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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.

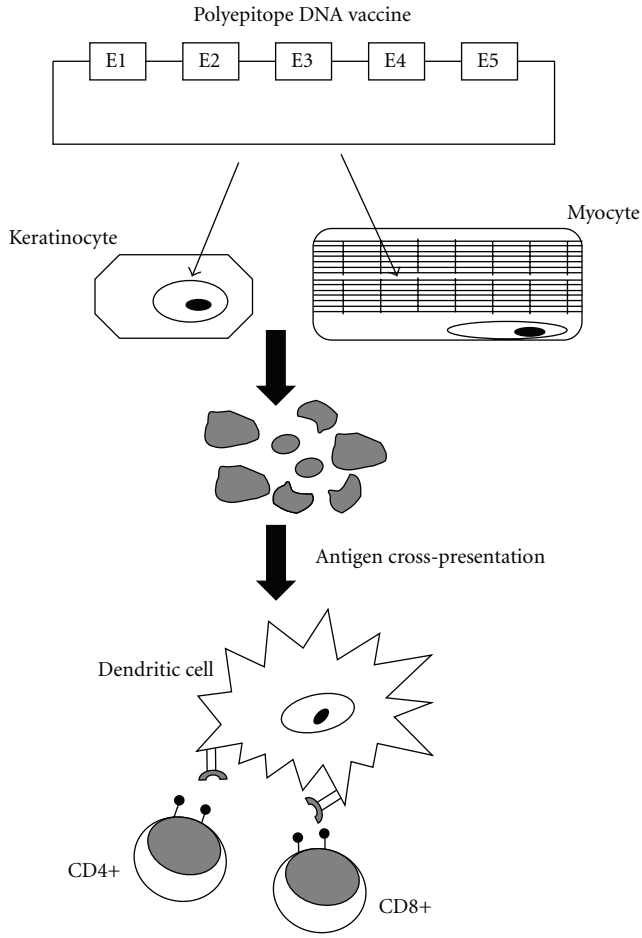

Figure 1: *Polyepitope DNA-based cancer vaccines and cross-antigen presentation.* After injection of the recombinant polyepitope DNA vaccine into myocyte or keratinocytes, antigenic materials is transferred to dendritic cells (antigen cross-presentation).

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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