ia antigens [9, 10]. Recently, unusually high levels of HLA-E have been detected in neoplastic cells [11, 12]. Although, HLA-E was first described as the ligand for CD94/NKG2A (inhibitory) and CD94/NKG2C (activating) NK receptors [13–15], thus mainly confining its regulatory function to NK cell population, emerging evidence (obtained by our and other groups) clearly support the notion that HLA-E can serve as a restricting element for the TCR of some $\alpha\beta$ T cells [16–20]. Along this line, this MHC class Ib molecule plays an important role in both natural and acquired immune responses [21].

2. HLA-E Does Bind Different Sets of Peptides

As indicated above, HLA-E is a conserved class Ib molecule characterized by a limited polymorphism. It binds nonameric leader peptides primarily derived from amino-acid residues 3–11 of the signal sequences of most HLA-A, -B, -C and -G molecules (e.g., VMAPRTLIL, VMAPRTLVL, VMAPRTLLL, VMAPRALLL, and VMAPRTLFL) [7]. Peptide binding stabilizes the HLA-E protein, allowing it to migrate to the cell surface. A functioning TAP molecule is required to transport these peptides into the endoplasmic reticulum (ER), where they can interact with HLA-E [7]. Although, HLA-E appears to bind a narrow peptide repertoire, recent evidences revealed that several proteins other than MHC class I molecules encode peptides that can bind to HLA-E. Among these peptides the best characterized are the VMAPRTLVL and the VMAPRTLIL peptides derived from the gpUL40 leader sequences ($gpUL40_{15\text{-}23}$) of two different human cytomegalovirus (CMV) strains (i.e., Toledo and AD169 strains). These peptides match exactly the leader sequence peptides of various HLA-A and -Cw alleles, respectively. Importantly, unlike the MHC-derived peptides, the gpUL40-derived ones are assembled with HLA-E via a TAP-independent mechanism [22]. In addition, peptides derived from the human heat shock protein 60 ($hsp60_{10\text{-}18}$, QMRPVSRVL) [23], the ATP-binding cassette transporter, multidrug resistance-associated protein 7 ($MRP7_{496\text{-}504}$, ALALVRMLI) [24], the human immunodeficiency virus (HIV) gag protein ($HIV\ p24_{14\text{-}22}$, AISPRTLNA) [25], the *Salmonella enterica* serovar Typhi GroEL protein ($GroEL_{15\text{-}23}$, KMLRGVNVL) [20], and gliadin (gliadin $\alpha 2$ $chain_{52\text{-}60}$, SQQPYLQLQ) [26] have also been shown to bind HLA-E.

Interestingly, binding to HLA-E has been demonstrated also for viral peptides previously shown to bind classical HLA class I molecules. For example, peptides from the Epstein-Barr virus (EBV) BZLF-1 protein ($BZLF\text{-}1_{39\text{-}47}$, SQAPLPCVL), the influenza matrix protein ($InflM_{59\text{-}67}$, ILGFVFTLT) [27], and the Hepatitis C virus (HCV) core protein ($HCV\ Core_{35\text{-}44}$, YLLPRRGPRL) [28] are viral peptides capable of binding to both HLA-A2 and HLA-E. Along this line, we have recently demonstrated that also peptides encoded by cellular-associated proteins can display a dual binding specificity (namely, they bind to both HLA-A2 and HLA-E). Thus, two alternative splicing isoforms of mutant peroxiredoxin 5 (Prdx5) encode two nonapeptides (Prdx5 splice variant $(Prdx5\Delta 2)_{52\text{-}60}$, AMAPIKTHL and Prdx5 splice variant $(Prdx5\Delta 2, 3)_{52\text{-}60}$, AMAPIKVRL) able to bind to both HLA-A2 and HLA-E and to allow their cell surface expression [29]. It is of note that the amino acid sequences of some of these HLA-E-binding peptides (derived from pathogen-associated, stress-related or normal proteins) differ markedly in sequence from the canonical MHC class I-derived leader sequence peptides.

3. HLA-E Is Recognized by T Cells via their $\alpha\beta$ TCR

$CD8^+$ CTLs expressing $\alpha\beta$ TCR generally recognize antigen peptides in association with MHC class Ia molecules [30]. MHC class Ia molecules are central to allow CTL-mediated discrimination between self and nonself. Thus, they present antigens, in the form of 8–10 amino acid-long peptides, to the $\alpha\beta$ TCR expressed by $CD8^+$ T cells. HLA class Ia-$\alpha\beta$ TCR interactions induce T lymphocytes to kill cells that are infected by viruses or some intracellular bacteria. MHC class Ia peptide epitopes, in most instances, are generated from the proteasomal degradation of cytosolic proteins and their loading into MHC class Ia is dependent on TAP function. The ability of MHC class Ib molecules to stimulate T-cell responses emerged only in recent years. Various experimental evidences revealed that MHC class Ib molecules may be recognized by T cells. Thus, while it is well established that HLA-E plays an important role in innate immunity, functioning as ligand for the CD94/NKG2 killer lectin-like receptors [13, 31] expressed by most NK cells [32] and a subset of CTLs [33–35], it is now clear that it can present peptide antigens for $\alpha\beta$ TCR-mediated recognition [16–20, 36]. Several studies have characterized HLA-E-restricted T cells. HLA-E dependent presentation of bacteria-derived antigens to human $CD8^+$T-cells has been documented. In particular, bacteria, including *Mycobacterium tuberculosis* (Mtb) and *Salmonella enterica* serovar Typhi GroEL, can elicit HLA-E-restricted T-cell responses [19, 20]. In addition, our group has recently demonstrated that an HLA-E-restricted $CD8^+$ T cell subset represents a significant component of the adaptive immune response to CMV in genetically predisposed individuals [36, 37]. Recently, other authors demonstrated that chronic hepatitis C is associated with increased intrahepatic HLA-E expression and showed that HCV gives rise to a peptide that binds to HLA-E and can be recognized by $CD8^+$ T cells via their TCR [38].

4. Role of HLA-E-Restricted CD8$^+$ T Cells in Infections

4.1. Nonclassically Restricted CD8$^+$ T Cells in the Host Response to Mtb. Mtb represents a leading cause of infectious disease morbidity and mortality worldwide. In addition to CD4$^+$ T-cells, also CD8$^+$ T lymphocytes appear essential for the containment of mycobacterial infection. The importance of HLA-E-restricted T cells in the host response to infection with Mtb has been defined by Heinzel and coworkers [19]. In particular, they found that these cells comprise the dominant CD8$^+$ T cell response in latently infected individuals. Remarkably, this finding represents the first compelling evidence of the ability of HLA-E to present pathogen-derived antigens and extended the function of HLA-E beyond its well-known role as an NK inhibitor through the interaction with the CD94/NKG2A heterodimer. Although, the recognized Mtb-derived peptides have not been identified so far, Mtb-derived antigen presentation was found to require proteasomal processing, but not TAP-mediated peptide transport.

4.2. HLA-E Restricted CD8$^+$ T Cells in the Host Response to HCV Infection. HCV is a single-stranded RNA virus belonging to the flaviviridiae family. HCV infection is a common cause of liver disease worldwide. In most instances, HCV is not eliminated by the host and results in chronic infection, which may develop into cirrhosis and hepatocarcinoma. The mechanisms favoring persistent infection are still poorly understood. However, numerous studies clearly indicate an association between an impaired immune response and clinical outcome. Patients who spontaneously recover from HCV infection typically mount vigorous multi-epitope-specific CD4$^+$ and CD8$^+$ T-cell responses that are readily detectable in blood samples. By contrast, patients with chronic hepatitis C tend to have late, transient and narrowly focused T-cell responses [39]. Recently, some authors suggested an important role for HLA-E in regulating antiviral immunity. With respect to HCV infection they demonstrated that chronic hepatitis C is associated with enhanced intrahepatic HLA-E expression [25]. In particular, they showed that HCV gives rise to the YLLPRRGPRL peptide (HCV core amino acid 35-44) that binds to, stabilizes HLA-E surface expression and protects cells from the NK cell-mediated cytotoxicity [25]. More importantly, they showed that, beyond its interaction with the CD94/NKG2A receptor, peptide-loaded HLA-E molecules can also be recognized by CD8$^+$ T-cells via their TCR [38]. Patients with chronic HCV infection were analyzed for their nonclassical CD8$^+$ T cell responses by using HLA-E transfected K562 cells, loaded with the appropriate HCV peptide, as antigen-presenting cells in an interferon (IFN)-γ ELISPOT assay. Interestingly, by this approach, they found that in nearly half of the HCV patients analyzed an HLA-E-restricted HCV-specific CD8$^+$ T-cell response could be detected. It is of note, that HLA-E –restricted IFN-γ secretion was associated with low viral load. Importantly, no IFN-γ production was found in CD8$^+$ T lymphocytes from healthy controls, confirming the specificity of this finding. Finally, they provided the first evidence for different functional roles of the 2 confirmed HLA-E allelic variants (i.e., HLA-E^{107R} and HLA-E^{107G}) in hepatitis C infection. Thus, when patients were stratified according to HLA-E genotype, they found that the frequency of HLA-E-restricted responses was higher in patients homozygous for the HLA-E^{107R} allele as compared to carriers of other HLA-E genotypes [38].

4.3. HLA-E-Restricted CD8$^+$ T Cells in the Host Response to CMV Infection. Human CMV is a β-herpesvirus that largely infects the human population, resulting in life long persistent asymptomatic infection, which, however, may cause severe morbidity in immunocompromised individuals. CMV infection is the most common viral complication following allogeneic hematopoietic stem cell transplantation and solid organ transplantation. Specific cytotoxic T-cell immunity represents a key factor to cointain CMV. Human CMV has evolved an impressive variety of strategies to escape from the recognition mediated by conventional (i.e., MHC class Ia-restricted) CTLs. Different CMV viral proteins (i.e., the unique short, US, proteins) are well known to inhibit the MHC class I expression in infected cells. In particular, the human CMV gene products US2 and US11 bind to nascent HLA class I chains resulting in their shuttle from the ER to the cytosol, where they undergo proteasome-dependent degradation. US3 binds to HLA class I molecules and retains them in the ER. Finally, the TAP-mediated transport of antigenic peptides to the ER is blocked by US6 [40]. As a consequence, conventional HLA class Ia-restricted CTLs may result, at least in part, inefficient in counteracting CMV infection. Interestingly, human CMV increases the surface expression of HLA-E, while downregulating the expression of many other MHC class I molecules [41]. Thus, human CMV itself, through the expression of gpUL40 protein, can supply peptides, which bind HLA-E in a TAP-independent fashion. This results in surface expression of HLA-E at even higher concentrations than in uninfected cells [22, 41, 42]. Notably, the upregulation of HLA-E in CMV-infected cells has been interpreted as a mechanism of viral escape from NK cells expressing the inhibitory CD94/NKG2A receptor.

Studies performed in our lab provided evidence that some CD8$^+$ T cells (via their TCR) recognize HLA-E when loaded with peptides (i.e., VMAPRTLIL and VMAPRTLVL) derived either from the gpUL40 proteins of different human CMV strains (e.g., AD169 and Toledo) or from the leader sequences of various classical HLA class I alleles [36]. In addition, we also obtained direct evidence *in vitro* that HLA-E-restricted CD8$^+$ T-cells can recognize and kill fibroblasts infected with human CMV strain AD169 [37]. These finding may have particular relevance in the immune defenses against CMV. Thus, HLA-E-restricted CD8$^+$ T cells could represent an additional type of effector cells playing a role in defense against a virus which can escape recognition mediated both by CTLs restricted by classical HLA class I molecules and by NK cells. Indeed, HLA-E-restricted CD8$^+$ T cells are able to kill infected cells despite the sharp downregulation of HLA class Ia, by recognizing the gpUL40-derived VMAPRTLIL peptide presented in the context of HLA-E [37].

It is of note that, different CMV strains have a mutated form of gpUL40 from which different HLA-E-binding peptides can be generated [22, 43]. For example, the open reading frame *UL40* encoded by the CMV Toledo strain contains the sequence VMAPRTLVL in its predicted leader peptide. This nonamer is identical to the HLA-E-binding peptide present in the signal sequence of the HLA-A*02 allele [22]. In agreement with these findings, also HLA-E-restricted $CD8^+$ T cells specific for the VMAPRTLVL peptide have been identified [36].

Donors that are able to develop an anti-CMV HLA-E-restricted $CD8^+$ T-cell response can be divided into two different groups according to their HLA-A and HLA-Cw haplotype (Table 1). In particular, "group 2" donors, from which HLA-E-restricted $CD8^+$ T cells specific for the VMAPRTLIL peptide could be derived, are characterized by a particular HLA class I haplotype that does not contain any VMAPRTLIL-bearing HLA-Cw alleles [36]. Thus, in these individuals this peptide represents a foreign antigen. On the other hand, donors who express HLA-Cw alleles carrying the VMAPRTLIL peptide, fail to generate HLA-E-restricted $CD8^+$ T cells specific for this self-peptide Thus, in these donors, HLA-E-restricted $CD8^+$ T cell precursors may have been negatively selected in the thymus by HLA-E/VMAPRTLIL self-complexes. Along this line, "group 1" donors, from which HLA-E-restricted $CD8^+$ T cells specific for both VMAPRTLIL and VMAPRTLVL peptides could be derived, do express in their haplotype neither VMAPRTLIL-bearing HLA-Cw alleles nor VMAPRTLVL-bearing HLA-A alleles [36]. Thus, these peptides represent foreign antigens for these donors. In summary, VMAPRTLIL represents a nonself peptide for "group 1" and "group 2" donors, and it is recognized with high avidity by HLA-E-restricted $CD8^+$ T cells from both groups. On the other hand, VMAPRTLVL represents a self-peptide for "group 2" donors, and is therefore recognized only by "group 1" donors. Taken together, these data suggest that the HLA class I host genotype, as well as the infecting CMV strain, may deeply affect the ability of different individuals to exploit HLA-E-restricted $CD8^+$ T cell-mediated defenses against CMV infection. Along this line, it is possible to speculate that only individuals carrying a particular MHC class I haplotype that lacks the VMAPRTLIL peptide (i.e., HLA-Cw*02 and/or HLA-Cw*07) are more resistant to severe CMV infection and/or reactivation occurring under certain pathological conditions. Because CMV UL40-derived VMAPRTLIL and VMAPRTLVL peptides are identical to those derived from the leader sequences of various HLA class I alleles, HLA-E-restricted CTLs may display a broad cytolytic activity against various HLA-E^+ allogeneic tumor cell lines belonging to different histotypes (a function referred to as NK-like activity) [33]. In addition, these effectors cells are characterized by the surface expression of HLA class I-specific inhibitory NK receptors (iNKRs) (i.e., KIR, CD94/NKG2A and ILT2/LIR1) [44, 45] and by mono-, oligoclonal TCR Vβ rearrangements, different in different donors [34]. Finally, by the use of HLA-E tetramers refolded with different peptides we also provided evidence that these cells are present *in vivo*, where they represent a sizeable fraction of CD8β^+T cells in some CMV seropositive individuals. These cells display an effector-memory surface phenotype ($CD27^-CD28^-CD45RA^+CCR7^-$) and express intracellular cytotoxic granules containing perforin and granzymes. In addition, functional analysis revealed that HLA-E-restricted $CD8^+$ T cells are capable of prompt production of IFN-γ upon specific peptide stimulation [37]. All the various phenotypic and functional data illustrated above, together with the fact that HLA-E-restricted $CD8^+$ T cells represent a oligo-, monoclonal expansion characterized by the expression of HLA class I-specific iNKRs, support the notion that they are effector-memory T cells possibly resulting from a chronic, antigen-driven stimulation [46]. The presence of these effector cells in the $CD8^+$ T-cell memory pool of some CMV seropositive individuals, strongly suggests that HLA-E-restricted $CD8^+$ T cells may actually play a relevant defensive role during CMV infection.

5. Possible Role of HLA-E-Restricted $CD8^+$ T Cells in Immune Responses against Tumors

HLA-E expression by tumor cells has been recently reported in several types of human cancers. Notably, HLA-E may be overexpressed in fresh lymphomas [47], ovarian carcinomas [48], gliomas [49], colon cancer [50], and melanomas [12]. Because of its capacity to bind to the inhibitory CD94/NKG2A receptor expressed by NK cells and a subset of T cells, HLA-E expression by neoplastic cells might favor tumor cell escape from immunosurveillance. Thus, it has been reported that NK cell activity against glioma cell cultures could be greatly increased when HLA-E was downregulated by RNA interference [49]. To date, the potential role of HLA-E as antigen-presenting molecule for tumor-specific $CD8^+$ T cells has been poorly addressed and tumor-derived peptides with binding specificity for HLA-E have not yet been identified. However, we recently showed that peptides specific for both HLA-E and classical HLA class I molecules (i.e., HLA-A2) could be generated by alternative splicing of the peroxiredoxin 5 (Prdx5) gene [29]. Prdx5 splice isoforms show widespread expression in normal and neoplastic cell lines belonging to different histotypes. As a result of the splicing events, Prdx5 isoforms encode two distinct nonapeptides (AMAPIKTHL or AMAPIKVRL) that bind to HLA-A2 and HLA-E molecules, stabilize HLA-A2 and HLA-E and allow their cell surface expression. Interestingly, we recently found that HLA-E^+ targets, loaded with these peptides, are recognized (although with low avidity), by HLA-E-restricted $CD8^+$ CTLs generated from healthy donors [29]. On the other hand, complexes composed by HLA-E and Prdx5 splice peptides are not recognized by the CD94/NKG2A inhibitory receptor and thus do not downregulate the NK cell function. Increased levels of Prdx5 can be detected in several diseases, such as osteoarthritis and tendon degeneration [51, 52]. Its upregulation has been reported during acute inflammation induced in rat lung by LPS, in chondrocytes by IL-1α and TNF-α and in human tendon cells during H_2O_2 exposure. In melanoma cells, Prdx5 expression can be upregulated by different

Table 1: Both the HLA class I host haplotype and the infecting CMV strain can affect the ability of different individuals to exploit an HLA-E-restricted CD8$^+$ T-cell-mediated defence against CMV.

Donors	HLA-Cw haplotype	HLA-A haplotype	VMAPRTLIL[1]	VMAPRTLVL[2]	HLA-E restricted CD8$^+$ T cells against CMV
Group 1	HLA-Cw*02, -Cw*07, -Cw*0809, -Cw*15, -Cw*1701, Cw*1703, and -Cw*18[3]	HLA-A*01, -A*03, -A*11, -A*29, -A*30, -A*33, -A*3401, -A*36, -A*74, and -A*80[4]	nonself peptide	nonself peptide	YES *(against both AD169 and Toledo strains)*
Group 2	HLA-Cw*02, -Cw*07, -Cw*0809, -Cw*15, -Cw*1701, Cw*1703, and -Cw*18[3]	HLA-A*02, -A*23, -A*24, -A*25, -A*26, -A*3402, -A*43, -A*66, and -A*69[5]	nonself peptide	self peptide	YES *(against AD169 strain but not against Toledo strain)*

[1]This peptide is contained in the UL40 open reading frame (ORF) from human CMV AD169 strain.
[2]This peptide is contained in the UL40 ORF from human CMV Toledo strain.
[3]These HLA-*CW alleles *do not* contain in their leader sequences the VMAPRTLIL peptide.
[4]These HLA-*A alleles *do not* contain in their leader sequences the VMAPRTLVL peptide.
[5]These HLA-*A alleles *do* contain in their leader sequences the VMAPRTLVL peptide.

forms of cellular stress. Along this line, HLA-E-binding peptides such as those derived from alternatively spliced Prdx5 isoforms, may be induced under stress conditions and could contribute to HLA-E stabilization leading to CD8$^+$-mediated recognition of tumor cells. It is therefore possible that when endogenous and environmental oxidative stress levels alter normal cellular processes and induce cell damage, as it might occur in cancer, peptides derived from Prdx5 variants may provide a signal that could activate MHC class Ib-restricted T cells, thus contributing to the elimination of stressed cells.

6. Concluding Remarks

While, the T-cell recognition of murine MHC class Ib molecules was described over a decade ago, the ability of human HLA class Ib molecules to stimulate T-cell responses is only now being recognized. Nevertheless, it is still unclear whether HLA-F and -G are involved in stimulating CD8$^+$ T cells. HLA-G-restricted T cells that specifically recognize pp65-derived peptides and kill HCMV-infected astrocytoma cells, have been generated in transgenic mice [3]. However, further studies are needed to evaluate how and whether such induction of the HLA-G-restricted, anti-HCMV response exists in humans. In addition, although it has been reported that HLA-H (HFE) can be directly recognized by the $\alpha\beta$ TCR of CD8$^+$ T-cells in a transgenic mouse model, it is usually unable to present antigenic peptides [53].

Conversely, during the past years a series of relevant discoveries unraveled several important functions of the poorly known MHC class Ib molecule HLA-E in humans. After the original report that HLA-E represents the ligand of CD94/NKG2 receptors, which are expressed by NK cells and CD8$^+$ T cells, it became evident that HLA-E can represent a restriction element for some T cells characterized by cytolytic activity [17]. In most instances, they appear to represent oligoclonal or even monoclonal cell expansions. A relevant work pointed out how these cells may kill a number of allogeneic normal or tumor cells (the so-called NK-like activity) upon recognition of HLA-E/peptide complexes on target cells [17]. This problem may represent a serious threat in transplantation [54, 55]. More recently, a number of studies revealed that HLA-E-restricted CTLs recognize a number of HLA-E-binding, pathogen-derived peptides, thus revealing the likely role exerted by these cells in host defenses. Remarkably, in certain viral infections (CMV and, possibly, HCV infections), HLA-E-restricted CTLs may play a role complementary to that of conventional (i.e., HLA class Ia-restricted) CTLs [37, 38, 56]. In particular, in human CMV infection, downregulation of HLA class Ia molecules and upregulation of HLA-E could mean that HLA-E-restricted CTLs might play a major role in antivirus defenses [37].

Finally, in view of the broad tissue expression of HLA-E, HLA-E-restricted CTLs should be investigated for their possible involvement in the rejection of HLA class Ia-deficient tumors. In this context, it should be stressed that frequently tumor cells may fail to express one or more HLA class Ia alleles or may display a general downregulation of HLA class Ia surface expression. Both these circumstances, might allow the induction of HLA-E-restricted CD8$^+$ T-cell responses against cancer-associated antigens. Along this line, the increased expression of HLA-E in different tumors and the recent demonstration in mice that the nonclassical Qa-1^b molecule (i.e., the murine HLA-E homologue) plays a prominent role as a restriction element for CD8$^+$ T cells in the adaptive immune response to TAP-deficient tumors [57], strongly suggest the need of intensive investigation in this field.

Abbreviations

CMV: Cytomegalovirus
CTLs: Cytotoxic T lymphocytes
EBV: Epstein-Barr virus
ER: Endoplasmic reticulum
HCV: Hepatitis C virus
HIV: Human immunodeficiency virus
HLA: Human leukocyte antigen
iNKRs: Inhibitory NK receptors
Mtb: *Mycobacterium tuberculosis*

MHC: Major histocompatibility complex
NK: Natural killer
Prdx5: Peroxiredoxin 5
TAP: Transporter associated with antigen processing
TCR: T cell receptor.

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Research Article

Development of Th1 Imprints to rBCG Expressing a Foreign Protein: Implications for Vaccination against HIV-1 and Diverse Influenza Strains

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We demonstrate here that immunizing naïve mice with low numbers of recombinant Bacille Calmette-Guérin (rBCG) expressing β-galactosidase (β-gal) generates predominant Th1 responses to both BCG and β-gal whereas infection with high numbers generates a mixed Th1/Th2 response to both BCG and β-gal. Furthermore, the Th1 response to both BCG and β-gal is stable when mice, pre-exposed to low numbers of rBCG, are challenged four months later with high numbers of rBCG. Thus the Th1/Th2 phenotypes of the immune responses to β-gal and to BCG are "coherently" regulated. Such rBCG vectors, encoding antigens of pathogens preferentially susceptible to cell-mediated attack, may be useful in vaccinating against such pathogens. We discuss vaccination strategies employing rBCG vectors that are designed to provide protection against diverse influenza strains or numerous variants of HIV-1 and consider what further experiments are essential to explore the possibility of realizing such strategies.

1. Introduction

A rational approach to vaccination against a pathogen is contingent upon a knowledge of the immunological correlates that discriminate between immunity able to contain the pathogen and immunity that fails to do so. Effective vaccination must guarantee the former type of immunity under circumstance where the latter type of immunity would be generated upon natural infection of the naïve individual [1]. Effective vaccination of people, against a diversity of pathogens, is achieved through guaranteeing a rapid and large antibody, Th2 response upon natural infection by the pathogen. Such vaccination is effective only when antibody is able to contain the pathogen and/or neutralize the toxins the pathogen produces. However, there are other situations, following infection by some intracellular pathogens or the development of cancer, where the correlates of protection are a predominant cell-mediated, CTL, Th1 response and where responses with a substantial Th2, antibody component, are associated with chronic or progressive disease [2]. In these cases, it would appear that effective vaccination must generate a Th1 imprint upon the immune system that is stable upon natural infection by the pathogen [3].

Mycobacterium bovis is responsible for tuberculosis of cattle. An attenuated form of *M. bovis*, named BCG, is currently the only available vaccine against *Mycobacterium tuberculosis*, the pathogen primarily responsible for human tuberculosis. Billions of individuals worldwide have been inoculated with BCG in attempts to provide protection against tuberculosis and leprosy. Moreover, these bacteria can be used as vectors that express antigens of diverse pathogens to raise immunity against these antigens. Several recombinant BCG vectors have been constructed for vaccination against a variety of bacterial, viral, or parasitic pathogens [4].

There are several advantages in using rBCG vectors. Not only does BCG act as an adjuvant, but its slow replication allows for persistent, low level delivery of antigen to the

host over a considerable time. Many observations show, in diverse systems, that chronic stimulation with low amounts of antigen can lead to the establishment of Th1 imprints [2, 3, 5–7]. Additionally, advances in expression and delivery of antigen by rBCG, hence increasing the availability of the encoded antigens to the host immune system, have allowed the development of vaccine vectors superior to those of the past [4]. We are also encouraged to explore the use of rBCG vectors in developing strategies of vaccination by the remarkable safety record of BCG as a vaccination agent [8].

A critical question, when considering the use of rBCG vectors to immunize against the encoded protein, is the relationship between the nature of the immune response to BCG and the nature of the response to the encoded protein. This is particularly critical in those cases where there are grounds for believing that the Th1/Th2 phenotype of the response to the encoded antigen is likely critical to the degree of protection provided by vaccination. It has been proposed that the Th1/Th2 phenotype of the responses to BCG and the encoded protein may be coordinately determined [1], a phenomenon referred to as coherence. We set out to explore whether, employing rBCG encoding β-galactosidase as a model rBCG vector, the Th1/Th2 phenotypes of the immune responses to BCG and to β-galactosidase are coherently regulated. Should this be the case, it would lay the foundation for further studies aimed at examining whether rBCG vectors could be employed to establish Th1 imprints against those pathogens preferentially susceptible to predominant cell-mediated, Th1 responses.

2. Materials and Methods

2.1. Mice. Female mice were used in all experiments. BALB/cJ mice were obtained from the animal colony at the Department of Microbiology and Immunology, University of Saskatchewan. All mice were housed at the animal care facility within the Department and were at least 6 weeks old at the time of immunization. Animal care and treatment were in accordance with standards approved by the Canadian Council on Animal Care.

2.2. Growth and Enumeration of rBCG. Dr. Emil Skamene of McGill University provided *M. bovis* strain BCG Montreal. The mycobacteria were propagated in Dubos medium containing 0.5% bovine serum albumin and 0.05% Tween 80. Bacteria were enumerated by plating various dilutions on Dubos agar plates after sonication for five seconds at a power output of 5 and 50% duty cycle to break up clusters of bacteria. Colonies were counted fourteen days after plating, and consequently numbers of bacteria are expressed as cfu.

2.3. Immunizations. Fourteen-day cultures of BCG or rBCG were used for injections. The cells were pelleted by centrifugation at 8000 g for 30 minutes and washed three times by repeated resuspension in saline containing 0.05% Tween 80 followed by centrifugation at 8000 g for 20 minutes. After the final wash, the mycobacteria were resuspended in 1/100 of the original volume of culture, and serial dilutions were performed from this stock as needed. BCG immunizations were performed by either intradermal (i.d.) or intravenous (i.v.) route. For i.d. injection, hair was removed from the abdomen using a commercial depilatory two days prior to injection. Injections were done using a 1 cc syringe with a 30-gauge needle attached. Mice were anesthetized, and the skin of the abdomen was gently stretched to prevent it from bunching up in front of the needle. With the bevel of the needle facing upward, the needle was inserted several millimeters horizontally into the skin and 25–50 μl of the BCG suspension containing the appropriate number of cfu was injected. For i.v. injection, the desired number of BCG cfu were injected in a 50 μl volume into the lateral tail vein using a 28-gauge needle.

Mice were immunized by the i.p. route with recombinant β-gal (Sigma Chemicals, St. Louis, MO), either as a soluble protein in phosphate-buffered saline (PBS) or adsorbed to the aluminum hydroxide gel adjuvant, Alhydrogel (Superfos Biosector, Vedbaek, Denmark) as indicated in the legend to Figure 6.

2.4. ELISPOT Assay for Detection and Enumeration of Antigen-Dependent IFNγ- and IL-4-Secreting Cells. The ELISPOT assay for enumeration of BCG-specific cytokine producing lymphocytes was performed on spleen cells as previously described in detail [9]. For detection of β-gal-specific cytokine-producing cells, the procedure was identical except that purified soluble β-gal (Sigma Chemicals) was used at 1 μg/well for stimulation of lymphocytes.

2.5. Analysis of Antimycobacterial or Anti-β-gal Antibodies by Enzyme Immunoassay. Blood from normal or infected mice was collected by tail bleeding into serum separator tubes (Becton Dickinson, Rutherford, NJ). After allowing the samples to clot for at least several hours, individual serum samples were harvested by centrifugation at approximately 5000 g for 60 seconds. Sera were stored at -70°C until use. The IgG1 and IgG2a serum antibody titers were determined using an ELISA. Immulon-4 96-well polystyrene plates (Dynatech Laboratories, Chantilly, VA) were coated with either BCG antigen or with soluble β-gal antigen at a concentration of 1 μg/well in 100 μl of PBS and the procedure was performed as described in [10]. The titer was determined by plotting optical density against the log of the dilution factor for each sample. The endpoint titer was assigned as the point on the line at which the optical density was 0.5 above the value for wells to which no serum was added.

2.6. Mycobacterial Expression Vectors. Dr. Mark Hanson of MedImmune, Gaithersberg, MA, kindly provided the mycobacterial expression vector pMV361::LacZ [11]. Vector pMV361 is a chromosomal integrating vector in which inserted genes are expressed as a fusion protein with the first six amino acids of the mycobacterial heat shock protein 60 (Hsp60).

2.7. Transformation of Competent M. bovis BCG. The method of Parish and Stoker [12] was used to prepare

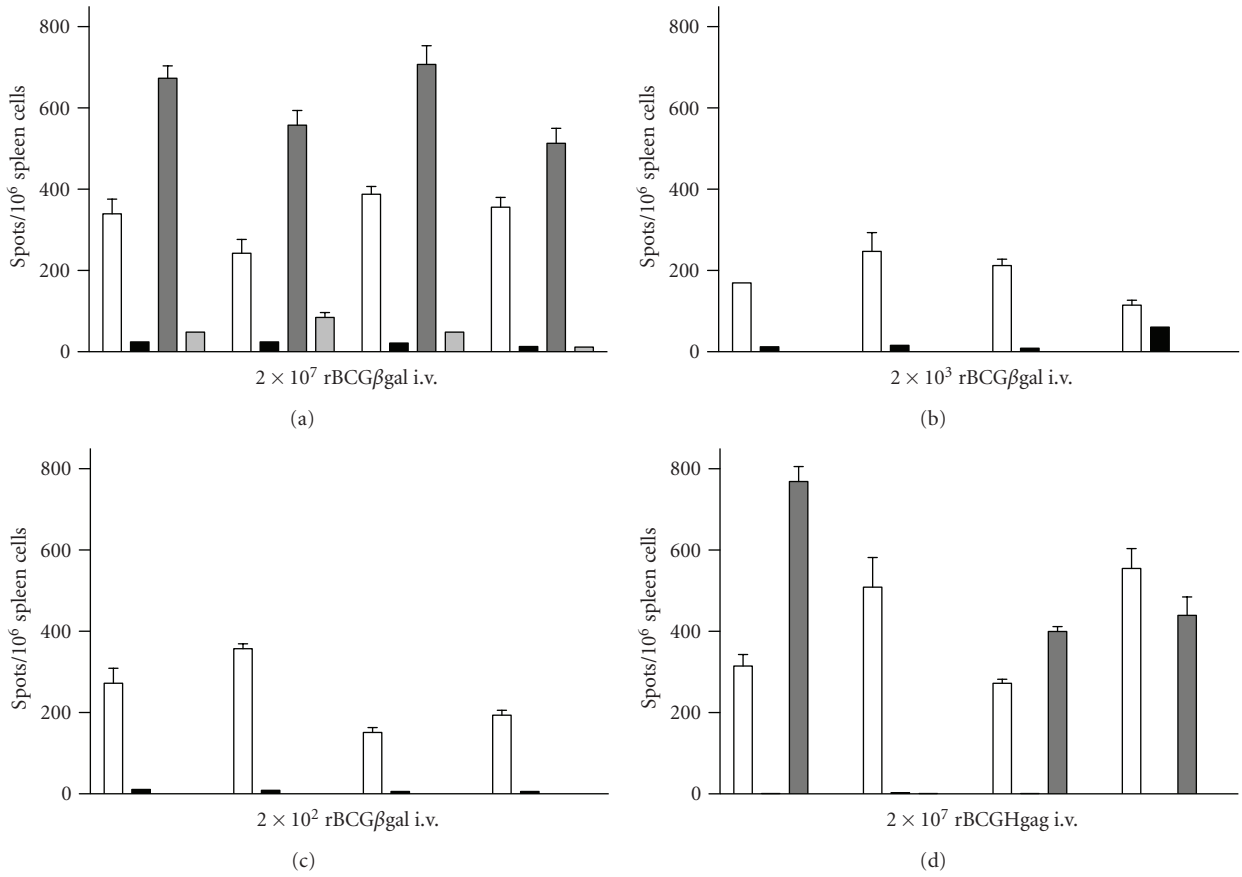

Figure 1: Immune response of four individual mice per group after i.v. immunization with different doses of recombinant BCG (rBCGβgal) or control rBCG expressing HIV gag protein (rBCGHgag). Immunization dose is indicated below each graph. Mice were immunized as indicated under each graph. After 9 weeks, the mice were killed and the number of β-gal- and BCG-specific IFNγ- and IL-4-producing cells in their spleens was determined by ELISPOT assay. The response in mice given a high dose of BCG transformed with a control vector containing the HIV gag sequence is also shown. Open bars—IFNγ response to BCG; Black bars—IFNγ response to β-gal; Dark gray bars—IL-4 response to BCG; Light gray bars—IL-4 response to β-gal. All values were corrected for background by subtracting the number of spots observed in wells without antigen. Results are representative of two independent experiments.

electroporation-competent BCG. Transformation of competent BCG was performed by electroporation using the Gene Pulser II (BioRad). Electroporation-competent BCG was removed from the freezer and immediately placed on ice for several minutes to allow samples to thaw. One microgram of plasmid DNA was added to the tube, and the contents were mixed and placed on ice for ten minutes. The mixture was transferred to a chilled, 2 mm gap electroporation cuvette (VWR scientific, Toronto, ON). The bacteria were subjected to a pulse of 3.5 kV, 25 μF, at a resistance of 1000 Ω [12]. The cuvette was incubated on ice for ten minutes before the contents were transferred to a separate tube and diluted with 3 ml of Middlebrook 7H9 media (Difco, Detroit, MI) with 0.5% Tween 80 and OADC supplement (Difco). The mixture was incubated at 37°C for 3 h to allow for antibiotic expression. Cells were harvested by centrifugation at 3000 g for ten minutes and plated on Middlebrook 7H10 agar plates with OADC and 10 μg/ml of kanamycin [12]. The plates were sealed with parafilm and incubated at 37°C for 21–28 days to allow colonies to grow.

3. Results

Figures 1 and 2 show the number of BCG- and β-gal-specific IFNγ- and IL-4-producing cells in the spleens of mice nine weeks after immunization with different doses of rBCG (either rBCGβgal or rBCGHgag). Immunization with both low and high doses of rBCGβgal induced not only BCG-specific but also β-gal-specific cytokine-producing cells, indicating that the β-gal is expressed in vivo at a level sufficient to induce an immune response. The immune response to different doses of rBCG given by i.v. (Figure 1) and i.d. (Figure 2) immunization is qualitatively different. Regardless of the route, high doses of rBCGβgal induce a mixed IFNγ and IL-4 response while lower doses induce a predominantly IFNγ response to both BCG and the expressed β-gal antigen. Although the numbers of β-gal-specific spot forming cells are low in rBCGβgal immunized mice, they represent a significant portion of the response to the rBCGβgal, particularly in mice immunized by the i.d. route. Mice immunized with BCG transformed with a control vector BCGHgag, not expressing β-gal (Figure 1(d)),

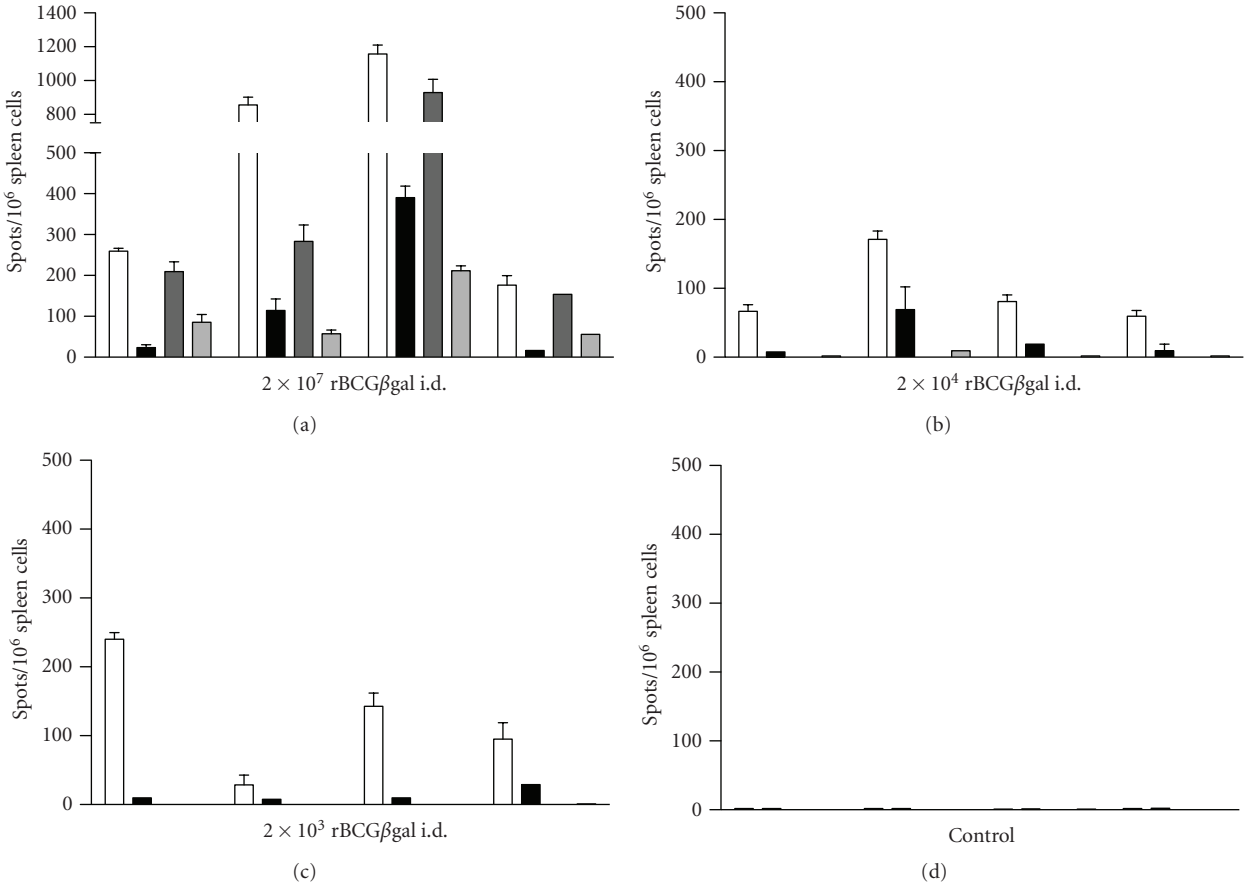

Figure 2: Immune response of four individual mice per group after i.d. immunization with different doses of recombinant BCGβgal. Mice were immunized as indicated under each graph, with an unimmunized control shown. After 9 weeks, the mice were killed and the number of β-gal- and BCG-specific IFNγ- and IL-4-producing cells in their spleens was determined by ELISPOT assay. Open bars—IFNγ response to BCG; Black bars—IFNγ response to β-gal; Dark gray bars—IL-4 response to BCG; Light gray bars—IL-4 response to β-gal. All values were corrected for background by subtracting the number of spots observed in wells without antigen. Results are representative of two independent experiments.

show no response to β-gal while cells from unimmunized control mice (shown in Figure 2(d)) do not respond to either the BCG-lysate antigen or to the β-gal antigen. Only mice immunized with the high doses of rBCGβgal had significant antibody titers to β-gal (data not shown), indicating that the antibody response is indeed attributable to immunization with rBCGβgal, and that only higher doses induce antibody production. Similarly, high doses of rBCG were required for induction of BCG-specific antibody production, demonstrating that the humoral response to β-gal follows that induced for the BCG.

We wished to determine if low-dose immunization with rBCGβgal could establish immune deviation such that the IFNγ-dominant response initially induced by the low-dose vaccination would be maintained upon subsequent challenge with a dose of rBCGβgal that induces a mixed IFNγ/IL-4 response in naïve mice. Mice immunized with different doses of rBCG by the i.d. route were challenged four months later with a high dose of rBCGβgal, also given intradermally. The immune response was assayed at 16 weeks postchallenge. Mice that were immunized with low doses by the i.d. route (Figure 3) maintained a dominant IFNγ response to BCG antigen and to β-gal after the challenge, which was distinct from the mixed IFNγ/IL-4 response of control mice that received only the challenge dose, demonstrating immune deviation toward a cell-mediated response. The ratio of IFNγ- to IL-4-producing cells (means within each group) are depicted in Figure 4. Even after challenge with the high dose of rBCGβgal, the mice that were immunized previously with a low dose of rBCG have a tenfold higher IFNγ response compared to control mice or those mice given only the high challenge dose. These results demonstrate that, once established, the dominant IFNγ response induced by low-dose vaccination is not easily deviated to an IL-4 response upon challenge. The anti-β-gal serum antibody titers from the mice of Figure 3 are shown in Figure 5. Note the prominence of IgG2a antibody in the serum of mice immunized with a low dose of rBCG before challenge, compared to the control mice given only the high dose. This is consistent with the prominence of IFNγ-producing cells in mice pretreated with low doses of rBCG, as class switching to IgG2a is associated with IFNγ production in mice [3].

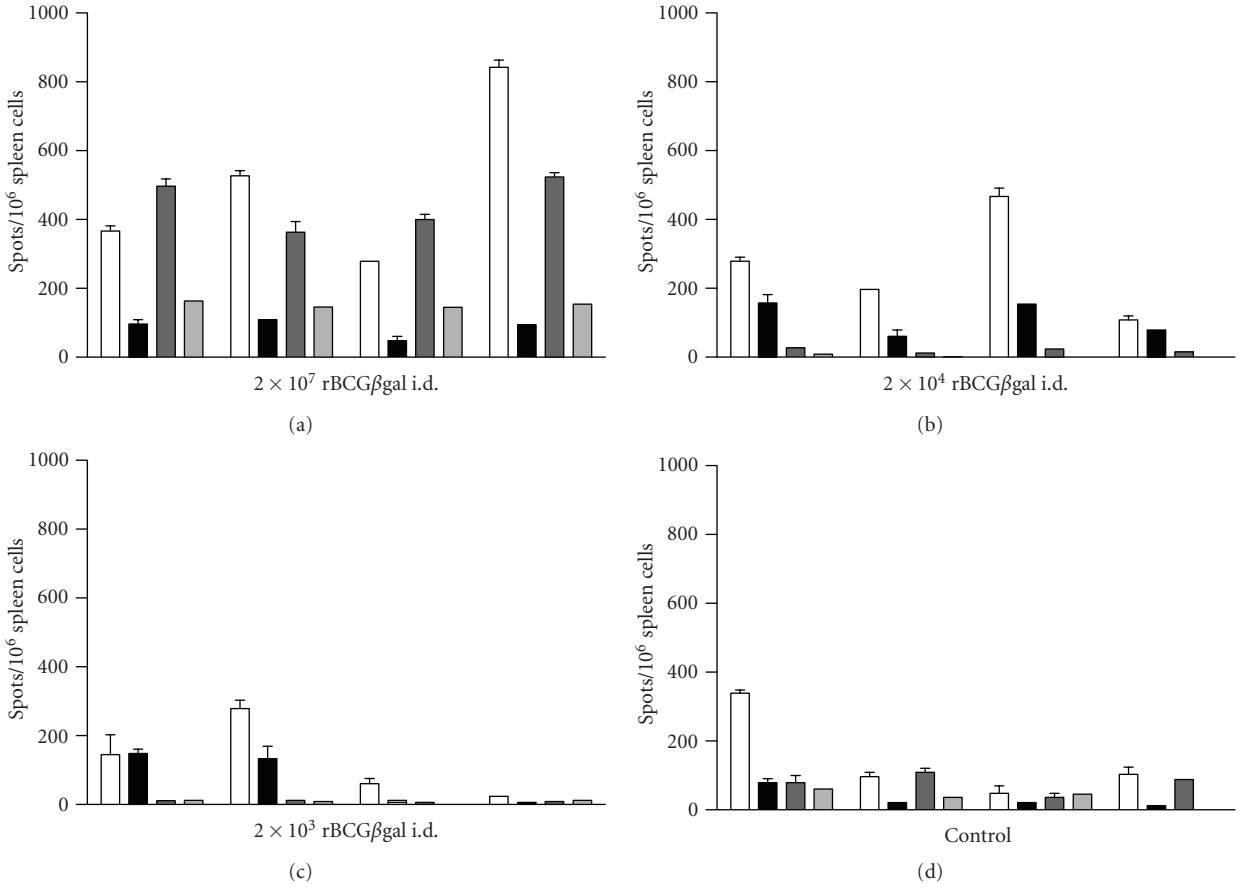

Figure 3: Immune response of individual mice vaccinated with different doses of rBCGβgal after subsequent i.d. challenge with a high dose of rBCGβgal. Each group of mice was initially given an i.d. vaccination dose of rBCGβgal as indicated below each graph with a control group receiving challenge dose only shown. After 4 months, the mice were challenged with approximately 2×10^7 rBCGβgal cfu given i.d. After an additional 16 weeks, the mice were killed and the number of β-gal- and BCG-specific IFNγ- and IL-4-producing cells in their spleens was determined by ELISPOT assay. Open bars—IFNγ response to BCG; Black bars—IFNγ response to β-gal; Dark gray bars—IL-4 response to BCG; Light gray—IL-4 response to β-gal. All values were corrected for background by subtracting the number of spots observed in wells without antigen. Results are representative of two independent experiments.

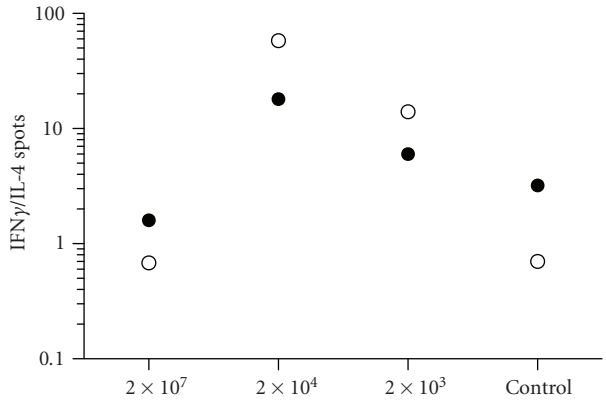

Figure 4: Ratio of IFNγ to IL-4 spots in the mice depicted in Figure 3. Ratios were calculated as the mean number of antigen-specific IFNγ spots/the mean number of IL-4 spots within each group of mice. Open circles and filled circles represent BCG- and βgal-specific IFNγ : IL-4 ratios, respectively.

We compared the immune response to β-gal in mice immunized with rBCGβgal and in mice immunized with purified β-gal using other methods that might be acceptable for immunization of humans. Figure 6 shows the immune response in mice immunized with purified β-gal given in alum as an adjuvant. In both cases, immunization was by the i.p. route. Soluble β-gal did not induce a significant immune response in any of the mice immunized with this preparation (data not shown). β-gal in alum, however, induced predominantly IL-4-producing cells in mice, at even the low dose used. This is not unexpected as alum is particularly effective at inducing Th2 responses [13]. However, these results exemplify potential problems in achieving a desirable Th1 response in humans with current vaccination procedures.

4. Discussion

Previous studies have shown that the Th1/Th2 phenotype of the immune response to BCG, generated in very young and

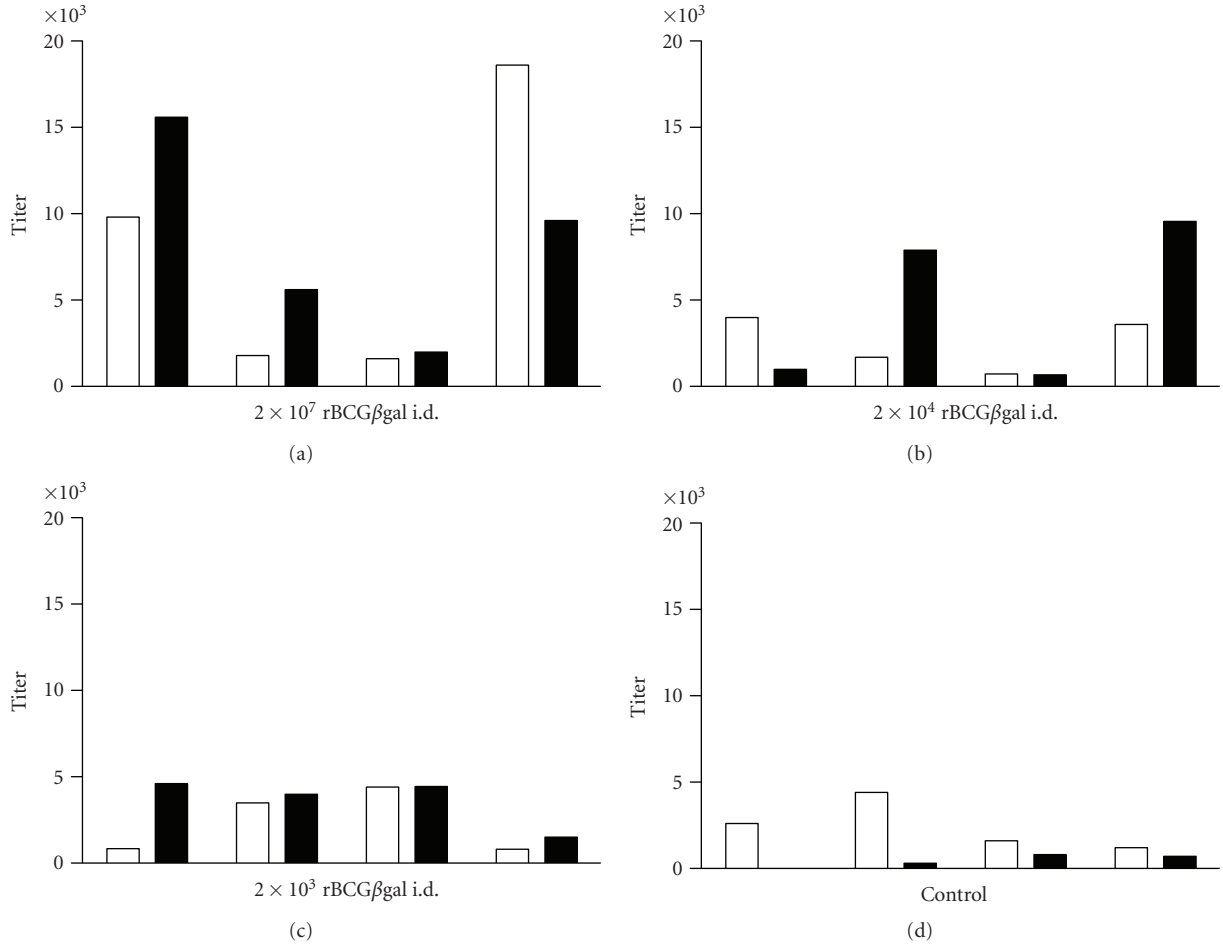

Figure 5: Serum antibody responses of mice to β-gal after immunization with different doses of rBCG by the i.d. route, and subsequent challenge with a high dose of rBCG by the same route. Mice were immunized initially with doses indicated below each graph with age-matched control mice receiving only challenge dose shown. After 16 weeks, all groups were challenged with 2×10^7 rBCG given i.d. Serum was collected at the time the mice were killed, 16 weeks after the challenge. Open bars show IgG1 titers while black bars depict IgG2a titers.

in adult mice, depends upon the dose of BCG administered, as assessed following infection by several different routes [10]. Infection with relatively low numbers of BCG generates a predominant Th1 response and with higher numbers a mixed Th1/Th2 response. Here we show that the Th1/Th2 phenotypes of the immune responses to BCG and to β-gal similarly depend upon the number of rBCG employed for infection and that the Th1/Th2 phenotypes of the responses to BCG and to β-gal appear to be coherent. Our studies show that rBCG can be used to preferentially generate a Th1 response to the encoded antigen by infecting with relatively low doses of rBCG. We now consider the potential use of this strategy in vaccination against HIV-1 and influenza viruses.

In looking at current views, it appears that there are two main, contradictory, positions as to how effective vaccination against HIV-1 might be achieved. Most suggest the combination of cell-mediated immunity, in the form of CTL and neutralizing antibody, will provide optimal protection. Thus, vaccination should ensure such a response is mounted upon natural exposure to the virus. It is well known that neutralizing antibody can be generated, but that HIV-1 has a means of generating viral variants very quickly. As such, neutralizing antibody generated against the original variant encountered will be effective against this particular variant, but usually is ineffective against newly arising variants that become dominant through the course of the infection.

The contrasting view we favor is that vaccination, in order to be effective, must guarantee a predominant Th1 response, associated with potent CTL generation, upon HIV-1 infection. We think three lines of reasoning support this view. Firstly, there are highly exposed and infected individuals who produce predominant and stable Th1, CTL responses, but who are symptom-free. A prime illustration of such a group is a cohort of female sex workers in Nairobi, whose immune state has been extensively characterized [14, 15]. What is additionally remarkable about this group of individuals is that they do not seroconvert and remain healthy over many years despite numerous exposures to major viral variants or clades of HIV-1 due to the nature of their work. Secondly, all individuals infected with HIV-1 are healthy during the period when their immune system

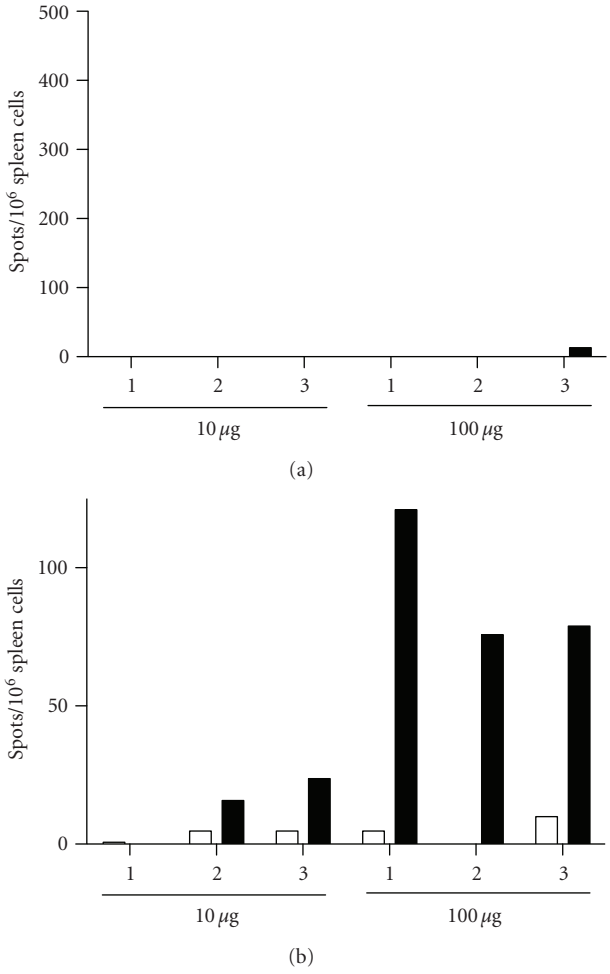

Figure 6: Number of IFNγ- and IL-4-producing cells in mice immunized i.p. with soluble β-gal (A) and β-gal adsorbed to alum as an adjuvant (B). Soluble β-gal was administered twice five days apart, while β-gal/alum was administered only once. Dose of β-gal administered in each experiment is indicated (10 μg or 100 μg). Mice were killed 11 days after the initial injection, and antigen-specific cytokine-producing spleen cells were enumerated by ELISPOT assay. Open bars represent IFNγ spots and black bars represent IL-4 spots. Results are representative of three separate experiments.

mounts a predominant Th1, CTL response, before they seroconvert and enter the progressive stages of disease [16]. Both observations strongly support the idea that a predominant Th1, CTL response can contain HIV-1. Why then not aim for having the best of both worlds, both CTL and neutralizing antibody, as most suggest? It is an assumption, and one that we feel is highly questionable, that this is possible. Distinct $CD4^+$ T cell subsets tend to inhibit each others' generation and often each others' effector activities [1]. It may not be possible to have the best of both worlds, involving a mixed Th1/Th2 response, with optimal production of CTL immunity and neutralizing antibody. We think it best to conclude, in accordance with natural situations in which the virus is contained, and infected individuals remain symptom-free, that a predominant Th1, CTL response is optimally protective. We employ this proposition as a starting point for considering how effective vaccination might be achieved.

In the case of influenza virus, that causes an acute infection, it seems antibody can be highly protective. Antibody is usually not produced in sufficient amounts upon infection of naïve individuals to prevent pathological symptoms, which is why immunization against the virus causing the current epidemic is effective in preventing illness. Individuals who have been infected during the last epidemic, but not immunized against the virus causing the current epidemic, are highly primed to produce antibody. The antibody produced is highly effective in neutralizing the influenza strain causing the previous epidemic, but not the virus causing the current epidemic. This makes eminent sense from an epidemiological point of view. It is just because the current influenza strain can stimulate the production of this antibody, ineffective in its own neutralization, that the virus can multiply unimpeded in the individual, primed by a previous infection, with the consequential spread of the virus through the population. An appreciation of these facts underlies the formulation of current policy. The aim is to achieve immunization against the strain causing the current epidemic as soon as it has been identified. This policy is sound, but its realization is extremely expensive and poses considerable logistical problems, clearly illustrated throughout the most recent influenza H1N1 pandemic.

The CTL response against influenza involves, in large measure, a response against peptides derived from viral proteins that do not vary greatly between strains, such as peptides derived from the nuclear protein, NP [17]. The possibility might therefore be considered that vaccination resulting in a sustained and effective CTL response upon infection by diverse variants might be effective against all variants. An intriguing report supports this possibility. DNA immunization of mice against NP results in a predominant Th1, CTL response on challenge with different viral variants and resistance [18]. Thus generating stable Th1 imprints against influenza might result in protection against diverse viral variants.

The observations reported here and our considerations on how vaccines against diverse clades of HIV-1 and strains of influenza might be achieved by causing Th1 imprints lead us to suggest that attempts at realizing a low-dose rBCG vaccination strategy is worthy of further exploration. We showed in this paper that infection with low numbers of rBCG generated not only predominant Th1 responses but also Th1 imprints as assessed by the predominant Th1 responses observed upon a subsequent challenge with a high number of rBCG that generate mixed Th1/Th2 responses in naïve animals. We consider the next important step in assessing the plausibility of this strategy will be to examine whether rBCG can provide protection against a pathogenic infection. There are mouse models of influenza infection. We hope to examine, employing established systems, whether infection of mice with low numbers of rBCG vectors, encoding conserved proteins of influenza virus, can generate Th1 imprints, as assessed by infection with a normally

pathogenic challenge of influenza virus, and whether such imprints provide protection against diverse viral variants.

Acknowledgments

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Research Article

β-Glucan Oligosaccharide Enhances CD8$^+$ T Cells Immune Response Induced by a DNA Vaccine Encoding Hepatitis B Virus Core Antigen

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DNA vaccination can induce specific CD8$^+$ T cell immune response, but the response level is low in large mammals and human beings. Coadministration of an adjuvant can optimize protective immunity elicited by a DNA vaccine. In this study, we investigated the effect of a synthetic glucohexaose (β-glu6), an analogue of Lentinan basic unit, on specific CD8$^+$ T cell response induced by a DNA vaccine encoding HBcAg (pB144) in mice. We found that β-glu6 promoted the recruitment and maturation of dendritic cells, enhanced the activation of CD8$^+$ and CD4$^+$ T cells and increased the number of specific CD8$^+$/IFN-γ^+ T cells in lymphoid and nonlymphoid tissues in mice immunized by pB144. Immunization with pB144 and β-glu6 increased the anti-HBc IgG and IgG2a antibody titer. These results demonstrate that β-glu6 can enhance the virus-specific CTL and Th1 responses induced by DNA vaccine, suggesting β-glu6 as a candidate adjuvant in DNA vaccination.

1. Introduction

Sugars are bioactive components of many plants and microorganisms. Polysaccharides and oligosaccharides of various origins (fungi, bacteria, plants, etc.) can be recognized by surface receptors of host cells, in particular macrophages and dendritic cells, and trigger host innate immune reactions [1]. Some polysaccharides and oligosaccharides extracted from plants, such as microgranular formulation of inulin, can regulate the immune response by acting as adjuvants to nonspecifically enhance cellular and humoral immune responses and activate the innate immunity through the alternative complement pathway [2].

The curative effects of *Ganoderma lucidum* and other mushrooms were recorded in the Compendium of Materia Medica. Their extracts have been used to prevent and treat diseases in traditional Chinese medicine. Polysaccharides and oligosaccharides are the major bioactive molecules in the extracts [3, 4]. Lentinan, a polysaccharide exacted from the fungus *Lentinus edodes*, possesses various biological activities, such as immune regulation, anti-infection and anti-tumoral activity [5, 6], and its usage is recorded in Chinese Pharmacopoeia [7]. Lentinan has potent ability to activate innate immune effector cells such as monocytes/macrophages and NK cells, and to stimulate cell-mediated immune response [4, 8]. Kupfahl et al. demonstrated that Lentinan enhanced the protective CD8$^+$ T cell response against Listeria monocytogenes in the spleen of mice [9]. This suggests that Lentinan may be used as an adjuvant to enhance host's immune response. However, it is very hard to extract pure Lentinan from the fruiting body of *Lentinus edodes* because the extracted fungal polysaccharides

and oligosaccharides have some drawbacks, such as the heterogeneous structures of the saccharides, poor solubility in water, low purity, and low yield [10]. These disadvantages limit their applicability in clinical practice.

Many bioactive polysaccharides from fungi and plants have the common structural signature of β-(1 → 6)-branched β-(1 → 3) gluco-oligosaccharides [11]. The basic unit β-(1 → 6)-branched β-(1 → 3) glucohexaose in Lentinan plays a vital role in antitumour activity in mice [12], and is endowed with the immunostimulatory effects of the whole polysaccharide. Gu et al. synthetized a β-(1 → 6)-branched β-(1 → 3) glucohexaose analogue containing a α-(1 → 3)-linked bond (β-glu6) (patent publication number: PCT/CN02/00478), and it shows similar effects as Lentinan on mouse spleen cell proliferation and TNF-α production [13]. The study by Dong et al. demonstrated administration of synthetic β-glu6 with hepatitis B surface antigen (HBsAg) significantly increased the antigen-specific antibody titer and the number of HBsAg-specific IL-4-producing T cell in spleen, and the ratio of anti-HBsAg IgG1/IgG2a was higher in mice immunized with HBsAg plus β-glu6 than that in mice receiving HBsAg alone, indicating a shift towards a Th2-biased response [14]. This suggests that β-glu6 can act as an adjuvant to enhance Th2 humoral immune response induced by a protein vaccine, but the adjuvant effect of β-glu6 on specific $CD8^+$ T cell response needs to be studied.

In a DNA vaccine, it is expected that DNA encoded antigens are subjected to intracellular proteasomal degradation, resulting in peptide fragments that can be presented by MHC class I antigens to $CD8^+$ T cells, thus mimicking viral infection [15]. Therefore, DNA vaccines have been extensively studied both for preventive and therapeutic approaches against microbe infections. Coadministration of DNA vaccine with adjuvants (such as cytokines, chemokines, CpG DNA, costimulatory molecules, ligands, etc.) is one of strategies to optimize $CD8^+$ T cells protective immunity [16–18]. Polysaccharides from microorganisms and plants can also be used as DNA vaccines adjuvants. For example, *Agaricus blazei Murill* extracts containing a great quantity of polysaccharides have an ability to enhance humoral and cellular immune responses induced by plasmid DNA encoding HBcAg [19]. In mice, Lentinan was found to increase Env-specific type 1 cytokine production and cytotoxic T-lymphocyte (CTL) activities induced by DNA vaccine, encoding human immunodeficiency virus envelope glycoprotein [20]. Since the adjuvant effect of β-glu6 on the polarization of Th1/Th2 balance and the induction of antigen-specific cytotoxic T lymphocytes upon administration of a DNA vaccine is still unknown, in the present study, we have investigated effect of β-glu6 on the antigen specific $CD8^+$ T cell response induced by a DNA vaccine encoding HBcAg.

2. Materials and Methods

2.1. Plasmid and Reagents. The eukaryotic expression plasmid pB144 was kindly provided by Prof. Yuan Wang, and was constructed by inserting a gene encoding HBcAg N'-end 144 amino acids into a vector (pcDNA3.1, Invitrogen, Carlsbad, CA, USA) under the control of the cytomegalovirus (CMV) immediate early promoter. COS-7 cells transiently transfected with pB144 significantly express HBcAg and efficiently secrete it into the cell culture supernatant [21].

2.1.1. Plasmid Purification. The pB144 plasmid was prepared according to the protocols of QIAGEN-TIP 2500 Plasmid Mega Kit (Qiagen Corporation, Maryland, USA). A single colony from a LB/Amp plate was inoculated into LB/Amp culture medium at 1/1000, and incubated at 37°C for 12–16 hours with vigorous shaking (approx. 300 rpm). The bacterial cells were harvested by centrifugation at 6000 x*g* for 15 minutes at 4°C. The bacterial pellet was resuspended in Tris·Cl-EDTA buffer containing RNase A (100 μg/ml) and lyzed with NaOH (200 mM)/SDS (1%). The lysate was neutralized by the addition of acidic potassium acetate (3.0 M, pH 5.0). The supernatant of lysate was applied to the QIAGEN-tip. The binding plasmid was eluted in a high-salt buffer, then the DNA was precipitated with isopropanol. The DNA was dissolved with sterile PBS (pH 7.0). The concentration of plasmid DNA was determined spectrophotometrically.

2.1.2. Reagents. FITC-CD4 (clone GK1.5), PerCP-CD8a (clone 53–6.7), APC-IFN-γ (clone XMG1.2), PE-CD11c (clone HL3), and the Cytofix/Cytoperm Plus kit (with GolgiPlug) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). The following reagents were purchased from Biolegend (San Diego, CA, USA): FITC-CD40 (clone HM40–3), FITC-CD86 (clone GL-1), FITC-MHC-II (clone M5/114.15.2), and PE-CD69 (clone H1.2F3). Brefeldin A was obtained from eBioscience (Boston, MA, USA). HISTOPAQUE-1083 and Deoxyribonuclease I were from Sigma-Aldrich (St. Louis, MO, USA). Anti-HBc ELISA kit and goat antimouse IgG-HRP were purchased from Huamei Bioengineering Co., Ltd. (Shanghai, China). Bovine anti-mouse IgG1 and anti-mouse IgG2a were purchased from the Binding Site Co., Ltd. (Birmingham, UK). Collagenase Type IV, 2-mercaptoethanol, RPMI-1640 medium, Fetal Bovine Serum (FBS), L-glutamine, penicillin, and streptomycin were obtained from GIBCO Invitrogen (Grant Island, NY, USA). Percoll was purchased from Pharmacia (Uppsala, Sweden). The peptide MGLKFRQL, representing an H-$2K^b$-restricted CTL epitope of the hepatitis B core antigen, was synthesized and generously provided by Dr. Rafi Ahmed (Emory University, Altanta, GA, USA).

2.2. β-(1 → 6)-Branched β-(1 → 3) Glucohexaose Analogue. β-glu6 glucohexaose was synthetized by Kong Fanzuo, Ning Jun, and Gu Jianxin, and identified with NMR, MS, and HPLC. ^{1}HNMR (CDCl3,400 MHz): δ5.269(d, J3.2 Hz, 1H, H-1α), 4.743(d, J8.0 Hz,2H,H-1β), 4.667(c, J8.0 Hz, 2H,H-1β), 4.440(d, J8.0 Hz, 2H, H-1β), 4.152–4.081(m, 4H), 3.919–3.256(m, 32 H); ^{13}C NMR(100 Hz, D_2O):δ102.69 (2 C-1β, JC1-H1 173 Hz), 102.6 (3C-1β,JC1-H1 173 Hz), 98.92 (1C-1α, JC1-H1 164 Hz), 85.34, 84.87,92.90 (C-3), 81.96, 75.85, 75.76, 75.74, 75.57, 75.54, 75.52,75.45, 75.40, 73.73, 73.34, 73.04, 72.12, 70.98, 70.75, 69.68,69.55, 69.49, 68.86, 68.37, 68.24, 67.78 (C-2,3,4,5,6); ESMS for

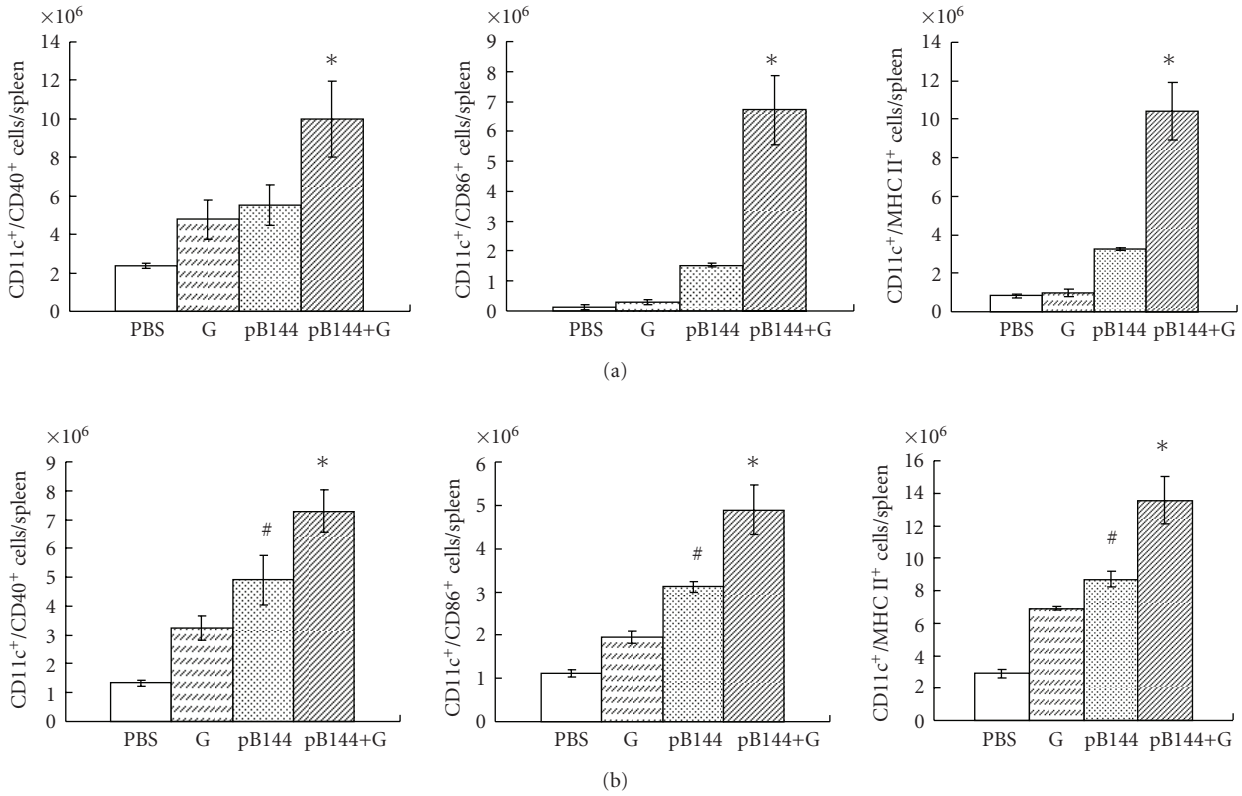

Figure 1: *Effect of the β-glu6 on pB144-induced CD11c⁺ DC maturation.* Mice were injected in the hind leg muscle with β-glu6 (G, 1 mg/kg), pB144 (100 μg/mouse), pB144 together with β-glu6 (pB144+G), or PBS. At day 5 after priming (a) and day 5 after boosting (b), spleen CD11c⁺ cells coexpressing CD40 (left panels), CD86 (middle panels), or MHC-II (right panels) were measured by flow cytometry. Data are expressed as mean ± SD of data from 3 mice/group. $^{*}P < .05$, pB144 +G versus all other groups; $^{\#}P < .05$, pB144 versus PBS control.

$C_{36}H_{62}O_{31}$ (992.5): 991.4 $[M\text{-}1]^+$. The details of the synthesis are illustrated in the related patent (publication number: WO03004507). The purity of the synthetic β-glu6 is more than 98%, as determined by HPLC. The endotoxin contamination in β-glu6 was under detection level by Limulus Amoebocyte Lysate (LAL) colorimetric assay (Cambrex; Walkersville, MD, USA).

2.3. Immunization of Mice with pB144 and β-glu6. Female inbred C57BL/6 mice (H-2^b), aged 6–8 weeks, were purchased from B&K Universal Group Limited (Shanghai, China), and kept under pathogen-free conditions. Mice were grouped as described below, each group consisting of 15 mice. Before immunization, mice were anesthetized with 0.75% sodium pentobarbital (75 mg/kg). One group of mice was inoculated with pB144 DNA (100 μg/mouse) and synthetic β-glu6 (1 mg/kg) in phosphate buffered saline (PBS) into the tibialis anterior muscle of each hind limb, as previously described [22]. These mice then received daily intramuscular injections of synthetic β-glu6 (1 mg/kg/day in 100 μl PBS) for six days. Another group of mice were immunized with pB144 DNA alone. Control mice were administered with the same volumes of PBS. At day 30 after the first immunization with pB144, all mice were boosted with a second antigen dose, using the same immunization schedule as in the priming. At days 5, 14, 35, and 40 after priming, three mice/group were sacrificed, and peripheral blood, spleen, and liver were collected.

2.4. Lymphocyte Preparation. Mice were anesthetized and sacrificed. The peripheral blood was collected from angular vein, using 4% sodium citrate as an anticoagulant. PBMC were obtained by density gradient centrifugation over HISTOPAQUE-1083 (Sigma-Aldrich), washed three times with RPMI-1640 and counted. Mice spleens were dissociated on a 200-gauge nylon mesh. Splenocytes were collected and treated with lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4) to eliminate red cells, washed and resuspended in RPMI-1640 with 10% heat-inactivated FBS (hereafter referred to as culture medium). Hepatic lymphocytes were isolated as previously described [23]. Briefly, the liver was perfused with 5 mL RPMI-1640 (containing 2% FBS, 2 mM L-glutamine, 100 U/mL penicillin and streptomycin, resp.) through the portal vein, then the inferior caval vein was cut and the medium was allowed to flow until the liver was free of red cells. The liver was then pressed through 200-gauge nylon mesh, fragments suspended in RPMI-1640 containing 0.05% collagenase IV and 0.001% DNase I, and digested at 37°C for 40 minutes. The liver cell suspension was collected, and mononuclear cells (MNC) were separated from parenchymal cells by centrifugation at 500 g for 10 minutes. Lymphocyte-rich

cell pellets were resuspended in 44% Percoll in complete RPMI-1640 medium, gently overlaid onto 56% Percoll and centrifuged for 10 minutes at 750 g at 4°C. The pellets were resuspended in erythrocyte lysing solution (lysis buffer with the addition of 170 mM Tris, pH 7.3), washed with RPMI-1640 and re-suspended in complete RPMI-1640.

2.5. Flow Cytometric Analysis of Surface Markers on Lymphocytes. Coexpression of CD11c with CD40, CD86, and MHC class II on spleen leukocytes was determined by flow cytometry. Coexpression of CD69 with CD4 and CD8 on lymphocytes of spleen, liver and peripheral blood was also assessed. Briefly, cells were stained with relevant fluorochrome-labelled mAbs for 30 minutes, washed with FACScan buffer (PBS containing 2% FBS and 0.1% sodium azide), then fixed with 4% paraformaldehyde. Fluorescence profiles were generated on a FACScan flow cytometer (BD Biosciences). For each sample, 100,000 cells were collected and histogram and density plots were produced by the CellQuest software package.

2.6. Detection of IFN-γ Producing T Cells by Intracellular Cytokine Staining. At day 5, 14, and 40 after primary immunization, IFN-γ production was detected by intracellular staining. Spleen cells, PBMC, and hepatic lymphocytes were plated separately (1×10^6 cells/well) in a 96-well plate (Corning Costar; Cambridge, MA) in a final volume of 200 μl. Cells were stimulated with a specific peptide corresponding to a K^b-restricted CTL epitope of the hepatitis B core antigen (final concentration 1 μg/mL) for 5 hours at 37°C in moist atmosphere with 5% CO_2, in the presence of Brefeldin A (final concentration 2 μg/mL). Cells were then stained with PerCP-conjugated anti-CD8a mAb for 30 minutes at 4°C, washed with FACS buffer (2% FBS, 0.1% sodium azide in PBS), treated with Cytofix/Cytoperm for 20 minutes, and washed with Perm/Wash Buffer (both from BD Biosciences). Cells were subsequently stained with APC-conjugated anti-mouse IFN-γ mAb for 30 minutes at 4°C, washed with Perm/Wash Buffer and FACS buffer, and fixed with 4% (w/v) paraformaldehyde. Sample data were acquired using a FACSCalibur flow cytometer (BD Biosciences).

2.7. Detection of Anti-HBc Antibodies. Blood was collected from the retroorbital plexus of mice after 4 weeks from the primary immunization and 2 weeks from boost, and serum were obtained. The titer of anti-HBc antibodies was measured by ELISA (anti-HBc ELISA kit; Diagnostic Reagent Center of Shanghai Municipal Infectious Diseases Hospital, Shanghai, China). Serum was serially diluted in PBS with 5% nonfat milk (starting from 1 : 100) and incubated in microtitre plates precoated with HBcAg for 1 hour at 37°C. Plates were then washed and further incubated (1 hour at 37°C) with 100 μL of HRP-conjugated goat anti-mouse IgG, bovine anti-mouse IgG1, or bovine anti-mouse IgG2a. After extensive washing, 50 μl of substrate were added to each well and incubated for 15 minutes at 37°C. The plates were read at 450 nm (reference wavelength 630 nm) with a BenchMark ELISA reader (Bio-Rad Laboratories, Hercules, CA, USA). The cut-off line discriminating between positive and negative antibody detection was set as 0.20 (OD 450/630). The endpoint titer of anti-HBc antibodies is reported as the reciprocal of the highest dilution at which the OD 450/630 reading is above 0.20.

2.8. Statistical Analysis. Data are presented as mean ± SD of values obtained from replicate mice within a single representative experiment. Each experiment was repeated at least three times with similar results. Results were analyzed with SPSS software and compared by ANOVA and the post-hoc analysis. A P-value of $< .05$ was considered as statistically significant.

3. Results

3.1. Effect of β-glu6 on PB144-Induced Maturation of $CD11c^+$ Dendritic Cells in the Spleen. Activated dendritic cells (DC) have a strong antigen-presenting capacity. The effect of β-glu6 on pB144-induced activation of DC in mice was studied. In the spleen of mice immunized intramuscularly with pB144 plus β-glu6, the number of $CD11c^+$ cells coexpressing CD40, CD86, or MHC-II was examined by flow cytometry. Compared with the mice immunized with pB144 alone, the populations of mature DC ($CD11c^+/CD40^+$, $CD11c^+/CD86^+$, and $CD11c^+/MHC\text{-}II^+$) in mice immunized by pB144 with β-glu6 at day 5 after priming were increased 1.8-, 4.4-, and 3.2-fold, respectively, as shown in Figure 1(a).

Compared to cells in naïve animals, at day 5 after boosting, $CD11c^+/CD40^+$, $CD11c^+CD86^+$, and $CD11c^+/MHC\text{-}II\text{-}^+$ DC increased about 3–3.5 times in the spleen of mice immunized with pB144 alone (from 1.3×10^6, 1.1×10^6, and 2.9×10^6 in naïve mice to 4.9×10^6, 3.1×10^6, and 8.7×10^6 in pB144 immunized mice), and about 4.5–5.6 times in the spleen of mice receiving pB144 plus β-glu6 (7.3×10^6, 4.9×10^6, and 13.6×10^6, resp.) (Figure 1(b)). No statistically significant difference was observed between naïve mice and mice receiving β-glu6 alone intramuscularly.

3.2. Effect of β-glu6 on PB144-Induced Recruitment and Activation of $CD4^+$ and $CD8^+$ T Cells. The recruitment and activation of $CD4^+$ T cells in the spleen of mice immunized by pB144 with or without β-glu6 were evaluated. At day 5 after priming, compared to the mice immunized with pB144 alone, the percentage of $CD4^+$ T cells in the mice immunized by pB144 with β-glu6 was higher in the spleen (from 21.5% to 27.3%) (Figure 2(a)). At day 5 after boosting, the percentage of $CD4^+$ T cells in the mice immunized by pB144 with β-glu6 increased from 24.8% to 29.3% in the spleen (Figure 2(a)). Both at day 5 after priming and at day 5 after boosting, the number of activated $CD4^+/CD69^+$ T cells in the spleens of mice immunized by pB144 with β-glu6 was about 1.7-fold higher than that in mice immunized with pB144 alone (Figure 2(b)). The recruitment of $CD4^+$ T cells in the liver and peripheral blood of mice immunized by pB144 with β-glu6 was of no significant difference from that in the mice immunized with pB144 alone, shown in

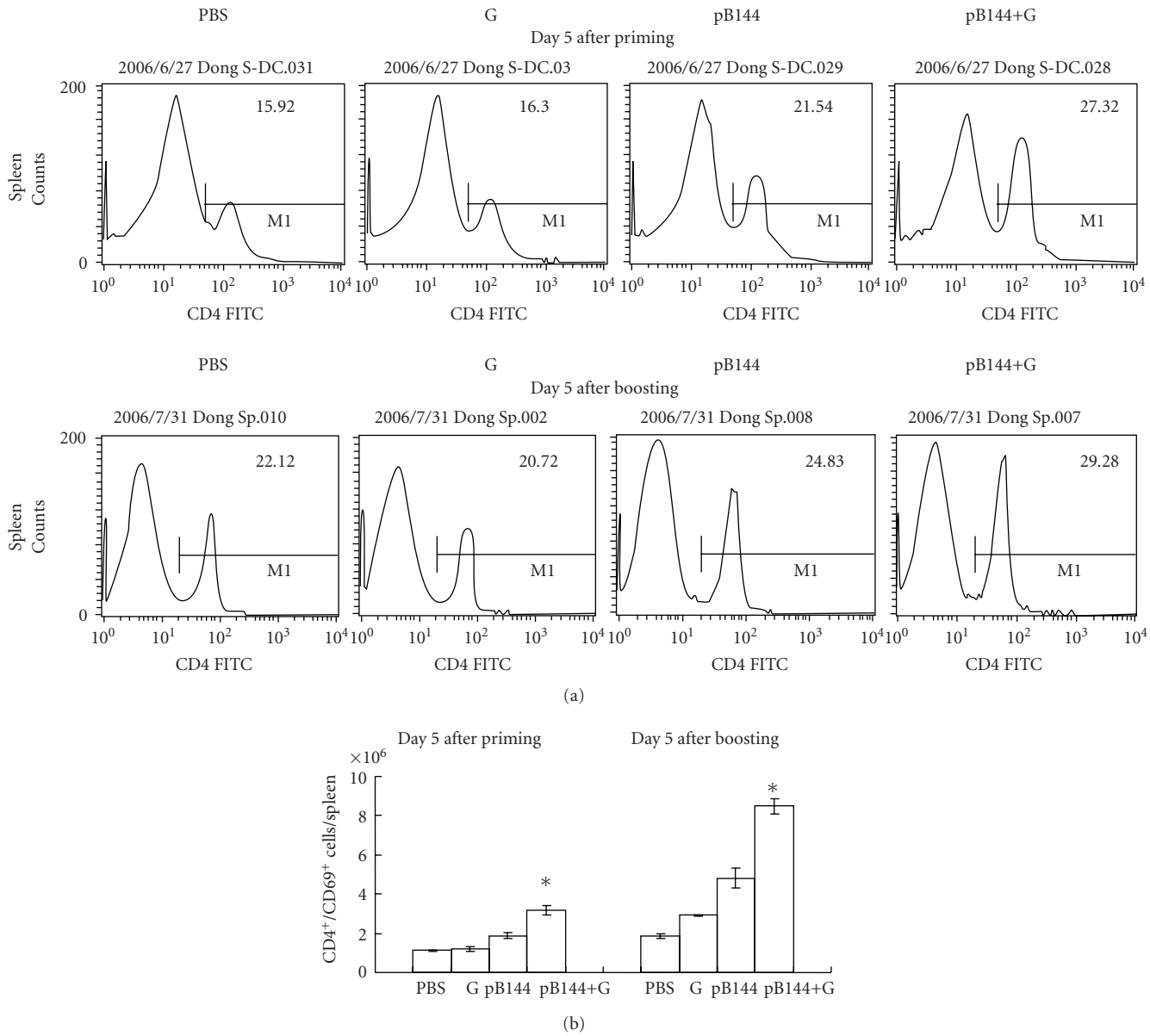

Figure 2: *Effect of β-glu6 on pB144-induced recruitment and activation of CD4⁺ T cells in the spleen.* Mice were immunized with β-glu6 (G), pB144, pB144+G, or PBS as described in Section 2. Five days after priming and five days after boosting, the percentage of $CD4^+$ T cells (a) and the number of $CD4^+/CD69^+$ T cells (b) in the spleen were evaluated. Data are expressed as mean of 3 mice/group. $^*P < .05$, pB144 + G versus pB144 alone.

the Supplementary Figure 1 (see in supplementary Material available online at doi:10.1155/2010/645213).

The recruitment and activation of $CD8^+$ T cells in the spleen of mice immunized by pB144 with or without β-glu6 were evaluated. At day 5 after priming, upon treatment with β-glu6, the number of $CD8^+$ T cells in the spleen of pB144-immunized mice was increased from 17.9×10^6 to 23.9×10^6 (Figure 3(a)). At day 5 after boosting, β-glu6 enhanced the pB144-induced recruitment of $CD8^+$ T cells into the spleen by 1.7-fold (from 18.9×10^6 to 31.7×10^6 cells/spleen, $P < .05$) (Figure 3(a)). At day 5 after priming, a higher number of $CD8^+$ T cells in splenocytes were activated by administration of pB144 with β-glu6, as compared to pB144 alone, judged by the expression of the CD69 as an activation marker (from 3.8×10^6 to 5.9×10^6 $CD8^+/CD69^+$ cells/spleen, $P < .05$), while activated $CD8^+/CD69^+$ T cells were increased to 8.3×10^6/spleen ($P < .05$ versus pB144 alone), at day 5 after boosting (Figure 3(b)). The number of $CD8^+$ T cells in the liver and peripheral blood was not statistically different among groups (Supplementary Figure 2).

3.3. Effect of β-glu6 on PB144-Induced Antigen Specific $CD8^+/IFN\text{-}\gamma^+$ T Cells. The effect of β-glu6 on antigen-specific $CD8^+/IFN\text{-}\gamma^+$ T cells in the mice immunized with pB144 was evaluated by intracellular cytokine staining, stimulated with the peptide MGLKFRQL, an H-$2K^b$-restricted

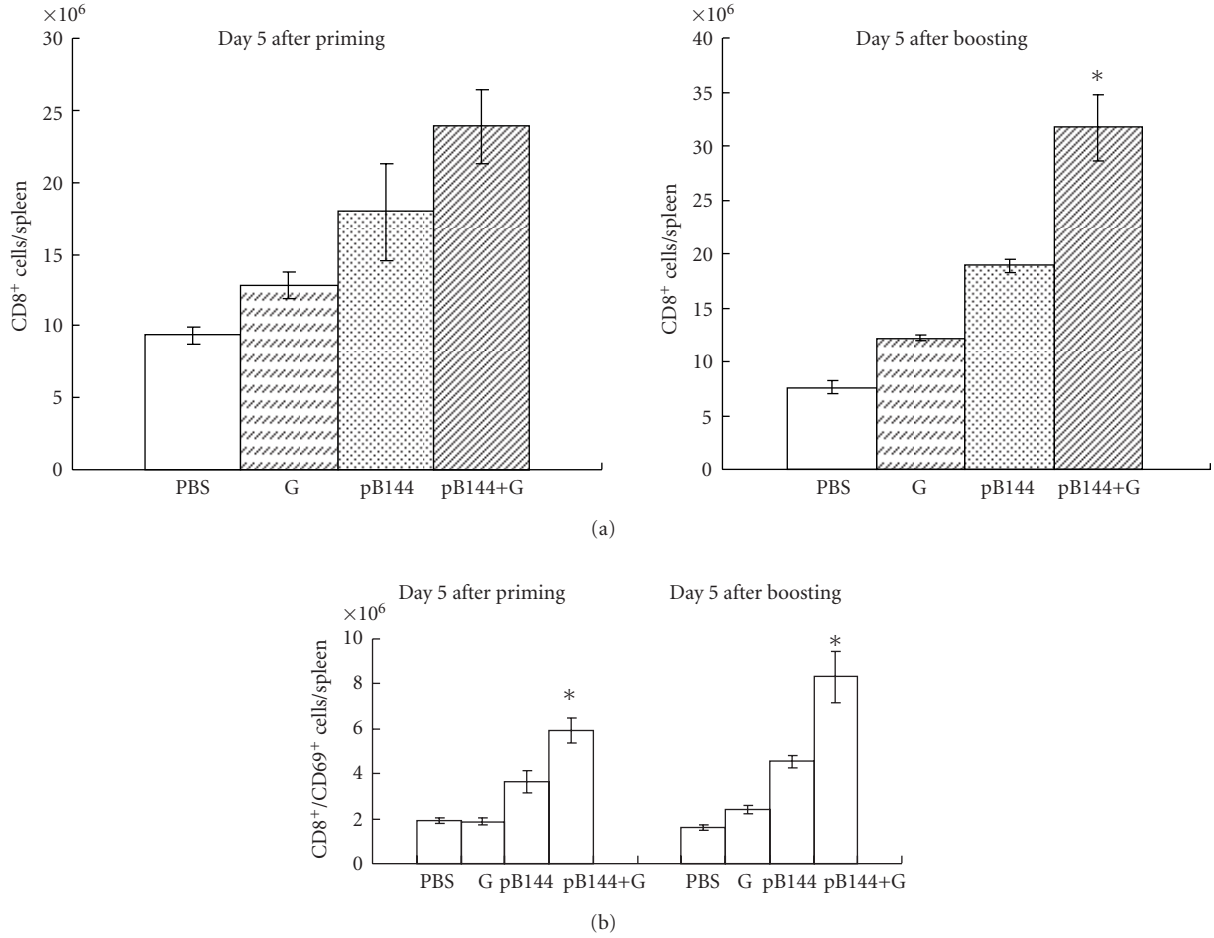

Figure 3: *Effect of β-glu6 on pB144-induced recruitment and activation of CD8⁺ T cells in the spleen.* Mice were immunized with β-glu6 (G), pB144, pB144+G, or PBS, as described in Section 2. Five days after priming and five days after boosting, the number of $CD8^+$ T cells (a) and $CD8^+/CD69^+$ T cells (b) in the spleen were evaluated. Data are expressed as mean ± SD of 3 mice/group. $^*P < .05$, pB144+G versus pB144 alone.

CTL epitope of the hepatitis B core antigen. At day 5 and 14 after priming, β-glu6 did not increase the number of $CD8^+/IFN\text{-}\gamma^+$ T cells in the spleen (Figure 4). However, at day 10 after boosting, the number of $CD8^+/IFN\text{-}\gamma^+$ T cells was 1.5-fold higher in splenocytes from mice immunized by pB144 with β-glu6, as compared to mice immunized with pB144 alone. β-glu6 increased the percentage of $CD8^+/IFN\text{-}\gamma^+$ T cells induced by pB144 in the liver (0.54 ± 0.08%), PBMC (0.74 ± 0.17%), and the spleen (1.5 ± 0.3%), as compared with that in mice immunized with pB144 alone (Figure 5).

3.4. β-glu6 Enhanced the PB144-Induced Antibody Production. The effect of β-glu6 on anti-HBc antibody response induced by the DNA vaccine was evaluated by ELISA. Anti-HBc antibodies (IgG) in sera from immunized mice were detected at 4 weeks after priming and 2 weeks after boosting. At 4 weeks after priming, anti-HBcAg antibody titers in mice immunized with pB144 alone (1 : 900) were lower than that in mice receiving pB144 plus β-glu6 (1 : 2700) (Table 1). No anti-HBcAg antibodies were detected in the serum of control mice injected with PBS or β-glu6 alone. At 2 weeks after boosting, anti-HBc IgG was higher in mice immunized by pB144 with β-glu6 (1 : 24,300) than that in mice immunized with pB144 alone (1 : 8100).

The IgG subclasses (IgG1 and IgG2a) of anti-HBc antibody were analyzed. At week 4 after priming, only low level of IgG2a was detected in mice immunized by pB144 with β-glu6. At week 2 after boosting, the mean titer of anti-HBc IgG2a was 1 : 4500 in the mice immunized by pB144 with β-glu6, and 1 : 3600 (mean) in mice receiving pB144 alone (Table 1). Anti-HBc IgG1 was under detection level in the all groups, even after boosting.

4. Discussion

Virus-specific $CD8^+$ T cells response plays an important role in the process of viral clearance. Herpes Simplex Virus (HSV) began to be cleared from all sites about 5 days after infection when HSV glycoprotein B-specific $CD8^+$ T cells first appear within infected tissues [24]. The failure of inducing a virus-specific $CD8^+$ T cell response contributes to the development

Table 1: Effect of β-glu6 on pB144-induced antibody production*.

Immunogens	Anti-HBcAg antibody titer ($Log_2 \pm$ SD)**					
	Week 4 after priming			Week 2 after boosting		
	IgG	IgG1	IgG2a	IgG	IgG1	IgG2a
G	*N.D.****	*N.D.*	*N.D.*	*N.D.*	*N.D.*	*N.D.*
pB144	9.81 ± 0.21	*N.D.*	*N.D.*	12.98 ± 0.18	*N.D.*	11.81 ± 0.55
pB144+G	11.40 ± 0.09****	*N.D.*	6.90 ± 0.49	14.57 ± 0.50****	*N.D.*	12.13 ± 0.6
PBS	*N.D.*	*N.D.*	*N.D.*	*N.D.*	*N.D.*	*N.D.*

*The sera from immunized mice were collected 4 weeks after priming and 2 weeks after boosting. Anti-HBcAg IgG, IgG1, and IgG2a were analyzed by ELISA. The initial dilution of each serum from immunized mouse was 1 : 100 and followed with serial of three-fold dilution for anti-HBc IgG detection, and two-fold dilution for anti-HBc IgG1, IgG2a analysis.

**The well with an absorbance $OD_{450} \geq 0.20$ (the blank well, ~0.020) was scored as positive. The anti-HBc antibody titers are expressed as the reciprocal of the highest dilution showing a positive reaction and calculated as the mean ± standard deviation (SD) of Log_2 for each group (6 mice/group).

***N.D. indicates that anti-HBc antibody in the sera of the mice is under detection level.

****$P < .05$, pB144 +G versus pB144 alone.

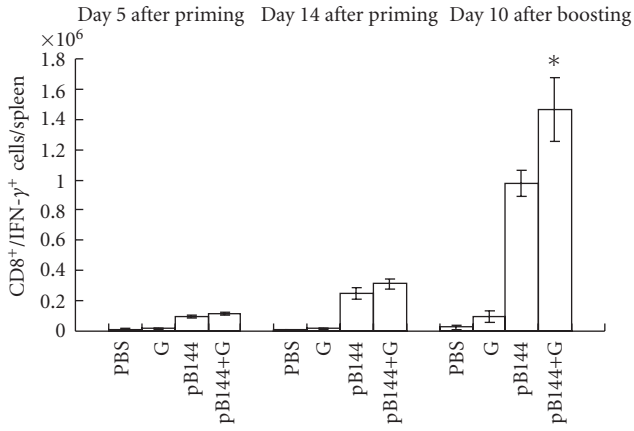

Figure 4: *Effect of β-glu6 on the $CD8^+/IFN\text{-}\gamma^+$ T cells induced by pB144 in the spleen.* Mice were immunized by pB144 with β-glu6, and boosted at day 30 after priming. At day 5 (left) and 14 (middle) after priming, and day 10 after boosting (right), splenocytes were isolated and cultured for 5 hours in the absence or in the presence of a specific peptide corresponding to K^b-restricted CTL epitope of HBcAg. Cells were then double-stained with PerCP-anti-CD8a and APC-anti-IFN-γ antibodies and analyzed cytofluorimetrically. Data expressed as mean ± SD of 3 mice/group. *$P < .05$, pB144+G versus pB144 alone.

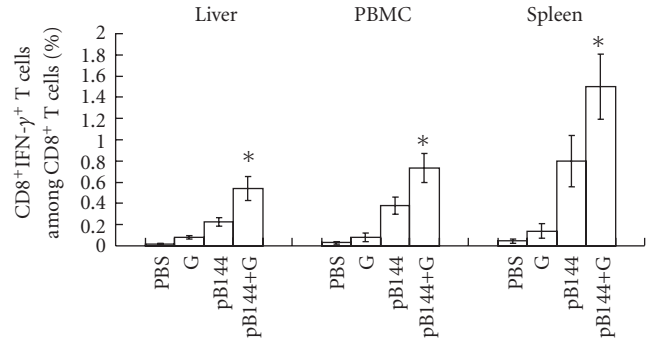

Figure 5: *Effect of β-glu6 on the $CD8^+/IFN\text{-}\gamma^+$ T cells induced by pB144 in different organs.* Mice were immunized with pB144 and treated with β-glu6, and boosted at day 30. Ten days after boosting, the liver lymphocytes, PBMC, and splenocytes were isolated and cultured for 5 hours in the absence or in the presence of a specific peptide corresponding to K^b-restricted CTL epitope of HBcAg. Cells were then double-stained with PE-anti-CD8a and APC-anti-IFN-γ antibodies and analyzed cytofluorometrically. Data are expressed as mean ± SD of 3 mice/group. *$P < .05$, pB144+G versus pB144 alone.

of pulmonary eosinophilia and disease augmentation in formalin-inactivated respiratory syncytial virus vaccine vaccinated individuals [25]. DNA immunization has the ability to induce a strong specific $CD8^+$ T cell response against a variety of infectious diseases [26–28].

However, clinical applications of DNA vaccines are limited by their low immunogenicity. Therefore, it is imperative to develop effective adjuvants for improving protective response in DNA vaccination. Adjuvants have been proven to optimize $CD8^+$ T cells response induced by DNA vaccine. For examples, cytokine plasmid-delivered IL-15 enhances the longevity of $CD8^+$ T cells induced by pB144 DNA vaccine [18]; soluble CD40L, a member of the tumor necrosis factor superfamily, augments $CD8^+$ T cell responses induced by human immunodeficiency virus DNA vaccines [16]. Some polysaccharides extracted from plants or microorganisms possess ability to improve the protective potential of a DNA vaccine against experimental infection [19, 29].

β-glu6 is a synthetic glucohexaose containing a structure of β-(1 → 6)-branched β-(1 → 3) with an α-(1 → 3)-linked bond, that is an analogue of Lentinan basic unit. Lentinan was extracted from *Lentinus edodes* being recorded in Compendium of Materia Medica for the treatment and prevention of diseases in traditional Chinese medicine. Lentinan can strengthen the cell-mediated immune response [30, 31] and activate some innate immune effector cells such as mononuclear macrophages and NK cells [11]. Administration of Lentinan before infection can mobilize host defence and reduce mycobacterial infection [32]. The β-(1 → 6)-branched β-(1 → 3) glucohexaose is a basic unit of Lentinan, endowed with potent anticarcinoma

activity. β-glu6, synthetic glucohexaose analogue, has stimulatory effects on mouse spleen cells (cell proliferation, TNF-α production), which is similar to Lentinan [12, 13]. Furthermore, β-glu6 has the advantages over Lentinan, such as possessing a defined structure, higher purity (>98% by HPLC), and availability.

Based on the study of β-glu6 as adjuvant enhancing effectivity of protein vaccine (Hepatitis B surface antigen) [14], we further investigated the adjuvant effect of β-glu6 on antigen-specific $CD8^+$ T cell response induced by DNA vaccine encoding HBcAg (pB144) that can be used as antigen candidate of DNA vaccine against HBV. We found β-glu6 possess ability to enhance the maturation of DCs, the recruitment and activation of T cells, and to increase the number of antigen-specific $CD8^+$/IFN-γ^+ T cells induced by pB144.

DCs function as both antigen-presenting cells and antigen-producing cells in DNA vaccine immunization. Thus, triggering DC activation can improve the efficacy of genetic vaccines [33]. Immunization of HIV-1 envelope (env) DNA vaccine alone recruited few DCs to the injection site and elicited low-frequency, Env-specific immune responses in mice [34]. As an endogenous ligand, polysaccharide degradation products of the extracellular matrix produced during inflammation may activate DCs via TLR4 [35]. In the previous study, it showed administration of β-glu6 (i.p.) alone has effect on DC recruitment and maturation in the spleen [14]. Maturation of bone marrow-derived dendritic cell (MDC) induced by HBsAg was enhanced with β-glu6 treatment, and in mixed lymphocyte reaction with MDC the proliferation of T cells was increased with the treatment of β-glu6 and HBsAg (unpublished data). In the present study, β-glu6 was able to enhance the DC maturation and migration to the spleen induced by DNA vaccines. We investigated the effect of anti-TLR2 and anti-TLR4 on β-glu6 immune activity, and found that the antibody against to TLR2 or TLR4 inhibited the activities of β-glu6 inducing TNF-α secretion in a mouse macrophage cell line, RAW264.7, suggesting that β-glu6 may activate innate immune cells via TLR2 or TLR4 signal pathway, though it needs to be confirmed in vivo. DC maturation may be related with β-glu6 enhancing the HBcAg-specific $CD8^+$ T cell response induced by DNA vaccine. Steffen Jung et al. observed that DC-depleted mice fail to mount CTL responses to infection with the intracellular bacterium Listeria monocytogenes and the rodent malaria parasite Plasmodium yoelii [36], suggesting that DC maturation play a role in specific $CD8^+$ T cell activation.

Antigen-specific $CD8^+$ and $CD4^+$ T cells play a vital role in control of viral infection. They can remove infected target cells through cytotoxic or noncytotoxic function, such as producing IFN-γ and other Th1 cytokines [37]. Christine Heufler' et al. found DC produced bioactive IL-12 upon antigen-specific interaction with T cells without any other stimuli, and DC-derived IL-12 was critical for optimal proliferation and IFN-γ production by activated Thl blasts [38]. The enhancement of DC maturation, migration, and the antigen presentation increased the number of antigen-specific $CD8^+$/IFN-γ^+T cells in DNA vaccine-immunized mice [39]. Booster injections with mature DCs raised $CD8^+$ T cell response in humans [40]. In the mice immunized by pB144 with β-glu6, the number of HBcAg-specific $CD8^+$/IFN-γ^+ T cells in lymphoid tissues (the spleen) and nonlymphoid tissue (the liver), were higher than that in the mice vaccinated by pB144 alone, suggesting that β-glu6 can amplify specific Th1 immune response induced by the DNA vaccine. With Lentinan being injected into mice intraperitoneally, the macrophage glutathione redox status and capability to produce IL-12 were improved, thus orienting toward type-1 immunity [41]. The effect of β-glu6 on the IFN-γ, IL-4 and DC-derived IL-12 production induced by pB144 needs to be further investigated.

In the present study, we found that β-glu6 increased the recruitment of $CD4^+$and $CD8^+$ T cells to the spleen, which was induced by pB144. With recruitment of antigen—nonspecific $CD4^+$ and $CD8^+$ T cells to lymphoid tissue, T cell-derived cytokines may help antigen-specific T cells augmentation.

When the antigen is endogenously produced upon intramuscular DNA vaccination, β-glu6 improved anti-HBc antibody production, in mice which the major IgG subclass of anti-HBc antibody was IgG2a and IgG1 were detected scarcely, and amplified the HBcAg-specific $CD8^+$ T cell response induced by DNA vaccine, that indicates a bias towards a Th1 immune response. In the previous study, β-glu6 improved anti-HBsAg IgG1 antibody production and the number of HBsAg-specific IL-4-producing T cells in spleen in mice immunized with HBsAg protein vaccine [14], indicating that β-glu6 can act as an adjuvant for a protein vaccine shifting towards a Th2-biased response. The roles of route of antigen entry, the physical form of antigen, the type of adjuvant and the dose of antigen in controlling the type of Th-cell differentiation have been reported [42]. Apparently, β-glu6 can amplify different types of responses, depending on the type of the vaccine utilized. Recent work suggests that dendritic cell subsets contribute significant polarizing influences on T helper differentiation, but how this comes about is less clear. Mosmann and Coffman indicated a single APC type may influence the Th1/Th2 ratio by providing different accessory signals to Th cells, depending on the physical nature of the antigen encountered [33, 43]. The physical type of vaccine and DC distinct roles in immunization may contribute to β-glu6 skewing Th1/Th2 immune response induced by vaccine that warrant to study.

Because mice is a species already known to respond very well to DNA immunization, the effect of β-glu6 has yet to be proved in another animal species refractory to DNA immunization, like nonhuman primates. Since Lentinan is recorded in Chinese Pharmacopoeia as an immunopotentiator, the plasticity of the adjuvant effects displayed by β-glu6, which contains the basic bioactive unit of Lentinan, makes it a suitable candidate adjuvant for different types of vaccines, providing its potential clinical application.

Acknowledgments

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Review Article

A New Insight into Hepatitis C Vaccine Development

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Chronic hepatitis C virus (HCV) infection remains a serious burden to public health worldwide. Currently, HCV-infected patients could undergo antiviral therapy by giving pegylated IFN-α with ribavirin. However, this therapy is only effective in around 50% of patients with HCV genotype 1, which accounts for more than 70% of all HCV infection, and it is not well tolerated for most patients. Moreover, there is no vaccine available. The efforts on identifying protective immunity against HCV have progressed recently. Neutralizing antibodies and robust T cell responses including both $CD4^+$ and $CD8^+$ have been shown to be related to the clearance of HCV, which have shed lights on the potential success of HCV vaccines. There are many vaccines developed and tested before entering clinical trials. Here, we would first discuss strategies of viral immune evasion and correlates of protective host immunity and finally review some prospective vaccine approaches against chronic HCV infection.

1. Introduction

More than 170 million people are currently affected by chronic hepatitis C virus (HCV) infection worldwide with the highest prevalence in Africa and Asia [1–3]. Since the adoption of the all volunteer blood donor system to screen blood donations in 1990s, the incidence of HCV infection has dropped dramatically. However, some populations remain highly susceptible including drug users sharing the same devices and patients that have received unsafe therapeutic injection or unsafe blood transfer [4]. Among all HCV infected individuals, 80% of them remain chronically infected [4, 5], 10%–20% of them develop cirrhosis, and 1%–5% of them acquire liver cancer over years [6]. Therefore, previous incidence as well as new incidence all account for future disease burdens. In developed countries, HCV infection has became the leading cause for the failure of liver transplants [1]. Up until now, there is no vaccine available for HCV infection. HCV-infected patients could receive anti-viral therapy by giving pegylated interferon-α (PEG-IFN) with ribavirin [7]. However, this therapy is long, expensive, toxic, and only effective in around 50% of patients for the most common genotype [8]. A regimen of 48-week therapy with PEG-IFN and ribavirin costing $25,000 USD is recommended for HCV genotype 1 and 24-week therapy for HCV genotype 2/3 [9]. There are many side effects associated with PEG-IFN, which have lead to early withdrawals or dose modification, including neutropenia, flu-like symptoms, neuropsychiatric disorders like depression, and autoimmune syndromes like autoimmune thyroiditis [8]. A sustained virological response (SVR) representing long lasting disappearance of viral RNA in the serum can be achieved in 80%–90% of genotype 2/3 but only around 40%–50% of genotype 1 [10, 11], which accounts for more than 70% of HCV infection in US [12, 13]. Therefore, the development of effective vaccines for HCV, especially therapeutic, is crucial in controlling chronic HCV infection.

HCV is an RNA virus with enveloped virion belonging to the family Flaviviridae. It contains a positive-sense single-stranded RNA genome that is 9,600 nucleotides in length. HCV genomic RNA is composed of one open-reading frame flanked by 5′ and 3′ noncoding region. The HCV polyprotein encoded by the only open-reading frame is approximately 3,000 amino acids in length and is cleaved into three structure proteins (core, E1, and E2), and seven nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [14]. According to international standardization and coordination of the nomenclature of variants of hepatitis

Table 1: HCV genotypes, subtypes, and their geographical distributions.

Genotypes	Subtypes	Geographical Distribution
1	a, b, c	Central Africa, Europe, North America
2	a, b, c, k	Western Africa
3	a, b, k	Southeast Asia
4	a	Central Africa
5	a	
6	a, b, d, g, h, k	Southeast Asia

C virus, HCV is classified into 6 clades or genotypes with 31%–33% diversity in nucleotide based on partial sequences of core/E1 and NS5B, or complete sequences. Each genotype is further divided into different subtypes with 20%–25% differences (Table 1) [15]. The importance of HCV genotype lies in its geographical distribution and treatment response to PEG-IFN and ribavirin [8]. Due to the low-fidelity of RNA-dependent RNA polymerase, NS5A, in viral replication, there are many quasispecies within one infected individual [16]. Primarily HCV infects hepatocytes of humans and chimpanzees. Various molecules including CD81 [17], scavenger receptor class B type I [18], Claudin-1 [19], low density lipoprotein receptor [20], and glycosaminoglycan [21] have been shown to be the receptors for HCV. The recent discovered receptor, occludin, however, is the crucial factor allowing HCV replication in mice [22].

The studies on HCV evasion from host immunity and host immunity in HCV patients that have spontaneously recovered have allowed us to address important immunological parameters related to protective immunity. Spontaneous recovery has been linked to multifunctional $CD4^+$ T cells, cross-genotype cytotoxic $CD8^+$ T cells, as well as cross-genotype neutralizing antibodies. These studies have advanced our understanding on protective immunity against HCV and provide a blueprint for HCV vaccine developments. There were many vaccines developed and tested in preclinical setting in the past. Among them, several vaccines have now advanced to clinical trials. Herein, we would examine the immune evasion strategies used by HCV, discuss correlates of successful host immunity against HCV infection, and review some prospective therapeutic vaccines to chronic HCV infection.

2. Immune Evasion by HCV

HCV can target many different effectors of the immune system, which enables its escape from host immune surveillance and ultimately leads to chronic infection. HCV can inhibit IFN-α production, inhibit NK activity, and produce escape mutants from antibody and $CD8^+$ T cell recognition. All these have aided to the development of chronic HCV infection.

Double-stranded RNA expressed by many RNA viruses during replication could be recognized by host pathogen-recognition receptors, such as TLR3 and RNA helicases (RIG-I and MDA-5), which lead to anti-viral responses. The recognition of dsRNA by TLR3 triggers its signalling pathways. In addition to the MyD88-dependent pathway, the MyD88-independent pathway leads to phosphorylation and nuclear translocation of IFN regulatory factor 3 (IRF-3) through adaptor protein TRIF [23–25]. The activation of transcription factor IRF-3 subsequently induces type 1 IFN production and other genes involved in host defence [26]. By comparison, RIG-I activates IRF-3 for type I IFN production through another pathway required a CARD-containing adaptor protein, Cardif. During replication, HCV NS3-4A protease recognizes and cleaves both TRIF and Cardif, which blocks the signalling pathways of TLR3 and RIG-I and ultimately inhibits the production of type I IFN [27–29].

The lack of type I IFN production in patients chronically infected with HCV may indirectly lead to a decrease in NK cell activity. Activated NK cells are important effectors in innate immunity against viral infection through the secretion of inflammatory cytokines like IFN-γ or the cytolytic ability like antibody-dependent cell-mediated cytotoxicity (ADCC) [30]. Type I IFN activates DCs which subsequently prime NK cells via the production and transpresentation of IL-15 [31, 32]. Thus, the lack of type I IFN production might have led to the lack of IL-15 production present in the serum, which eventually causes the decrease in total number of NK cells, especially cytotoxic $CD16^+CD56^{dim}$ NK cells, in chronic HCV- infected patients [33, 34].

Antibody responses may not be sufficient to protect individuals from HCV infection since neutralizing antibodies are rarely found in acute HCV patients but are found in the majority of chronically infected patients at relatively high titers (>320) [35, 36]. The failure of neutralizing antibodies in controlling HCV infection could be caused by several different factors. HCV can bind to very low-density lipoprotein (VLDL), which facilitates the uptake of HCV by hepatocytes via the interaction between ApoB and scavenger receptor class B type I, helping HCV avoid recognition by neutralizing antibodies [37]. E2 is highly glycosylated with 11 N-linked glycans located at the conserved region outside hypervariable region 1 (HVR1), which is targeted by most antibodies. These glycosylation sites are conserved across all HCV genotypes and subtypes. Three glycans located at the CD81-binding site of E2 decrease its immunogenicity and eventually protect viruses from antibody neutralization [38]. HCV can also infect surrounding cells through a direct cell-cell contact mediated by CD81 and Claudin-1, which can also avoid itself from the clearance of neutralizing antibodies [39]. Moreover, HCV can evolve into many quasispecies representing closely related but heterogenous RNA sequences within one individual during the course of infection. The number of quasispecies identified within a single sample ranges between 3–10 variants and the sequence variation occurs mainly in the HVR1 [40–42]. Studies following the evolution of HCV in one single patient well illustrated the development of quasispecies in chronic patients. Neutralizing antibodies to broad genotypes of HCV caused by continuous mounting immune response to evolving HCV

could be detected in this patient. However, these antibodies could not neutralize the dominant HCV isolate from this patient at the time of sample collection. Therefore, the antibody response failed to resolve HCV infection [41]. Furthermore, the presence of interference antibodies could diminish the function of true neutralizing antibodies. Two important epitopes located at E2 envelope glycoprotein have been identified (Table 2). Whereas epitope I located at residue 412–426 is an important neutralizing site and conserved between different genotypes, epitope II at 434–446 varies among different genotypes and generates antibodies interfering with the antibody to epitope I. When analysing the appearance of antibodies specific to these two epitopes, chronic HCV patients developed antibodies to nonprotective epitope II first. The appearance of antibodies to protective epitope I only appeared at very late time point together with equal abundant interference antibodies. Indeed, when antibodies specific to epitope II were depleted from patient plasma, this plasma, which contained antibodies to epitope I, could now provide better neutralizing capacity to a variety of genotypes [43]. In addition to the constant mutation occurred in HCV, the induction of interfering antibodies is yet another strategy of HCV to escape from immune response. Since these interference antibodies appear earlier than the protective antibodies, a vaccine effective in generating antibodies to epitope I would be critical against HCV infection.

The emergence of escape mutations in $CD8^+$ T cell epitopes requires a balance between virus infectivity and host immune response. The low-fidelity of RNA-dependent RNA polymerase, NS5A, is generally known to be the reason for the emerging of many quasispecies within HCV- infected individuals [16]. The rapid accumulation of mutated variants could be tracked back to the slow immune response generated against HCV, which has allowed many mutations to accumulate in vivo [40, 44]. The emerging of mutational variants in $CD8^+$ T cell epitopes has been carefully described both in chimpanzees [44, 45] and in humans [46–48]. Firstly, when the mutation rate was analysed, amino acid substitutions within $CD8^+$ T cell epitopes occurred more frequently compared to other regions [47]. Secondly, the lack of $CD4^+$ T cell help in chronic phase has prevented the effectiveness of $CD8^+$ T cells to clear the virus [49]. When chimpanzees with resolving HCV infection were rechallenged after $CD4^+$ T cell depletion, they developed chronic HCV infection. The inability of HCV- specific $CD8^+$ T cells to control HCV viremia correlated with the emerging of mutations in $CD8^+$ T cell epitopes. Therefore, these ineffective $CD8^+$ T cells have provided the selective pressure on shaping mutational variants of HCV. Recently, studies analysing different HCV variants within one chronically infected individual have shown that some HCV variants were actually emerging early during acute infection. Despite poor viral production while infecting hepatocytes, these variants survived due to poor recognition by host $CD8^+$ T cells [50]. Therefore, a fine balance between virus infectivity and host immune response could shape HCV mutants present in chronically infected individuals.

3. Protective Immunity to HCV

Despite the ineffectiveness of the host immune system to eradicate HCV, studies on patients that have spontaneously recovered from HCV infection and vaccinated chimpanzees that have recovered from HCV challenge have allowed us to address the important immunological correlates related to HCV clearance. Some reports have shown that the clearance of HCV could be associated with certain host genetic background including host HLA types, cytokine and chemokine expression (e.g., IL-10, IL-28B, and CCR5) [51–56]. For example, HCV clearance is often linked to patients with HLA-B27 allele in their MHC class I locus [55, 57]. Since MHC class I is directly associated with antigen presentation to $CD8^+$ T cells, this implies the importance of cytotoxic T cells in HCV eradication. Many studies published recently have provided critical immune parameters on protective immunity against HCV.

$CD8^+$ T cells are the most important effectors in controlling HCV infection. It was first recognized in earlier studies analysing acute HCV infection in chimpanzees. Few chimpanzees have resolved HCV spontaneously while most of them became chronically infected. When comparing these two groups of chimpanzees for the development of neutralizing antibodies and cytotoxic $CD8^+$ T cells, spontaneously recovered chimpanzees exhibited a strong $CD8^+$ T cell response toward multiple viral epitopes and across multiple MHC class I restrictions (Table 2) [58]. When these spontaneously recovered chimpanzees were challenged with HCV for the second time, they recovered more rapidly, within 14 days compared to 40 days in primary infection. Specific $CD8^+$ T cell number inversely correlated with HCV viral load in the blood. Additionally when $CD8^+$ T cells were depleted before the third challenge, a prolonged HCV infection was observed [59]. This was further supported by similar findings in human studies. When patients with chronic HCV infection were investigated, a general lack of broad specificity and cytotoxicity in $CD8^+$ T cells toward HCV epitopes was observed (Table 2) [60]. A more detailed study on HLA-matched individuals with chronic infection or spontaneously resolving infection was set to investigate the breadth, magnitude, phenotype, and function of HCV-specific $CD8^+$ T cells. Individuals with resolved HCV infection contained stronger $CD8^+$ T cell responses (17/20 versus 9/20; $P = .019$) and specific $CD8^+$ T cells to broader range of epitopes (Table 2; mean 2.3 versus 1; $P = .039$) with higher frequency in circulation (mean 584 versus 95 per 1 million PBMC; $P = .027$) measured by IFN-γ secretion in response to HCV peptides [61]. These specific $CD8^+$ T cells proliferated vigorously in response to antigens and expressed memory $CCR7^{hi}CD45RA^{low}CD27^+CD28^+$ phenotype [60, 61]. Notably a predominant expression of IL-7 receptor (CD127) on HCV-specific $CD8^+$ T cells was identified in patients who recovered from infection [62]. Additionally cross-genotype $CD8^+$ T cells could limit the escape of HCV and help the clearance of viruses [57]. Thus, a robust multispecific and cross-genotype $CD8^+$ T cell response to different epitopes implies a successful response against HCV infection.

Table 2: Important epitopes of HCV recognized by T cells and B cells.

Ref.	Antigen	Epitopes (aa sequence, restriction molecules)	Functional properties
CD8 epitopes			
Cooper et al. [58]	E1	306–315 (CSIYPGHITG, Patr-A*0402); 366–375 (GNWAKVLVVL, Patr-C*0601/C*0602)	cytotoxic epitopes identified in recovered champanzees
	E2	621–629 (TINYTIFKI, Patr-B*2001); 651–665 (RCDLEDRDRSELSPL, Patr-A*0601)	
	P7	781–791 (KWVPGAVYTFY, Patr-A*0601)	
	NS2	997–1008 (INGLPVSARRGR, Patr-A*0402)	
	NS3	1629–1637 (GAVQNEITL, Patr-B*1701)	
	NS5A	2055–2065 (MWSGTFPINAY, Patr-A*0601)	
Dazert et al. [57]	NS5B	2841–2849 (ARMILMTHF, HLA-B27)	IFN-γ secreting and protective epitope
Lauer et al. [61]	core	41–49 (GPRLGVRAT, HLA-B7); 88–96 (NEGCGWMGW, HLA-B44); 111–119 (DPRRRSRNL, HLA-B7)	IFN-γ secreting epitopes identified in recovered individuals
	E1	207–214 (CPNSSIVY, HLA-B35); 322–330 (MMMNWSPTT)	
	E2	541–551 (NTRPPLGNWFG, HLA-B57); 610–619 (YRLWHYPCTI, HLA-Cw7)	
	NS2	831–840 (LSPYYKRYIS, HLA-A25); 941–960 (LGALTGTYVYNHLTPLRDWA); 957–964 (RDWAHNGL, HLA-B37)	
	NS3	1070–1089 (ATCINGVCWTVYHGAGTRTI); 1073–1081 (CINGVCWTV, HLA-A2); 1175–1183 HAVGLFRAA, HLA-A68); 1359–1367 (HPNIEEVAL, HLA-B35); 1395–1403 (HSKKKCDEL, HLA-B8); 1406–1415 (KLVALGINAV, HLA-A2); 1435–1443 (ATDALMTGY, HLA-A1); 1610–1627 (CLIRLKPTLHGPTPLLYR)	
	NS4	1695–1702 (IPDREVLY, HLA-B35); 1745–1754 (VIAPAVQTNW, HLA-A24); 1751–1770 (VFTGLTHIDAHFLSQTKQSG); 1758–1766 (ETFWAKHMW, HLA-A25); 1771–1790 (GIQYLAGLSTLPGNPAIASL); 1801–1809 (LTTSQTLLF; HLA-B57); 1966–1976 (SECCTPCSGSW, HLA-B37); 1987–1995 (VLDSFKTWL, HLA-A2)	
	NS5	2162–2170 (EPEPDVAVL, HLA-B35); 2225–2233 (ELIEANLLW, HLA-A25); 2461–2480 (TSRSACQRQKKVTFDRLQVL); 2594–2602 (ALYDVVTKL, HLA-A2); 2629–2637 (KSKKTPMGF, HLA-B57); 2819–2828 (TARHTPVNSW, HLA-A25); 2912–2921 (LGVPPLRAWR, HLA-B57)	
Wedemeyer et al. [60]	core	35–44 (YLLPRRGPRL, HLA-A2); 132–140 (DLMGYIPLV, HLA-A2)	cytotoxic and IFN-γ secreting epitopes identified in chronic & recovered individuals
	NS3	1073–1081 (CVNGVCWTV, HLA-A2); 1406–1415 (KLVALGINAV, HLA-A2)	
Urbani et al. [62]	NS3	1073–1081 (CVNGVCWTV, HLA-A2); 1406–1415 (KLVALGINAV, HLA-A2)	Differential expression of CD127 on IFN-γ secreting $CD8^+$ T cells
CD4 epitopes			
Day et al. [67]	NS3	1248–1262 (GYKVLVLNPSVAATL, HLA-DRB1*0401); 1579–1597 (SGENLPYLVAYQATVCARA, HLA-DRB1*0401)	$CCR7^+CD45RA^-CD27^+$ tetramer-positive T cells identified in recovered individuals
	NS4	1770–1790 (SGIQYLAGLSTLPGNPAIASL, HLA-DRB1*0401)	

Table 2: Continued.

Ref.	Antigen	Epitopes (aa sequence, restriction molecules)	Functional properties
CD4 epitopes			
Lasarte et al. [68]	core	99–112 (SPRGSRPSWGPTDP, HLA-DR); 146–159 (GAARALAHGVRVLE, HLA-DR)	Epitopes recognized by IL-2 secreting Th cells in IFN-α treatment responders
Schulze zur Wiesch et al. [66]	NS3	1209–1219 (VFTDNSSPPVV, HLA-DRB3*0201); 1251–1260 (VLVLNPSVAA, HLA-DRB1*0101/0401/1104 & DRB3*0101); 1542–1550 (YMNTPGLPV; HLA-DRB1*0701); 1587–1598 (VAYQATVCARAQ; HLA-DRB1*1001)	Broad specificity to NS3/4/5 proteins identified by proliferation and IFN-γ secretion in recovered individuals
	NS4	1775–1785 (LAGLSTLPGNP, HLA-DRB1*0401/0404/0407/1104); 1913–1922 (VQWMNRLIAF, HLA-DRB1*1104); 1915–1924 (WMNRLIAFAS, HLA-DRB1*1001)	
	NS5	2273–2286 (EILRKSRRFAQALP, HLA-DRB1*1104); 2423–2436 (SYSWTGALVTPCAA; HLA-DRB1*0701); 2577–2588 (ARLIVFPDLGVR, HLA-DRB1*0404/0407); 2944–2954 (YLFNWAVRTKL, HLA-DRB1*1104)	
Antibody epitopes			
Law et al. [72]	E2	396-424/436–447/523–540 (conformational epitope)	Conserved cross-genotype, neutralizing antibody epitope
Meunier et al. [70]	E1	313–327 (ITGHRMAWDMMMNWS)	Conserved cross-genotype, neutralizing antibody epitope
Perotti et al. [71]	E2	412–423/528–535 (conformational epitope)	Conserved cross-genotype, neutralizing antibody epitope
Zhang et al. [43]	E2	412–426 (QLINTNGSWHINSTA)	Conserved cross-genotype, neutralizing antibody epitope
	E2	434–446	Unconserved interfering antibody epitope

Robust $CD4^+$ T cells with broad specificity and function predict a spontaneous recovery in individuals with acute HCV infection. $CD4^+$ *T* helper cells are important in shaping adaptive immune effectors like B cells and $CD8^+$ T cells. Even with the critical role of $CD8^+$ T cells in controlling HCV infection, a broad specificity of $CD8^+$ T cells to HCV could be found in some patients with chronic HCV infection [63]. The difference between spontaneous resolving and chronic persistence seems to lie on the quality of the $CD4^+$ T cell response [62–65]. When studying the specificity of $CD4^+$ T cells in acute HCV infected individuals, individuals with spontaneously resolving HCV infection have $CD4^+$ T cells specific to many different HCV epitopes compared to chronically infected individuals (Table 2) [62, 66, 67]. A similar study was reported in patients responding to IFN-α treatment (Table 2) [68]. When the function of $CD4^+$ T cells was analysed, multifunctional $CD4^+$ T cells with the capacity to secrete IL-2 and IFN-γ seemed to correlate better with HCV clearance during acute HCV infection. In contrast, acute HCV- infected individuals became chronically infected when their specific $CD4^+$ T cells secreted no IL-2 [62, 65]. Therefore, multi-specific $CD4^+$ T cells capable of secreting IL-2 and IFN-γ are critical in the generation of quality $CD8^+$ T cell responses necessary for HCV eradication.

In spite of the general lack of protection with neutralizing antibodies to HCV, cross-genotype neutralizing antibodies seem to render protection against HCV infection. HCV-infected patients can develop anti-HCV antibodies to HCV core, NS3, NS4, and NS5 proteins as measured by a third generation of anti-HCV assays [69]. Neutralizing antibodies to HCV, however, are detected using E1 and E2 expressing HCV pseudotype particles (HCVpp) [35]. Since E1 and E2 proteins are present on the surface of HCV virions and are critical for viral entry into hepatocytes, specific antibodies to certain E1 and E2 regions, mostly IgG isotype, have neutralizing capacity [35, 36, 70]. New findings using human monoclonal antibodies derived from HCV infected patients demonstrated that specific antibodies against certain E1 and E2 epitopes have in vitro cross-genotype neutralizing capacity to HCVpp (Table 2) [70–72]. In addition, when these cross-genotype neutralizing antibodies were given to humanized mice following intravenous HCV challenge, these antibodies could offer passive protection and prevent HCV replication in vivo [72]. Furthermore, a study on intravenous drug users with resolved HCV infection demonstrated the contribution of broad neutralizing antibodies in HCV clearance. When these individuals with resolving infection experienced a secondary HCV infection, the majority of

them (83%) would clear the virus spontaneously compared to 25% of them in primary infection. When the specificity of neutralizing antibodies was analysed, neutralizing antibodies reacting to a broad range of genotypes were found in patients spontaneously recovered instead of those who became chronically infected [73].

Studies on patients responding to IFN-α-based therapy have revealed the importance of innate immunity in HCV clearance. Several genome-wide association studies on chronic HCV-infected patients have identified a strong genetic association of IL28B gene, which encoded for IFN-λ3, on the responsiveness to standard IFN-α and ribavirin therapy. Three different studies analysing patient populations at Australian [74], Japan [75], and United States [56, 76] have demonstrated that the polymorphism at the upstream of IL28B is associated strongly with sustained virological response. Patients with genotype expressing more IL28 mRNA respond better to standard IFN therapy. This genetic variation of IL28B has also been shown to be associated with individuals who were infected by HCV and experienced spontaneously viral clearance [56]. When different geographic populations are compared, C allele (rs 12979860) occurs most often in individuals from Asia, then Europe, and least common from Africa origin. Since 36.4% of non-Africa individuals and only 9.3% of Africa individuals spontaneously clear HCV, it further confirms the association of C allele to HCV clearance [76]. IFN-λ3 together with IFN-λ1 (IL-29) and IFN-λ2 (IL-28B), which act through the receptor complex consisting of IL-28Rα and IL-10Rβ and then signal through JAK/STAT pathway, has very similar anti-viral effects as type I interferon (IFN-α and IFN-β). Although the importance of IFN-λ3 on the immune system to combat HCV infection remains mostly unknown, IFN-λ can inhibit HCV replication in hepatoma cells (Huh-7.5) [77]. In addition, IFN-λ can enhance antiviral activities of IFN-α and vice versa, which suggest the possible mechanism of IL-28B polymorphism in the responsiveness of PEG-IFN and ribavirin therapy [77, 78]. A pilot study on chronic HCV patients received pegylated IFN-λ alone or with ribavirin has showed some promising results in HCV RNA reduction after 4 weekly subcutaneous injections [79]. Overall, these data suggest the benefit of IFN-λ in controlling HCV infection.

4. Vaccine Approach

The development of vaccines against HCV has been hampered greatly by the availability of research tools. Due to the limited tissue tropism and host selection, HCV could be generated in vitro in tissue culture system only very recently. Without tissue culture techniques, there would not have been enough viruses for vaccine antigens and immunological bioassay. The development of HCV pseudotype particles by genetically expressing *E*1&*E*2 in retrovirus vector [80] has successfully filled the gap before the discovery of cell culture HCV (HCVcc) [17]. Moreover, it facilitates the identification of various receptors for HCV entry. Currently, various vaccines are primarily tested in chimpanzees and humans. Through the identification of different receptors for HCV entry, it allows the construction of a humanized mouse model, which expresses HCV entry receptors like occludin [22]. Although it is still early in the development, the availability of small animal models could accelerate the preclinical screening for potential vaccine candidates.

After the diagnostic kits for HCV became available, the implementation of HCV screening during blood transfusions and organ transplants has dramatically decreased the numbers of new cases of HCV infection. However, chronic HCV infection still presents in many individuals. This emphasizes the importance of therapeutic vaccination against HCV infection. One of the challenges dealing with chronic infection is to rescue impaired T cells. Thus, the goals of therapeutic vaccines are to generate broad and multispecific $CD4^+$ *T* cells, to activate cytotoxic $CD8^+$ *T* cells and finally to generate cross-genotype neutralizing antibodies. Due to the variability of HCV, a combination approach including vaccination, anti-viral therapy or immune modulation might be necessary. Many vaccines have been tested by both nonprofit and profit organizations. Most of them are still at preclinical stage with some advanced into phase I or II trials to determine safety and efficacy of the candidate vaccines in a small group of patients. Earlier vaccine approaches aiming to generate neutralizing antibodies against E1 failed to show efficacy in chronic HCV patients in spite of its effect on antibody production. Consequently, the recombinant E1 with alum adjuvanted vaccine has been discontinued after an unsatisfactory outcome in its phase II trial [88]. Therefore, most of HCV vaccines are focused on generating cytotoxic $CD8^+$ *T* cells in addition to antibody responses. Different vaccines have been developed over years including epitope vaccines, vector vaccines, recombinant protein vaccines, and DNA vaccines. A review on the progress and efficacy of vaccines currently in clinical trials is summarized in Table 3.

4.1. Epitope Vaccines. HCV peptide-based vaccines with different adjuvants are among the earliest vaccines aiming to induce Th1 and cytotoxic T cell response in chronic HCV patients. One of these, IC41, contains 5 conservative peptides from core, NS3 and NS4 proteins, which are conserved within HCV genotype 1 and 2, and include 4 known HLA-A2 epitopes and 3 promiscuous $CD4^+$ epitopes. In a randomized, dose escalating phase I trial, 128 HLA-$A2^+$ healthy volunteers received 4 subcutaneous vaccinations every 4 weeks. IC41 adjuvanted with poly-L-arginine was well tolerated by these healthy volunteers [89]. When this vaccine was given to 60 chronic HCV nonresponders, there were 67% of patients with specific T cell proliferation, 33% with specific IFN-γ-secreting $CD4^+$ T cells, and 25% with specific IFN-γ-secreting $CD8^+$ T cells. Three responders with the strongest IFN-γ-secreting T cells had a transient decline in serum HCV RNA (>1 log) [81]. Since the response was not efficient in controlling HCV viral load, IC41 would require further modifications by using more intense regimens and stronger adjuvants or could be incorporated into the combination therapy with PEG-IFN and ribavirin [90]. Other two peptide vaccines composed of peptides derived from conservative region of HCV with ISA51, an

Table 3: Main vaccines in clinical trials for HCV.

Vaccine	Subject	Stage	Outcome	Ref.
Peptides (core, NS3, NS4)/poly-L-arginine (IC41)	60 HLA-A2$^+$ chronic HCV nonresponders	II	67% responding to peptide plus adjuvant treatment versus 17% to peptide alone; 3 patients with transient decline of serum HCV RNA (>1 log)	[81]
Peptide (core)/ emulsified with ISA51	26 chronic HCV patients	I	Well tolerated with no severe toxicity; 15/25 responder; 2/25 with 1 log decline on HCV RNA	[82]
Peptides (NS3)/Virosome	30 healthy volunteers	I	No result released	NCT004 45419
MVA-HCV NS3/NS4/NS5B (TG4040)	15 chronic HCV patients	I	Well tolerated; 6/15 with decline on HCV RNA (0.5–1.4 log)	[83]
HCV gpE1/E2 glycoproteins/MF59	60 healthy volunteers	I	No result released	NCT005 00747
Recombinant yeast transfect with HCV NS3-core fusion protein (GI5005)	Chronic HCV patients	II	Well tolerate and showed better virology response in chronic patients after triple therapy	[84]
HCV core protein/ ISCOMATRIX	30 healthy volunteers	I	Well tolerated with mild local redness; all developed antibody response, 7/8 showed cytokine production & 2/8 showed cytotoxic T cell response in the group with highest antigen dose (50 μg)	[85]
NS3/4A DNA vaccine (ChronVac-C)	12 chronic HCV patients	I/IIa	Safe, immunogenic with transient effect on serum viral load	[86]
Recombinant core protein & core/E1/E2 DNA vaccine (CIGB-230)	15 chronic HCV patients	I	Safe, immunogenic, and stabilized liver function with persistence detection of HCV RNA	[87]

emulsified incomplete Freud adjuvant, were shown to be safe in HCV-infected patients [82, 91]. Preliminarily, 26 patients received subcutaneous injection of a conserved peptide derived from HCV core (C35-44, YLLPRRGPRL) biweekly. 15 of 25 patients showed an increase in peptide-specific CD8$^+$T cell response measured by IFN-γ production and 2 patients demonstrated 1 log decrease in HCV viral load after 12 vaccinations. The clinical efficacy would require further validation in phase II trial [82]. In addition, another phase I trial with virosome-based vaccine containing NS3 peptides derived from HCV is ongoing. It is a single-blinded placebo-controlled randomised trial with 30 healthy volunteers to evaluate dose-dependent safety and vaccine-induced immune response (ClinicalTrials.gov Identifier: NCT00445419). No data from this clinical study have been released at this time. Overall, the response with peptide-based vaccines shows good tolerability but their efficacy remains to be optimised.

4.2. Vector Vaccines. HCV vaccines delivered by attenuated virus vectors could induce effective CD4$^+$ and CD8$^+$ *T* cell responses. Modified Virus of Ankara (MVA), a highly attenuated poxvirus strain, is immunogenic and safe compared to other strains of poxvirus due to the lack of several genes coding for immunomodulatory proteins, such as the soluble receptors for IFN-γ, type I IFN, TNF-α, and CC-chemokines [92]. It has been used in several different vaccine designs, such as HIV, tuberculosis, colorectal cancer, and melanoma [93–96]. Owing to its high immunogenicity and cross-reactivity, individuals immunized with vaccinia virus or MVA-based vaccines have a strong antivector response. However, this preexisting immunity would not affect the induction of immunity against vectored antigens despite lower amount of specific T cells and antibodies to vectored antigens were observed [97]. Vaccines based on MVA vector expressing HCV antigens including NS3, NS4, and NS5B have been shown to induce IFN-γ-secreting CD4$^+$ T cells and specific CD8$^+$ T cells capable of secreting IFN-γ and killing in vitro and in vivo when tested in HLA-A2.1 and HLA-B7.2 transgenic mice [98]. The phase I trial in 15 chronic HCV patients who received 3 weekly injections demonstrated that MVA-HCV (TG4040) was well tolerated, and 6 of 15 patients showed a decline in HCV viral load (0.5–1.4 log) associated with significant increase in IFN-γ-secreting T cells [83]. Currently, a phase II trial has been proposed to treat chronic HCV patients in combinational therapy with PEG-IFN and ribavirin.

4.3. Recombinant Protein Vaccines. Recombinant HCV proteins require a strong Th1 adjuvant in order to generate specific T cell response to HCV. HCV E1/E2 glycoproteins emulsified with MF59, a proprietary oil-in-water emulsion adjuvant, have shown to induce a strong CD4$^+$ *T* cell response with significant production of neutralizing antibodies to E1

and E2 in nonhuman primates [99]. No information has been released from one double-blinded placebo-controlled randomized trial with 60 healthy volunteers to evaluate dose-dependent safety and vaccine-induced immune response (ClinicalTrials.gov Identifier: NCT00500747). The vaccine, GI-5005, designed to treat chronic HCV infection is containing heat-killed yeast cells expressing conserved NS3-core fusion protein. Because of its yeast components, this vaccine can induce robust $CD4^+$ and cytotoxic $CD8^+$ T cell responses. In preclinical studies, vaccinated mice exhibited strong Th1 with IL-2 and IFN-γ production and cytotoxic activity to NS3 and core proteins measured by in vitro killing assay and in vivo tumor challenge experiment. Biweekly repeated administration could effectively improve their specific immune response [100]. With this successful pre-clinical result, phase I trial has also demonstrated its safety and shown to induce immune response in chronic HCV patients [101]. Phase II trial was designed to compare a combined therapy with GI-5005 and PEG-IFN/ribavirin to PEG-IFN/ribavirin alone in chronic HCV patients. At the end of 48-week treatment, patients received GI-5005 and PEG-IFN/ribavirin had 74% of response rate determined by HCV RNA less than 25 IU/mL in contrast to 59% without GI-5005. In addition, clinical tests have suggested a better liver function by ALT normalization after the combined therapy [84]. Another vaccine based on conserved HCV core protein is adjuvanted with ISCOMATRIX, an adjuvant composed of saponin, cholesterol, and phospholipid to form sphere particles with 40 μm in diameter. The first phase I trial in 30 healthy volunteers provided the evidence for its safety and tolerability. As to the efficacy measured by immune response, 8 volunteers who received the highest antigen dose, 50 μg, all showed antibody response to core protein, 7 with cytokine-producing T cells, and 2 with $CD8^+$ T cell responses measurable by intracellular IFN-γ staining [85]. A phase Ib trial is prepared to evaluate its safety and immune response in chronic HCV- infected patients.

4.4. DNA Vaccines. DNA vaccines using naked DNA delivered by electroporation have been designed to treat chronic HCV patients. Due to the heterogeneity of HCV subtypes in most chronically infected patients, a DNA vaccine was designed to include the most conserved region including NS3 and NS4A. Through extensive codon modification, the DNA can be effectively expressed in vivo and elicit a Th1 response and cytotoxic response. These primed $CD8^+$ *T* cells could effectively eliminate NS3/4A expressing hepatocytes and tumor cells in mouse model [102, 103]. This DNA vaccine (ChronVac-c) has been given to 12 patients with chronic HCV infection through intramuscular electroporation. Preliminary results suggested that the vaccine is safe and immunogenic after 4 monthly vaccinations. Two out of three patients received the highest dose of 1500 μg showed a decrease in serum HCV RNA (1.2 & 2.4 log). Moreover, after completing the vaccination, three patients who received standard IFN-α-based therapy had an accelerated clearance in HCV viral load. Therefore, ChronVac-c has been proposed to treat chronic HCV patients in combination with standard IFN-α-based therapy [86]. Another DNA vaccine currently in phase I clinical trial is CIGB-230, a mixture of recombinant HCV core protein and core/E1/E2-expressing plasmid DNA. Vaccination in 15 chronic HCV patients showed that this vaccine is safe, partially immunogenic, and able to stabilize liver histology despite persistent detection of HCV RNA [87].

The lessons from studying protective immunity against acute HCV infection have taught us the importance of multifunctional $CD4^+$ T cells toward a broad spectrum of viral epitopes. With the help of these $CD4^+$ T cells, the body can then generate functional cytotoxic T cells to eliminate virus-infected hepatocytes and produce neutralizing antibodies to prevent HCV from entering into uninfected cells [62–64]. The prototype of ideal vaccine would have to meet these requirements. HCV E1/E2 glycoprotein emulsified with MF59 can induce a strong $CD4^+$ T cell response and neutralizing antibody to E1 and E2 [99]. Vaccination with defective alphaviral particles with RNA encoding for HIV gag has shown to generate a strong gag-specific $CD8^+$T cell response [104]. Through different adjuvant and antigen combinations, Lin et al. [105] have formulated a prime-boost regimen. Mice were first primed with E1/E2 glycoprotein, CpG, and MF59 to induce robust Th1 response, and followed by the boost vaccination with defective alphaviral particles with RNA encoding for HCV E1/E2/NS3-5 to generate a strong cytotoxic $CD8^+$ T cell response. With this protocol, mice also generated neutralizing antibodies to E1/E2 capable of neutralizing heterogeneous HCV isolates. Although this vaccine approach remains to be evaluated in other pre-clinical trials, this would set a prototype for the next generation of HCV vaccines.

5. Conclusion

Various immunological parameters favoring HCV clearance have gradually been identified. Together with the knowledge on the strategies deployed by HCV, we now have a good picture on the war against chronic HCV infection. The issue would lie on how to use the information in various vaccine platforms the scientists worldwide have built for years. In addition to vaccine development, efforts on developing anti-viral drugs are underway. Several targets for drug development have been proposed including NS3-4A serine protease, NS5B RdRp, HCV 5′-NCR, HCV viral entry and fusion, and p7 ion channel. Among them, NS3-4A serine protease inhibitor has gone into clinical trial with edges on blocking viral replication and enhancing viral recognition by innate immunity. Since the nature of HCV in chronic infected patients is changeable, we would have to modify our strategy accordingly. A combination therapy including vaccination, anti-viral therapy like NS3-4A protease inhibitor, and immune modulation like IFN-α or IFN-λ would need to be tailored to meet individual requirements. With the help of NS3-4A protease inhibitor, antigen-presenting cells, especially DCs and Kupffer cells, and infected hepatocytes can now sense HCV infection by TLR3 and RIG-I pathways, which consequently activates innate and adaptive immune responses. Since the immune response to HCV is skewed in chronic HCV patients, it can be

redirected toward Th1 and cytotoxic T cell responses through the work of vaccines and immune modulators. Hence, the availability of multiple vaccines and treatment options is critical in treating chronic HCV patients.

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Review Article

Tumor Microenvironment and Immune Effects of Antineoplastic Therapy in Lymphoproliferative Syndromes

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Lymphomas represent a wide group of heterogenic diseases with different biological and clinical behavior. The underlying microenvironment-specific composition seems to play an essential role in this scenario, harboring the ability to develop successful immune responses or, on the contrary, leading to immune evasion and even promotion of tumor growth. Depending on surrounding lymphoid infiltrates, lymphomas may have different prognosis. Moreover, recent evidences have emerged that confer a significant impact of main lymphoma's treatment over microenvironment, with clinical consequences. In this review, we summarize these concepts from a pathological and clinical perspective. Also, the state of the art of lymphoma's anti-idiotype vaccine development is revised, highlighting the situations where this strategy has proven to be successful and eventual clues to obtain better results in the future.

1. Introduction

Tumors are in permanent state of chronic inflammation, and lymphoproliferative syndromes are not only a collection of tumoral cells or a simple genetic disease. Tumoral cells may give and receive instructions from other structural components, the tumor microenvironment, which is composed by extracellular matrix, stromal cells, neoangiogenic vessels and overall, the cells and cytokines that constitute the tumoral immune response. These elements constitute a complex signalling network where a delicate balance exists between microenvironment and tumoral cells. The products of mutated or deregulated genes contribute to the growth and invasion of tumoral cells, as well as to the expression of proteins with the ability to stimulate the immune response. The immunogenic capacity of the tumor can be evaluated by means of the study of the reactive infiltration, which is mainly composed by innate immune cells, especially macrophages, also granulocytes, eosinophils and mast cells, and adaptive immune cells, especially cytotoxic T cells—CTLs-, the most important suppressor of tumoral growth and target for vaccine approaches.

The hypothesis of immunesurveillance postulates that one of the principal functions of the immune system would be recognizing neoplastic cells and eliminating them before they form tumors [1]. This affirmation implies that in the absence of an effective immune system there is a high risk of developing cancer. Truly, there is evidence involving the immune system in the protection from certain tumors, especially those associated with viral infections, tumors related with elderly, transplanted and immunosuppressed patients, and those lymphomas associated with Epstein-Barr virus (EBV), Kaposi's sarcoma, and human immunodeficiency virus (HIV).

Immune system is a nonlinear complex system, and its main function in cancer is acting as an effective suppressive

system of tumors. However, as it occurs in the immunology of infectious diseases, an adequate immune response with enough magnitude to eradicate the microorganism or harmful pathogen is necessary. Nevertheless, the system can behave in an ineffective manner, as the appearance of tumors in immunocompetent population shows. So, along with the concept of immunosurveillance as immune defensive process, the concept of immunostimulation also arises [2], meaning that the immune response might not only be ineffective but it might contribute actively to tumoral progression. Among different molecular distinctive alterations of the lymphoproliferative syndromes, the role of the microenvironment has been studied extensively, and lymphocytes (cytotoxic T cells—CTL- and native killer—NK-), macrophages, dendritic cells, and neutrophils constitute potential effectors of the antitumoral immunity. Nevertheless, in the last few years a huge amount of evidence has emerged suggesting that these cells can also promote the growth and the development of the neoplasia, and that the immune system not only can affect the tumor, but the tumor itself may also alter the host immune system.

The ability of the immune system to act as a double-edge weapon, protective or stimulating, indicates that tumoral clearance requires the effective coordination of the different elements of the immune system in an appropriate balance in quantity and quality. Therefore, current cancer research in lymphoproliferative syndromes and other tumors aims to develop methods to increase the effectiveness of host antitumor immune response. This inevitably leads to consider tumors as more than an accumulation of neoplastic cells; they might be more properly considered as a functional tissue immunologically mediated and formed by a complex tissue network in which neoangiogenesis, infiltrating immune competent cells, stromal cells, and a differentiated and specific extracellular matrix constitute the tumor microenvironment with the capacity of regulating cancer development [3]. The interplay between the host immune system, malignant cells, and all other components of tumoral stroma determine proliferation, invasion, angiogenesis, and remodelling of extracellular matrix and metastasis.

2. Cellular Microenvironment and Hematopoiesis

Lymphoid neoplasms are functionally connected tissues dependent on the microenvironment, determining morphology and tumor classification, clinical behaviour, prognosis, and immune response to the tumor [4]. Perhaps one of the greatest exponents of the maxim that tumors constitute caricatures of normal tissues from which they arise might be the lymphoproliferative syndromes. In physiological conditions, the production of cellular elements corresponding to the immune system is an elaborated process, into which a series of main cells evolve in a sequential way, in a process of differentiation of each of the hematopoietic series. Its shortcomings are implied in the pathogenesis of hematological malignancies [5].

The hematopoietic microenvironment is constituted by a three-dimensional complex and highly organized structure, which serves to regulate the location, proliferation, and function of the hematopoietic cells. This is established by stromal cells, extracellular matrix (ECM), cytokines, and chemokines. Among stromal cells, there are macrophages—derived from hematopoietic stem cells, fibroblasts, adipocytes, and osteoblasts. All of them are derived from mesenchymal stem cells in the bone marrow and from immature myeloid cells in the stromal tumor, along with the endothelia of the neoangiogenic tumor microvessels. The hematopoietic microenvironment not only has great importance in the physiology of hematopoiesis and the physiopathology of some leukaemia, but also in the formation of the intratumoral cell microenvironment, which structure corresponds to the ECM. ECM represents a biophysical filter that offers protection, nutrition, and cell innervation, giving way for immune response, angiogenesis, fibrosis, and tisular regeneration. Its disruption supposes a functional loss for nutrition, elimination, cell denervation, regenerative capacity, and wound healing. This also causes the loss of the immune response to pathogens, tumor cells, and toxins.

Stromal tumor cells derive from progenitors of the bone marrow, which are mobilized across the circulation until joining the tumor microenvironment [6], where they will develop across different cellular lines in endotelia, fibroblasts, histiocytes, and macrophages and finally constitute the tumoral stroma. Tumoral microenvironment is involved in the regulation of tumoral cell growth and the metastatic potential of the tumor, so it is determinant in the response to treatment [7]. The collaboration of one of these cells in particular, the macrophage, turns out to be essential in the process of migration, invasion, and tumor metastasis [8]. It seems that stem cells derived from the bone marrow represent the precursors of metastasis in distant sites, being those in charge to activate a suitable microenvironment, preparing an ideal niche to receive the tumoral cells [9]. Myeloid cells, with CTL suppressive activity, have a special importance because they are recruited by the soluble factors liberated by the proper tumor [10]. There, and in the shape of intratumoral inflammatory monocytes, $CD11b^+$ exert their powerful immunosuppressive action in a multistep process that could be interfered in each single step with the subsequent restoration of immune reactivity.

3. Antitumoral Immunity

Tumor cells can express strange molecules recognized by the immune system and the expression patterns of these antigens vary among different tumors. These tumor antigens can be tumor-specific antigens (TSA), which are exclusively expressed in tumor cells and easily evoke immune responses; characteristic tumor antigens are TSA expressed only in one or a few tumor clones, harbouring peculiar typical mutations of these tumors; tumor-associated antigens (TAA) expressed both in normal and tumoral cells are often unable to induce immune response owed to tolerance mechanisms. Finally viral antigens represent viral strange tumor proteins produced by oncogenic virus [11]. Tumor antigens recognized by T cells (generally $CD8^+$ lymphocytes) represent the

principal target of antitumoral immunity and are presented by MHC class I molecules; that is to say, that tumoral cells behave as antigen presenting cells (APC), presenting their own antigens to T cells. Naturally, professional APC can also present antigens to $CD4^+$ lymphocytes through MHC class II molecules. Tumors can be destroyed by means of specific CTL and an increase in tumoral immunogenicity is accompanied by the specific rejection of the tumor.

Most tumor antigens identified by specific CTL are products of aberrant gene expression and, surprisingly, are not mutated. Products of oncogenes and mutated tumor suppressor genes may also be presented in association with MHC class I and/or class II. Both $CD4^+$ and $CD8^+$ T cells can respond to the products of these genes such as mutated ras gene, p53 and bcr-abl; however, it appears that these responses are poorly protective. Other tumor antigens are encoded by both RNA and DNA viruses that are implicated in tumor development. Tumor cells synthesize and process viral peptide complexes that are presented bound to MHC class I, and therefore stimulate specific T cell responses. These antigens encoded by viruses are not tumor specific but are shared by all tumors induced by the same type of virus. In particular, the protective function of the immune system in controlling virus-induced tumor DNA is given by the high frequency of these tumors in immunosuppressed patients, such as EBV-associated lymphoma.

Effector systems of tumoral immunity are varied and exert different mechanisms of action. The main elements are T cells and especially $CD8^+$ cytotoxic T lymphocytes (CTLs) that destroy tumor cells via the triggering of apoptosis, and providing effective antitumor immunity *in vivo*. They are predominantly CD8 and carry out their function of surveillance by means of the recognition and destruction of potentially malignant cells, which express peptids derived from mutated cellular proteins or oncogenic viral proteins, presented in affiliation to MHC class I molecules. Tumor infiltrating lymphocytes (TIL) are mononuclear cells that infiltrate solid tumors. These include tumor-specific CTL. The $CD4^+$ helper T cells are traditionally considered as noncytotoxic, although new evidence is emerging against this concept [12]. Actually, at least four distinct CD4 T-cells subsets have been described: Th1, Th2, Th17, and Treg cells, each one with a unique cytokine secretion pattern and function [13]. Of course one of their primary roles is providing cytokines for the development of CTL, in addition to being able to secrete TNF and IFN-gamma, which can increase the expression of MHC class I by the tumor cell and therefore increase its sensitivity to CTL lysis. Immunoregulatory cytokines such as IL-10 and TGF-beta play an important role in immune tolerance, and it seems that suppressor effect of T $CD4^+CD25^+$ is independent of cytokines.

Among natural cytotoxic cells, natural killer cells (NK cells) can be activated directly by contact with the tumor or as a result of the stimulus provided by cytokines such as interferons, TNF, IL-2, and IL-12, released by tumor-specific T lymphocytes and macrophages; therefore, their activity is endowed with some degree of specificity. In addition, lymphokine-activated killer cells (LAK) are a group of NK cells derived from peripheral blood cells or TIL in patients with high concentrations of IL-2 and show a high capacity, nonspecific in this case, to lyse tumor cells.

Macrophages are cellular mediators capable of lysing tumor cells by releasing a large amount of lysosomal enzymes and reactive oxygen metabolites. Once activated they also produce cytokines such as tumor necrosis factor (TNF) that exerts its cytotoxic activity triggering apoptosis in a similar way to that mediated by Fas; it has indirect effects on tumor vasculature and vascular thrombosis and produces free radicals from which normal cells are protected by the secretion of superoxide dismutase, but not tumoral cells. Dendritic cells (DC) and other antigen presenting cells (APC) are dispersed between tissues as sentinels or alarm systems ready to detect the presence of foreign antigens. While in the tumor microenvironment IL-12 production tends to be suppressed, resulting in a decrease in Th1 activity, DCs represent probably the most important regulators of naïve T cells, with a great capacity to produce and release IL-12. In their process of polarization, DCs are under the influence of inflammatory mediators such as prostaglandins produced by macrophages, fibroblasts, and tumor cells. A new route of junction between innate and adaptive immunity through the interaction between DC and NK cells has been suggested [14]. Finally, it is believed that antibodies are less important than the T cells to mediate antitumor immune response. However, there are Ab responses specifically against viral Ag, as in patients with EBV-associated lymphomas.

The tumor microenvironment consists of a specific mixture of immune cells that express a distinctive profile for each tumor type [15], from which the efficacy of the immune response against the tumor is eventually derived. Especially polynuclear neutrophils, dendritic cells, macrophages, NK cells, and mast cells play an important functional role in preneoplastic and tumoral tissues. Differences in gene expression profiles of malignant cells in lymphoproliferative syndromes do not always determine the aggressiveness of the lymphoma, while recent contributions determine the increasing importance role of cellular microenvironment in prognosis and disease progression. Each phase of tumor development progresses according to specific signals. So, while the activation of the immune response in advanced stages may be beneficial to the host, its activation during early stages can stimulate tumor development. Although lymphoid cells infiltrating the tumors are often considered as cytotoxic to tumor cells, these cells often contribute to the oncogenic process, tumor growth, and dissemination. Specific cells are responsible for activating specific processes within the tumor tissue, as occurs with mast cells and tumor neovascularization. Dendritic cells and macrophages may provide growth factors to malignant cells, sometimes instigated by viral sequences from stromal cells and not tumor cells themselves. The same cell in different microenvironments can act differently, as befits its power of dialogue and dynamic response to stimuli from the stromal environment.

4. Immune Response in Lymphoproliferative Syndromes

Lymphoproliferative syndromes are mainly distinguished by specific clinical factors and characteristic molecular alterations of low- and high-growth fraction lymphomas. Lymphomas with smaller fraction of growth include follicular lymphoma (FL), marginal zone lymphoma (MZLs), mantle cell lymphoma (MCL), and chronic lymphocytic leukaemia B (B-CLL), which as a group share a paradoxical combination of advanced clinical stages associated with a low clinical aggressiveness. By contrast, lymphomas with high-growth fraction, including diffuse large B cell lymphoma (DLBCL) and Burkitt lymphoma (BL), are frequently associated with clinically localized stage but a high clinical aggressiveness. Each of these groups appears to accumulate specific molecular proliferative and apoptotic changes [16], but differences in gene expression profiles of malignant cells do not always determine the aggressiveness of the lymphoma.

A wide repertoire of specific cell subpopulations constitutes the tumor microenvironment of each lymphoproliferative syndrome [17], with important diagnostic, prognostic, and therapeutic implications [4] (Table 1). The nature, role, and specificity of effector cells that are capable of inhibiting the growth of T and B-cell lymphomas *in vitro* and *in vivo* in immunocompetent individuals have been studied extensively. The number and especially the activation status of infiltrating cells appear to be independent of the degree of malignancy in Hodgkin's lymphoma and various B and T cell non-Hodgkin's lymphomas [18]. The reactive microenvironment determines not only histological morphology and immune phenotype, but also the clinical outcome of lymphoproliferative syndromes. Some of them, such as HL, slow growing tumor as FL, fast growing tumor as DLBCL or T cell lymphomas, are going to be revised briefly in the next paragraphs (Table 1).

4.1. Hodgkin Lymphoma. As if tumoral entities were sculpted by the immune system, the presence of a characteristic inflammatory background not only distinguishes Hodgkin Lymphoma (HL) from other lymphomas, but even more, this is the main characteristic that makes HL a separate entity itself allowing its diagnosis. Tumor cells of HL-PLN and the TCRBCL are the same or very similar, nevertheless we classify these two diseases differently depending on the accompanying tumor microenvironment. Thus, HL and other germinal centre-derived lymphomas can be differentiated through their cellular microenvironment [19].

Regardless of the classic clinical and pathological features, some studies have shown that the presence of activated CTLs (granzyme B^+) is associated with an unfavourable follow up of HL patients [20]. There is a predominance of activated $CD4^+$ T cells in the background of the tumor [21] and a high number of cytotoxic T lymphocytes [22] around the Hodgkin/Reed Sternberg cells (H/RS). $CD4^+$ T cells produce Th2 cytokines that could contribute to local suppression of the cellular immune response mediated by Th1. However, the categorization of $CD4^+$ T cells in Th1 and/or Th2 is an oversimplification [23] as regulatory T cells with $CD4^+CD25^+$ phenotype not only play a regulatory role of autoimmunity, but also have suppressive effects on the development of antigen-reactive lymphocytes associated with the tumor [24]. Functional and molecular characterization of these cells has been facilitated by the identification of markers such as FOXP3, which acts by converting naïve T cells $CD4^+CD25^-$ into the regulatory phenotype $CD4^+CD25^+$ [25]. These regulatory T cells can inhibit the production of IL-2 as well as upregulating the expression of IL-2Ra (CD25), delaying or blocking the activation of $CD8^+$ cells and NK cells against tumor antigens [26]. In HL, the immunosuppressive properties of regulatory T cells appear to be particularly important because of its large effect on cellular cytotoxicity represented by CTLs and NK cells. The presence of low numbers of $FOXP3^+$ cells and a consequent high rate of TIA-1^+ cells in the infiltrate represents an independent prognostic factor negatively affecting the survival of the disease. Furthermore, when the disease relapses and progresses, larger number of TIA-1^+ cells and lower proportion of $FOXP3^+$ on the reactive background of the tumor are also prone to be seen [27]. There is a significant loss of intratumoral $CD4^+$ T cells (an inversion of CD4/CD8 ratio) and a decrease of intratumoral activated CTLs in HIV-infected HL patients [28]. All these data are of interest due to the possibility to significantly expand tumor-induced $CD4^+$ Tregs by the application of therapeutic cancer vaccines [29].

A plausible explanation of the extensive inflammatory infiltrate present in HL could be the secretion of a variety of cytokines produced by both tumor cells and the surrounding stromal tissue. H/RS cells produce and secrete high amounts of chemokines, including TARC and MDC, which attract lymphocytes expressing the CCR4 receptor [30]. In addition, immune cells themselves can produce cytokines responsible for proliferation and tumor survival.

Within the complexity of the interactions between the inflammatory reaction and HL tumor cells, immune cells present in the infiltrate can modulate apoptosis and proliferation of tumor cells [31]. The antiapoptotic profile observed in H/RS cells is associated with a general increase in infiltrating $CD4^+$ T cells and a general decrease in infiltrating $CD8^+$ T lymphocytes, NK cells, and dendritic cells. The progression of G1/S tumoral phase and the high rate of proliferation are also strongly associated with higher infiltration of the overall immune response against the tumor [32]. These results point to the regulation of proteins involved in apoptosis and proliferation of tumor cells by direct interactions between these cells and the surrounding inflammatory microenvironment. This opens up new approaches for research and treatment of HL through the modulation of host immune response.

4.2. An Example of Low-Grade B-NHL, Follicular Center Cell Lymphoma (FL). FL is recognized as a disease of functional B cells, in which T-cell costimulation is essential in the maintenance and ongoing development of B-cell secondary follicles [33, 34].

Table 1: Type of immune infiltration in Lymphomas and prognosis.

Type of lymphoma	Microenvironment	Prognosis
HL	↑↑ activated CTLs (Granzyme B^+)	Unfavourable
	↑↑ TIA-1^+ cells	
	↓↓ $FOXP3^+$ cells	
FL	Type 1 immune response pattern	Longer survival
	↑↑ T lymphocytes and regulatory T cells ($FOXP3^+$)	Favourable outcome
	Type 2 immune response pattern	Shorter survival
	↑↑ tumor-associated-macrophages (TAM, $CD68^+$) and NK cells ($CD57^+$)	Poor prognosis
DLBCL	↑↑ activated $CD4^+$ T cells, dendritic cells and macrophages	Better prognosis
	Infiltrate greater than 20% of $CD4^+$ cells, including $CD45RO^+$	
	↑↑ $FOXP3^+$	
	Higher expression of Th1 than Th2	
	↓↓ IL-6 (Th2 response) during the first weeks after the therapy	Predict complete remission
	↓↓ TILs-$CD8^+$, ↑↑ activated CTLs	Poor prognosis
T and NK cell	↑↑ monocytes	Poor prognosis
	↓↓ $FOXP3^+$	Unfavorable
ALCL	↑↑ Granzyme B^+	Unfavorable
	↑↑ Granzyme B^+ and lack of expression of ALK	Poor prognosis

Since the FL represents the tumoral counterpart of germinal centre B cells and resembles its follicular architecture, the development of this lymphoma may be closely linked to interactions with cellular components of the microenvironment, including dendritic and T cells inside the follicle. It is said that the relationships between tumoral cells and microenvironment can follow three distinct patterns: a loss of interconnection with the immune response to the tumor, a dysfunctional environment, and a friendly, regulated coexistence of the malignant and immune cells [35]. FL seems a good example of the latter pattern, a disease usually indolent and with a long median survival in which at least 15% or greater may experience spontaneous remission, sometimes after acute viral illness and with rapid responses after vaccine therapy. In addition, tumor microenvironment but not tumor cells could be the fundamental key to choosing the most appropriate chemotherapeutic regimen for these patients [36].

At molecular level, survival of patients with FL appears to correlate with the characteristics of nonmalignant immune cells present in the tumor at diagnosis through two patterns of gene expression [15]. Type 1 immune response pattern is associated with longer survival and includes a complex mixture of T cells and other immune cells, while type 2 pattern is associated with shorter survival and includes genes that encode no markers of innate immune cells, primarily macrophages. The differences in the biology of the host immune response determine the clinical course and prognosis of patients with LF, and not the genetic alterations of the tumor cells themselves.

At cellular level, the relationship between cellular elements of specific and nonspecific cell-mediated immunity implies that FL is an immunologically functional disease in which an interaction between the tumor cells and the functional composition of the microenvironment determines their clinical behaviour [37]. The general mechanisms involved in FL tumor immunity have been principally attributed to $CD4^+$ T helper lymphocytes, $CD8^+$ cytotoxic T-cells (CTLs), NK cells, and macrophages. The presence of modulating $FOXP3^+$ T-cells has also proved to have an important role in the host immune response [38]. Taken as a whole, the results of these studies have highlighted the existence of two principal immune facts in which the presence of T lymphocytes and regulatory T cells is related to a favorable outcome, whereas the presence of tumor-associated macrophages (TAM) and NK cells is more usually associated with a poor prognosis. Dysfunctional immune profiles in the tumor microenvironment of FL seem to be attributed to the state of functionality of regulatory T cells, the presence of a particular subset of $CD57^+$ cells, and the reprogrammed immune cells such as TAMs [37, 38].

The favorable clinical impact of the high number of Tregs in FL may be due to a direct inhibitory effect on neoplastic B-cells [39] and the inhibition of tumor-infiltrating leukocytes that can facilitate tumor progression by secreting various growth factors and proteases. However, in epithelial carcinomas these cells correlated inversely with clinical outcome [40], representing the dominant immune escape mechanism early in the tumor progression but not in late phases [41]. These different behaviors seem to be secondary to different mechanisms of immune response regulation from Tregs in FL and in solid tumors. Tregs have numerous lymphoid targets, including $CD8^+$ T cells, B cells, NK cells, and dendritic cells. When the control of the immune response is misguided, Tregs cells can induce immunosuppressive mechanisms through the attenuation

of tumor-specific $CD8^+$ T-cell killing and restricted NKT cells.

In addition to $FOXP3^+$ Tregs cells, $CD57^+$ cells appear to represent another marker of general immune dysfunction in FL [42]. Unlike T cells and macrophages, a higher infiltration of $CD57^+$ cells appears to be related to unfavorable clinicobiological factors in FL patients [38]. CD57 is expressed on NK cells, one of the major effector cells in cellular cytotoxicity together with CTLs. The nonspecific inflammatory infiltrate ($CD57^+$ cells and $CD68^+$ macrophages) seems to be mainly involved in the control of growth and expansion of tumoral cells whereas the specific immune infiltrate ($CD4^+$ and $CD8^+$ T lymphocytes) seems to be mainly involved in the host immune response against the tumor and the main clinical features. Both systems seem to emerge directly associated with the capacity to disseminate tumoral cells, as shown by the greater infiltration of T lymphocytes observed in low-grade FL with spontaneous regression [43], the relatively low absolute number of T cells observed on transformation [44], and the role of naïve and memory T cells in downregulating tumor proliferation rate [45].

Although in a non-restrictive cohort of FL patients was considered, the presence of $CD68^+$ TAM tended to be associated with an indolent clinical behavior and longer survival [46], $CD68^+$ TAM appeared to be associated with an unfavorable outcome for FL patients [47]. The specific subsets of activated macrophages evaluated by the expression of STAT1 may be considered as prototypic type 2 polarized macrophages reprogrammed to induce *in situ* immune suppression. TAMs seem to have a dual nature that appears to be specific to the tumor type. Depending on the microenvironment, they may either exhibit antitumor cytotoxic activity or facilitated tumor growth and progression while reinforcing the Th2-biased immune response [48]. Polarized M1 and M2 (or alternatively activated) macrophages differ in terms of receptor expression, effector function, and cytokine and chemokines production. Regardless of functional defects or absence of activation, in most of the cases TAM do not exhibit cytotoxic activity and facilitate tumor growth, angiogenesis, and metastasis, that is, Th1 immunosuppressive response. TAM products act in two manners to support tumor progression, on one hand, they support tumor growth angiogenicity and extracellular matrix degradation, and on the other hand, they suppress potential antitumor activities [49].

4.3. An Example of High-Grade B-NHL, Diffuse Large B Cell Lymphoma (DLBCL). Recent molecular studies show that survival of patients with DLBCL is influenced by immune cells, fibrosis, and angiogenesis of tumor microenvironment [50]. Stromal-1 signature genes encode components of the extracellular matrix and antiangiogenic factors, while stromal-2 signature genes encode markers of endothelial cells and key regulators of angiogenesis. This survival model reflects the character of nonmalignant cells in DLBCL, including TAMs and myeloid-derived suppressor cells.

At immunophenotypical level, the component of nonmalignant infiltrate can vary among the different subtypes of DLBCL, with the greatest exponent provided by the T cell/histiocyte rich B-cell lymphoma. The presence of an increased number of activated $CD4^+$ T cells [51] as well as dendritic cells and macrophages seems to predict a better prognosis of DLBCL. The DLBCL negative for gene expression of MHC II have few $CD8^+$ T cells infiltrating the tumor [52] and a high percentage of activated CTLs, both of them representing a powerful adverse prognostic factor [53]. A specific subgroup of patients with DLBCL defined in terms of host response has been identified [54]; in this type of response, an increased expression of NK/T cell, monocyte-macrophages, and dendritic cells (DCs) markers as well as inflammatory mediators can be observed. Moreover, those cases with an infiltrate greater than 20% of $CD4^+$ cells, including $CD45RO^+$, show a trend towards better survival. Although it is possible that the role of the microenvironment as a whole can be dual, depending on the tumor, the patient, and the functional status of the host immune system.

An effective cytotoxic response represented by a dense CTL infiltrate and numerous accompanying reactive cells including a high number of $FoxP3^+$ Treg cells seems to be accompanied by better prognosis in DLBCL [55]. The presence of interdigitating dendritic cells associated with infiltrating T cells is involved in coordinating the immune response. However, tumor cells also seem able to modulate the maturation of dendritic cells so they can remove the ability of these cells to process and present tumor antigens. A higher expression of Th1 than Th2 response has also been observed in patients who achieved complete remission [56], and a significant decrease in IL-6 (Th2 response) during the first weeks after therapy in patients with aggressive NHL seems to predict complete remission [57]. In these patients, a germinal center phenotype ($bcl\text{-}6^+/CD10^+$) is accompanied by a lower level of circulating IL-6 compared to activated phenotypes.

4.4. T and NK Cell Lymphomas. Frequently, in T-NHL, the microenvironment cellularity represents the bulk of the tumor, and the clinicobiological manifestations of disease reflect a deregulated immune response rather than the effect of tumoral cells. In PTCL as well as in AITL, a follicular helper T cell tumor, once again, there is not an association between gene clusters and their histological subtypes [58]. However, cells present in tumor microenvironment promote tumorigenesis and suppress host immunity. T cell lymphomas characteristically present a great number of monocytes that promote survival of malignant cells [59]. Another lymphoma characterized by the significant presence of reactive lymphocytes around tumor cells is anaplastic large cell lymphoma (ALCL), where infiltration of a high percentage of activated cytotoxic cells (Granzyme B^+) is an unfavorable prognostic marker [60], especially when combined with the lack of expression of ALK. The mechanisms by which tumor cells escape the CTL attack have been scarcely investigated, although among the postulated mechanisms, the downregulation of MHC I molecules, the expression of IL-10, the expression of FAS-L in tumor cells, overexpression of Bcl-2, and also the expression of PI9, an inhibitor of proteolytic activity of granzyme B, have been considered [61]. The $FoxP3^+$ Tregs cells

predicted improved clinical outcome in extranodal NK/T lymphomas, whereas a decreased number of these cells are more common in patients with poor performance status [62].

Therapeutic Applications of Immune Response. The immune system appears to be essential for therapeutic success if we consider that it can eliminate definitively residual cancer cells that remain after chemotherapy. In this sense, therapy applied during tumor escape phase can inhibit suppressive mechanisms of tumor-induced tolerance, boost T and/or B cells, or stress tumor cells in such a way that tumor cells become immunogenic and sensitive to lysis [63]. The simple reduction of the tumor mass by chemotherapy or surgical removal may also reduce its immunosuppressive properties, reversing tumor-induced immune tolerance and restoring the antibody- and cell-mediated immune responses [64].

On the relationship between the tumor and the immune system, the immunosuppressive side effects of massive chemotherapy should be reevaluated. A bidirectional interaction between tumor and inflammatory/immune cells is ultimately responsible for orchestrating the immunosuppressive network at the tumor site [65]. The manipulation of one of these partners may consistently influence the other. Looking for new modalities of cancer treatment, the induction of a potent and specific immune system has been described as a logical and reasonable strategy for controlling tumor evolution. The different strategies that have been used to improve immunity against tumors include vaccination to provide antigens to the patients' immune system, providing costimulatory signals on tumor cells, induction of cell death with cytotoxic drugs, sustaining immune effectors with NK, NKT, or DC adjuvant, and improving efficiency of cross-priming [66]. If anticancer immune responses dictate long-term therapeutic success, then local signs of antigen priming (DCs) or NK and T cell responses would correlate with favorable responses.

In conclusion, different entities of lymphoproliferative syndromes, independently whether they are HL or NHL, B or T cell lymphomas, and fast or slow growing tumours, have specific patterns of immune responses associated with their morphologic aspect, their immunophenotype, their clinicobiologic course, outcome and the probable response to the therapeutic drug used in their clinical management. Among the nonspecific immune response, a clear distinction can be made between activated and nonactivated CTL, with different significance in various lymphomas [4]. There is a specific machinery to control the tumor microenvironment, represented fundamentally by Treg cells, FoxP3$^+$, CD57$^+$ T cells, and TAMs. Chemotherapy can inhibit a tumor-promoting immune reaction but may in fact be an example of immunotherapy depressing mast cells and macrophages that secrete factors with the ability of promoting tumor growth. In the search of new modalities of lymphoma treatment, the induction of a specific immune response through effective immunotherapy appears to be a promising route of help for these patients and their clinicians.

5. Immune Effects of Antineoplastic Therapy in Lymphoproliferative Syndromes

We will discuss new evidence about immune microenvironment changes after antineoplastic treatment in lymphomas. Lymphomas represent a wide group of heterogenic diseases with different clinical behaviours, and nowadays, the three main options in lymphoma's armamentarium remain to be chemotherapy, radiotherapy, and passive immunotherapy (monoclonal antibodies). All these treatments have a relevant impact on the surrounding stroma and microenvironment.

5.1. Chemotherapy—The Anthracyclines Model. Chemotherapy remains the treatment modality of choice for most lymphomas, especially in advanced stages. Different cytotoxic drugs destroy tumor cells by apoptosis, a process mediated by the activation of caspases and exposure of phosphatidylserine residues in the outer leaflet of the cell [67]. Apoptosis destroys billions of cells in an adult lifetime as a consequence of physiologic tissue renewal and cell turnover without leading to any adverse inflammatory or autoimmune phenomena. Thus, programmed cell death has been traditionally considered as immunologically "bland" or nonimmunogenic. However, this theoretical assumption has not been confirmed in basic and translational research. Rather, it seems that apoptosis is a heterogeneous process, that under some circumstances may lead to immunogenic effects. Recent studies focus on apoptosis and tumor suppressor pathways in cancer and suggest that some chemotherapeutics may induce tumoral destruction which improves cancer cell recognition by the immune system [68, 69].

Anthracyclines remain one of the drugs of choice against lymphoproliferative diseases for Hodgkin's and Non-Hodgkin's Lymphomas and is included in most of the first line chemotherapy schedules. There is now clear evidence that anthracyclines may promote apoptosis in cancer cells with immunogenic effects through several mechanisms (Figure 1).

(1) Calreticulin. Anthracyclines facilitate the translocation of intracytoplasmic protein calreticulin (CRT) to the cell surface, inducing the apoptotic cell antigen presentation to Antigen Presenting Cells (APCs), in particular dendritic cells (DC), and stimulating specific antitumor T cell responses [70].

(2) High-Mobility Group Box 1 (HMGB1). Another immunogenic determinant of cell death is the pro-inflammatory factor HMGB1. HMGB1 is a nuclear protein that is released after necrotic cell death and, as recently reported, from dying cells during late stage apoptosis. After cell death induced by anthracyclines and alkylating agents, HMGB1 may be released in the stroma and act as a neo-antigen representing an immunogenic endogenous "danger signal", initiating an inflammatory response through binding Toll-Like Receptor

4 (TLR4) on DC (but not other DC receptors, such as RAGE or TLR2) [71]. MyD88 is one downstream effector of TLR4, and nowadays there is clear evidence which supports that immunogenicity triggered by anthracyclines is exclusively dependent on an intact TLR4-MyD88 signalling pathway [72].

(3) DNA Damaging Agents. Alkylating chemotherapeutic agents, such as cyclophosphamide, induce the expression of NKG2D ligands. NKG2D acts as an activating receptor on NK cells, $\gamma\delta$ T cells, NKT cells, and memory $CD8^+$ T cells, giving raise to the possibility that DNA damage response may induce immune system activation [68, 73].

(4) Secondary Necrosis. Although not specific for anthracyclines, when massive chemotherapy cell destruction occurs, the mechanisms of controlled apoptosis are overwhelmed and a secondary necrosis occurs triggering an inflammatory response mediated by the intracellular inflammation mediators release, as uric acid, heat shock proteins (HSP), and IL-12 [74].

(5) Cross-Presentation. Cross-presentation is a mechanism favoured by some antineoplastic drugs such as anthracyclines or gemcitabine. These drugs allow tumor antigens to be presented to MHC class I pathway through APCs, a pathway previously thought to be restricted to class II pathway. This mechanism allows tumor antigen presentation to both CD4 T and CD8 T cells which will subsequently identify and destroy the remaining tumour cells [75], but paradoxically apoptotic cells lead to secretion of VEGF that promote the proliferation of endothelial cells and other survival factors that stimulate extracellular matrix with many other implications.

(6) Eradication of Tumor Cells by Chemotherapy. Immune-inhibitory molecules released by tumor cells, such as interleukin-10 (IL-10) or tumor growth factor-β (TGF-β) which inhibit T cell activation, or IL-10, IL-6, and vascular endothelial growth factor (VEGF), involved in the maturation and differentiation of dendritic cells (DCs), are downregulated as a result of the use of chemotherapy after an effective cell destruction [76].

Therefore, emerging evidence led Lake and Robinson to announce a paradigm shift in the way of understanding the effects of chemotherapy on the surrounding stroma [76, 77]. CT can induce a highly potent immune response by increasing antigen (neoantigens) threshold and presentation (via APCs), with enhancement of T-cell response and generation of memory T cells. Other chemotherapeutics like cyclophosphamide, etoposide, and taxanes (docetaxel and paclitaxel) have also proved to have an immunogenic effect in preclinical models [78, 79]; however, evidence is scarce and further investigation is required. These new concepts may serve to consider chemotherapeutics like anthracyclines as less empirical and more specific drugs, and then customizing treatments taking into account its potential effects on microenvironment.

5.1.1. Dendritic Cells and GM-CSF. Among different molecules and cells activated as a result of immunogenic cancer cell death mediated by chemotherapy, antigen presenting cells (APCs), in particular dendritic cells (DC), seem to play an essential role. This is particularly true because tumor reactive T cells are often anergic because of inappropriate antigen exposure or owed to self-recognition. On the contrary, immunogenic tumor cell death mediated by some chemotherapies is characterized by a temporal sequence of events including early translocation of calreticulin to the cell surface, and thereafter interaction of CRT with multiple receptors on DC with apoptotic bodies phagocytosis, release and exposure of heat shock proteins, and late release of HMGB1 [68]. HMGB1 is able to bind to the TLR4 receptor on DC, which allows tumor derived antigens to be processed and presented along with MHC and costimulatory molecules on the surface of DC [68, 69]. These mechanisms altogether serve to trigger DC-mediated specific antitumor response, which may be enhanced by the use of costimulatory molecules. Costimulatory molecules provide additional or second signals for lymphocyte activation beyond those provided through the antigen receptor.

GM-CSF is one of the most important cytokines in cancer microenvironment [80]. GM-CSF has pleiotropic properties, including the mobilisation, differentiation, and function of dendritic cells, possibly by reversing the host's immune tolerance to its own tumor associated antigens, and by initiating (priming) immune responses for which immunologic memory has not been established, that is, activating the so-called naïve T cells. The proliferation of naïve lymphocytes during the first encounter with an antigen (primary immune response) generates both effector T and B cells and memory T and B cells. Memory cells enable a quantitatively and qualitatively superior secondary immune response to be mounted after a subsequent encounter with the same antigen [81].

GM-CSF has been studied in the clinical setting in relapsed follicular lymphoma, along with Rituximab, showing promising results. In a phase 2 trial, combination of rituximab with GM-CSF attained 70% overall response rate (ORR) and 39% complete response rate (CRR), which compares favourably with rituximab as single therapy against relapsed follicular lymphoma (CRR of 6%) [82]. This synergism can be explained, at least in part, by the antibody-dependent cellular cytotoxicity (ADCC) of Rituximab, which is enhanced by GM-CSF [83]. Interestingly, a molecular substudy was executed, demonstrating that this immunotherapy schedule increases the counts of numerous immune cells, particularly monocytes and granulocytes. However, there were no differences between CR and non-CR patients in the mean and ratio pre- or postimmunotherapy counts [82]. Some groups argue that maybe a better way of analyzing the impact of these therapeutic approaches would be determining the magnitude of accumulation of the effector cells at tumor sites, and not only blood levels, with semiquantitative measures through radiolabeling autologous granulocytes or mononuclear white blood cells with indium-111 labeled oxine [84]. The latter would be a highly interesting approach

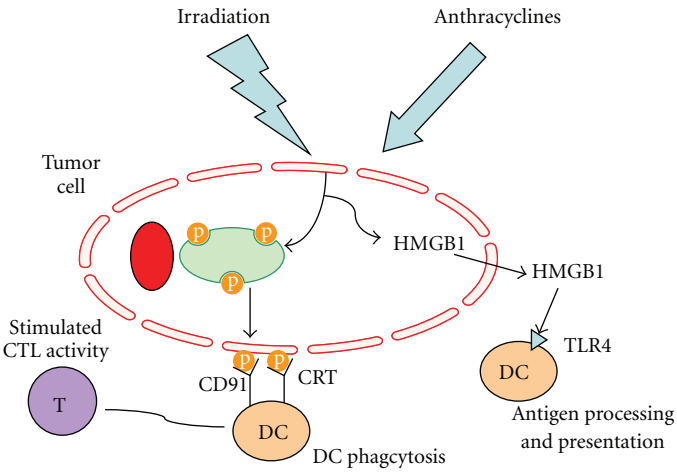

Figure 1: Immunogenic effects of anthracyclines and radiation. CRT—Calreticulin; DC—Dendritic cell; CTL—Cytotoxic T lymphocytes; TLR4—Toll-like receptor 4; HMGB1—High-mobility group box 1.

to monitor immune response *in vivo*, especially if it correlates with clinical efficacy.

5.2. Radiotherapy. Radiation therapy has been the backbone of lymphoma's treatment during decades. Though indications have diminished in the last years, radiotherapy still remains the treatment of choice for curative purposes in localized low-grade non-Hodgkin's lymphomas and Hodgkin's lymphoma, and even in high grade non-Hodgkin's lymphomas as a consolidation treatment after CT in sites of initial bulky disease.

Intrinsic radiosensitivity of malignant lymphocytes is extremely high; however, the underlying mechanisms which explain it are not fully elucidated. Recently, new evidence is emerging about some changes induced by radiation at a molecular level, which may provoke a type of cell death highly immunogenic [85]. Outside the field of treatment, radiation therapy can activate cells of the immune system to produce proinflammatory mediators of genomic instability [86]. Curiously, outcome of the inflammatory response triggered by radiation can be beneficial or detrimental depending upon the context, which is related with the type of macrophages activated (M1-proinflammatory or M2-woundhealing) [87]. Moreover, ionising radiation has different immune effects regarding the dose administered, so in the case of low doses, the final effect is mostly protumorigenic [88]. On the contrary, at higher doses with cytotoxic activity, cell death may induce tumoral neoantigens which can be embraced by dendritic cells, and thus activate an effective adaptive immune response [89]. As with anthracyclines, the two critical mediators of this process seem to be translocation of calreticulin to the cell surface and release of HMGB1 by the dying cells [85] (Figure 1). Both of them trigger danger signals which activate immune mechanisms. In addition, surviving cancer cells after radiation show increased expression of death receptors, adhesion molecules (ICAM-1), and major histocompatibility complex class I (MHC-I), which activate APCs [90, 91]. Once APCs are activated, essentially dendritic cells (DCs), they migrate to the tumor-draining lymph nodes, where naïve T cells can be activated through interaction with tumor-derived antigens presented by DCs. Preclinical studies have also revealed that irradiation of the tumour site may induce release of chemotactic cytokines, that regulate the transit of leukocytes, especially effector T cells, from blood into tumors [92].

Sequence of immune events generated by radiotherapy is critically important, since radiation of loco-regional lymph nodes, which is a common procedure in daily practice, may alter and disrupt the possibility of an effective immune response by depleting naïve T cells.

Immunogenicity of radiation therapy opens a new window of clinical research. Theoretically, molecules like anti-CTLA4 monoclonal antibodies, or costimulators such as GM-CSF, Interferons, or IL-2, may serve as boosters, amplifying immune effectors triggered by radiotherapy. So, if these new concepts are finally confirmed in the clinical setting, it will probably translate into a new way of administering radiotherapy in the coming future.

5.3. Passive Immunotherapy: The Rituximab Era. More than any other discovery, widespread use of monoclonal antibodies (mAbs) in daily practice has dramatically improved clinical results in terms of disease-free survival and overall survival in many types of lymphomas. This is especially true for rituximab, a chimeric monoclonal antibody targeting the CD20 antigen found on both normal B cells and on most low-grade and some high grade B-cell lymphomas [93]. It is effective as a single agent in induction and maintenance therapy, though it is primarily used in combination with standard chemotherapies in the treatment of patients with non-Hodgkin's B-cell lymphomas and chronic lymphocytic leukemia [93]. Although its mechanisms of action are not fully elucidated, rituximab can induce killing

of $CD20^+$ cells (95% of malignant B lymphocytes) via multiple mechanisms. Direct effects of rituximab encompass complement-dependent cytotoxicy (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC), which are retained as the major mechanisms of action of rituximab on B-cell lymphomas. The indirect effects include structural changes, B-cell apoptosis, and sensitization of cancer cells to chemotherapy [83, 93].

The complement system can trigger three protease cascades known as the classical, mannose binding lectin (MBL) and alternative pathways. All three pathways converge at the C3 and C5 levels, leading to the formation of a membrane attack complex (MAC) that, if remains open, will directly induce targeted cell lysis by osmotic mechanisms [94]. Specifically, rituximab activates the classical complement cascade by interacting with C1q through its Fc region, exposed after binding with CD20 on the B-cell surface [95], forming MACs and subsequent cytolysis. Along with CDC, rituximab-mediated ADCC is important for the elimination of malignant B lymphocytes. ADCC triggers tumor cell killing through interaction between the Fc region of CD20 binding rituximab and FcγRs. The final effect is releasing of inflammatory and cytotoxic immune modulators, which lead to phagocytosis of targeted cancer cells by monocytes/macrophages and granulocytes/neutrophils, or lysis mediated by NK cells using the granzyme-perforin system [83, 96, 97]. Some cytokines may aid ADCC to enhance cytotoxicity and avoid antibody-targeted tumor resistance to innate immune cells. Again, GM-CSF has demonstrated *in vitro* enhancement of cytotoxicity upon lymphoma cells through upregulation of monocyte FcγRs [98]. Interleukin-2 (IL-2) activates selective immune effector cell responses associated with antitumor activity, since IL-2-activated NK cells strongly enhance activity of rituximab through ADCC in primary B-cell NHL [99]. Moreover, IL-2 acts as a chemokine, inducing activation and traffic of monocytes and NK cells to tumors. Other cytokines as IL-12 also synergize the rituximab effect by upregulating γ-interferon and other immune mediators, increasing NK cell lytic activity *in vitro* [100].

CDC-resistant cells may be sensitive to ADCC, and the same occurs with ADCC-resistant cells, that can be destroyed by CDC activation [83]. Nowadays, it is widely accepted that ADCC and CDC, the main mechanisms of action of rituximab against lymphoid cells, act synergistically by enhancing cytotoxicity in cancer cells through the ability of complement to promote inflammation and induce activation of innate immune effectors.

Besides the pure immunogenic effects of rituximab, other cytotoxic effects have been studied, in particular apoptosis induction and direct growth arrest. CD-20-rituximab crosstalk can redistribute lipid grafts of the cytoplasmic membrane and subsequently transactivate the Src family tyrosine kinase and the Fas-pathway, which results in initiation of downstream signaling pathways leading to a caspase-dependent apoptosis [18, 35, 101]. Moreover, rituximab downregulates the p38 mitogen-activated protein kinase (MAPK), nuclear factor (NF)-$\kappa\beta$, ERK-1/2, and Akt survival pathways, thus inhibiting the expression of antiapoptotic gene products (Bcl-2/Bcl-xL, and others) [102, 103]. Rituximab also induces caspase-independent apoptosis, through mechanisms still under investigation [104].

Inhibition of antiapoptosis related pathways sensitizes B-cell NHL to undergo apoptosis and facilitates the proapoptotic effect induced by chemotherapy [105, 106]. The combination of rituximab and CHOP chemotherapy (cyclophosphamide, doxorubicin, vincristine, and prednisone) is proving a highly effective combination in the treatment of NHL [93], with better clinical results of each treatment modality alone. It seems that synergism of this chemoimmunotherapy schedule relies, at least in part, in chemosensitization of drug-resistant NHL cells mediated by rituximab via selective downregulation of antiapoptotic factors through the type II mitochondrial apoptotic pathway [105, 106]. Moreover, new concepts about immunogenic apoptosis induced by chemotherapy may also contribute to explain the success of chemoimmunotherapy combinations.

6. Vaccines in Lymphoproliferative Diseases

Concept of vaccination is based on the fact that deliberate exposure to a harmless version of a pathogen generates memory cells, but not the pathologic sequelae of the harmful agent itself. In this way, the immune system is primed to mount a secondary immune response with strong and immediate protection against a new encounter with the pathogen in the future [107]. Active immunotherapy has been traditionally considered a promising approach in lymphoproliferative diseases, especially in low-grade lymphomas. In this sense, follicular lymphomas have demonstrated a high sensitivity to passive immunotherapy with Rituximab and Interferons, either alone or combined with chemotherapy [93]. Also, the indolent course of these diseases, with prolonged spontaneous remissions in up to 23% of patients, seems to be ascribed to immune regulation [108]. Finally, survival of patients with FL appears to correlate with gene expression signatures of tumor infiltrating lymphocytes (TILs) [109].

Vaccine strategies targeting LF have largely focused on using the tumor immunoglobulin molecule expressed on the surface of malignant B cells as an antigen. Antibodies can be produced as circulating or stationary molecules. The latter type has a hydrophobic transmembrane sequence that anchors the molecule in the B-cell membrane, where it functions as the B-cell receptor. Antibodies consist of two identical heavy chains and two identical light chains that are held together by disulfide bonds. The variable regions of the heavy and light chains of the tumor immunoglobulin contain unique determinants known as idiotype (Id) that are a collection of antigenic determinants selectively expressed in tumor cells and serve as tumor-specific antigens [110, 111]. Thus, idiotype vaccination can potentially induce effective polyclonal antibody and T-cell responses against malignant clonal B cells.

Induction of clinically relevant tumour-specific immunity was less frequent in animals with sizable tumour

burden [112]. Therefore, probably the best clinical setting for optimizing immunogenicity and achieving meaningful clinical results comes after a complete response after antineoplastic treatment. This is consistent with the way vaccines work in infectious diseases, without the harmful agent present at vaccination. Preclinical studies revealed that the tumour-specific Id is a weak self-antigen [110, 111]. To enhance immunogenicity, Id vaccine formulations require conjugation to a strongly immunogenic carrier protein, such as keyhole-limpet hemocyanin (KLH) [110]. In addition, using an immunological adjuvant as GM-CSF facilitates activation and recruitment of mature dendritic cells and induction of tumor-specific $CD8^+$ T cells [111]. Thus, most of the following clinical trials use KLH^+ GM-CSF to overcome immune tolerance.

6.1. Clinical Trials with Idiotype Vaccines. Kwak et al. conducted the first study of Id vaccination in humans [113]. It was a pilot study in which 41 FL patients, in complete response or minimal residual disease after chemotherapy, were immunized with subcutaneous injections of autologous purified tumor-derived immunoglobulin conjugated to KLH along with a standard emulsion adjuvant (Syntex adjuvant formulation 1). Results were successful in terms of biological efficacy, with a demonstrated 41% of specific anti-Id antibody, and clinical efficacy with 17% of cellular proliferative responses [113]. These promising data led the Biological Resources Branch, Development Therapeutics Program of the National Cancer Institute to initiate phase 2 trials to confirm safety, clinical efficacy, and good manufacturing practices (GMP) in order to start an eventual commercialization [114, 115]. Most phase 2 studies confirmed that vaccines were well tolerated and induced humoral and cellular immune responses with some clinical effects (clinical and molecular remissions). Results of phase 1 and 2 trials also suggested that biological and clinical efficacy may induce a clinical benefit, which is the capacity to influence on disease-free and overall survival. Inogés and coworkers showed that patients with FL in second CR after chemotherapy (not containing Rituximab) and being successfully vaccinated in biological terms had longer CR (more than 13 months) than the duration of the same patient's first CR obtained with standard chemotherapy with or without Rituximab [116]. These data, though achieved in a limited number of patients, are critically important because they suggest once again that the best clinical setting to employ vaccination is when there is minimal or no residual disease [116]. Hence, these encouraging results achieved with the Id-KLH^+ GM-CSF led to the initiation of three phase 3 trials to clarify its eventual clinical benefit in FL patients (Table 2).

6.2. Phase 3 Trials of Idiotype Vaccines. (i) First study sponsored by Genitope included FL patients treated with 8 cycles of first line CT with CVP schedule. Patients who achieved a complete or partial response were randomized in a 2 : 1 fashion to seven Id vaccine doses or a control arm with KLH and GM-CSF. Finally 192 patients were vaccinated and 95 received control treatment. Regarding the main endpoint, statistical significance was not found in terms of progression-free survival among both arms. However, it was observed that patients failing to mount an Id-specific humoral response had significantly worse results [117, 118].

(ii) Recently, Freedman et al. communicated the final results of another phase 3 trial sponsored by Favrille's in which patients treated with four doses of Rituximab who entered in CR, PR, or SD were randomized to an Id vaccine group ($n = 174$ patients) and a control group treated with GM-CSF ($n = 175$ patients). This trial not only failed in showing a better disease-free survival for vaccinated patients, but even demonstrated a statistically significant difference in favour of the control group treated with GM-CSF [119].

(iii) In the 2009 American Society of Clinical Oncology (ASCO) Annual Meeting, Schuster et al. presented phase 3 results on vaccine BiovaxID [120]. This trial, sponsored by Biovest, included patients in CR or CR unconfirmed after 6 cycles of CT with PACE or Rituximab plus CHOP. Again, randomization was done in two groups, Id vaccine (experimental) and KLH plus GM-CSF (control). Main objective was disease-free survival. Unfortunately, this study was halted in April 2008 with only 31,2% of patients included, owed to rituximab irruption and dominance in FL guidelines and clinical trials. Of 177 patients included in this trial, 60 relapsed while waiting for their vaccine, so conclusions were drawn from only 117 patients, 76 in the experimental and 41 in the control group. Median survival was statistically significant favouring treatment arm (44,2 versus 30,6 months; $P = .047$) and the main endpoint showed a 13,6-month increase in median disease-free survival for Id vaccine group [120].

6.3. Pitfalls and Clues in Vaccine Development. Though soluble protein idiotypic vaccination has provided proof of principle of biological and clinical efficacy, and even clinical benefit in some small clinical studies in FL, results of the three phase 3 trials mentioned above are disappointing and failed. However, there are many circumstances that may alter final results of these randomized trials.

(i) Two of the trials included patients irrespective of the quality of the response after CT. As suggested in preclinical models, disease's situation at vaccination seems to be crucial. When there is a sizable tumour burden, vaccines are less likely to be effective maybe because, among other mechanisms, remaining malignant cells still have the ability to secrete cytokines to evade immune recognition. Accordingly, it must be underscored that better clinical results have been obtained in clinical trials where a CR was previously achieved [114].

(ii) The Favrille's phase 3 trial employed four doses of Rituximab before vaccination [119]. Nowadays, in daily clinical practice, it is preferred using chemoimmunotherapy schedules at first line in fit patients. Hence, four doses of Rituximab may be considered as a suboptimal schedule with few complete responses. Besides this, Rituximab causes B cell depletion in normal and malignant cells, hence interfering in the initiation of humoral response. Final results of this

Table 2: Phase 3 trials of idiotype vaccines.

Author/Sponsor	Idiotype	Comparison	Pretreatment	Patient status prevaccination	End Point	Results
Levy et al. [118] Genitope	Recombinant	2/1 randomization in first line	8 cycles of CVP	First CR or PR	PFS	P = n.s
Freedman et al. [119] Favrille	Recombinant	2/1 randomization in first line	4 doses of Rituximab	First CR, PR or SD	TTP	P = n.s
Schuster et al. [120] Biovest	From hybridoma	2/1 randomization in first line	6 cycles of PACE or CHOP-R	Firsr CR or CRu	DFS	P = .045

phase III trial suggest poorer results for the experimental arm and probably this is the consequence of an early vaccination, before B-cell counts after rituximab treatment were recovered.

However, there is still scarce evidence in vaccination after rituximab-containing immunochemotherapy schedules. Neelapu et al. [121] communicated data of a pilot trial in 26 patients with mantle cell lymphoma treated with EPOCH-R, followed 12 weeks later with five monthly vaccinations of autologous tumor-derived Id-KLH$^+$GM-CSF. As expected, after chemoimmunotherapy, peripheral blood B cells were completely depleted in all patients. Recovery was detected at 6 months, returning to baseline levels at 12 months. CD4$^+$ T cell counts decreased only slightly after CT and recovered 3 months later, by the start of vaccination. CD8$^+$ T cell counts did not change substantially. Curiously, after rituximab administration, antibody responses against KLH and Id were detected in 74% (17 out of 23) and 30% (7 out 23) of patients, respectively. Humoral responses were delayed and correlated with the recovery of B cells following the administration of rituximab, especially after the fourth or fifth vaccination.

The results of this pilot study are extremely important, because it demonstrates that vaccination after rituximab treatment is feasible and can induce delayed humoral responses. Taking into account that Id vaccine production takes some months, it would be interesting to design clinical trials in which Id-vaccine was administered between 6 and 12 months after a chemoimmunotherapy schedule containing rituximab.

(iii) Control group in the three phase 3 trials used KLH$^+$GM-CSF or GM-CSF. It is uncertain whether these compounds may induce an immune response against lymphoid cells by themselves. In particular, GM-CSF is a cytokine with highly immunogenic properties that has even demonstrated clinical efficacy in the clinical setting in FL, in combination with Rituximab, and in other solid neoplasms [122]. So, it is arguable if KLH$^+$GM-CSF or GM-CSF alone represents an ideal control group with neutral immune effects.

(iv) Follicular lymphoma is such a heterogenic disease with a different and unpredictable evolution. Moreover, host's immune response to vaccines is also heterogeneous in every single patient. Therefore, there are many sources of uncontrollable variability that make idiotypic vaccination in FL such a difficult strategy to reach success in randomized clinical trials, where the methodology remains rigorously dictated by statistics and clinical benefit in the overall population.

(v) As previously mentioned, several mechanisms may explain the low clinical effectiveness reported. One of the main reasons lies in the inability of immune cells to infiltrate and become activated after an encounter with tumor antigen *in vivo*. Moreover, it seems that tumors do not express costimulatory molecules or produce the inflammatory microenvironment necessary to activate effector cells with the ability to eradicate tumors [123, 124]. Therefore, the development of methods to activate antitumor immune cells by stimulating APCs and generate long-term memory cells, probably with the aid of costimulators, is one of the future challenges for definitively integrating tumor vaccines into the antineoplastic arsenal. In this sense, GM-CSF has demonstrated clinical activity when used alone [125] (melanoma) and in combination with other agents (follicular lymphoma, colorectal and breast cancer [82, 126, 127]). Among these protocols, GM-CSF administration is prolonged, ranging from 5 to 14 days, yet in vaccine trials GM-CSF is commonly used in a short course of three or four doses. So, safety and efficacy data encourage the prolonged administration of maintenance boosters (GM-CSF, Interleukin-2, etc.), especially once there is biological evidence of an immune response successfully triggered.

(vi) Regardless of the method used, Id vaccine production is expensive and time consuming. In fact, the NCI/Biovest study loosed more than 30% of patients included because of a relapse while waiting for the vaccine production. This is especially worrying because it predicts serious difficulties in an eventual extensive clinical use. Other sources of vaccination are under development with membrane proteoliposomes, or tumor cell-based vaccines transduced with GM-CSF, CD40-activated or HSP-96 [111]. These new formulations are under clinical investigation, mostly in phase 1 trials and have the advantage of targeting multiple tumor antigens with a shorter production time.

(vii) Therapeutic armamentarium in LF is changing, and knowledge of the immune effects of the new therapies employed may be of critical importance for clinical trials with Id-vaccines. Recently, Yttrium-90 Tositumomab Tiuxetan (Zevalin^R) has been approved by the FDA in first line of LF, as consolidation after CT [128]. Zevalin^R is a CD-20-directed radiotherapeutic antibody with several mechanisms of action. In addition, in the 2009 annual meeting of

the American Society of Hematology (ASH), results of a phase III trial in low-grade lymphomas, comparing CHOP-R and Bendamustine-R, have been communicated, providing better progression-free survival (median PFS 54,9 versus 34,8 months; HR 0.57, $P = .00012$) and a better toxicity profile for the experimental arm [129]. These data could oust the standard CHOP-R regimen in brief, so anthracyclines could be out of first line treatment in the coming future.

Even though clinical results in many types of lymphomas, including LF, that have improved over the years owed to introduction of rituximab and chemoimmunotherapy schedules, there is still room for improvement; yet many patients relapse and finally die as a consequence of their disease. Thus, once confirmed proof of principle of biological and clinical efficacy of vaccine-therapy, these results might not be overlooked nor neglected by physicians, since it may translate into a prolonged disease-free survival, and eventually the recovery of some LF populations. Although history of vaccines in Oncology has been extremely disappointing, reminding the myth of Minotaurus, with every little step forward followed by a new frustration, new insights into this strategy may hopefully obtain better and surprising results, and so finding out the Ariadne's thread which eventually leads to see the end of this complex and challenging labyrinth.

7. Conclusions and Final Remarks

Tumorigenesis is a multistep process leading to the progressive transformation of normal human cells into highly malignant derivatives. Thus, mechanisms of oncological diseases are extremely complex, with several alterations at multiple sites. Hanahan and Weinberg, in an effort to synthesize the huge body of knowledge in cancer research, postulated six essential alterations in cell physiology [130]. Among them, the insensitivity to antigrowth signals, evasion of apoptosis, sustained angiogenesis, and tissue invasion and metastasis seem to be intrinsically related to microenvironment dysregulations. Lymphomas constitute an excellent model for microenvironment translational research. These tumors might be considered as a functional tissue immunologically mediated and formed by a complex tissue network in which the imbalance of homeostasis between the host immune system, malignant cells, and all other components of tumoral stroma determine proliferation, invasion, angiogenesis, and remodelling of extracellular matrix and metastasis. Moreover, the distinctive profile of immune cells in the surrounding stroma leads to a wide repertoire of specific cell subpopulations which constitute the specific tumor microenvironment of each lymphoproliferative syndrome. In the last few years, the critical importance of these findings and correlation with prognosis and clinical results has been recognized.

Recent evidence has emerged that confers new properties to antineoplastic treatments against lymphomas, in relation with microenvironment changes. This is the case of some chemotherapeutics like anthracyclines or radiotherapy that may induce tumoral destruction with the ability of improving cancer cell recognition by the immune system, and thus enhancing the possibility of a successful immune response. In addition, these "new discoveries" in the mechanisms of action of classic antineoplastic treatments might be the basis of the synergism of the new combined chemoimmunotherapy strategies in lymphomas that include passive immunotherapy with the monoclonal antibody Rituximab. Finally, active immunotherapy with anti-idiotype vaccines, though still far from daily practice integration, has demonstrated clinical efficacy in some subpopulations of patients. Fine tune approaches in vaccine development and a better design of vaccine clinical trials are needed to definitely elucidate the role of active immunotherapy in lymphoproliferative syndromes.

Conflict of Interests

Authors declare no conflict of interests.

Acknowledgment

T. Álvaro and L. de la Cruz-Merino have contributed equally to this article.

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Review Article

Immunomodulatory Effects of dsRNA and Its Potential as Vaccine Adjuvant

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dsRNA can be detected by pattern recognition receptors, for example, TLR3, MDA-5, NLRP3 to induce proinflammatory cytokines responsible for innate/adaptive immunity. Recognized by endosomal TLR3 in myeloid DCs (mDCs), dsRNA can activate mDCs into mature antigen presenting cells (mAPCs) which in turn present antigen epitopes with MHC-I molecules to naïve T cells. Coadministration of protein and synthetic dsRNA analogues can elicit an antigen-specific Th1-polarized immune response which stimulates the $CD8^+$ CTL response and possibly dampen Th17 response. Synthetic dsRNA analogues have been tested as vaccine adjuvant against viral infections in animal models. However, a dsRNA receptor, TLR3 can be expressed in tumor cells while other members of TLR family, for example, TLR4 and TLR2 have been shown to promote tumor progression, metastasis, and chemoresistance. Thus, the promising potential of dsRNA analogues as a tumor therapeutic vaccine adjuvant should be evaluated cautiously.

1. Introduction

Pathogens on invading host cells express molecules that are broadly shared by all microbes and distinct from host. These include lipopolysaccharide, peptidoglycan, flagellin and microbial nucleic acids, and collectively are referred to as pathogen-associated molecular patterns (PAMPs) [1–3]. Pattern recognition receptors (PRRs) of the host when recognizing PAMPs trigger a release of inflammatory cytokines and type I interferons (IFNs) [4, 5]. PRRs are evolutionally conserved and have been investigated extensively [4]. From the initial investigation of Toll-like receptor (TLR) family [6] to the recent discoveries of retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) [7, 8] and the nucleotide-binding domain and leucine-rich repeat containing gene family (NLRs, also known as NOD-like receptors) [9, 10], all the evidence point to an important role in host defense. TLRs are membrane bound receptors that sense PAMPs on the cell surface or in endosomes [11] while RLRs and NLRs recognize microbial molecules in the host cytosol [12]. The individual members of these PRRs families are characterized by their ligand specificity, cellular localization and activation of unique downstream signaling pathways. Immunity against different microbial pathogen primarily depends on the recognition of the specific PAMP of the pathogen by the corresponding PRR.

Double-stranded RNA (dsRNA), a virus replication intermediate and a signature of infection, is sensed by TLR3, two members of RLRs family, that is, the RNA helicases RIG-I and melanoma differentiation-associated gene 5 (MDA-5), and the NLR pyrin domain (NLRP) 3 protein of NLR family. These trigger the release of inflammatory cytokines, that is, activating innate immunity which shapes adaptive immune response [11, 13, 14]. The primary cytokines involved in this response are type I IFNs including IFN-α and IFN-β [15].

In this review, the mechanism of innate and adaptive immunity induced by dsRNA and the potential application of dsRNA as a vaccine adjuvant against viral infection and anticancer immunotherapy is elaborated.

2. dsRNA Induced Signaling

2.1. The TLR3-Mediated Signaling. TLRs are type I integral membrane glycoproteins which have a trimodular structure composed of an extracellular domain, a single transmembrane domain and an intracellular Toll/IL-1 receptor (TIR) domain [11, 16, 17]. Twelve members of TLR family have been identified in mammals [11]. The extracellular N-terminal domains of TLRs contain 16–28 leucine-rich repeats (LRRs) [16, 18] in a horse-shoe structure [17]. Each individual LRR is composed of 20–30 amino acids with a conserved characteristic repetitive sequence pattern rich in leucines, the "LxxLxLxxNxL" motif and two or more repeats in tandem, form curved solenoid structures suitable for protein-protein interactions [19]. The extracellular domain of human TLR3 comprises of 23 LRRs in a horse-shoe shaped structure. The convex face of the extracellular domain of TLR3 is glycosylation-free and contains many positively charged residues while the concave face is largely glycosylated and negatively charged [20, 21]. The dsRNA binding site of TLR3 is located in two regions near the N-terminus and C-terminus. When dsRNA interacts, two ectodomains of TLR3 are connected by dsRNA in an "m" shape to form a TLR3-dsRNA 2 : 1 complex [22]. When combined with TLR3, dsRNA spans the whole "m" consisting of two "horse-shoes" of the ectodomains of TLR3 (Figure 1A). This satisfies the minimum requirement of 40–50 base pairs of dsRNA allowing stable binding to TLR3 inducing signaling [16, 23, 24]. With the secondary structure, the transmembrane domain of TLR3 is a single α-helix and the endodomain is composed of a five-stranded β-sheet surrounded by five α-helics that forms the TIR domain [25]. The B-B loop that connects β-strand B with α-helix B in the TIR domain is considered the essential structure that directly interacts with the adaptor protein TIR domain-containing adaptor inducing IFN-β (TRIF) [26]. In addition to the B-B loop, three boxes of conserved residues that reside in TIR domain are involved in TLR3 signaling [25, 27].

TLR3 can be found both intracellularly and on the cell surface in human fibroblasts and epithelial cells. However, it is predominantly located in intracellular vesicles, for example, endosomes, in most cell types including dendritic cells and macrophages [28, 29]. TLR3 is activated by extracellular dsRNA that is released from dsRNA viruses or is produced during single-stranded RNA viruses' replication or comes from application of synthetic dsRNA analogues [4]. It is largely unknown how extracellular dsRNA are delivered to the intracellular vesicles containing TLR3. Studies have suggested that CD14 may play an important role in dsRNA uptake [20, 30]. Once internalized into the endosome, dsRNA binds to its adaptor protein TLR3 and activates several signaling pathways. Upon binding to dsRNA, the B-B loop of the TLR3 TIR domain combines with TIR domain of TRIF activating several transcription factors, including nuclear factor-κB (NF-κB), interferon regulatory factor 3 (IRF3), and activating protein 1 (AP-1) [25].

There are two pathways to activate NF-κB mediated by receptor-interacting protein 1 (RIP1) and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) (Figure 1A). TRAF6 is a ubiquitin ligase and plays a role in RIP1 polyubiquitination [31, 32]. Polyubiquitinated RIP1 is recognized by ubiquitin receptors, the transforming growth factor β-activating kinase (TAK) binding protein (TAB) 2 and 3, which in turn activate TAK1 [33]. IκB kinase-related kinase α (IKKα) and IKKβ are phosphorylated by the activated TAK1. This leads to phosphorylation and degradation of IκB, an inhibitor of NF-κB, and eventually results in the translocation of NF-κB to cell nucleus. This is followed by the activation of specific gene promoter A20. TAK1 also activates the 2 other classes of kinase, JNK and p38 and these activate the family of AP-1 transcription factors [27]. The third signal comes from TRIF activating IRF3 (Figure 1A). TRIF activates the kinase complex TRAF family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1) and IKKε through its adaptor protein, NF-κB activating kinase (NAK)-associated protein 1 (NAP1), leading to the phosphorylation and nuclear translocaton of IRF3 consequently inducing the expression of IFN-β [34]. In addition to NAP1, TRAF3 is also part of the TBK1/IKKε complex that is involved in the TRIF-mediated IRF3 activation [35]. Another signal from TLR3 is related to the phosphorylation of two specific tyrosine residues (Tyr^{759} and Tyr^{858}) in the TLR3 TIR domain when TLR3 interacts with TRIF (Figure 1A). Phosphorylated Tyr^{759} recruits phosphatidylinositol 3-kinase (PI3K) which then activates kinase Akt required for phosphorylation and activation of IRF3 in nucleus [36]. The phosphorylation of Tyr^{759} and also Tyr^{858} results in degradation of IκB leading to NF-κB release and this induces phosphorylation of NF-κB which partially activates it [37]. Tyrosine kinase c-Src also plays a role in Akt activation [38]. The unique signal pathway of TRIF is able to induce mammalian cell apoptosis (Figure 1A). TRIF interacts with Fas-associated cell death domain (FADD) protein through RIP1 which in turn activates procaspase-8 to initiate cell apoptosis [25, 39].

2.2. The RLRs-Mediated Signaling. RLRs are cytoplasmic viral RNA sensors with three recognized members including RIG-I, MDA-5 and laboratory of genetics and physiology 2 (LGP2). All belong to the DExD/H box RNA helicase family [40]. In addition to the central helicase domain, RIG-I and MDA-5 have two caspase recruitment domains (CARDs) at its N-terminus which are responsible for downstream signaling cascade through the interaction with a CARD-containing adapter, mitochondrial antiviral signaling adapter (MAVS, also known as IPS-1, VISA, or Cardif) located in the outer mitochondrial membrane [41]. LGP2, lacking the N-terminal CARD, works as a negative regulator of RLRs signaling. Although only a single CARD physically interacts with MAVS, both CARDs are essential for downstream signaling [42, 43]. The RIG-I activation is self-inhibited by the C-terminal regulatory domain (RD), also referred as the C-terminal domain (CTD) or repressor domain, through intramolecular association between RD and both the CARD and the helicase domains [42–44]. RD is also responsible for the binding affinity to the dsRNA and 5′-triphosphated single-stranded RNA (5′ppp-ssRNA) of viral RNAs with

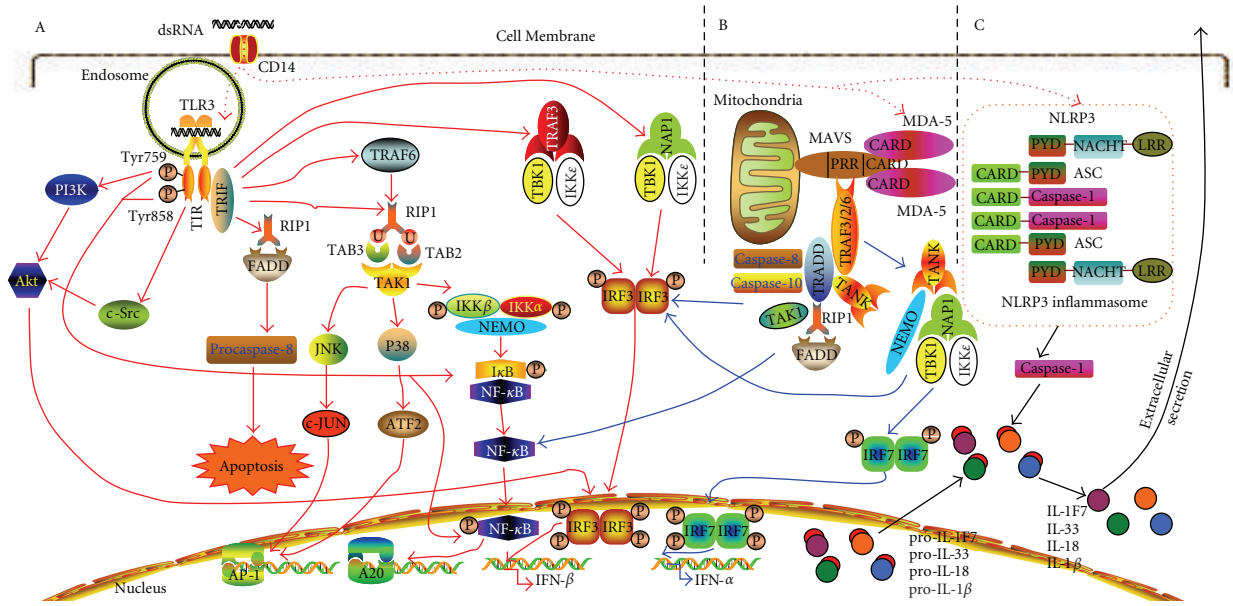

FIGURE 1: dsRNA induced signal pathways. (A) *dsRNA signaling through TLR3*. dsRNA that is internalized into endosome binds to TLR3. The mechanism of dsRNA internalization is unknown, CD14 may play a role. TLR3 possesses two dsRNA binding sites near the N-terminus and C-terminus. When combined with dsRNA, four dsRNA binding sites from two TLR3 molecules that are linked by the sole dsRNA molecule in an "m" shape, and the B-B loop of the TLR3 TIR domain combines with the TIR domain of TRIF. The interaction of TRIF with RIP1 or TRAF6 results in polyubiquitination of RIP1, the latter binds ubiquitin receptors TAB2 and TAB3 which activates TAK1. Activated TAK1 induces phosphorylation of IKKα and IKKβ leading to phosphorylation and degradation of IκB. The release and translocation of NF-κB to cell nucleus then occurs which results in the specific gene promoter A20 being activated. TAK1 also starts an activation sequence beginning with JNK and p38, which activates c-JUN and ATF2 and this activates the AP-1 transcription factors family. TRIF activates TBK1 and IKKε through NAP1 and this results in phosphorylation and nuclear translocaton of IRF3 resulting in IFN-β production. TRAF3 also binds with the TBK1/IKKε complex inducing IRF3 activation. Combination of TRIF results in phosphorylation of Tyr^{759} and Tyr^{858} in the TLR3 TIR domain which subsequently induces the phosphorylation of, and degradation of IκB leading to NF-κB release. Phosphorylated Tyr^{759} recruits PI3K and activates kinase Akt for full phosphorylation and activation of IRF3 in nucleus. Tyrosine kinase c-Src also plays a role in Akt activation. TRIF interacts with FADD through RIP1 and activates procaspase-8 to initiate cell apoptosis. (B) *dsRNA signaling through MDA-5*. dsRNA is recognized by MDA-5 in cytosol. MDA-5 interacts with MAVS located on the outer mitochondrial membrane via CARD-CARD interaction leading to the dimerization of the MAVS N-terminal CARD domains which subsequently binds TRAF3 directly through the interaction between the TRAF domain of TRAF3 and the TRAF-interacting motif in the PRR of MAVS. Then the RING domain of TRAF3 forms a scaffold to assemble the noncanonical IKKs signal complex composed of TANK, TBK1, IKKε, NAP1 and NEMO. This complex activates the signal-dependent phosphorylation of IRF3 and IRF7 to form a functional homodimer or heterodimer which translocate to the nucleus to induce expression of type I IFN. TRAF2 and TRAF6 also associates with MAVS and is involved in activation of P38 MAPK and promotion of IL-12 and type I IFN production or activation of NF-κB, JNK and P38 signaling. In addition, MAVS recruits TRADD and interacts with TRAF3, TANK, FADD and RIP1 to activate both IRF3 and NF-κB signaling. (C) *dsRNA signaling through NLRP3 inflammasome*. NLRP3 recognizes dsRNA by a series of LRRs at the C-terminus. The NACHT domain at the middle of NLRP3 is related to self-oligomerization and the formation of inflammasome. Studies have suggested dsRNA may activate the NLRP3 inflammasome. Activation of NLRP3 results in the interaction of NLRP3 PYD with ASC PYD, and in turn ASC CARD associates with pro-caspase-1 CARD and thereby assembles the NLRP3 inflammasome. Once incorporated into NLRP3 inflammasome, pro-caspase-1 is activated by proteolytic cleavages to remove the CARD prodomain. The active caspase-1 in turn cleaves the IL-1β, IL-18, IL-33 and IL-1F7 precursors into their active forms and these active cytokines are secreted extracellularly.

a common core RNA binding site specifically adapted to distinct and unique patterns [44–47]. It is also responsible for RIG-I dimerization. MDA-5 RD preferentially binds dsRNA with blunt ends but does not associate with dsRNA with either the 5′ or 3′ overhangs [13]. The central helicase domain displays cooperative RNA binding properties [43, 48, 49]. The RD of MDA-5 does not exhibit self-inhibitory activity [42, 50]. MDA-5 is negatively regulated by dihydroxyacetone kinase (DAK) and other possible regulators [51].

RIG-I binds 5′-triphosphate RNA in single- or double-stranded forms [52–54] or short dsRNA of 300–1000 bp without a 5′-triphosphate [54] while MDA-5 recognizes long dsRNA of more than 1000 bp in length and the synthetic dsRNA analogue polyinosinic-polycytidylic acid [poly (I:C)] [54–56]. Upon sensing RNA, both RIG-I and MDA-5 are activated and initiate downstream signaling through the common pathway via adaptor protein MAVS (Figure 1B). MAVS consists of a CARD at the N-terminus, a proline-rich region (PRR) in the middle and a transmembrane domain at the C-terminus attached to the outer surface of mitochondria [57]. Activated RIG-I and MDA-5 associate MAVS via a CARD-CARD interaction which leads to the

dimerization of the MAVS N-terminal CARD domains. Once activated, binding to TRAF3 occurs directly through the interaction between the TRAF domain of TRAF3 and the TRAF-interacting motif (TIM) in the PRR of MAVS [58–60]. Following the association of TRAF3 with MAVS, the RING domain of TRAF3 forms a scaffold to assemble noncanonical IKKs signal complex composed of TANK, TBK1, IKKε, NAP1 and NF-κB essential modulator (NEMO). This complex then activates the signal-dependent phosphorylation of IRF3 and IRF7 to form a functional homodimer or heterodimer and translocates to the nucleus [61, 62]. The association of MAVS and TRAF2 or TRAF6 has also similarly been demonstrated [63]. TRAF6 is essential in the activation of NF-κB, JNK and P38 signaling [64] while TRAF2 is involved in activation of P38 MAPK which promotes IL-12 and type I IFN production [65]. Moreover, TNFR-associated death domain (TRADD) is recruited to MAVS following virus infection and interacts with TRAF3, TANK, FADD and RIP1 as well as activating both IRF3 and NF-κB signaling [66]. Another protein-termed stimulator of interferon genes (STING, also known as MITA or MYPS) expressed either on the outer micochondrial membrane [67] or endoplasmic reticulum [68] directly interacts with RIG-I but not MDA-5. It subsequently recruits TBK1 activating IRF-3. STING has been reported to associate with major histocompatibility complex class II (MHC II) and mediates apoptotic signals via ERK activation [69]. It was recently reported that RIG-I binds to an adaptor apoptosis-associated speck-like protein containing a caspase-activating and recruitment domain (ASC) to trigger caspase-1-dependent inflammasome activation by a mechanism independent of MAVS, CARD9 and NLRP3. This suggests that RIG-I is able to activate the inflammasome in response to certain RNA viruses [70].

2.3. The NLRs-Mediated Signaling via Inflammasome. NLRs are intracellular PRRs sensing PAMPs and danger signals or danger-associated molecular patterns (DAMPs) released by injured cells [71]. The NLR family has 23 members in humans and at least 34 members in mice [72]. NLRs are multidomain proteins with tripartite structure composed of an N-terminal effector region, a central NACHT (Neuronal Apoptosis inhibitory protein, NAIP; Class II transactivator, CIITA; plant Het product involved in vegetative incompatibility, HET-E; Telomerase-associated protein 1, TP-1) domain (also known as NOD domain), and a C-terminal region for PAMPs recognition. The C-terminal region is characterized by a series of LRRs and is implicated in ligand sensing and NLRs autoregulation but the precise mechanism is not clear [71]. The NACHT domain is a member of signal transduction ATPases, part of the P-loop NTPase family [73] and is related to self oligomerization and the formation of inflammasome [71]. The N-terminal region contains several protein interaction modules, such as acidic domain, baculoviral inhibitory repeat (BIR)-like domain, CARD and pyrin domain (PYD). Accordingly, NLRs are further divided into subfamilies as NLRA, NLRB, NLRC and NLRP. An additional subfamily, NLRX, is characterized by the presence of an N-terminal domain with no strong homology to any known domains of other NLR subfamily member [74].

Upon recognition of PAMPs, toxins, or danger signals by NLRs, a large protein complex termed the inflammasome composed of NLRs, ASC and pro-caspase-1 is activated. This protein platform activates pro-caspase-1 into its active form of caspase-1 and this hydrolyzes pro-IL-1β and pro-IL-18 into their mature biologically active forms which are secreted extracellularly to play a role in immune response [14, 71]. Limited studies have suggested dsRNA is an activator of NLRP3 inflammasome [75–78] although this has been disputed [79]. NLRP3 senses PAMPs either from bacteria, such as lipopolysaccharide (LPS), muramyl dipeptide (MDP), bacterial pore-forming toxin and bacterial DNA and RNA, or from viruses such as viral ssRNA or dsRNA, dsRNA analogue poly (I:C). It also senses other compounds such as imidazoquinoline antiviral drugs R837 and R848, nonmicrobial signals encompassing uric acid crystals, calcium pyrophosphate dehydrate (CPPD), asbestos, silica, extracellular ATP, alum adjuvant and fibrillar amyloid-β [14, 71, 72, 80]. It is believed that NLRP3 activation results in the interaction of NLRP3 PYD with ASC PYD which in turn causes ASC CARD to associate with pro-caspase-1 CARD assembling the NLRP3 inflammasome (Figure 1C) [81]. Besides NLRP3, ASC and pro-casepase-1, human NLRP3 inflammasome contains a CARD-containing protein, CARD inhibitor of NF-κB-activating ligands (Cardinal, also known as CARD8). However, there is no homologue of Cardinal in the mouse [81–83]. Allen et al. (2009) utilized small heteroduplex RNA (shRNA) to knockdown the expressions of ASC, NLRP3 and Cardinal respectively in human THP-1 monocyte cell lines. The IL-1β production triggered by lentivirus infection was significantly attenuated by the addition of shRNA of ASC or NLRP3. In contrast, secretion of IL-1β was not affected by knockdowns of Cardinal. Thus, it has been suggested that Cardinal may not affect NLRP3 inflammasome function [77]. Furthermore, Cardinal does not bind to NLRP3 in the inflammasome [84].

The activation of the NLRP3 inflammasome has been demonstrated through a number of mechanisms. However, the direct ligand for NLRP3 has yet to be defined. It has been proposed that the activation of the NLRP3 inflammasome requires both microbial molecules and a second signal such as extracellular ATP or pore-forming molecules [72]. Alternatively, it has also been suggested that reactive oxygen species (ROS) may be the common NLRP3 inflammasome activator since the most striking features associated with NALP3 activators like potassium efflux and the induction of frustrated phagocytosis all leads to ROS production via NADPH [71, 85, 86]. In particular, the activation of NLRP3 inflammasome triggered by virus infection or by poly (I:C) requires sensing viral RNA or poly (I:C), lysosomal maturation, cathepsin B and ROS generation [77]. The assembly of NLRP3 inflammasome leads to the activation of pro-caspase-1 and consequently the maturation of pro-IL-1β and pro-IL-18 (Figure 1C).

Caspases belong to a conserved metazoan aspartate-specific cysteine proteases family with 11 members in human (caspases 1 to 10, and 14) and 10 members in murine species

(caspases 1, 2, 3, 6, 7, 8, 9, 11, 12, and 14) [87, 88]. All caspases are synthesized as inactive zymogens containing a prodomain and are divided into two subfamilies: initiator caspases and effector caspases based on the length of their prodomains. Initiator caspases (caspases 1, 2, 4, 5, 8, 9, 10, 11, 12) are involved in the interaction with upstream adapter molecules and possess long prodomains that contain either the death effector domain or CARD. Effector caspases (caspases 3, 6, 7) that possess short prodomains are activated by upstream caspases and are able to cleave multiple cellular substrates involved in apoptosis [87]. Caspase-1, along with caspases 4, 5, 11, and 12 are often referred to as proinflammatory caspases. Once incorporated into NLRP3 inflammasome, pro-caspase-1 is activated by proteolytic cleavage to remove the CARD prodomain. The active caspase-1 in turn cleaves the IL-1β, IL-18, IL-33 and IL-1F7 precursors into their active forms and these active cytokines are secreted extracellularly and become immunoreactive (Figure 1C) [89].

It was recently reported that influenza virus infection results in the activation of NLR inflammasomes in the lung. Although NLRP3 is required for inflammasome activation in certain cell types, adaptive immunity to influenza virus is ASC and caspase-1 dependent rather than NLRP3 dependent, suggesting a central role of ASC inflammasomes. The investigators concluded that influenza virus infection stimulates NLRP3-dependent and NLRP3 independent inflammasomes in a cell type-specific manner [90]. Moreover, it has been suggested that some viral RNA can activate inflammasome via interaction between RIG-I and adaptor ASC independent of NLRP3, MAVS, and CARD [70].

3. Modulation of Adaptive Immunity by dsRNA

3.1. Activation of DCs. Activation of dendritic cells (DCs) occurs upon sensing dsRNA [91]. DCs express a repertoire of PRRs including TLRs (TLR3 is not expressed in plasmacytoid DCs), RIG-I and MDA-5 (absent in plasmacytoid DCs), as well as NLRs, and are able to recognize a range of pathogenic microbes [92–94]. The interaction of PAMPs and PRRs on DCs induces the maturation and activation of DCs via transcription, translation and secretion of inflammatory cytokines and chemokines through the signal pathways as described above. The activated DCs, characterized by enhanced antigen presentation capacity and referred to as antigen-presenting cells (APCs), migrate to draining lymph nodes and interact with T and/or B lymphocytes initiating the immunity process. Among the cytokines triggered and secreted, type I IFN plays a major role in the cross-priming of $CD8^+$ T-cells by promoting the expression of costimulatory molecules of DCs [95]. The proliferation and differentiation of the lymphocytes are mediated by signals from the activated DCs which comprise of the co-presentation of MHC molecules and pathogen-derived peptides. Additionally, signals from costimulatory molecules including CD80 and CD86, as well as the instructional signals, for example, IL-12p70 for Th1, IL-4 for Th2, and IL-6 and IL-23 for Th17 from the presenting DCs [91] are also present. dsRNA receptors which include TLR3 and RIG-I/MDA-5 are expressed in myeloid DCs (mDCs) and primarily produce IL-12 and IFN-β when recognition of dsRNA occurs [94]. However, poly (I:C) with different molecular weights have differential effects on the maturation of DCs [96]. Interestingly, co-culture of bone marrow-derived DCs with protein and poly (I:C) reduced the antigen uptake by DCs. However, the reduced uptake of antigen did not affect CTL priming by DCs suggesting that the reduction in uptake of soluble antigen in the presence of poly (I:C) is independent of TLR-mediated DC activation [97].

3.2. Activation of Th1 Cells. Newly primed $CD4^+$ T-cells are programmed by various cytokines and other factors from DCs and other innate immune cells to differentiate into Th1 or Th2 or Th17 effector cells or regulatory T-cells (Treg) [98]. Th1 lymphocytes are produced by the naïve $CD4^+$ T-cells (Th0) interacting with IL-12 from mDCs to stimulate the expression of signal transducer and activator of transcription (STAT) 1 and subsequently that of T box expressed in T-cells (T-bet). The latter is the critical transcription factor of Th1 cells [99]. Activated Th1 cells produce cytokines like IL-2 and IFN-γ that are cofactors in $CD8^+$ CTLs activation and synergistically activate mDCs acting via a feedback loop. Signals from Th1 cells are essential for $CD8^+$ T-cell proliferation and function [99]. It is believed that dsRNA is capable of inducing robust IL-12p70 production which reduces the threshold of Th1 response and herein promotes Th1-biased adaptive immunity through TLR3 and JNK pathways [98, 100]. TNF-α, type I IFN and IL-18 also play important roles in the induction of Th1 response by dsRNA [101]. Type I IFN can activate mDCs directly by inducing phenotypic maturation which includes but is not restricted to upregulation of MHC Class I, class II, CD40, CD80, CD86 and higher expression of CD83 [102]. Besides inducing mDCs maturation and activation, IFN-α/β upregulates the expression of chemokine receptor CCR7 to sensitize mDCs to CCL19 and CCL21 which promote the migration of mDCs from peripheral tissues towards the T-cell area of lymphoid organs [102]. Type I IFN is also necessary for the generation of a Th1 $CD4^+$ adaptive T-cell response whereas IL-12p40 and type II IFN are not. Therefore, the activation of Th1 cell response induced by dsRNA is possibly mediated by its capacity of inducing robust type I IFN production [92]. However, at low concentrations of dsRNA (0.1–1 μg/ml), human lymphocytes express prototypic Th2 cytokine IL-4 [103]. Indeed, when coadministered with protein antigen, in addition to the induction of robust Th1 biased immunity, dsRNA is also capable of enhancing Th2 antigen-specific immune response [104–106].

3.3. Activation of Cytotoxic T Lymphocytes. When stimulated by dsRNA along with specific antigen, activated DCs are able to induce antigen-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) activation through cross-presentation and cross-priming mechanism [91]. During viral infection of mDCs, dsRNA is produced during replication in the infected mDCs

and the latter in turn activates mDCs through TRIF, MAVS, NLRP3 inflammasome and possibly another undefined signal pathway(s) to become APCs. APCs present endogenous antigens including those from intracellular viral origin with MHC class I molecules to naïve $CD8^+$ lymphocytes along with costimulatory factors and instructional signals to activate the lymphocytes becoming MHC-I restricted CTLs. However, in most cases, the virus does not invade mDCs directly but instead infects cells other than mDCs. In such cases, the extrinsic viral antigen and dsRNA can be taken up into mDCs and these mDCs in turn present the antigen epitope with MHC-I molecules to $CD8^+$ lymphocytes inducing a CTL response. This mechanism is referred to as cross-priming and inducible by TLR3-TRIF signaling and MDA-5-MAVS signaling [107, 108]. Type I IFN produced through these signal pathways also enhances the cross-priming ability of mDCs possibly via augmenting their capacity to deliver costimulatory signals [102] or directly stimulation of $CD8^+$ T-cells [109]. The mechanism of mDCs uptake of the extrinsic antigens involves phagocytosis of particulate antigen, pinocytosis of soluble antigen and receptors mediating cross-presentation such as Fc receptor, mannose receptor and Dectin-1 [110]. The ability of cell-associated poly (I:C) with antigen to induce robust cross-priming responses in naïve mice is completely lost in the Tlr3-deficient chimera mice [107]. Immunized with protein along with alum and poly (I:C), the expansion of antigen-specific $CD8^+$ T-cell can be reduced in both MAVS-deficient and TRIF-deficient mice and entirely abrogated in the bideficient mice [108]. Type I IFN produced through TRIF or MAVS signal pathway also enhances cross-priming by mDCs [102, 109]. A recent report suggested that $CD8^+$ T-cells can be activated by dsRNA directly triggering TLR3 [111]. Priming of IFN-γ-producing $CD8^+$ T-cells by dying tumor cells failed in the absence of a functional IL-1 receptor 1 and in Nlpr3-deficient or caspase-1-deficient mice unless exogenous IL-1 was present [112]. This suggests that the NLRP3 inflammasome is involved in the activation of CTLs.

3.4. Activation of Th17 Cells. Th17 cell is a proinflammatory lymphocyte belonging to Th cell subset [113, 114]. This subset preferentially produces IL-17, IL-17F, IL-22, and IL-21, but not IFN-γ or IL-4 [115]. Naïve T-cells are induced by transforming growth factor (TGF)-β to differentiate into two reciprocal subsets, that is, Th17 cells and Treg cells under different polarizing signals. IL-6 is the polarizing signal of Th17 which switches the transcriptional program initiated by TGF-β to induce the development of Th17 cells and blocks the development of Treg cells [116–118]. IL-23 plays a role in amplifying and stabilizing the phenotype of Th17 cells [119]. However, receptors for IL-23 are not expressed in naïve T-cells. In fact, Th17 cells can express such receptors if induced by TGF-β acting through ROR-γt which is a unique transcription factor of Th17 (the human counterpart of murine ROR-γt is ROR-c) [120]. ROR-γt is the key transcription factor that orchestrates the differentiation of Th17 effector cell lineage by inducing transcription of the IL-17 gene in naïve helper T-cells and is also required for the development of IL-17 producing cells in the presence of IL-6 and TGF-β [121]. IL-6 is involved in upregulation of IL-23R mRNA expression, and IL-6 and IL-23 synergistically augment its protein expression [122]. Therefore, IL-23 acts on T-cells that are already committed to the Th17 lineage rather than inducing Th17 differentiation.

Th17 cells are engaged in the neutrophil related inflammation against infections of fungi and certain extracellular bacteria [115]. Most parenchymal cells express IL-17 receptors that interact with IL-17 that are expressed primarily from Th17 cells to produce proinflammatory factors such as IL-1, IL-6, IL-8, tumor necrosis factor (TNF) and matrix metalloproteinases [115, 123]. Th17 cells can also induce chemokine production which attracts numerous effector T-cells into inflammatory area promoting the inflammatory response. Thus, inappropriate regulation of Th17 cells activities is associated with chronic inflammation and severe immunopathologic conditions such as autoimmunity [115]. Various studies have suggested that TRIF signal pathway is involved in Th17 response. TRIF signaling in mDCs might induce IL-12 and IL-23 production and play a role in Th17 activation [124]. However, the TLR3 pathway activated by dsRNA induces activation of IRF3 and IRF7 which exclusively induce p35 and p28 but not p19. These in turn would induce IL-12 (p35 and p40) and IL-27 (Epstein-Barr virus-induced gene 3 and p28) but not IL-23 (p19 and p40) [125]. Stimulation of endosomal TLR3 by poly (I:C) can induce mDCs to produce both IL-12p70 and IL-27; the former promotes Th1 cells to produce IFN-γ which can inhibit Th17 cells generation and the latter inhibits Th17 cells differentiation in a STAT1-dependent manner. Thus, poly (I:C) is likely to dampen Th17 responses [126]. TRIF-dependent type I IFN production along with its downstream signaling pathway negatively regulates Th17 development and constrains Th17-mediated autoimmune inflammation in mice [127]. It has also been reported that poly (I:C) can induce synthesis of both IL-17 and IL-21 and drive the differentiation of naïve Th cells into an IL-21 but not into an IL-17-producing phenotype without affecting the levels of transcription factors T-bet, GATA-3, or retinoic acid receptor-related orphan receptor C [128]. Thymic stromal lymphopoietin (TSLP) is a hemopoietic cytokine capable of conditioning mDCs and orientating the differentiation of naïve T-cells towards a Th2 profile. mDCs activated by a combination of TSLP and poly (I:C) are capable of priming naïve $CD4^+$ T-cells to differentiate into Th17-cytokine-producing cells with a central memory T-cell phenotype without changing the Th2 polarization property of TSLP [129].

4. Potential Application of dsRNA as a Vaccine Adjuvant

4.1. The Adjuvant Properties of dsRNA. Innate immunity shapes adaptive immunity. Activated immune cells present specific antigen epitope associated with MHC-I/II molecules along with costimulatory and instructional signals to naïve T-cells to stimulate activation, differentiation

and proliferation of immunoreactive T-cells. As a potent activator of both innate and adaptive immunity, dsRNA simultaneously administered with a foreign antigen can act as an immunoadjuvant to induce specific adaptive immunity against the foreign antigen [91, 130].

Upon dsRNA stimulation, type I IFN production by DCs is critical for the adjuvant property of poly (I:C) [92]. Type I IFN is considered to be the major player linking innate to adaptive immunity. Besides activating DCs in an autocrine or paracrine manner, type I IFN is capable of inducing an antigen-specific $CD8^+$ T-cell response, a $CD4^+$ Th1 cell response and enhances the primary antibody response [2].

Salaun et al. (2009) demonstrated that proliferation and IFN-γ production of antigen-specific $CD8^+$ T-cells in the mice immunized by antigen GP33 (an H-$2D^b$ restricted peptide derived from Lymphocytic Choriomeningitis virus glycoprotein 33–41) with poly (I:C) adjuvant is abrogated when this regimen is administered in $TLR3^{-/-}$ mice [131]. The mRNA of TLR3 is undetectable in either $CD8^+$ effector or $CD8^+$ effector memory T-cells. $CD8^+$ T-cell proliferation and the ability of INF-γ production are not affected by direct stimulation with poly (I:C) and specific TCR. Therefore, it appears that the adjuvant effect of poly (I:C) may be TLR3-dependent without any direct effect on $CD8^+$ T-cells [131]. However, Ngoi et al. (2008) experimenting on (C57BL/6) mice that were $TLR3^{-/-}$ and TRIF-deficient, with the poly (I:C) (InvivoGen) but using a different antigen, staphylococcal enterotoxin A, showed that $CD8^+$ T-cells expansion was not impaired, that is, a type I IFN production in response to poly (I:C) occurred [132]. Splenocytes from naïve wild-type mice can produce IL-10 in a dose-dependent manner upon stimulation with poly (I:C) in the absence of antigen while IL-10 production was impaired in $TLR3^{-/-}$ mice. Although these IL-10 producing cells may be innate immune cells, the IL-10 produced acts as a suppressive signal for adaptive immunity. Thus, the presence of TLR3 may suppress the development of adaptive immunity [132]. Indeed, the activation of NK cells, involved in innate immunity, is inducible by poly (I:C) via both MAVS- and TRIF-dependent pathways [133]. Kumar et al. [108] reported that in MAVS-deficient or TRIF-deficient mice immunized with OVA, alum and poly (I:C), the antigen-specific $CD8^+$ T-cell expansion was reduced in either MAVS-deficient or TRIF-deficient mice and was entirely abrogated in the doubly deficient mice. Hence, the adjuvant effects of poly (I:C) requires a cooperative activation of TLR and cytoplasmic RNA helicase pathways [108]. Contamination of proteins used as antigens in these studies with other TLR ligands [93] or the contamination of $CD8^+$ T-cells with other immune cells like innate cells might be a possible explanation of the discrepancy.

With respect to human $CD8^+$ T-cells, TLR3 mRNA expression has been detected in human effector and effector memory cells but not in naïve and central memory T-cells [134]. The addition of poly (I:C) significantly increased the quantity of IFN-γ released by effector and/or effector memory $CD8^+$ T-cells in response to PHA in a dose-dependent manner. However, poly (I:C) by itself did not detectably induce IFN-γ release by any of the purified $CD8^+$ T-cell subsets. Furthermore, the addition of poly (I:C) had no effect on the cytolytic activity of CTL. Therefore, it is likely that the adjuvant effects and the corresponding mechanism of poly (I:C) are different in human and mouse cells.

4.2. dsRNA as Vaccine Adjuvant against Viral Infection. Lau et al. (2009) reported that mice vaccinated with H5N1 influenza vaccine with PIKA (a stabilized dsRNA) as adjuvant experienced a maximum three-fold increase in antibody titer comparable to that produced by mice immunized by vaccine with complete Freund's adjuvant [135]. Vaccination significantly reduced the virus titer in the lung of mice challenged after immunization with the H5 vaccine and PIKA adjuvant. Without a specific vaccine, sole PIKA administration was also capable of reducing pulmonary viral titer in mice [135]. This immunoprotective property of dsRNA against influenza virus was further demonstrated by other studies using another synthetic dsRNA analogue poly ICLC comprising of poly (I:C) stabilized with L-lysine and carboxymethylcellulose [136, 137]. PIKA was also able to induce activation and proliferation of B cells and NK cells. When HBsAg was coadministered, an increase in HBsAg-specific IgG production was noted. However, PIKA did not activate $CD4^+$ and $CD8^+$ T-cells [138]. Nonetheless, most studies suggested that poly (I:C) based dsRNA analogues displayed Th1 adjuvant property capable of activating antigen specific $CD4^+$ and $CD8^+$ T-cells when administrated as vaccine adjuvant [92, 105, 131, 132, 139, 140]. Another dsRNA analogue, poly (I:C12U) (Ampligen), was effective in inducing mDCs maturation and promoted Th1 cytokine IL-12 production while significantly decreased suppressive cytokine IL-10 production compared to that induced by poly (I:C) in healthy donors [140]. Intranasal administration of an Ampligen adjuvanted H5N1 vaccine resulted in the secretion of vaccine-specific IgA and IgG in nasal mucosa and serum respectively and protected mice against homologous and heterologous viral challenge [141]. Immunization of mice by hepatitis C virus nonstructural protein 3 and poly (I:C) emulsified with Montanide ISA 720 have demonstrated that the protein-based vaccine adjuvant, poly (I:C) was capable of eliciting Th1-biased adaptive immunity although the protein-based vaccine alone favors Th2-polarization. Additionally, the adjuvant potency of poly (I:C) emulsified with Montanide ISA 720 was much stronger than that of dispersed delivered poly (I:C) which suggests that protection of poly (I:C) from rapid degradation by ribonucleases was crucial for the adjuvant property of poly (I:C) [105]. Other groups have also suggested an Th1 adjuvant role of poly (I:C) [92, 139]. More importantly, induction of $CD4^+$ Th cells by poly (I:C) is required for memory in $CD8^+$ T lymphocytes [142]. $CD8^+$ CTL memory play important roles in vaccine design for prevention of viral infection. Therefore, it seems that the adjuvant property of distinct dsRNA molecules may be different as suggested by Avril et al. [96].

Several synthetic dsRNA analogues are commercially available such as poly (I:C), poly (I:C12U), poly ICLC, poly (A:U) and PIKA. Poly (I:C) is a mismatched dsRNA with one

strand being a polymer of inosinic acid, the other a polymer of cytidylic acid. It was discovered in 1967 by Hilleman's group [143] who also discovered IFN induction by dsRNA [144] before the discovery of its molecular receptors of TLR3 [145] and MDA-5 [56, 146]. However, toxicity concerns prevent the clinical utility of poly (I:C) [147–149]. Thus, most studies using poly (I:C) as an immunoprotective or vaccine adjuvant have been undertaken in animals. Attempts to design dsRNA analogues that can induce IFN with less toxicity have resulted in poly (I:C13U) being modified to poly (I:C12U) [150]. Poly (I:C12U) differs from poly (I:C) in that every 13th cytosine of the dsRNA, cytosine (C) is replaced by uracil (U). Uracil is unable to bond hydrogen to the hypoxanthine of the partner poly (I) strand and therefore results in a mismatched base which would be more readily degraded than the parent molecule [147]. This poly (I:C) analogue has been applied in clinical trials for chronic fatigue syndrome and AIDS. It was generally well-tolerated via intravenous administration with insomnia and dry skin the most commonly reported adverse events [151]. It also exhibited profound and uniformly suppressive effects on HIV expression in vivo [152]. Poly ICLC was introduced by Levy et al. in 1975 and comprised of poly (I:C) with poly-L-lysine and carboxymethylcellulose [153]. This complex is 5–10 times as resistant to hydrolysis by primate serum as the parent poly (I:C) and has a thermal denaturation temperature about 40°C higher than that of poly (I:C). It was able to induce significant levels of serum IFN in monkey and chimpanzee under conditions in which poly (I:C) itself induced no IFN [153]. In an early clinical trial in patients with malignancy, toxic reactions composed of fever, nausea, hypotension, thrombocytopenia and leukopenia, erythema, polyarthralgia with myalgia [154]. PIKA is a stabilized dsRNA greater than 100 base pairs in length [135]. Clinical information regarding PIKA is limited and the available data are only from animals [135, 138, 155]. Stahl-Hennig et al. (2009) reported that poly (I:C) and its analogues poly ICLC and poly (I:C12U) are effective adjuvants for the induction of protein-specific cellular immune responses [104]. Among the three molecular analogues, poly ICLC was the most potent adjuvant in monkeys. This may be attributed to poly ICLC being more stable against primate serum nucleases. Another study using HIV gag as antigen showed that both poly (I:C) and poly ICLC were able to induce antigen-specific $CD4^+$ T-cell response without IL-4 and IL-17 secretion, confirming Th1-polarized adjuvanticity. This adjuvant role is type I IFN-mediated [92]. Gowen et al. [156] revealed that poly (I:C) treatment significantly protected $TLR3^{-/-}$ mice from the lethal Punta Toro virus infection despite deficiencies in cytokine induction while poly (I:C12U) was unable to protect $TLR3^{-/-}$ mice from lethal challenge. It failed to produce IFN-α, IFN-β, and IL-6. However, in wild-type mice, poly (I:C12U) treatment was able to promote IFN-α, IFN-β, and IL-6 production and conferred protection from Punta Toro infection [156]. These results suggested that both TLR3 and MDA-5 are required for poly (I:C) to elicit immune responses but poly (I:C12U) requires only TLR3. This conclusion was verified later by Trumpfheller et al. (2008) [139]. Distinct forms of poly (I:C) with different molecular weights or poly (I:C12U) are not equivalent in their biological behavior [96].

4.3. dsRNA as Vaccine Adjuvant against Cancer. DCs and macrophages are major sensor cells to invading pathogen and transformed cells via germ-line encoded PRRs. Upon sensing pathogens or tumor cells, activation involves these cells becoming APCs triggering innate immunity and thereby shaping adaptive immunity through cross-priming to eliminate the invading microbes and tumor cells. Cancer cells are malignantly transformed cells of the host and are capable of expressing antigens that are not expressed or in trace amount in healthy host and are referred to as tumor associated antigens (TAAs) [157]. Adaptive immunity is the major mechanism to eliminate cancer cells in the late stage of host defense responses by the generation of tumor-specific immunity [158]. Various reports have demonstrated that the recruitment of tumor infiltrating lymphocytes, especially $CD8^+$ T-cells, is closely related to prognosis of the patients [159–161]. However, the anticancer immunity of the tumor-bearing host is usually weak or anergic due to either the weak antigenicity of TAAs or because of suppressive immunity in the host. Thus, enhancing the immune response, in particular TAA-specific CTL response and overcoming the immune suppression is crucial for anticancer immunity. Indeed, Provenge (also known as sipuleucel-T or APC8015) (Dendreon, Seattle, WA), a new cancer vaccine for advanced prostate cancer, was recently approved by FDA at April 29, 2010 [162]. Provenge works by ex vivo stimulation of isolated autogenous DCs of the patient with a fusion protein of full-length human prostatic acid phosphatase (PAP) and granulocyte-macrophage colony stimulating factor. This stimulation of DCs results in activation of APCs. The PAP activated APCs are suspended in lactated Ringer's solution, after removing the excessive antigen, and then infused into the patient resulting in innate and adaptive immunity against cancer cells [163]. Clinical trials have shown statistically significant prolonged median survival times [163–165]. This is the first FDA approved therapeutic tumor vaccine [166].

TLRs have been involved in the immunotherapy for cancer. Agonists of TLRs with the capacity of priming and shaping adaptive immunity have aroused significant interest in the development of cancer immunotherapy, in particular imiquimod, unmethylated cytosine preceding guanosine motif oligodeoxynucleotides (CpG ODNs), and dsRNA which act as agonistically with TLR7, TLR9, and TLR3, respectively [167].

Activation of TLR3 by dsRNA was capable of inducing either anticancer immune response or cancer cells apoptosis via TLR3 receptor expressed on a variety of cancer cells [168–173]. The two mechanisms of dsRNA against cancer work synergistically. Apoptosis of cancer cells presents the immune system with a new repertoire of TAAs in a TLR3 activation context that is favorable to the development of long-term anticancer immune responses. Poly (I:C12U) is capable of inducing phenotypic and functional maturation of DCs generated from peripheral blood monocytes of advanced ovarian cancer patients with sustained bioactive

IL-12p70 production. DCs primed with tumor lysate and matured with poly (I:C12U) are capable of generating Th1-biased specific anticancer responses in peripheral blood T-cells derived from cancer patients in the presence of ascites medium containing immunosuppressive cytokines. Using ovarian cancer ascites as an in vitro model, $CD8^+$ T-cells derived from ascites fluid primed with tumor antigen loaded DCs matured with poly (I:C12U), exhibited cytotoxic activity with the capacity of lysis of autologous tumor cells [140, 174]. Another synthetic poly (I:C) derivative, poly ICLC, more effective as an type I IFN inducer in humans but also associated with more clinical side-effects [175], has been involved in a variety of clinical trials of malignancy treatment in the last 30 years [176–180]. When poly ICLC was used either in monotherapy or in combination therapy, it exhibited immunomodulatory effects [181] and enhancement of IL-2-induced NK lytic activity in cancer patients [179]. However, sole poly ICLC administration did not improve the survival of cancer patients [177, 179, 180]. Despite the unfavorable results of poly ICLC in tumor therapy, auspicious results were noted when used as a cancer vaccine adjuvant in mice [182]. When poly ICLC was administered with tumor antigen-derived peptide epitopes as a cancer vaccine adjuvant in a murine brain tumor model, it was capable of enhancing antigen-specific CTL response. This facilitates the infiltration of antigen-specific T-cells into the tumor site, promotes tumor homing of antigen-specific T-cells and improves the survival of tumor-bearing mice by inducing long term antitumor protection [182]. A phase I/II clinical trial using type I polarizing DCs loaded with peptides in combination with poly ICLC in patients with recurrent malignant glioma is currently being conducted [183]. Polyadenylic polyuridylic acid [poly (A:U)] is another type of synthetic dsRNA analogue that has been used in combination with chemotherapy for locally advanced gastric cancer after curative surgery patients compared with chemotherapy alone [184] despite of its inefficiency as a single adjuvant [185]. A prolonged overall and recurrence-free survival was noted. Poly (A:U) is capable of inducing Th1 cell generation and antibody production in mice when coadministered with protein [186]. In vivo targeted delivery of tumor associated epitope to APCs in conjunction with poly (A:U) resulted in correction of the ineffective response to idiotypic epitopes, control of tumor growth, establishment of immune memory and protection against tumors bearing antigenic variants [187]. The immunoadjuvant effects of poly (A:U) is believed to signal TLR3 and TLR7 [188].

TLR3 agonists may be an double-edged sword in cancer treatment [189]. TLR3 expresses not only on immune cells sensing dsRNA and triggering immune response but also on tumor cells exhibiting other functions [190]. It is well known that viral infection is closely related with carcinogenesis and approximately 20% of all cancers are associated with infectious agents [191] such as human papillomavirus [191] and hepatitis viruses [192]. Prevention of viral infection is able to significantly reduce the occurrence rate of cancer [193–195]. Inhibition of virus replication reduced the development of cancer dramatically even in chronically viral infected patients [194, 196]. In this scenario, it is suspected that dsRNA, as intermediate of viral replication, is involved in the carcinogenesis. Long before the discovery of TLR3, researchers have found that dsRNA, such as poly (A:U) treatment is capable of enhancing carcinogenesis in animals [197, 198]. Studies have suspected that activation of TLRs in cancer cells could promote tumor progression and chemoresistance by activation of NF-κB to induce upregulation of antiapoptotic proteins and to inhibit proapoptotic proteins [190]. Consistently, several groups have reported that TLR agonists stimulate the proliferation and suppressor function of Treg cells and so attenuate the antitumor effects [199, 200]. However, most studies were conducted by activation of the TLRs other than TLR3 [190, 201, 202]. Recent reports suggested that TLR3 expression is much higher in metastatic cancer cells in comparison with primary cancer cells [169]. In human hepatocellular carcinoma cells, TLR3 can be expressed both on cell surface and in cytosol and only activation of the cytoplasmic TLR3 can induce cancer apoptosis accompanied by the down-regulation of antiapoptotic protein [171]. In situ stimulation of TLR3 and synergistic molecule CD40 can transform ovarian cancer-infiltrating DCs from immunosuppressive to immunostimulatory cells thus exhibiting therapeutic potential of TLR3 activation [203]. Activation of TLR3 in nasopharyngeal carcinoma cells can inhibit cell migration by downregulation of chemokine receptor CXCR4 suggesting antimetastasis activity of endogenous human TLR3 expression in cancer cells [204]. Thus, it seems that activation of endogenous human TLR3 expressed by cancerous cells may induce direct pro-apoptotic activity of the tumor cells [168]. Additionally, mRNA escaping from damaged tissue or contained within endocytosed cells could serve as an endogenous ligand for TLR3 [205].

5. Perspectives

Double-stranded RNA and its synthetic analogues, such as poly (I:C) and poly (A:U) have long been known to be potent type I IFN inducers and immunomodulators [144]. However, the fact TLR3 recognized dsRNA and activates NF-κB signal pathway was only discovered in 2001 [145]. Subsequently, other dsRNA receptors, RIG-I/MDA-5 and NLRP3 have been uncovered [7, 75, 206]. Studies of the interaction of dsRNA and its receptors have focused on immune cells. It is unknown if there are any endogenous ligands that share these receptors with exogenous dsRNA. Additionally, dsRNA receptors especially TLR3 are found expressed ubiquitously in the body. It is reasonable that not all types of cells are involved in sensing viral infection and eliciting immune response. Thus, the role of these receptors in other type of cells deserves further exploration.

dsRNA is able to induce both innate and adaptive immunity to eliminate the invading virus. However, the virus may evolve protective mechanism that enables it to destroy the dsRNA-induced signaling thereby protecting itself by evasion from the immune response of the host [207, 208]. Thus, overcoming viral protective mechanism(s) is desirable.

There is insufficient information regarding TLR3 activation in cancerous cells. Recent report suggested that

poly (I:C) binds to endo/lysosomal MDA-5 and activates apoptotic caspases in melanoma cells to induce their self degradation by autophagy and apoptosis [209]. The possibility that other dsRNA receptors may be present in tumor cells deserves further investigation. Having such knowledge would be very helpful for development of therapeutic tumor vaccine with dsRNA analogue adjuvant. In addition, the effects of dsRNA signaling on Treg cell are not known. It appears that there needs to be a balancing act between the possible carcinogenesis and the immune stimulating property of dsRNA when dsRNA analogues are considered as immunoadjuvants in tumor immunotherapy.

Although dsRNA have displayed favorable immunostimulatory and protective properties, many questions remain to be answered. Further investigations to uncover its roles in viral infection, carcinogenesis or anticancer actions deserve consideration.

Abbreviations

AP-1: activating protein 1
ASC: apoptosis-associated speck-like protein containing a caspase-activating and recruitment domain
CARD: caspase recruitment domain
dsRNA: double-stranded RNA
FADD: Fas-associated cell death domain
IFN: interferon
IKK: IκB kinase-related kinase
IL: interleukin
IRF: interferon regulatory factor
LRR: leucine-rich repeat
MAVS: mitochondrial antiviral signaling adapter
MDA-5: melanoma differentiation-associated gene 5
NACHT: (NAIP, CIITA, HET-E, TP-1) domain
NAIP: Neuronal apoptosis inhibitory protein
CIITA: Class II transactivator
HET-E: plant Het product involved in vegetative incompatibility
TP-1: Telomerase-associated protein 1
NAP1: nuclear factor-κB activating kinase-associated protein 1
NEMO: nuclear factor-κB essential modulator
NF-κB: nuclear factor-κB
NLRP3: nucleotide-binding domain and leucine-rich repeat containing gene family pyrin domain 3
PI3K: phosphatidylinositol 3-kinase
PRR: proline-rich region
PYD: pyrin domain
RIP1: receptor-interacting protein 1
TAB: transforming growth factor β-activating kinase binding protein
TAK: transforming growth factor β-activating kinase
TANK: TRAF family member-associated NF-κB activator
TBK1: TRAF family member-associated NF-κB activator-binding kinase 1
TIR: Toll/IL-1 receptor domain
TLR3: Toll-like receptor 3
TRADD: tumor necrosis factor receptor-associated death domain
TRAF6: tumor necrosis factor receptor-associated factor 6
TRIF: Toll/IL-1 receptor domain-containing adaptor inducing IFN-β.

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Review Article

Strategies for Cancer Vaccine Development

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Treating cancer with vaccines has been a challenging field of investigation since the 1950s. Over the years, the lack of effective active immunotherapies has led to the development of numerous novel strategies. However, the use of therapeutic cancer vaccines may be on the verge of becoming an effective modality. Recent phase II/III clinical trials have achieved hopeful results in terms of overall survival. Yet despite these encouraging successes, in general, very little is known about the basic immunological mechanisms involved in vaccine immunotherapy. Gaining a better understanding of the mechanisms that govern the specific immune responses (i.e., cytotoxic T lymphocytes, CD4 T helper cells, T regulatory cells, cells of innate immunity, tumor escape mechanisms) elicited by each of the various vaccine platforms should be a concern of cancer vaccine clinical trials, along with clinical benefits. This review focuses on current strategies employed by recent clinical trials of therapeutic cancer vaccines and analyzes them both clinically and immunologically.

1. Introduction

Cancer is the second leading cause of death in the United States, exceeded only by heart disease (23.1% versus 26.0% of total deaths, resp.). Currently, 1 in 4 deaths in the United States is due to cancer. According to American Cancer Society statistics, an estimated 1,479,350 new cases and 562,340 deaths from cancer are expected during 2009, with a slightly higher incidence and death rate in the male population. Prostate, lung, and colorectal cancers are the most common types of cancer in men; breast, lung, and colorectal cancers are most common among women. Altogether, lung, breast, prostate, and colorectal cancers account for 49% of cancer-related deaths in the U.S. population [1]. Overall, except for lung cancer in women, incidence and mortality rates have steadily decreased for all 4 types of cancer in both men and women, probably due to both an increase in early diagnosis and improvements in therapy and combination therapies (surgery, radiotherapy, chemotherapy, and, lately, targeted therapy). But despite these encouraging advances, cancer is still a major public health problem worldwide, requiring new strategies and treatment modalities to optimize patient outcomes.

In this context, immunotherapy has always been an attractive and potentially efficient treatment for cancer patients [19]. Tumor immunotherapy can generally be classified as (a) passive (or adaptive), consisting of administration of cells or antibodies ex vivo, and (b) active, represented by vaccines, aimed at eliciting a specific immune response against tumor-specific antigens (TSAs) and tumor-associated antigens (TAAs). Prophylactic and therapeutic vaccines represent one of the most intriguing approaches in the multidisciplinary treatment of cancer patients. Compared to all other standard modalities (surgery, chemotherapy, radiotherapy, and adaptive immunotherapy), an effective vaccine-based immune response against tumor may be the only cancer treatment with the potential to last a lifetime. Theoretically, vaccinated patients could mount an immune response able to either cure tumor or keep it under constant restraint (i.e., immune surveillance), delaying tumor recurrence and prolonging survival.

One of the major problems in developing an efficient cancer vaccine is the lack of TSAs and the weakness of immune responses against TAAs, usually recognized by the immune system as self-antigens. During the last decades, various strategies for therapeutic cancer vaccines have been

proposed to overcome this weak immune response against TAAs, including cell-based vaccines, DNA- or RNA-based vaccines, protein- or peptide-based vaccines, and vector-based vaccines [20]. The common rationale for all these modalities is the activation of antigen-presenting cells (APCs) and the stimulation of an antigen-specific cytotoxic T lymphocyte-(CTL-) mediated immune response. Dendritic cells (DCs) are the most potent APCs, and various strategies have been used to enhance their ability to activate T cells. This review focuses on the state of the art of these modalities and analyzes the most promising phase II/III clinical trials, emphasizing vaccines directed against carcinomas (Table 1). Despite recent achievements, one criticism of some of these clinical trials has been the lack of immunological data supporting the significant improvements in time to progression and overall survival (OS) observed. An effort should be made to define the specific components of each immune response as a consequence of anticancer vaccination. In this context, both the specificity and the identification of potential escape mechanisms (i.e., increase of Treg number or function, balance between positive and negative regulators of antitumor responses, such as CD28, cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), programmed death-1 molecule (PD-1) and its ligands PD-L1 and PD-L2) should be investigated. Increasing our understanding of how these modalities modulate the CTL response is vital to developing novel and effective antitumor vaccines.

The goal of therapeutic cancer vaccines is to "teach" the patient's own immune system to specifically recognize and eliminate tumor cells. The potential target for the immune response can be either TSAs (antigens present only on tumor cells) or TAAs (antigens present mostly on tumor cells but also on some normal cells). Theoretically, TSAs are the ideal target for cancer immunotherapy because of their specificity. They are largely composed of mutant proteins caused by somatic mutations in the original sequence of the protein. A major advantage of targeting TSAs is that many of these proteins have been demonstrated to be essential for tumorigenesis and cancer progression [21]. On the other hand, a major drawback of targeting TSAs is the fact that most of the mutations identified are unique to each tumor, potentially requiring the development of personalized immunotherapy for individual patients. In contrast, TAAs are commonly expressed on tumors with the same histology and are shared among tumors of different origin. A major limitation of targeting TAAs is that they are weakly immunogenic due to the tolerance for self-antigens acquired by the immune system in its developmental stages [22].

In the last decades, several different mechanisms have been proposed to "instruct" DCs, the most potent APCs known, to induce Th and CTL responses against tumor antigens, thus breaking immune tolerance. Antigen-loading techniques include (a) infecting DCs with viral, bacterial, or yeast vectors, (b) pulsing DCs with proteins or peptides, (c) loading DCs with tumor cells or tumor-cell lysates, and (d) transfecting DCs with DNA or RNA (Figure 1) [20].

Encouraged by positive preclinical and clinical data [23–27], further studies are currently ongoing to evaluate the possibility to enhance vaccine-induced immunity by combining vaccines with low doses of chemotherapeutic agents (i.e., cyclophosphamide, doxorubicin, docetaxel) or radiation therapy, that showed synergistic immunotherapeutic effects when given in proper sequence.

2. Vaccines with Viral, Bacterial, or Yeast Vectors

As mentioned above, one of the major difficulties in cancer immunotherapy is to develop a strategy to overwhelm the characteristically weak immune response of the host against TAAs. Several vectors can be used to deliver recombinant genes (including genes expressing TAAs, costimulatory molecules, or cytokines) into APCs. Recombinant vector-based vaccines may induce the immune system to generate a strong inflammatory response, directed mainly towards vector proteins. In turn, this inflammatory response may lead to an increased immune response against the genes of interest that have been inserted into the vector. One advantage of using vectors as vehicles for TAAs is that this type of delivery of a recombinant protein is much more immunogenic than administering the protein with adjuvants [28, 29].

Vectors used in cancer immunotherapy include viral, bacterial, and yeast vectors. The choice of vector can have important consequences for the subsequent immune response against TAAs because each vector has its own characteristics and is potentially able to uniquely stimulate the host immune system. A further factor that must be taken into account in the development of an efficient vector-based vaccine strategy is the balance between the stimulation of innate versus adaptive responses, Th1 versus Th2 responses, or the preferred activation of subsets of cells mainly committed to regulatory (Tregs, Tr1, and Th3) or proinflammatory functions (Th17).

Poxviral vectors are among the most heavily exploited in vaccine development. The prototype is vaccinia virus, which was used successfully to eradicate smallpox [30]. The poxvirus family is composed of double-stranded DNA viruses that replicate within the cytoplasm of infected cells. This feature is important for the safe use of poxviruses as recombinant vaccines, since no genetic sequence from the virus will be inserted into the host cell genome. However, owing to concerns about the use of replicating vectors in potentially immunocompromised patients and immune responses generated against the vector by immunocompetent patients, developing safe, nonreplicating viral vectors has been the focus of extensive research. Other attenuated poxviruses have been identified and are currently available for clinical use. Fowlpox, an avipoxvirus, can infect mammalian cells abortively, but recombinant-encoded genes are transcribed [31]. The drawback is that recombinant fowlpox usually generates a weaker immune response in humans than vaccinia and is thus often used for booster vaccinations after a primary vaccination with recombinant vaccinia. Modified vaccinia Ankara (MVA) is a highly attenuated strain of vaccinia that was developed by hundreds of passages of vaccinia virus in chick embryo fibroblasts. MVA can infect mammalian cells and undergo DNA replication in them

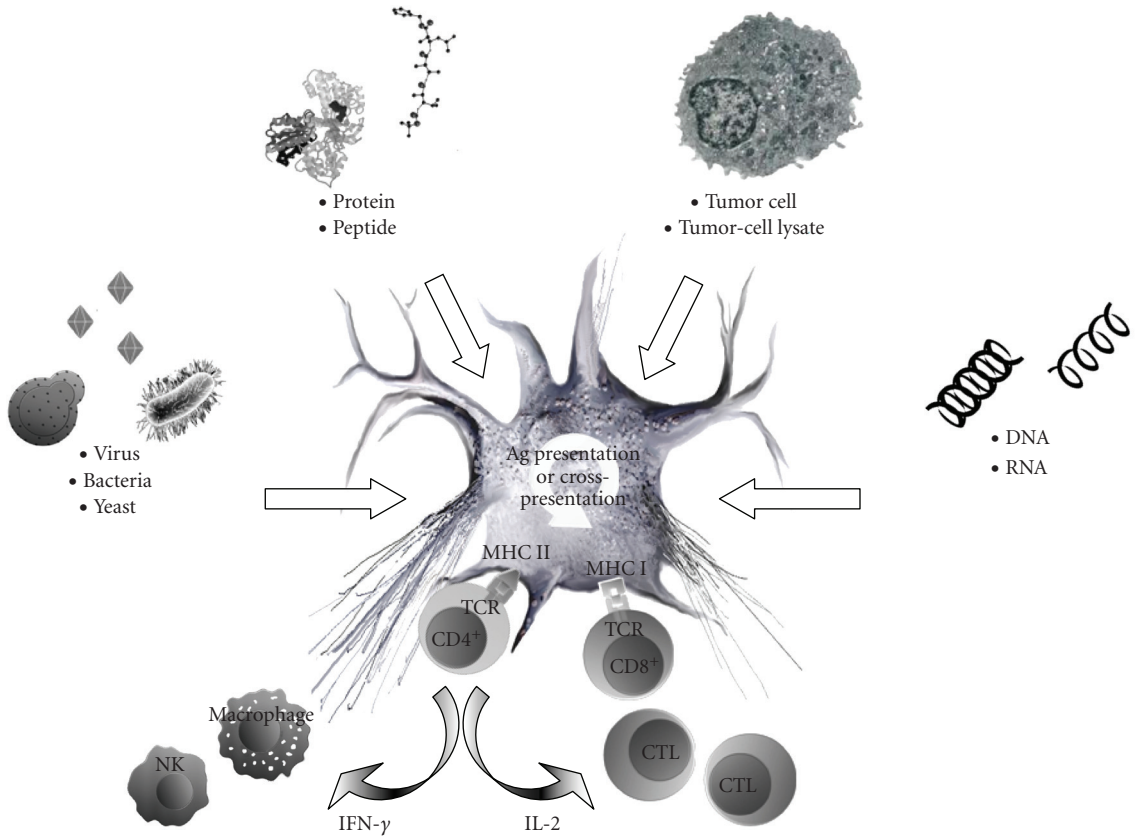

Figure 1: Various modalities employing dendritic cells for the development of therapeutic cancer vaccines.

but has lost the ability to produce infective viral particles [32]. Preclinical and clinical studies have demonstrated the superiority of a priming vaccination with recombinant vaccinia followed by multiple boosts with recombinant fowlpox, over different dosing schedules or the continuous use of either vector alone [33–36].

The large genome of poxviruses (approximately 130 kb for mammalian poxviruses and 300 kb for avian poxviruses) allows for insertion of more than 10 kb of foreign DNA.

Moreover, gene products are usually expressed at high levels, resulting in a potent cellular immune response. As mentioned previously, poxviruses can also be modified to express one or more T-cell costimulatory molecules along with the transgene for a TAA, or cytokines such as GM-CSF. Tumor recognition by CTLs is a complex mechanism that requires several different signals. DCs provide T cells with antigenic signal 1 through the specific interaction between the peptide-MHC I complex and the T-cell receptor. A costimulatory signal 2 is needed for the activation and expansion of T cells. Finally, DCs provide an additional polarizing signal 3 through the release of different cytokines, driving the immune response toward type-1 or type-2 immunity. Therefore, costimulatory molecules are critical in the generation of potent T-cell responses, particularly toward weak antigens such as TAAs. The most studied costimulatory signals involve the interaction between B7.1 (CD80) expressed on APCs and CD28 or CTLA-4 on T cells, between intercellular adhesion molecule-1 (ICAM-1 or CD54) on APCs and leukocyte function-associated antigen-1 (LFA-1) on T cells, and between LFA-3 (CD58) on APCs and CD2 on T cells [37].

PSA-TRICOM vaccine (prostate-specific antigen plus a TRIad of COstimulatory Molecules; PROSTVAC) consists of a priming vaccination with recombinant vaccinia- (rV-) PSA-TRICOM and booster vaccinations with recombinant fowlpox- (rF-) PSA-TRICOM. Each vaccine consists of the transgenes for PSA, including an agonist epitope [38], and 3 immune costimulatory molecules (B7.1, ICAM-1, and LFA3; designated TRICOM). The efficacy of PSA-TRICOM has been evaluated in 2 phase II clinical trials in patients with metastatic hormone-refractory prostate cancer (mHRPC). In the first multicenter clinical trial, 122 patients with Gleason scores of ≤ 7 were randomized 2 : 1 to receive PSA-TRICOM plus GM-CSF ($n = 82$) versus an empty-vector placebo ($n = 40$). A vaccinia-based vector was used as prime, followed by 6 boosts with a fowlpox-based vector. Vaccinated patients had a greater 3-year OS compared to the placebo arm (30% versus 17%, resp.) and an improvement in median OS of 8.5 months (24.5 months versus 16 months, resp.; $P = .016$). T-cell responses to vaccine or vector were not evaluated in this trial [2–4].

In a concurrent phase II clinical trial at the National Cancer Institute employing the identical PSA-TRICOM vaccine, 32 patients (representing all Gleason scores) were

Table 1: Overview of 4 different vaccination strategies employed in clinical trials.

VACCINE	PHASE	TUMOR	PTS*	NOTE	REF.
Vaccines with viral vectors					
PSA-TRICOM	II	Prostate	122	8.5 mos OS improvement versus placebo.	[2–4]
	II	Prostate	32	>16.4 mos OS improvement in HPS>18 mos group.	[5]
PANVAC-VF	III	Pancreatic	255	Failed >OS. Pts with life expectancy <3 mos.	[6]
Vaccines with peptides					
Provenge	III	Prostate	512	4.1 mos OS improvement versus placebo.	[7, 8]
Oncophage	III	Melanoma	322	Prolonged OS in M1a or M1b subpopulation.	[9]
	III	Renal	818	No difference in DFS and OS.	[10]
gp100 : 209-217(210 M)	III	Melanoma	185	Significant improvement in RR and PFS.	[11]
Stimuvax	IIB	Lung	171	17.3 mos OS improvement versus BSC in locoregional stage IIIB.	[12]
Vaccines with tumor cells or tumor-cell lysates					
OncoVAX	III	Colon	254	Significant improvement in DFS and OS in stage II.	[13–15]
Reniale	III	Renal	558	Significant improvement in DFS and OS.	[16, 17]
GVAX	III	Prostate	626	Failed to improve OS versus docetaxel.	[6]
	III	Prostate	408	Failed. Higher death rate in combination arm (vaccine + docetaxel) versus docetaxel alone.	[6]
Vaccines with RNA					
mRNA from PCa cell lines	I/II	Prostate	19	Immunological responses.	[18]

*PTS: patients enrolled.

randomized to 1 of 4 cohorts. Cohort 1 received no immune adjuvant; cohort 2 received recombinant human GM-CSF protein; cohort 3 received 10^7 plaque-forming units (pfu) rF-GM-CSF; cohort 4 received 10^8 pfu rF-GM-CSF. All patients were primed with rV-PSA-TRICOM s.c. on day 1 and then received monthly boosts of rF-PSA-TRICOM until progression. Patients who remained on-study after 12 months had booster vaccinations every 3 months. With a median follow-up of 44.6 months, the median OS for all 32 patients on-study was 26.6 months, compared to

a median Halabi nomogram-predicted survival of 17.4 months (an improvement of 9.2 months) [5]. No major differences were observed among the 4 cohorts. The subanalysis of patients with a Halabi-predicted survival (HPS) of <18 months showed a minimal difference between actual OS and HPS. However, patients with HPS >18 months had a significant increase in actual OS (>37.3 months, median not reached, with 8 of 15 patients still alive at 44.6 months) compared to HPS (20.9 months). PBMCs from patients pre and post vaccination were analyzed by ELISPOT assay to evaluate the specific immune response against the HLA-A2 PSA peptide PSA-3 [39]. Thirteen of 29 patients analyzed had an enhanced (≥2-fold) PSA-3-specific T-cell immune response post vaccination. Furthermore, patients with a postvaccination ELISPOT response to PSA epitope >6-fold seemed to live longer, compared to patients with a postvaccination ELISPOT response to PSA epitope <6-fold ($P = .055$). We also analyzed Treg function pre and post vaccination. Among patients who survived longer than predicted, Treg suppressive function decreased in 10/13 (77%) after 3 vaccinations versus pre vaccination. In contrast, among patients whose survival was less than predicted, Treg function increased in 6/8 (75%) after 3 vaccinations versus pre vaccination. These data strongly suggest that Tregs play a significant role in the modulation of antitumor immune response [40].

PANVAC-VF, another poxviral-based vaccine, consists of a priming vaccination with rV encoding CEA(6D), MUC1(L93), and TRICOM plus booster vaccinations with rF expressing the identical transgenes. CEA(6D) and MUC1(L93) represent carcinoembryonic antigen and mucin 1 glycoprotein, respectively, with a single amino acid substitution designed to enhance their immunogenicity [41, 42]. A phase III study in patients with advanced pancreatic cancer treated with PANVAC-VF as second-line therapy showed no improvement in survival [6]. The vaccine is currently under evaluation in several different types of CEA- or MUC1-expressing carcinomas and in patients with a life expectancy >3 months. In our experience, PANVAC-VF was well tolerated in a pilot study enrolling 25 patients with metastatic carcinomas. After vaccination, CAP1(6D)-specific CD8 immune responses were detected in 3/8 patients by ELISPOT, CAP1(6D)-tetramer, and intracellular IFN-γ staining. We also evaluated CD4 immune responses in 15 patients included in the study, using CEA protein as antigen. Six of 15 patients with undetectable levels of IFN-γ pre vaccination showed measurable levels in response to CEA protein. Four of 14 patients were positive for the generation of MUC1-specific T cells post vaccination [43].

The rationale for the use of microbes such as yeast as delivery vehicles for TAAs is based on the ability of these agents to activate a proinflammatory response through the interaction of pathogen-associated molecular patterns with pattern-recognition receptors, such as Toll-like receptors, expressed on APCs. These interactions play a central role in the activation of innate and adaptive immunity [44]. Over the years, several different bacterial and yeast vectors, such as *Escherichia coli*, *Salmonella*, *Shigella*, *Yersinia*, *Listeria monocytogenes*, and *Saccharomyces cerevisiae*, have been investigated for use as vaccine vectors.

The development of genetic engineering technology and efficient fermentation procedures has made large-scale, cost-effective production of these vectors possible and is one of the major advantages of their use in antitumor vaccines. Unfortunately, development of yeast-based vaccines has lagged behind that of cell-, protein-, and viral-based vaccines, and clinical experience has been limited to phase I/II studies [45]. One such vector currently being evaluated is a whole, heat-killed, recombinant *S. cerevisiae* yeast (Tarmogens GI-4000, GlobeImmune) intended to generate a T-cell response to eliminate tumor cells expressing the 7 most common mutations in the ras oncogene product. A randomized, double-blind, placebo-controlled phase IIa clinical trial has enrolled 100 patients with resected pancreatic cancer, with half receiving adjuvant gemcitabine plus placebo and half receiving adjuvant gemcitabine plus GI-4000 [46].

3. Vaccines with Proteins or Peptides

The use of proteins or peptides to stimulate a specific immune response against cancer has long been investigated and covers a broad spectrum of possibilities employing single agents or combinations of proteins, heat-shock proteins (HSPs) [47], peptides and agonist peptides [48–51], anti-idiotype antibodies [52, 53], and fusion proteins [54]. These protein- or epitope-based vaccines have 2 main advantages over the use of tumor cells or lysates: (a) production, storage, and distribution are faster and more cost-effective, and (b) the identification and administration of TSAs is preferable since tumor-cell preparations mostly contain self-proteins with no therapeutic benefit and are potentially capable of generating an autoimmune response. On the other hand, this approach has certain drawbacks: (a) first is the weak immunogenicity of a single protein or, especially, a single epitope; (b) tumors can easily escape immune recognition through antigen mutation or loss; (c) their use is HLA-restricted (mainly for epitope-based vaccines) and limited to a subset of patients (usually HLA-$A2^+$); (d) they have a poor ability to induce balanced activation of CD4 and CD8 subsets, which is thought to be essential for effective, long-lasting antitumor immunity. To date, in fact, most epitope-based vaccines induce HLA-A2-restricted responses that efficiently kill tumor cells but are characterized by a limited lifespan in the absence of CD4 helper T cells. Protein-based vaccines are capable of generating stronger CD4 responses (MHC class II-restricted), but at the cost of less effective induction of CTLs [55, 56]. Most of the issues described above could be easily overcome by the use of longer peptides or the combination of several different epitopes in the same vaccine, while the relatively poor immunogenicity of peptides could necessitate that they be administered with adjuvants or loaded onto DCs [57, 58].

The use of specific proteins or peptides as targets for immunotherapy clearly requires a careful choice of the targeted TAAs and their epitopes, involving knowledge of their structural and functional characteristics. Single-peptide

epitopes composed of 8 to 10 amino acids are able to induce a CTL response by binding to MHC class I molecules expressed on APCs. Each epitope is composed of conserved anchor residues (mostly at position 2 and the C-terminal position) needed to bind to the cleft of MHC I molecules and residues that are specific for T-cell recognition. Theoretically, changes in the former do not affect the specificity of the latter, and they have been used as a strategy to increase the immunogenicity of several different epitopes (agonist epitopes) [38, 41, 42, 50, 59]. Furthermore, the ideal TAA should be widely expressed in different tumor types and also play a central role in oncogenic processes or in cancer cell survival, to avoid immune escape by mutations or loss of antigens by tumor cells.

Identification of novel TAAs can be achieved through 2 experimental processes: direct immunology (starting from patient-derived autologous tumor-specific CTL clones specific for an unknown epitope) and reverse immunology (starting from a predicted epitope). The former has been used since the discovery of the first tumor-specific CTL epitope, MAGE-1 [60]. Direct immunology is further subdivided into genetic or biochemical approaches. Briefly, in the genetic approach, a patient-derived CTL clone is screened by using target cells transfected with tumor-derived cDNA libraries. Subsequently, the increased release of cytokines in the supernatant due to the recognition by the tumor-specific CTL clone allows one to select the cells that contain the antigen-encoding cDNA; these are then subcloned and rescreened to finally identify the cDNA that encodes the specific antigen. The biochemical approach consists of the purification of peptides eluted from MHC class I molecules of antigen-expressing cells by high-performance liquid chromatography fractionation. Antigen-negative target cells expressing the appropriate HLA molecule are used to load these peptides and tested for CTL recognition. Positive fractions are analyzed by mass spectrometry to identify the amino acid sequence of the epitope recognized by CTLs [61]. The need for expensive specialized equipment, plus the labor-intensive method, probably accounts for the increasing use of reverse immunology. Over the years, a growing understanding of HLA-specific peptide-binding motifs has led to the development of several computer algorithms for amino acid sequences with predicted binding capacity. Reverse immunology consists of two different phases: in the epitope prediction phase, proteins are analyzed for the presence of potential epitopes by the use of prediction algorithms. Subsequently, in the epitope validation phase, the candidate peptides are tested by binding and stability assays *in vitro*. Nevertheless, differences between the processing machinery in normal and tumor cells might be liable for the lack of activity against tumor cells of several CTLs raised against high-affinity binding TAAs [62]. Nowadays, indeed, the most recent algorithms also take into account the proteasomal processing and transporters associated with antigen processing- (TAP-)translocation, 2 other fundamental processes in the antigen-presentation pathway. Despite many efforts, the use of epitope-based vaccines has not advanced beyond phase I or II clinical trials, probably due to the drawbacks described above. To date, the best results have been achieved with the use of fusion protein- or HSP-based vaccines.

Provenge (sipuleucel-T, Dendreon Corporation) is in late-stage development for the treatment of mHRPC. Sipuleucel-T is an immunotherapy product designed to stimulate T-cell immunity against prostatic acid phosphatase (PAP). It consists of autologous APCs isolated by leukapheresis, cultured with a PAP-GM-CSF fusion protein, and reinfused into the patient. The time from apheresis to infusion of final product is approximately 48 hours. The efficacy of Provenge was evaluated in 2 randomized, double-blind, placebo-controlled phase III clinical trials (D9901 and D9902A) [7, 8]. D9901 enrolled 127 patients with asymptomatic mHRPC, who were randomly assigned 2 : 1 to receive 3 infusions of Provenge ($n = 82$) or placebo ($n = 45$) every 2 weeks. Enrollment in D9902A was stopped at 98 patients after D9901 showed encouraging results in terms of disease progression, and the study was amended to become D9902B (IMPACT), enrolling 512 patients with OS as the primary endpoint. An integrated analysis of 225 patients in D9901 and D9902A (147 in the vaccine arm and 78 in the placebo arm) demonstrated a survival benefit for patients treated with Provenge versus placebo (23.2 months versus 18.9 months, resp.), with a 33% reduction in the risk of death. The only immunological data to emerge from these studies are limited to the correlation between the upregulation of CD54 molecules on the cell surface of sipuleucel-T-treated APCs and OS, whereas no data are available about a specific immune response against PAP. At the American Urological Association 2009 Annual Meeting, Dendreon Corporation announced that the phase III IMPACT clinical trial had met its primary endpoint of significantly improving OS by 4.1 months compared to placebo {25.8 months versus 21.7 months, respectively, $P = .032$, HR = 0.775 [95% CI: 0.614, 0.979]}. The U.S. Food and Drug Administration (FDA) will respond to the existing Dendreon's amended Biologics License Application (BLA) for the licensing of Provenge in men with metastatic castrate-resistant prostate cancer (CRPC) by May 2010. If approved, Provenge will be the first active cellular immunotherapy to decisively demonstrate a survival benefit for cancer patients.

Oncophage (vitespen, Antigenics), an autologous tumor-derived HSP gp96 peptide complex, has been evaluated in 2 phase III clinical trials in stage IV melanoma patients and in renal cell carcinoma (RCC) patients at high risk of recurrence after nephrectomy [9, 10]. Oncophage consists of a purified preparation of the HSP gp96 from tumor. HSPs are noncovalently bound to peptides derived from self- and tumor-specific proteins. Immunization with gp96 peptide complexes leads to their uptake by DCs through CD91 (an HSP receptor) and stimulation of cognate T cells. In the first phase III clinical trial, 322 patients with stage IV melanoma were randomized 2 : 1 to receive Oncophage or a treatment of the physician's choice. The first 4 injections were administered weekly and subsequent injections were given every other week. Results from this trial suggested a survival benefit in the subpopulation of patients with M1a or M1b disease who were able to receive 10 or more doses of vaccine. In the second phase III trial of 818 patients

with postnephrectomy RCC, no difference in recurrence-free survival or OS was observed between patients receiving Oncophage versus no treatment, although a trend toward a decrease in recurrence-free survival was reported in stage I or II disease in the experimental arm.

In a prospective randomized multicenter phase III trial, 185 patients with locally advanced stage III or stage IV melanoma were randomized to receive high-dose (HD) IL-2 alone (94 patients) or a synthetic peptide from the gp100 melanoma-associated antigen [gp100 : 209-217(210M)] plus an adjuvant (Montanide ISA) followed by HD IL-2 (91 patients). Overall response rate (RR, 22.1% versus 9.7%, $P = .0223$) and progression-free survival (PFS) (2.9 months versus 1.6, $P = .0101$) were significantly improved in the experimental arm compared with the HD IL-2 arm, respectively. Median OS was 17.6 months in the HD IL-2 + vaccine arm versus 12.8 in the HD IL-2 alone arm ($P = .0964$) [11].

Stimuvax (BLP25 liposome vaccine, L-BLP25, Oncothyreon partnered with Merck KGaA) is a cancer vaccine designed to induce an immune response against the extracellular core peptide of MUC1, a type I membrane glycoprotein widely expressed on many tumors (i.e., lung cancer, breast cancer, prostate cancer, and colorectal cancer). Stimuvax consists of MUC1 lipopeptide BLP25 [STAPPAHGVTSAPDTRPAPGSTAPPK(Pal)G], an immunoadjuvant monophosphoryl lipid A, and three lipids (cholesterol, dimyristoyl phosphatidylglycerol, and dipalmitoyl phosphatidylcholine), capable of enhancing the delivery of the vaccine to APCs.

A randomized phase IIB clinical trial evaluated the effect of Stimuvax on survival and toxicity in 171 patients (88 in the L-BLP25 arm and 83 in the best supportive care arm (BSC)) with stage IIIB and IV nonsmall-cell lung cancer (NSCLC), after stable disease or response to a first-line chemotherapy [12]. Median OS was 17.4 months in the L-BLP25 arm and 13.0 months in the BSC arm, respectively, with a nonsignificant improvement of 4.4 months in the experimental arm ($P = .112$). T-cell proliferation assays were conducted on 78 of 88 patients enrolled in the L-BLP25 group, before and after immunization. Sixteen patients showed a MUC1-specific T-cell response (only two with a locoregional stage IIIB disease). No severe toxicities were reported. After a median follow-up of 53 months, updated survival data reported a median OS of 30.6 months in the Stimuvax arm versus 13.3 months in the BSC arm, in the subgroup of patients with locoregional stage IIIB (65 patients, of whom 35 were randomized to the vaccine arm and 30 were randomized to the BSC arm) ($P = .09$) [63]. Although nonsignificant, considering the magnitude of the difference and the prolonged follow-up, these results suggest a signal of efficacy for the vaccine.

Based on these data, Merck is currently conducting three large phase III clinical trials of Stimuvax. START (Stimulating Targeted Antigenic Responses To NSCLC) is a double-blind, placebo-controlled, randomized, multicenter phase III clinical trial that will enroll patients with unresectable stage IIIA or IIIB NSCLC, after stable disease or response to a platinum-based chemo-radiotherapy. This study will involve more than 1,300 patients.

The INSPIRE study (Stimuvax trial In Asian NSCLC Patients: stimulating Immune REsponse) will enroll approximately 420 patients with unresectable stage III NSCLC across China, Hong Kong, South Korea, Singapore, and Taiwan. STRIDE (STimulating immune Response In aDvanced brEast cancer) is a randomized, double-blind, controlled, multicenter Phase III study designed to evaluate the efficacy of Stimuvax, in combination with hormonal therapy, in patients with inoperable, locally advanced, recurrent, or metastatic breast cancer.

4. Vaccine with Tumor Cells or Tumor-Cell Lysates

Theoretically, tumor-cell vaccines have at least 3 advantages over the single-target approaches discussed above in terms of eliciting an immune response: (a) different and unknown antigens can be targeted at the same time; (b) the immune response is not HLA-restricted; (c) the variety of both MHC class I and class II epitopes processed is likely to be able to stimulate both an innate (NK cells, macrophages, and eosinophils) and adaptive ($CD8^+$ and $CD4^+$ T cells) response.

The first important distinction is between vaccines using autologous (patient-specific) or allogeneic (nonpatient-specific) tumor cells. Second, these cells may be unmodified, modified for expression of MHC, costimulatory molecules, or cytokines, or used in combination with adjuvants such as GM-CSF and Bacille Calmette-Guerin (BCG). Third, these cells can be used in the form of tumor-cell lysates [64].

The mechanism for priming naïve T cells in response to whole-cell or lysate vaccination is still unclear. Tumor antigens are probably phagocytosed by DCs and cross-presented to $CD8^+$ cells by MHC class I molecules. In some models, a $CD4^+$ response seems to be required for effective tumor rejection [65, 66]. A mesothelin-specific $CD8^+$ T cell response has been shown in a clinical trial employing vaccination with GM-CSF-secreting pancreatic cancer cell lines. The results of this study provide the first direct evidence that a cross-priming mechanism mediated by professional APCs is involved in a postvaccination induction of $CD8^+$ T cell response [51].

In the past 20 years, several different vaccines derived from whole tumor cells or tumor-cell lysates have been evaluated in preclinical models and clinical trials. OncoVAX (Vaccinogen) is composed of autologous irradiated tumor cells, with or without BCG as an adjuvant. In a multicenter phase III clinical trial, 254 patients with stage II and III colon cancer were randomly assigned, after curative resection for primary tumor, to receive OncoVAX or no adjuvant treatment [13]. The 5.8-year median follow-up showed a 20.4% reduction in risk of disease progression in patients receiving OncoVAX compared to the control group. Analysis by stage showed no significant benefit of OncoVAX in stage III disease, whereas a statistically significant improvement in recurrence-free survival in stage II was reported, with a 41.4% reduction in relative risk of disease progression ($P = .018$) in the OncoVAX arm. The OS rate for the OncoVAX-treated group was higher compared to control,

with an 11.1% and a 33.3% relative risk reduction in all patients and stage II patients, respectively [14]. Besides the clinical data and a prospective study of medical and economic benefits, the only immunological mechanism proposed by the authors was the presence of a significant delayed cutaneous hypersensitivity response to tumor cells after the third and fourth OncoVAX treatments (which lack BCG), as a measure of the immugenicity of the treatment, potentially correlated with long-term survival [15].

Reniale (LipoNova) is a vaccine designed to treat RCC. It is based on a lysate of autologous tumor cells, preincubated with IFN-γ to increase the antigenicity of these cells, and tocopherol acetate to protect cell membranes during the incubation process. A randomized, open-label, multicenter phase III clinical trial compared adjuvant treatment with Reniale after radical nephrectomy versus radical nephrectomy alone in nonmetastatic RCC (pT2-3b, pN0-3, M0) [16]. Prior to surgery, 558 patients at 55 institutions in Germany were enrolled in the trial and were randomized to receive 6 s.c. vaccinations at 4-week intervals, or no adjuvant therapy (control group). The intention-to-treat (ITT) population consisted of 379 patients in the primary analysis (177 patients in the vaccine group and 202 patients in the control group). Progression-free survival at 5 years for patients at all tumor stages was 77.4% in the Reniale group and 67.8% in the control group ($P = .0204$). Interestingly, patients with a higher risk (T3 subgroup) showed greater benefit from adjuvant treatment with Reniale, with a 5-year PFS of 67.5% in the vaccine group and 49.7% in the control group. A secondary ITT analysis on 477 patients (233 patients in the Reniale group and 244 patients in the control group) showed a statistically significant advantage in the experimental arm in terms of PFS ($P = .0476$); there was no statistically significant difference in OS between the 2 arms ($P = .1185$). However, a per-protocol analysis of 352 patients revealed a statistically significant increase in PFS ($P = .024$) and OS ($P = .0356$) in the vaccine arm [17]. No immunological data from this study have been reported.

GVAX for prostate cancer (Cell Genesys) is an allogeneic vaccine composed of 2 irradiated human prostate cancer cell lines, LNCaP and PC-3, modified by ex vivo transduction with an adenoassociated viral vector encoding the human GM-CSF gene. A preclinical study has demonstrated that s.c. administration of these cells invokes a local immune response, characterized by a local infiltration of neutrophils, $CD4^+$ T cells, and apoptotic cells. The irradiated tumor cells persist and secrete high levels of GM-CSF at the injection site for >21 days. Theoretically, secretion of GM-CSF by allogeneic tumor cells improves the antigen presentation of TSAs and TAAs through recruitment and maturation of DCs at the site of immunization. DCs then migrate to draining lymph nodes and activate antigen-specific $CD4^+$ T cells, characterized by the production of both Th1 and Th2 cytokines. Moreover, DCs may efficiently capture apoptotic tumor cells and cross-present multiple TAAs on MHC class I molecules for recognition by host $CD8^+$ T cells, as demonstrated by the ability of GM-CSF-secreting tumor cells to generate T-cell responses to multiple TAAs capable of targeting antigenically related but distinct tumors [67]. Based on encouraging clinical and immunological responses in 5 phase I/II clinical trials with nearly 200 prostate cancer patients [68–70], 2 phase III trials were initiated. VITAL-1 completed patient accrual in 2007, enrolling 626 patients with mHRPC randomized to receive GVAX as monotherapy for up to 6 months or standard docetaxel chemotherapy. The primary endpoint of the trial was improvement in OS. In 2008, Cell Genesys terminated the trial based on the results of a futility analysis conducted by the study's Independent Data Monitoring Committee (IDMC), which indicated a <30% chance of meeting the primary endpoint. VITAL-2 was a phase III trial designed to compare GVAX plus docetaxel versus docetaxel alone in mHRPC. The primary endpoint of VITAL-2 was also improved in OS. The trial was initiated in 2005 and enrolled 408 patients. In 2008, Cell Genesys announced its decision to terminate VITAL-2, as recommended by a safety review in which the IDMC reported an imbalance in deaths between the 2 treatment arms (67/114 deaths in the GVAX plus docetaxel arm and 47/114 deaths in the docetaxel-alone arm). In this case, despite encouraging preclinical and immunological data, GVAX failed to meet the defined endpoints of both phase III clinical trials [6].

Further clinical trials, employing GVAX cancer immunotherapies, are underway and include pancreatic and breast cancers. A randomized three-arm clinical trial is currently evaluating the efficacy and toxicity of GVAX for pancreatic cancer (GM-CSF secreting allogeneic pancreatic cancer vaccine) administered either alone or in combination with either a single intravenous dose or daily metronomic oral doses of cyclophosphamide for the treatment of patients undergoing chemotherapy and radiation therapy for stage I or II disease, surgically resectable.

Recently, studies of combination therapies of GVAX vaccine and CTLA-4-blocking antibodies have shown activity in melanoma and ovarian carcinoma, representing a potential new strategy to enhance vaccine-mediated antitumor effects [71].

5. DNA and RNA Vaccines

DNA-based vaccines are a recently developed strategy that has proven capable of activating strong immunity against weak TAAs. Several approaches have been developed and evaluated for enhancing the potency of DNA-based vaccines, including improved delivery systems (Gene Gun, cationic liposomes) [72, 73], simultaneous administration of cytokines (GM-CSF or IL2) [74], and the use of separate plasmids encoding nonself-antigens (i.e., hepatitis B surface antigen) [75]. The immunogenicity of DNA-based vaccines can also be enhanced by various modifications of plasmid-encoded antigens [76, 77].

Recently, several phase I/II clinical trials employing DNA-based vaccines targeting different TAAs (i.e., PSA, PAP, gp100, CEA, hsp65) have been conducted in patients with prostate cancer [78, 79], melanoma [80, 81], colorectal cancer [75], and head and neck carcinomas [82]. In all these trials, DNA-based vaccines were administered either as

monotherapy or in association with different delivery systems and adjuvants. In terms of immune response, most of these trials showed a low immunogenicity of TAAs. The small sample size of these phase I/II studies precludes achieving a statistical correlation between development of an immune response and clinical outcomes in vaccinated patients. Evidence of clinical benefit must be evaluated in larger studies.

mRNA-based gene transfer vaccines are another attractive immunotherapeutic approach to cancer treatment [83, 84]. This method, based primarily on transient transfection of nondividing cells, is regarded as pharmaceutically safe because the transfected mRNA does not integrate into the host genome [85]. In addition, high transfection efficiency can be achieved by electroporation [86, 87]. mRNA, which can be effectively overexpressed in target cells, is generated by in vitro transcription from a bacteriophage promoter-equipped plasmid DNA. It is composed of a cap structure at the 5′ end, the coding RNA for target antigen, and a tail of poly-adenosine (polyA tail) [88]. The target antigen used can be a single peptide PSA [89] or CEA [90], allogeneic cancer cell lines [18, 91, 92], or autologous tumor mRNA [93]. The mRNA-based vaccine containing the mRNA-coding TAA is transfected into DCs and translated into proteins. After protein processing, the antigen can be loaded on MHC molecules for antigen presentation, thus activating an antigen-specific CTL response [94].

Clinical trials have been performed employing mRNA-transfected DCs or injecting mRNA directly into patients with prostate cancer [18, 89, 95], RCC [96], ovarian cancer [97], lung cancer, breast cancer [90], pediatric brain cancer [98], neuroblastoma [99], and melanoma [100, 101]. A phase I clinical trial was performed using PSA-mRNA-transfected DCs in patients with metastatic prostate cancer [89]. When the effects of repeated vaccinations with PSA-mRNA-transfected DCs were examined, the results demonstrated that the vaccine was able to increase PSA-specific CTL responses.

In a phase I/II clinical trial in androgen-resistant prostate cancer, patients were vaccinated with DCs transfected with mRNA from 3 allogeneic prostate cancer cell lines (DU145, LNCaP, and PC-3) [18]. Twelve of 19 patients showed specific T-cell responses; 10 of those 12 had a positive response in IFN-γ by ELISPOT assay and 9 had a specific T-cell proliferation response. Two $CD8^+$ CTL clones were generated from a patient who showed a positive response in both the ELISPOT and proliferation assay. The CTL clones demonstrated specific killing of tumor mRNA-transfected DCs and PC-3 cells. Of the 19 patients on-study, 11 showed stable disease and 10 developed specific T-cell responses; only 2 of 8 patients with disease progression showed T-cell responses. These results demonstrate a correlation between immune response and clinical response.

In another clinical trial, patients with metastatic RCC received a vaccine consisting of DCs transfected with total RNA extracted from clear cell carcinoma, with or without $DAB_{389}IL2$ prevaccination [97]. The results showed a significant increase in the frequency of tumor-specific $CD4^+$ and $CD8^+$ T cells as well as a decrease in Treg frequency. This trial demonstrated that mRNA-transfected DCs can increase immune response, and that this immune response in combination with depletion of Tregs can have a synergistic effect on antitumor immunity.

6. Conclusions

The promising results of recent phase II/III clinical trials may herald a new era for cancer vaccine immunotherapy. However, in spite of exciting improvements in the activity and efficacy of various vaccine platforms, including objective response, disease-free survival, progression-free survival, and overall survival, there is still much to learn about the immunological mechanisms by which these results can be improved. Further research is required to improve our understanding of CTL antigen-specific activation, decreased Treg numbers and functionality, NK activation, antigen cascade, and the impact of tumor escape.

A paradigm shift is necessary in order to improve the design of immuno-oriented clinical trials, increase understanding of the balance between proinflammatory and immunosuppressive responses in antitumor immunity, and define new criteria for the immunological evaluation of antitumor activity and clinical outcomes. Such knowledge would not only improve the efficacy of cancer vaccines but would help to guide decisions regarding patient selection, vaccine scheduling, and the combination of vaccines and other treatment modalities such as surgery, radiotherapy, chemotherapy, and targeted therapy.

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Review Article

Bridging Innate and Adaptive Antitumor Immunity Targeting Glycans

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Effective immunotherapy for cancer depends on cellular responses to tumor antigens. The role of major histocompatibility complex (MHC) in T-cell recognition and T-cell receptor repertoire selection has become a central tenet in immunology. Structurally, this does not contradict earlier findings that T-cells can differentiate between small hapten structures like simple glycans. Understanding T-cell recognition of antigens as defined genetically by MHC and combinatorially by T cell receptors led to the "altered self" hypothesis. This notion reflects a more fundamental principle underlying immune surveillance and integrating evolutionarily and mechanistically diverse elements of the immune system. Danger associated molecular patterns, including those generated by glycan remodeling, represent an instance of altered self. A prominent example is the modification of the tumor-associated antigen MUC1. Similar examples emphasize glycan reactivity patterns of antigen receptors as a phenomenon bridging innate and adaptive but also humoral and cellular immunity and providing templates for immunotherapies.

1. Introduction

It is a widely held view that the engagement of both innate and adaptive cellular immune responses is necessary for efficient immunotherapy of cancer [1, 2]. Using vastly different molecular and cellular strategies, they operate in concert targeting tumor antigens [3–5] albeit cancer cells escape this immune surveillance via a variety of mechanisms [6, 7]. Consequently, in contrast to the long-held belief that most cancers are weakly immunogenic [8], antitumor immune surveillance mechanisms prove much more prevalent. The observation of naturally occurring tumor reactive B [9, 10] and T lymphocytes [11–15] along with tumor reactive innate immune cells that include natural killer (NK) cells, granulocytes, and macrophages, raises hopes that immunotherapy may succeed in targeted patients, specifically those with either regional or minimal residual disease and in the preventive setting [16–20].

While tumor cells are not usually in the focus of the immune system, Burnet and Thomas hypothesized that the immune system can recognize nascent transformed cells and can eliminate primary tumor formation [21]. The process of immune editing validates the operative nature of immune surveillance, suggesting that at some point antitumor immune surveillance was working and that immune editing is a form of escape from immune surveillance. Such observations also lead to the hypothesis that downstream suppressive mechanisms may dominate antitumor immune responses, permitting malignant cells to evade an effectively primed immune response. As we come to understand immune surveillance mechanisms better, these mechanisms should prove a useful template for immunotherapy design, but also instructing on which patient populations might benefit the most from immunotherapy approaches. Immune surveillance, as a model and rationale for immunotherapy, requires that cancer cells are recognized as "nonself" or may be "perturbation of self." Often the ability of inducing a rejection strength response has been associated with appearance of xenoantigenic determinants but now the other dimension of immunogenicity—the danger signal [22] is recognized as even more important.

Natural immunity is the first actor on stage in immune surveillance processes, with polyspecific IgM antibodies, typically reactive with "xenotypic" or "neotypic" carbohydrate antigens, playing a role in surveillance [9, 23]. Antitumor immunity mediated by naturally occurring antibodies was first noticed in mice [23]. Naturally occurring antibodies to Tumor-Associated Carbohydrate Antigens (TACAs) are observed in cancer patients and are attributed to patient longevity [9, 24]. Whether the B cells producing these antibodies, coupled with T cells, represent an inducible component of cancer immunosurveillance—and one that might be therapeutically exploited—remains unknown. Tumor reactive activated B cells can collaborate with effector T cells in adoptive cellular therapies of cancer. Activated B cells can be used as effective antigen presenting cells (APCs) for T cell sensitization. In this context tumor-primed B cells might collaborate with effector T cells in adoptive cellular therapies of cancer. Other B cell subsets (Bregs, IL-10 producing, etc.) may rather impair T cell responses.

Unlike the clonally organized adaptive immunity, the *cells in the innate system bind to* nonself determinants quasi-specifically, using pattern-recognition receptors. A prominent antigen type that fits this bill are carbohydrates. Due to dysregulation of glycoslyation events, carbohydrates are often altered and/or overexpressed as multivalent molecular species [25] on the tumor cell surface, possibly representing a type of danger signal [22] to the immune system. In fact the Gal (Galα1-3Gal) antigen, is the major target of preexisting antibodies in tissue rejection in xenotransplantation [26] validating carbohydrates as targets for tissue rejection and carbohydrate expression patterns as danger signals. These xenoreactive natural antibodies comprise 1% of circulating IgG. Furthermore, anti-Gal antibodies can be exploited for clinical use in cancer immunotherapy by targeting autologous tumor vaccines engineered to express the Gal antigen to APC, thereby increasing vaccine immunogenicity [26].

In this paper, focus is placed upon the role carbohydrate binding receptors and cells expressing them play in bridging innate and adaptive immune responses to cancer cells. Carbohydrate antigens are targets of the immune surveillance machinery and these responses provide a template for cancer immunotherapy. Of particular importance is how carbohydrate reactive lymphocytes and cells of the innate immunity might mediate cellular responses. This includes the role glycopeptides and carbohydrate mimetic peptides might play in integrating innate and adaptive antitumor responses. Certain peptides act as molecular mimics of carbohydrates in that carbohydrate-binding proteins specifically recognize them but may also activate tumor reactive T cells [27–29]. Naturally occurring carbohydrate mimics include peptide regions from MUC1 [30]. Carbohydrate mimetic peptides as mimics of glycopeptides, glycoproteins, and carbohydrate structures provide an interesting bridge to facilitate B and T cell interactions. While making for vaccine candidates, they also provide a tool to probe carbohydrate immunology paradigms because the synergistic interaction of effector T and B cells require common recognition of identical tumor-associated antigen(s).

2. Carbohydrates and the Immune Surveillance Paradigm

The success of vaccines in infectious disease prevention, together with the evidence for immune control over tumor growth, are major arguments in favor of immunotherapy or cancer vaccine approaches. Although the main processes involved in these two aspects of immunity are mostly the same, the context is different. A major difference between microbial pathogens and tumors as potential vaccine targets is that cancer cells are derived from the host, and express mostly self-antigens present in normal cells. In terms of antigenic properties and "danger" signals, an infection is an external perturbation followed by a reaction to it as the system is returning to its previous or very close equilibrium. Cancer, for its part, is a gradual transformation of the very equilibrium state of the system. It can be seen as a disease of "selfish" tissue proliferation that causes pathological immune tolerance as its own provision. Now it is clear that the difference between self and nonself is defined by a complex set of tolerance mechanisms beyond the absence of antigen specific reactivity. Understanding tolerance as active, threshold-dependent, and redundant helps rationalize its reshaping and repair, rather than breaking, as a tumor immunotherapy objective [31].

The task of designing immunotherapies that can effectively impact on the survival of cancer patients remains challenging. Two important elements in this challenge are defining antigenic formulations that target multiple antigens associated with tumor cells [31], and to understand the therapeutically effective range (frequency) of effector cells for antitumor protection [14, 32]. Sugars display high density on tumor cells and often play a critical role in immune recognition [33–37]. In addition, often times they are neoantigens as many are embryonic in nature. As suggested recently, it's the antigen stupid [20], one needs not look beyond carbohydrate targets, because TACAs prove to be broad-spectrum antigens or "universal tumor antigen" targets for immune surveillance [9, 31] by antibodies, cells of the innate immune system and lymphocytes. Despite clinical evidence for glycoantigens as important cancer targets they are still largely neglected. Notes of caution come mostly in the context of low immunogenicity of carbohydrates and their dual role as "danger" or immune suppressive signals [38]. Nevertheless, sufficient body of evidence indicates that the unique immunomics of tumor-associated glycoantigens may yield clinically important biomarkers and treatment targets for the management of human cancer [39].

Adaptive immune responses have long been considered the territory of antigenic proteins because they are T cell dependent (TD), whereas carbohydrates are characterized as T-cell-independent (TI) (either Type 1 or Type 2) antigens [40]. Typically, adaptive responses depend on the recognition of protein sequences by T helper (Th) cells. In addition, thymus-independent responses may be related to a more or less sequestered compartment of the antibody repertoire [41, 42]. Yet, T cells reactive with processed glycopeptides as well as glycolipids have been described [43]. The running paradigm is that albeit CD1 presentation to NKT cells is

possible [44], pure carbohydrate forms are believed not to be associated with T cell presentation. Nevertheless, some types of carbohydrates are processed and presented to T cells by class II MHC [45, 46]. Carbohydrate antigens displaying helical shapes [47], which mimic helical peptides, are suggested to bind directly to at least the MHC Class II grove, indicating that carbohydrate antigens devoid of lipids and protein can directly influence T cell proliferation [48]. Model carbohydrates with a zwitterionic structure appear to affect T cell activation, being responsible in preventing abscesses induced by pathogenic bacteria [49]. Taken together, these advances illustrate an indisputable viewpoint that carbohydrate recognition by the adaptive and innate immune system is an indispensable stage of most immune responses.

The induction of an effective response to tumors mainly depends on innate and adaptive immunity coordinated by Dendritic Cells (DCs). DCs in particular are well equipped to distinguish between self- and nonself-antigens by the invariable cell-surface receptors such as C-type lectin receptors (CLRs) and Toll-like receptors (TLRs). CLRs are adept at recognizing glycoproteins in general, while typical pattern recognition receptors, such as TLRs, detect various molecular patterns typical only for microbial antigens. Uptake of antigen by CLRs leads to presentation on MHC class I and II molecules. It is likely that the crosstalk between TLRs and CLRs, differentially expressed by subsets of DCs, account for the different pathways to peripheral tolerance, such as deletion and suppression, and immune activation [38]. Several pathogens specifically target CLRs to subvert this communication to escape immune surveillance, either by inducing tolerance or skewing the protective immune responses [50]. It is likely that tumor cells do the same as they adapt their glycan shield to immune effector cells.

Lessons can be learned from immune surveillance mechanisms associated with natural antibodies. Apes, old world monkeys, and humans do not express the Gal epitope on their tissues, and therefore have circulating life-term-generated anti-Gal antibodies due to antigenic stimulation by bacteria of gastrointestinal flora [51, 52]. Human anti-Gal antibodies are of IgM and IgG isotypes [51, 53, 54]. Anti-Gal antibodies responsible for hyperacute rejection of pig-to-primate xenotransplantation are mostly IgM and apparently work through a complement dependent mechanism. If the hyperacute rejection is prevented, after a delay an acute vascular rejection will occur, in which IgG antibodies play a major role [55, 56]. All IgG subclasses are present in the postimmune serum [56] and rejection is dependent on complement and Fc receptor [57]. Therefore, as expected, exposure to the respective antigen will trigger a secondary response mostly of specifically induced and cross-reactive IgG.

Anti-Gal antibodies are purported to augment the uptake and presentation of tumor antigens by antigen-presenting cells to induce higher immune responses [58, 59]. This uptake mechanism might relate to TACAs as well. Two TACAs that are highly thought of as immune targets are the Thomsen-Friedenreich (TF or T) antigen (Galb1-3GalNAca) and the Tn (GalNAca) antigens [60]. Human blood serum contains natural TF- and Tn antibodies whose subpopulations may bind the corresponding antigens on human tumor cell lines [61, 62]. The high level of anti-TF and Tn IgG, observed in some patients with cancer, may be a sign of an ongoing immune response, as indicated by the switching of antibody to the IgG-class. They may be also cross-reactive antibodies raised to other structurally similar carbohydrate forms. Blood Group B individuals, for example, may show reactivity to Tn antigen [63], and some anti-Gal antibodies are cross-reactive with the Blood Group B antigen [64] and Figure 1.

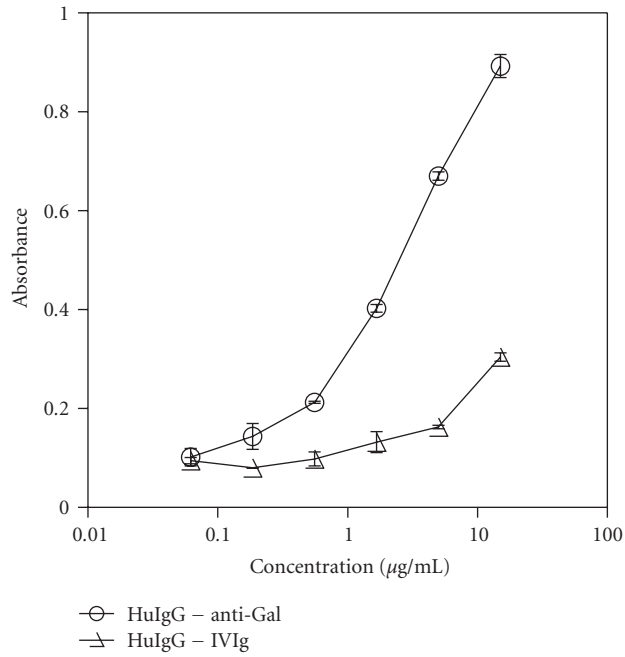

Figure 1: A. ELISA plates were coated with blood type B antigen and reactivity of Gal purified human antibodies detected. Human IVIg was used as control.

A high level of anti-TF and Tn antibody titers in long-term cancer survivors are supportive of the idea that patients with a disease of an early stage or with a minimal residue are more responsive to active immunotherapy [65, 66]. The detectable spontaneous immune responses to TF and Tn antigens are not necessarily efficient since the expression of these antigens correlates with worse prognosis mostly because of increased metastasis [67]. The reason may be an escape from the control by immune responses to TACA like TF, Tn, and sialyl-Tn enforced by the biological function of these antigens, since immunoediting would select cells that suppress the expression of TACA. It is also possible that the correlation with higher grade and metastasis is due to the observation only of these tumors that have been resistant to immunoediting and, respectively, the lowered malignancy of the immunoedited tumors. It would be interesting to differentiate between primarily TACA negative tumors and secondarily negative due to immunediting. It is likely that specific suppressive influence of the tumor on the production of TF antibodies is associated with the stage and grade of the

tumor. The observation of positive correlation between the level of TF antibodies and the count of lymphocytes in TF-responders appears to reflect the adaptive immune response and provides a further explanation for the involvement of anti-TF IgG in cancer-associated immunosuppression. However, the possible protective mechanism of TF antibodies in cancer has yet remained unclear as is the role antibodies play in the natural anticancer defense system.

3. T Cells and Glycans

The paradigm of T cell recognition of foreign antigens is principally established by studies of the host immune response to proteins and peptides. However, the demonstration that T cells can recognize nonprotein antigens has modified ideas on the chemical nature of molecules recognized by T cells [68]. In the early years, it was suggested that hapten-specific T cells recognize hapten-modified peptides [69]. Chemical haptens and metal ions interact with proteins and thereby become recognizable by T and B lymphocytes. At the same time, they induce the production of proinflammatory cytokines and chemokines by various cell types due to triggering of innate immune responses. This is an important prerequisite for the activation of the adaptive immune system and the development of diseases like allergic contact dermatitis and adverse drug and autoimmune reactions. But they may also provide concepts relevant for immunotherapy of cancers, as hapten model systems are not that dissimilar to peptides modified with small carbohydrate antigens. Hapten-specific T cells can detect antigenic structures in two ways. They can recognize the hapten only in context with a specific amino acid motif or independent of the carrier peptide.

In the last decade, a number of researchers report that both $CD4^+$ and $CD8^+$ T cells can recognize glycopeptides carrying mono- and disaccharides in a MHC-restricted manner, provided the glycan group is attached to the peptide at suitable positions [70–82]. For such glycopeptides, the primed T cells recognize the glycan structure with high fidelity. These observations are very important in understanding the complexity of the antitumor response targets especially in terms of abnormal expression.

Due to an incomplete formation of glycan sidechains resulting from premature glycosylation events, the restrictive distribution of short glycans such as the TF, Tn, and sialyl Tn (NeuAca2-6GalNAca) in normal tissues and their extensive expression in a variety of epithelial cancers make them excellent targets for immunotherapy. Immunodominant glycopeptide remnant epitopes as well as glycosylation changes on self-proteins can generate antitumor responses. For example, anti-sTn T helper type 1 (Th1) antigen-specific T-cell response as determined by interferon-γ, has been noted in patients immunized with a sialyl-Tn-conjugate [83].

The carbohydrate-based design for T cell antigens is strongly supported by several HLA/peptide complexes resolved by crystallography [74, 80]. The structure of different crystals describes the core of the peptide(s), as critical for TCR recognition with a "cavity" corresponding to the CDR3 region that often accommodates aromatic amino acid residues, similar in size and conformation to small glycan molecules, as TF, or the monomer Tn. The ability of TACA-specific T cell clones to recognize glycan antigens in the context of different peptide sequences is very relevant to validate this imunotherapeutic approach. The ability of T cells to recognize mono-and disaccharides attached to peptides with Ser or Thr might indicate that T cells might be degenerate in recognizing glycopeptides (Table 1). In some cases CTL, generated upon immunization with glycopetide, preferentially kill target cells treated with glycopeptide compared to those treated with the core peptide. In other cases it does not matter [81], and in some cases it has been suggested that other glycan receptors are involved in T cell targeting [84]. Polyclonal CTL have been observed to kill target cells expressing glycolipid [43]. It has been suggested that glycopeptide-specific-restricted CTL and unrestricted glycan-specific CTL belong to different T cell populations with regard to TCR expression [70]. Such results demonstrate that hapten-specific unrestricted CTL responses can be generated with MHC class I-binding carrier peptides.

The development of designer glycopepitdes based upon high affinity binding to MHC Class I or Class II is an acceptable practice for enhancing T cell responses [72, 82, 85]. The design of such glycopeptides usually starts with some concept of a target sequence and then modifying it based upon amino acid substitution guided by some sense of change in binding affinity of the peptide core for MHC. Central to these peptides is a Ser or Thr at position 4–6 to which a glycan antigen is attached (Table 1). While promiscuous MHC binders are possibly the optimal carriers for the carbohydrate epitopes, it is not clear where to choose potential peptide cores from. The use of epitopes from widespread viruses, like influenza virus, may not be the best solution. Instead of providing a recall immune response, such peptide cores may be recognized by virus specific T cells as altered peptide ligands and actually prevent the immune response. Another adverse phenomenon that can be anticipated in this case is original antigenic sin.

An alternative to glycopeptides are carbohydrate mimetic peptides (CMP), which are non-glycoslyated but can induce T cells reactive with glycopeptides or induce T cells that are MHC unrestricted. CMPs function as xenoantigens and, consequently, can overcome tolerance to carbohydrate self-antigens. CMPs from natural proteins are known for some time. Peptides from MUC1 are shown to be naturally occurring CMPs [30, 86–89]. In particular, MUC1 derived peptides are considered mimics of the Gal-epitope [86, 89]. In our own studies CMPs, as defined by cross-reactivity to TACA reactive antibodies and lectins, induce Th1 responses [90] and antitumor $CD8^+$ T cells [91]. Molecular modeling studies indicate that amino acids can themselves be structurally similar to mono-and disaccharides on glycopeptides [29]. CMP reactive T cells can also be activated by the TACA that they mimic [90]. Unlike unconjugated carbohydrate antigens, we have shown that unconjugated multivalent antigen peptide forms of CMPs prime for subsequent memory of unconjugated carbohydrate antigens, facilitating long-term surveillance through recall of carbohydrate immune

Table 1

MHC Alleles	Total Binders	Binders having Ser at Position			Binders having Thr at Position		
		4	5	6	4	5	6
HLA-A∗0201	1497	92	64	87	62	81	66
H2-B	16	1	0	2	0	1	0
H2-D	92	4	5	7	3	2	2
H2-Db	249	28	12	19	10	5	10
H2-Kb	319	13	4	15	18	6	20
H2-Kd	325	20	15	9	14	18	20
HLA-A	5325	268	265	414	170	272	252
HLA-B	2963	150	178	283	170	141	126

responses [92]. This effect is a major advantage that would minimize the need for constant boosting. Furthermore, we observed that CMPs mediate cognate B and T cell interactions as CMPs can induce antibodies in a host model with deficiency in IgM production that typically do not respond to carbohydrate antigens [93]. These studies stress the role of B cells as APCs as part of the CMP effect in vivo. Particular B cell subsets may affect differentially tumor reactive T cell involvement in this process. More importantly, the type of TACA mimicked by the CMPs are expressed in mice. Consequently, these studies are obtained in a toleragenic model, further suggesting that tolerance is broken upon CMP immunization.

4. Bridging the Divide between TACA Reactivity and CD4$^+$ T Cells

Tumor immunotherapy with nonmutated tumor-associated antigens attempts to break tolerance in a manner reminiscent of models for autoimmune diseases. When autoimmune diseases are induced in animal models, they are often self-limiting, which is rarely the case with spontaneous autoimmune pathology. It may be possible to use a similar model of induced autoimmune inflammation as a strategy for an effective tumor immunotherapy since cancer patients in general are not genetically prone to autoimmunity. Although it has been expected and conceptually implicated in different approaches, this notion was directly addressed only recently [94]. The dissection of tumor immunity indicates roles for B and T lymphocytes that include CD4$^+$ T cell responses of the T-helper type-1 (Th1) phenotype [95]. There is adequate evidence to prove a central role of the CD4$^+$ T cell in antitumor immunity [96–98]. This is possible through direct communication between the tumor cell and CD4$^+$ T cell through interferon gamma (IFNγ), IFNγ receptor interactions or through tumor stroma interactions [97].

Delayed Type Hypersensitivity (DTH) reactions are a means to monitor CD4$^+$ T cell responses to the immunizing agent and in some cases DTH responses in cancer patients correlate with survival. It has been postulated that the Th1 cell is the "inducer" of a DTH response since it secretes IFN$\boldsymbol{\gamma}$, while the T-helper type-1 (Th2) cell is either not involved or acting as a downregulator of the cell mediated immune response [99]. Although typically inducing T cell independent responses, surprisingly, some carbohydrate targeting vaccines have induced also DTH responses in humans [100–102].

Early studies using xenogenized cells suggest that the mechanisms of antiparental tumor protection involve specific induction of a DTH response mediated by the inflammatory Th1 subset of L3T4+ T lymphocytes and IFN-activated macrophages [103]. Tumor cells can mediate DTH responses, necessary for tumor regression [104]. Typically, at the time of immunization, DTH-effector T cells are activated by binding to complexes of antigen peptides and MHC molecules on APCs. Subsequently, at skin challenge with antigen, DTH is elicited involving at some point recruitment of antigen specific memory T cells into the tissues and recognition of Ag/peptide-MHC complexes on presenting cells. This leads to a characteristic late 24- to 48-hours effector responses.

The earliest events in DTH have only recently been shown to depend on B-1 B cell produced IgM, immune complexes and complement [105]. B-1 cell-derived anti-hapten IgM antibodies generated within 1 day (d) of immunization combine with local challenge antigen to activate complement and recruit T cells [106]. These latter findings overturn three widely accepted immune response paradigms by showing that (a) specific IgM antibodies are required to initiate contact sensitivity (CS), which is a classical model of T cell immunity thought exclusively due to T cells, (b) CS priming induces production of specific IgM antibodies within 1 d, although primary antibody responses typically begin by day 4, and (c) B-1 cells produce the 1-d IgM response to CS priming, initiating the DTH response, while B cells are generally considered suppressive in tumor immunity due to their production of IL-10 [107]. Finally, these findings indicate a possible mechanistic scenario for interaction between anticarbohydrate and Th1 responses.

As clinical correlates have highlighted carbohydrate reactive IgM responses to cancer cells in humans [24, 108], attention to antibody subsets is warranted to further understand and develop strategies to augment these responses, which might further impact on tumor reactive

cellular responses. Natural polyreactive antibodies that bind to tumor cells have been studied on several occasions. They are germline encoded antibodies mostly from $CD5^+$ B cells (the B1 genotype), they bind to various tumor antigens, induce apoptosis of tumor cells and detect not only malignant cells but also the precursor stages [10]. Natural polyreactive IgM autoantibodies, encoded by unmutated germline Ig V genes, represent a major fraction of the normal circulating IgM repertoire [109]. Such antibodies fulfill the definition of autoantibodies as they are self-reactive but they have broad reactivity and bind mostly to altered antigens. Even in mouse models, nonimmunized mice of widely differing genetic backgrounds have detectable IgM antibodies to tumor cell surface carbohydrates, their natural resistance to this tumor being related to their serum levels [110].

Because B1 cells can strongly activate T cells and induce Th1 cell differentiation by antigen presentation [111], we have been testing for CMP mediated T cell responses. Although cognate, MHC-restricted interaction of Th cells with antigen-presenting B cells provides effective help to a resting B cell, substantial B cell responses are also observed with preactivated T cell clones in a noncognate fashion (bystander help without specific Ag recognition) [112]. We have recently shown that immunization of mice with a carbohydrate mimetic peptide reactive with GD2 reactive antibodies induce GD2 reactive IgM antibodies [113]. This CMP also induces a DTH response to GD2 positive D142.34 cells, while no response was observed against the GD2 negative expressing cell line B78.H1 [114]. Consequently, our results on DTH induction can be interpreted in the following way. The anti-GD2 IgM induced by CMP plays the role of an initiating factor for a DTH response perpetuated by T cells cross-reactive with CMP and an unknown antigen on the tumor cells line, which have been stimulated during the priming with CMP.

This observation suggests that the dual character of a CMP carrying T cell epitopes, but also mimicking an unrelated TI-2 carbohydrate epitope, provides for long term IgM responses by promoting other aspects of cooperation between particular B cell subpopulations and CMP specific T cells reminiscent of epitope spreading [115]. One concept is that cognate interactions occur because the CMP is presented by MHC Class II on the B cell, stimulating concomitant peptide reactive T cells [28]. It is interesting to note that a very recent study by Deola et al. found another mechanism for noncognate T cell2-B cell interaction involved in the propagation of CTL longevity [116] and dependent on CD27. This antigen is considered a marker of human memory B cells. It is expressed also on human peripheral IgM "memory" cells that are related to marginal zone B cells. In their study, Deola et al. also demonstrate that the T-B contact initiates a chemotactic mechanism for a targeted serial noncognate T-B cell contacts. Such an intensive interaction in the absence of specific antigen recognition may be a prerequisite for a much more important role for T cell derived signals to bystander B cells.

The highly protective antibody responses mounted particularly by B1 cells clearly indicate a crucial role for this subset of B cells in TACA-targeting immunotherapy, but targeting B1 cells may actually skew Th1 responses for any antigen [111] which is highly desirable in antitumor cell response. DTH responses require both B1 and $CD4^+$ T-cell cognate recognition [117]. Precisely establishing the role of B1 cells in this respect relates not only to a possible long-term IgM response but, more importantly, circumventing a possible involvement of $CD1d^{hi}CD5^+$ Bregs (B10 cells) [118].

These observations further suggest that we pay more attention to the role of the major B cell subpopulations, and that carbohydrate reactive IgM antibodies in particular may help to bridge cellular responses. Expanding the pool of memory B and T cells by CMP vaccination or to activate residual TACA memory B and T cells might be of benefit for the course of immunization. Multivalent CMPs target B1 cells, which are hypothesized to facilitate the Th1 responses observed with the CMPs [93]. Collectively, these observations suggest that targeting the "natural memory" B cell repertoire might provide novel mechanisms to prevent recurrence of disease mediated through $CD4^+$ T cell responses. Although the mechanisms by which immunological memory is maintained after infection or vaccination are related to TD responses [119], similar mechanisms may also apply to cancer vaccines that target TACAs. Therefore, we hypothesize that CMP's immunotherapeutic potential is related to their capacity to stimulate B cell compartments that bridge innate and adaptive immunity. The translational character of the proposed mechanisms depends on the specifics of the human immune system. The central issue in this respect is the identity and physiology of the B cell subpopulation(s) that best relate CMP specificity to Th1 stimulation with potential to expand an epitope-spreading cascade. The human equivalents of mouse B1a, B1b and MZ B cells are still to be identified, if at all possible.

Another major mechanism involving B cells, along with other antigen presenting cells, in the initiation of adaptive responses is the formation of immune complexes including by natural antibodies. A number of possible mechanisms of immune complex modulatory role have been proposed (reviewed in [120]) including the central event of immune complex retention by follicular DCs [121]. An essential feature of antibodies in this respect is the isotype distribution. It affects the involvement of different Fc and complement receptors and the subsequent functional effects. Among them of special interest in tumor immunotheray is antigen crosspresentation.

5. TACA Mediated Crosspresentation That Activates Cellular Responses

Much has been learned about the maze of signaling events and cast of molecular characters activating cellular responses, principally by studying antigen trafficking. In broad strokes, antigenic peptides are presented to cytotoxic T ($CD8^+$) and T helper ($CD4^+$) cells by two separate pathways—intracellular (originating in the cytoplasm, in complex with MHC class I) and extracellular (after internalization, in complex with

MHC class II). The same T cell signaling machinery picks between peptides presented in MHC class I or class II, guided by the TCR specificity and with the help of the coreceptors CD8 or CD4 [122]. The two processes, though, serve quite different roles. The economy of the solution goes further as the same type of pAPC serve as initiators, licensing the T cell response in both cases. Utilizing the same or very similar cellular and molecular mechanisms by both pathways necessitates that pAPC are susceptible to infection with any intracellular parasite or, alternatively, that the external pathway intersects or mimics sometimes the internal. The fact that CTL responses can be mounted to those rare viruses that do not infect pAPC is a key argument in favor of the interaction between the two pathways [123], or, as it has been branded, crosspresentation [124].

After acquiring antigen by the external pathway, cross-presenting pAPC can activate or cross-prime naïve $CD8^+$ T cells with respective specificity initiating CTL responses not only to viruses but also to subunit [125] and DNA vaccines [126, 127] and tumor cell derived antigens [128–130]. The crosspresentation of self -antigens, for example, from phagocytosed apoptotic cells including tumor cells, may play also a role in cross-tolerization of autoreactive $CD8^+$ T cells in the periphery [131–133].

The concept of crosspresentation has not been accepted without some resistance [134, 135]. Still, as long as interrupting Th responses due to pAPC killing by CTL is avoided, cross-priming is compatible with current views and unavoidable in the explanation of some immune phenomena. Understanding the observed CTL responses to tumor specific antigens [136–141] as well as the induction of CTL responses to tumors by appropriate vaccines [139, 142, 143] relies heavily on presenting of external antigens in class I. The hypothetical suppressive effect on other T cell responses due to killing of pAPC can be avoided, at least to some extent, by specialization of a particular subset of pAPC [144].

For carbohydrate antigens, crosspresentation can make use of CD1 or pattern recognition receptors. Antigen presenting cells interact with antigens through an array of pattern recognition receptors (PRR—mostly wide range of C-type lectins—CLR, TLRs, etc.) as well as through complexes with antibodies and complement utilizing Fcγ and complement receptors. All of these capture mechanisms lead to internalization and presentation in class II. Most pathways of antigen internalization by DC were considered potentially permissive also of crosspresentation. Many were proven to be so.

The cross-presenting immature mouse DC express a wide range of CLRs from type IV and type II [145]. Type IV CLRs include type I transmembrane proteins with multiple carbohydrate recognition domains (CRD) like CD205 (DEC-205) with still unknown specificity and CD206 that binds terminal mannose [146]. Expressed preferentially on $CD8^+$ DC, CD205 acts as a recognition receptor for dying cells, potentially providing an important pathway for the uptake of self-antigen in central and peripheral tolerance [147]. Its ligand specificity is still unknown. The mannose binding CD206 may be exclusively involved in the internalization and crosspresentation of soluble antigens [148]. Consequently, mannose has been used as a delivery vector for antigens in vaccine design [149, 150] and has been considered in terms of undesirable immunogenicity [151]. Some ligands of CD206 trigger the DC expressing it to respond with an anti-inflammatory immunosuppressive cytokine pattern [152].

Type II CLRs are also known as asialoglycoprotein receptor family. Among its members role in crosspresentation has been found for CD207 and CD209 (DC-DSIGN) [153, 154] recognizing broad set of sugars including fucose, mannose, N-acetylglucosamine, and sulfated sugars [155–158]. Another member of this family is dectin-1. Its primary pathogen associated pattern is β-glucan [159]. The pathogen recognition is usually in conjunction with TLR2 and TLR4 triggering [160] to yield either proinflammatory cytokine secretion when phagocytosis is hindered or immunosuppressive signal when the bound particles are internalized [161, 162]. This context helps interpret the meaning of the finding that dectin-1 participates in the uptake of cellular antigens by human monocyte-derived DC and the cross-tolerization to the antigens associated [163]. Another member of this family, which is structurally related to dectin-1, is DNGR-1 (CLEC9A)—a novel, highly specific marker of mouse and human DC subsets that can be exploited for CTL cross-priming and tumor therapy [164]. Like dectin-1 and CD205, it has been found expressed on $CD8^+$ mouse DC and it binds and internalizes for loading on class I apoptotic cell antigens [165]. Like CD205, its, so far unknown, intracellular ligand is protease sensitive [147, 165].

Thus, although most of the known CLR appear capable of mediating crosspresentation, the outcome is, as a rule, tolerogenic DC phenotype [166]. It seems that the signals are interpreted in terms of the coengagement of appropriate TLR receptors, which not only switch on the cross-presenting function for some of the CLRs, but also modify the immune context of T cell stimulation [33], for example, to immunogenic.

CLRs are a part of a general scavenging mechanism. The same receptors, that do the first rough sorting of dangerous nonself, recognize also out of place "self" as an equally dangerous signal. Several examples like CD205 and dectin-1 indicate that there may be a general overlap of the PRR for pathogen associated molecular patterns (PAMPs) and receptors of apoptotic cells as well as those for danger-associated molecular patterns (DAMPs). Common structural themes shape in this broad recognition processes. crosspresentation is associated with carbohydrate epitopes for the extracellular PAMPs and hydrophobic regions for both PAMPs and DAMPs. Another common feature is the polyspecificity of these interactions captured in the term "patterns". Polyspecificity is a basis for a functional mimicry, which in this case is not in the sense of a switched signal but in the context of converging signals from a class of ligands conveying common biological meaning [167]. The overlap of carbohydrate and intracellular hydrophobic binding sites may suggest a biological function of carbohydrate/protein mimicry—a structural marker of changes in the internal environment.

6. Antibody Mediated Crosspresentation

The most intrinsic immune component participating in crosspresentation not so much as an input signal, but more as a feedback, are the antigen specific antibodies. At some point in the infectious cycle, most intracellular pathogens reside in the extracellular space, where they are vulnerable to antibody action. Thus formed immune complexes target them to Fc receptors the crosslinking of which can have profound effects both as signal transduction in the APC as well as presentation pathway of viral antigens [168, 169]. The formation of immune complexes is the fundamental basis of using the Gal epitope to facilitate vaccine development [143]. Although $CD8^+$ DCs constitutively cross-present exogenous antigens in the context of MHC class I molecules, $CD8^-$ DCs are actually also capable of doing so after activation via crosslinking of FcγRs [170]. It was shown that crosspresentation of tumor antigens after capturing through FcγR on DC is much more efficient than after internalization of dead cells or loading with peptide [171]. The increased presentation was not due to enhanced tumor cell uptake or to DC maturation. Recent results with NY-ESO-1 peptide further reinforced the evidence for a key role of antibodies in tumor immunity through providing for crosspresentation to CTL [130]. These examples stressed the importance of raising specific humoral responses to tumors, but in antiviral responses even natural antibodies are found to mediate presentation to CTL [172]. Autoantibodies, on the other hand, are also found to contribute to autoimmunity promoting self-reactivity in CTL [132].

Since they are represented mostly by IgM isotype, natural antibodies effect is dependent on complement deposition in the immune complexes and complement receptors [173]. The highly efficient crosspresentation of antigen in immune complexes containing IgG [174] seems to depend on FcγRIIA, which, unlike CLRs, is not downregulated in the process of maturation [175]. In immature DC this pathway is actually counterbalanced by the inhibitory FcγRIIB whose levels diminish in the process of maturation and, somewhat counter intuitively, mature DC cross-present through the FcγR pathway better than iDC [175]. It has been proposed that an enhancement of antitumor immunity may by possible by transient blockade of FcgRIIB [176]. The high efficiency of CTL priming by immune complexes has implications both for the design of tumor vaccines and for the mechanism of action of monoclonal antibodies used in immunotherapy. In the mouse system the hierarchy of the activity of the IgG isotypes of a class switched antitumor antibody was found to be IgG2a $\geq$ IgG2b $>$ IgG1 $\gg$ IgG3 and this followed the ratio of their affinity with the preferred activating FcR (FcγRIII for IgG1, FcγRIV for IgG2a and IgG2b) relative to the affinity with the inhibitory FcγRIIB [177].

In humans, IgG1 and IgG3 have higher affinity for the FcRs than IgG2 and especially IgG4. In addition, there are allelic variants of activating Fc-receptors that will significantly change the affinity for certain antibody isotypes. FcγRIIIA allele that contains a valine in position 158 (FcγRIIIA158V) has a higher affinity for IgG1 and IgG3 than the receptor that has phenylalanine (FcγRIIIA158F) at that position [176]. Furthermore, FcγRIIA131H has a histidine at position 131 and a higher affinity for IgG2 than the 131R allele, which contains an arginine at the same position. This allelic polymorphism defines different efficiency of the antibody mediated crosspresentation and may warrant genetic testing for targeting the most responsive group of patients when immunotherapy is planned.

It is interesting to speculate in this context on the possible role of TACA reactive antibodies in tumor immunotherapy. As thymus independent antigens they induce mostly IgM and IgG3 (IgG2 in humans). Nevertheless, as for natural antibodies in viral infections, TI-2 responses seemingly affect cellular responses significantly. In animal tumor models, some TACA targeting immunizations yielded cellular responses to the tumor [91, 178]. Although in the report by Wierbicki et al. the authors consider cross-reactivity of the carbohydrate mimotope peptide used with a peptide from CD166, the results are not conclusive. It is possible that in these cases the anti-TACA antibodies induced crosspresentation of tumor antigens associated with the TACA epitopes and, thus, initiated an epitope spreading loop. Further studies are necessary to determine the immunotherapeutic potential of this approach and its possible optimization, for example,in terms of suppression of the inhibitory FcγRs or therapeutic application of class switched TACA monoclonals. Furthermore, due to the low reactivity with the other FcR, the polymorphism in FcγRIIA in humans would have a very strong impact on the effects of IgG2 antibodies. It is also interesting to speculate that sometimes targeting of the antigen to sizeable B cell populations leads to a very efficient immunization for all branches of the adaptive immune response. Although historically only activated B cells are known to present efficiently to naïve T cells as opposed to resting B cells, recently it became clear that this rule should be mapped on to the B cell population landscape where it is modified [111]. Furthermore, targeting of antigen to CD19 appears as particularly efficient in tapping the B cell APC properties, apparently with little dependence on the B cell functional state. In the case of MUC1, such targeting leads to an efficient breaking of self-tolerance and priming of both Th and CTL responses [179]. Elegant as it is, this strategy relies on an epiphenomenon, distantly reminding one of superantigen action. It is interesting to establish the degree of equivalence of this targeting technique to antigen specific internalization by BCR. Another technical detail is the apparent blocking of FcR interactions by the bulky protein attached that would normally occur in the case of immune complexes.

The targeting of different B cell populations may expand the potential to control the outcome of immunization and may be a mechanism contributing to the observed diverse effects of carriers on the immunogenicity of haptens. As self-antigens induce tolerance, vaccination with nonself antigens that molecularly mimic self-antigens may overcome tolerance and lead to generation of antitumor immune responses. Crosspresentation mediated by antibodies may be implicated in the insufficiently understood phenomena following immunization with carbohydrate mimicking peptides. Anti-MUC1 responses may offer an interesting model

in this context. MUC1 is a highly glycosylated type I transmembrane glycoprotein with a unique extracellular domain consisting of a variable number of tandem repeats (VNTR) of 20 amino acids (PDTRPAPGSTAPPAHGVTSA). Antibodies and T-cell recognizing MUC1 antigens have been isolated from the blood of breast cancer patients [180, 181]. Using peptide vaccination anti-MUC1 antibodies were induced in mice [182]. Mimicry can occur also naturally, although the frequency of this phenomenon is still unknown. It was demonstrated that Galα1-3Galβ1-4GlcNAc-R (Gal epitope) and peptides derived from MUC1 antigen cross-react with anti-Gal antibodies [89]. Based on the immunogen and the immunization regimen and the background IgG isotype, the cross-reaction may lead to the production of certain IgG isotypes that can actually determine the vaccination outcome and the type of the immune responses. This concept suggests that there are two ways for presentation of peptides upon peptide immunization to facilitate cellular responses. The first follows extracellular loading onto Class II, the second through immune complexes formed with antibodies, with the efficiency of loading dependent on the isotype.

In a series of manuscripts McKenzie's group showed that anti-Gal antibodies reacted with MUC1 antigens and that anti-MUC1 antibodies reacted with Gal sugar. According to McKenzie's group previous publications on Gal cross-reactivity, again suggests that a low titer carbohydrate cross-reactive antibody is functional to protect patients from recurrence of the disease. So what evidence exists that crosspresentation might be operative? On the one hand anti-Gal antibodies are cross-reactive with MUC1 derived peptides. Figure 2 illustrates that human anti-Gal antibody is cross-reactive with a MUC1 derived peptide with the sequence NH2-CPAHGVTSAPDTRPAPGSTAP (MAP format) relative to pooled human IgG (IVIg). The MUC1 peptide is also cross-reactive with the lectins jacalin (JAC), and Peanut agglutinin (PNA) (Figure 3) associated with binding of TACAs expressed on a variety of tumor cells [183, 184]. JAC and PNA share as their preferred ligand the TF antigen, but differ in their finer specificities for modifications of this determinant and in their specificities for cancerous epithelia. In this context the MUC1 derived peptide is a CMP that mimics both the Gal epitope and TF antigen. While antibodies to MUC1 occur naturally in both healthy subjects and cancer patients it is difficult to ascertain their origin; for example, if anti-MUC1 antibodies are initially anti-TF or (most probably) anti-Gal antibodies.

The specificity of natural and induced MUC1 antibodies has defined minimal epitopic sequences, indicating that antibodies directed to more than one region of the MUC1 peptide core can coexist in one and the same subject. The most frequent minimal epitopic sequence of natural MUC1 IgG and IgM antibodies is found to be RPAPGS, followed by PPAHGVT and PDTRP. In some studies, MUC1 peptide vaccination induced high titers of IgM and IgG antibodies predominantly directed, respectively, to the PDTRPAP and the STAPPAHGV sequences of the tandem repeat. In recent studies we have identified a common cross-reactivity with Gal antigen of P10s—a CMP mimicking GD2 (WRYTAPVHLGDG), and an immunodominant epitope of

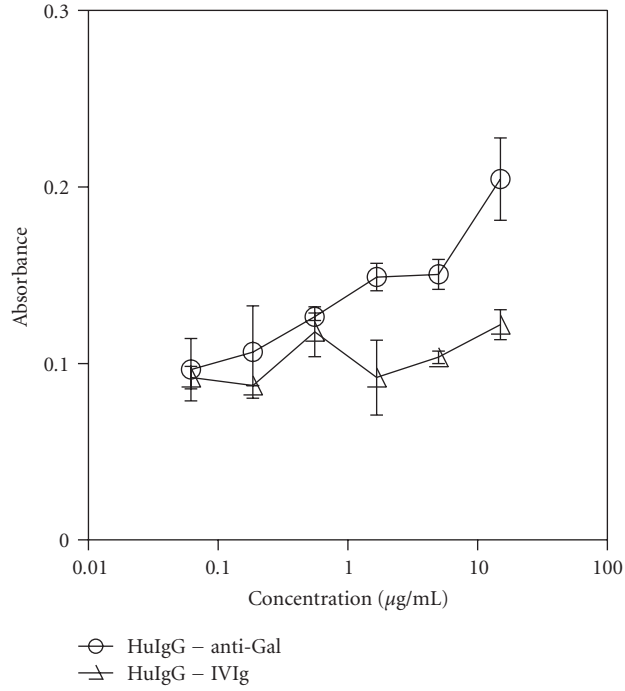

Figure 2: Plates were coated with MUC1 peptide and reactivity of human anti-Gal antibodies were measured. Human IVIg was used as negative control.

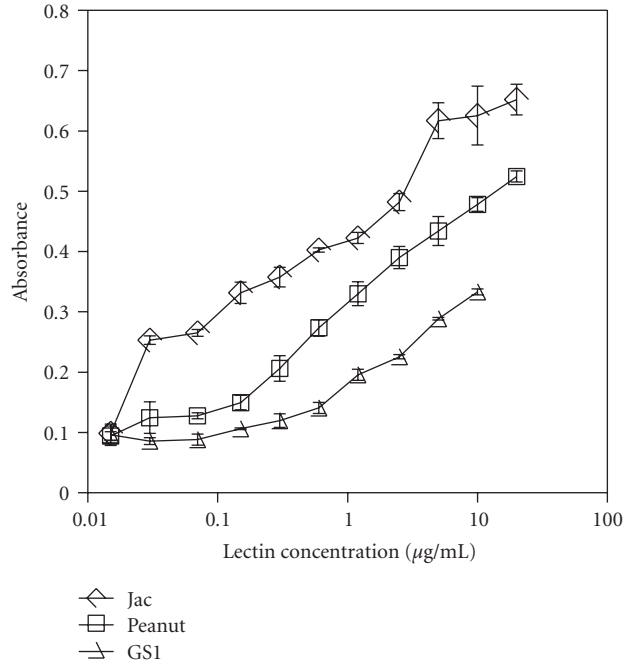

Figure 3: ELISA plates were coated with MUC1 peptide. Wells were then blocked with blocking buffer (PBS + Ca/Mg and 1% BSA) and various concentrations of Lectins were added. Plates were incubated for two hours washed and binding was visualized by Streptavidin-HRP.

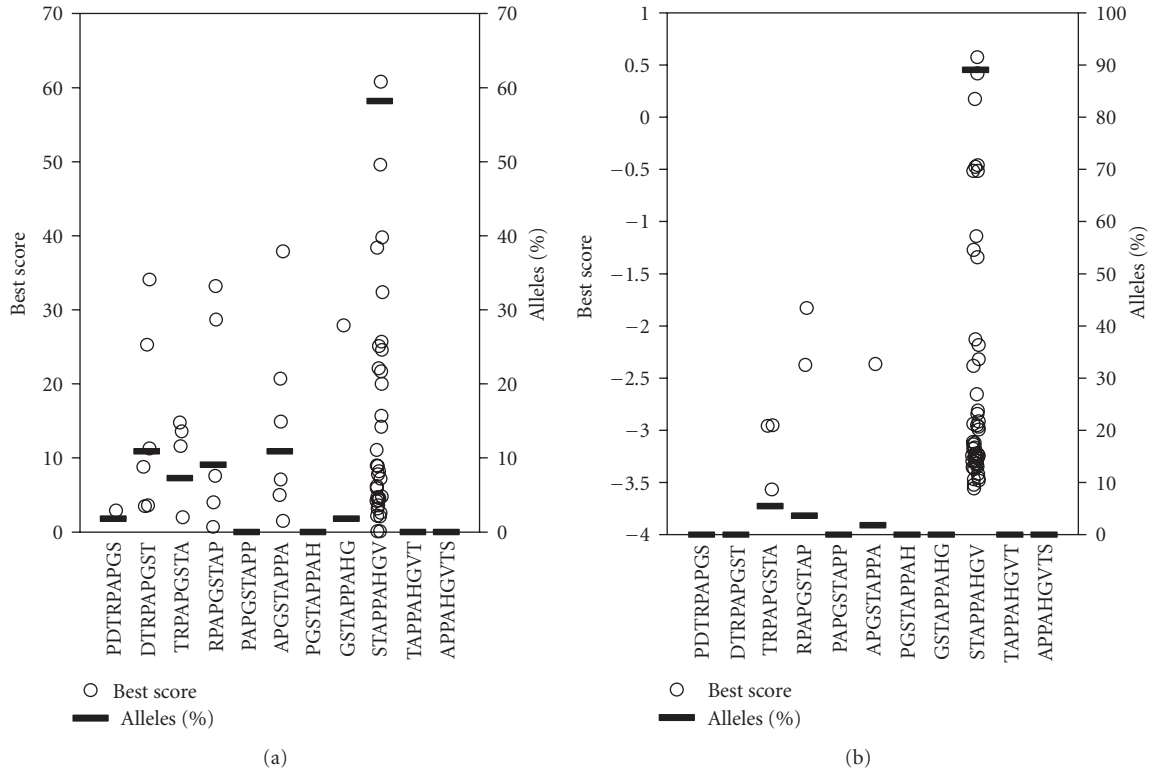

Figure 4: Using the Immune Database and Analysis Resource (IEDB, http://www.immuneepitope.org/) [187] the possible CTL epitopes in the sequence of the tandem repeat PDTRPAPGSTAPPAHGVTS were predicted. (a) Using consensus score only the best sequences for each allele are marked together with their score as well as the percentage of MHC class I alleles (secondary axis) for which the respective sequence is the best scoring sequence in the tandem repeat (the lower score indicates stronger binding); (b) A combined score using the ann algorithm for MHC calss I binding prediction, TAP transport and proteasome cleavage prediction is shown. The presentation is similar to (a). Again only the best-scoring sequences for each allele are shown. The higher scores indicate higher probability of presentation.

MUC1 (STAPPAHGVTS). Interestingly the peptides display compositional similarity as well as an alanine-proline dipeptide known to mimic structurally different sugar epitopes. We also observe that P10s is bound by antibodies that cross-react with TF and the Tn antigens. In this context, one could argue that the P10s CMP might be an antigenic "analog" of the STAPPAHGVTS sequence and further suggest that the TF and Tn mimicry of MUC1 lies within the STAPPAHGVTS sequence. In fact, the STAPPAHGV peptide has been defined as a CTL reactive peptide sequence [66, 185].

In mice, MUC1 peptide immunization resulted in cellular responses with reported little humoral response [86, 185]. In this context, it can be rationalized that peptide immunization resulted in activating cellular responses typical of peptide loading pathways. In contrast, MUC1 peptide induced strong cellular response in mice transformed to a strong antibody response in human immunization. It was argued that preexisting anti-Gal antibodies caused a deviation of the immune response in humans compared with mice that do not have anti-Gal antibodies [86]; attributed to cross-reactivity of natural anti-Gal antibodies to MUC1. It was shown that the Gal-CMP can induce humoral responses in Gal knockout mice, emulating the human condition. McKenzie's group clearly showed that anti-Gal antibodies cross-reacted with a mannan conjugated MUC1 peptide fusion protein lead to little cellular response, but a huge anti-MUC1 antibody responses in research subjects. They argued that such an immune response is based on macrophage Fc receptor binding to the immune complex, leading to processing via the exogenous pathway; MHC class II presentation and stimulation of helper T cells and antibody production. In the absence of anti-Gal antibodies, mannan binds to mannose receptor on macrophages and goes through the class I presentation to stimulate $CD8^+$ cells.

An alternative explanation is based on the fact that the MUC1 peptide is a nonself antigen in the mouse system and the strong CTL epitopes it contains direct the immune response. In the human system there is a strong tolerance mechanism precluding CTL response. It is interesting why the antibody response is not similarly suppressed. This may be due to cross-reactivity with the Gal antigen since the abundant anti-Gal memory B cells should exist with lower requirements for stimulation [186]. Using the Immune Database and Analysis Resource (IEDB, http://www.immuneepitope.org/) [187] the possible CTL

and Th epitopes in the sequence of the tandem repeat PDTRPAPGSTAPPAHGVTS were predicted. In this carbohydrate mimotope-like stretch of the MUC1 sequence there is no significant class II binder predicted, although some very antigenic regions are found after the tandem repeats, for example in the region of aa1040–1060 (data not shown). For CTL epitopes both a consensus algorithm for predicting the MHC affinity for multiple alleles [188] and a combined prediction of proteasome cleavage, TAP transport and MHC affinity [189] were applied to determine the relative antigenicity of all 9-amino acid frames within the tested sequence. In this way, not only the potential MHC affinity of each frame but also the probability for the generation of this epitope in vivo was predicted. The STAPPAHGV epitope was clearly the best binder and a promiscuous epitope (Figure 4(a)). When considering the proteasome processing and TAP transport, even stronger immunodominance of this epitope is predicted (Figure 4(b)). In the context of self, as in MUC1 expressed in humans, whether on tumor or normal cells, this finding may rather be indicative of an essential self-epitope inducing clone deletion, anergy or suppression. If this epitope is really visible to the immune system, it is possible that its dominance is not by chance, since evolution would have evaded otherwise similar self-target. It would be interesting to speculate that an aberrant glycosylation in this case may affect the recognition and deviate the specificity to nontolerized clones. In the case of MHC class II, similar promiscuous and strong binders among self antigens may represent targets of regulatory T cells [190, 191] provided the affinity is bellow a threshold that would actually promote central deletion of the high affinity clones.

So what are the consequences of this mimicry? On the one hand, reactivity with natural Gal antibodies would suggest that immune complexes will be formed and that they are taken up by macrophages and DCs, if the isotype of the complex lends to efficient uptake. In this context uptake would lend to activation of cellular responses if permitted by the tolerance mechanisms. If this was operative one could speculate that lactating women would have a skewed carbohydrate reactive repertoire that would also be cross-reactive with high molecular weight mucins because of their inherent mimicry of TF, Tn and milk sugars. In fact, this has been the observation. Anti-MUC1 IgG antibodies are increased in lactating women [192, 193]. However such studies do not differentiate between MUC1 specific versus anti-Gal cross-reactive antibodies. Their presence can either lead to phagocytosis of immune complexes of IgG1 with MUC1 domains lending to crosspresentation and reduced breast cancer risk or not if the predominate anticarbohydrate fraction is IgG2. It is also possible that IgG/IgM ratios can exacerbate the progression of disease.

These observations further highlight that the immune system has already taken advantage of glycan cross-reactivity to teach us that immune surveillance is operative in that self-vaccination, representative of anti-Gal antibody mediated crosspresentation of mucin antigens can lower the risk of cancer. We just need to learn to trust what we observe clinically, understand it and replicate it within the proper setting.

7. Conclusion

Immunotherapy represents a fourth-modality therapeutic approach against human neoplasms. It remains very attractive in light of the shortcomings of malignant tumor management by conventional surgical, radiation, and chemotherapies. The hope for immunotherapy in the context of vaccines is the notion that the immune system can mount a rejection strength response against neoplastically-transformed cells. Cancer vaccines are entering a new phase of enthusiasm. Part of this is recognizing when vaccines are most functional. In particular, therapeutic vaccines are not viable for large tumors but play a more important role in regulating micrometastases (i.e., adjuvant setting) with emphasis on prevention of recurrence of disease. It is perceived that in order to be effective, cancer vaccines must either break tolerance or activate a ”cryptic” population of T cells that escaped tolerance by virtue of their low affinity for antigens expressed by the tumor.

The concept of vaccines cannot be transferred mechanistically from infectious diseases because cancer cells express mostly self-antigens. While an infection is a perturbation of an equilibrium, which is restored by the immune system, cancer is a gradual transformation of the very equilibrium involving the immune system. Therefore, exploiting immune surveillance mechanisms is a challenge to our understanding of tolerance as a system property. Focusing on advances in cellular and molecular immunology, ultimately the knowledge of tolerance/surveillance mechanisms will help develop strategies that effectively and safely augment antitumor responses.

Carbohydrate determinants, both as epitopes as well as higher order patterns, are among the most ubiquitous tumor associated antigenic targets. The clinical importance of targeting TACAs is highlighted by: (1) the success of carbohydrate-based vaccines against infectious diseases; (2) the role of TACAs in autoimmune phenomena and tissue rejection and (3) the beneficial clinical correlates observed in cancer patients with anti-TACA antibodies. The limitations of the anti-tumor response to them might prove to some extent subjective, that is, rather a limitation of our understanding of surveillance. Carbohydrate moieties can and do participate in CTL epitopes and represent targets for cytotoxicity. Anticarbohydrate responses potentially bridge innate and adaptive immunity, T cell dependent and independent responses, humoral and cellular mechanisms. Their potential to affect directly and indirectly antitumor responses is still poorly understood and underutilized. The higher order-structures of TACA provide danger signals to the immune system recognizable by antibodies. Carbohydrate targets focus the activity of innate activating and tolerizing pattern recognition receptor together with antibodies (natural and induced) and, possibly, T cells. This is, at least, a prerequisite for an organizing role in tumor surveillance. However, we hypothesize that tolerance to TACA- conjugate vaccines is related to compartmentalization of the repertoires that generate TI responses to self or non-self carbohydrate antigens. One consequence of this hypothesis is that immunologic carriers in TACA-based vaccines may play another role besides

recruiting T cell help. They may redirect or perhaps highjack the immunogen from one compartment to another which would negatively impact on their ability to induce the type of desired immune response. Carbohydrate-conjugate vaccines are inherently designed to induce Th2 responses, while most anti-tumor targeting concepts focus on Th1 responses.

In terms of future directions, the design of immunogens eliciting long-lasting anti-TACA IgM responses would greatly improve the therapeutic utility of TACA-targeted vaccines. In some respects, we have thrown the baby out with the bath water. IgM antibodies are typically dismissed as being effective. However, natural IgM antibodies prove effective as proapoptotic molecules as part of the immune surveillance. To generate sustained immunity to TACAs, we have developed immunogens based on CMPs - a strategy whose clinical promise is supported by our preliminary studies. Carbohydrate mimicking peptides are agents with a potential to address the essential role of carbohydrates in tumor surveillance, but their application would depend on a systemic view of their pharmacodynamics beyond the mechanistic concepts borrowed from pathogen vaccines.

We have observed that sustained low serum titers of anti-TACA antibodies, elicited by CMP, are sufficient to inhibit the growth of tumors in therapeutic and prophylactic mouse models. Consequently, we might be barking up the wrong tree in developing high affinity responses. It is debatable if glycosylated T cell epitopes are better than naked peptide versions. It is clear that glycosylated peptide versions will be limited to loading onto dendritic cells and can not be incorporated into vector technologies. CMPs can be used in multiple formats. They induce cellular responses, including CMP- and TACA-reactive Th1 $CD4^+$ and tumor-specific $CD8^+$ cells. Most of all, CMPs can prime for memory responses to TACAs, which might be related to the B1b cell compartment. The relative specificity of mimics and the unusual dual immunological character (peptide epitopes/carbohydrate mimotopes) makes CMP novel tools to understand and manipulate immune responses to tumor cells. Thus, some, still enigmatic, immune effects of CMPs are definitely related to an organizing role in T/B/NK cell cooperation. These include: a role for anticarbohydrate responses in cross-priming, Th1 stimulation, DTH and NK antitumor activity, possibly through DC and B cell compartments that bridge innate and adaptive immunity.

A more fundament, set of studies are required to understand how the ratio of IgM to IgG might affect tumor progression. Augmentation of IgM titers to TACA are perceived to correlate with tumor growth inhibition while evidence is available that IgG might promote tumor progression. This might be of particular importance to better understand how carbohydrate reactive antibodies emerge in pregnant versus lactating women. But understanding these responses can provide insight as to how immune surveillance might be exploited in cancer vaccine design. Carbohydrate reactive antibodies and T cells may promote or prevent tumor growth by antigenic modulation or by cytotoxic killing of tumor cells. The emergence of IgM/IgG ratios may serve as a potential early endpoint for the effectiveness of cancer vaccines expressing TACA types.

TACAs might themselves associate with MHC. Molecular recognition is about the ordered arrangement of atoms and not on the molecular species. Consequently, helical carbohydrates might mimic helical peptides in associating with MHC Class II molecules or extended carbohydrates might mimic the beta structure type arrangement of peptides in the MHC Class I binding site. Interestingly, T cells that see carbohydrates or glycans might be unrestricted in that the T cell receptor might see antigen directly, devoid of MHC. Such T cells unfortunately might compete with antibodies for the same epitope. Such T cells are suggested to interact with MUC1 but have not been characterized. Yet such T cells could play an important role in immune surveillance.

The existence of cross-reactive antibodies to glycans suggests that cross-presentation might be operative for some antigens. More studies should be directed on the nature of cross-presentation of natural antibodies. IgG1 can theoretically cross present, but IgG2, which are typically thought of as carbohydrate reactive, might not. How does the ratio between these isotypes affect cross-presentation and how do natural polymorphisms of Fc receptors affect the outcome of cancer patients? Polymorphisms should be able to be exploited to improve vaccine efficacy. A better understanding of these mechanisms should contribute to designing MUC1 derived peptides to engage natural carbohydrate reactive antibodies to improve upon T cell targeting of MUC1.

Pattern recognition is fundamental to innate immune surveillance. These receptors typified by lectins, are "hard-wired" in the germ line. The innate immune response not only provides a first line of defense against invading microbes but also instructs the adaptive immune response. The discovery of Toll-like receptors has influenced thinking on how the innate immune response affects adaptive immunity. Interestingly all of these pattern receptors like carbohydrate antigens but TACA are still understudied in cancer immunotherapy circles.

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