T cell costimulatory molecules in anti-viral immunity: Potential role in immunotherapeutic vaccines

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In order to develop successful vaccines against chronic viral diseases it is widely thought that a cell-mediated immune response is required to eliminate or control intracellular viruses. For a protective immune response it is important to induce long term immunological memory. The initial activation of T cells requires both an antigen specific signal, involving the recognition of a peptide/major histocompatibility protein complex by the T cell receptor as well as additional costimulatory signals. In chronic viral diseases, T cell responses, although present, are unable to eliminate the infection. By providing antigens and costimulatory molecules together, investigators may be able to increase and broaden the immune response, resulting in better immunological control or even elimination of the infection. Recent progress in understanding the function of costimulatory molecules suggests that different costimulatory molecules are involved in initial immune responses than are involved in recall responses. These new developments have important implications for therapeutic vaccine design. In this review the authors discuss the function of T cell costimulatory molecules in immune system activation and their potential for enhancing the efficacy of therapeutic vaccines.

Key Words: Immunity; Therapeutic vaccines; Lymphocytes; Vaccination; Viral infection

Les molécules costimulatoires des lymphocytes T dans l’immunité antivirale : Le rôle potentiel des vaccins immunothérapeutiques

L’activation des lymphocytes T est nécessaire pour éliminer ou contrôler les virus intracellulaires. Cette activation exige à la fois un signal antigénique précis, exigeant la reconnaissance d’un multimère complexe d’histocompatibilité peptide-majeur par le récepteur des lymphocytes T et des signaux costimulatoires supplémentaires. En cas de maladies virales chroniques, les réponses des lymphocytes T, bien qu’elles soient présentes, ne réussissent pas à éliminer l’infection. En fournissant à la fois les antigènes et les molécules costimulatoires, les chercheurs pourraient accroître et étendre la réponse immunitaire, ce qui assurerait un meilleur contrôle immunologique ou éliminerait même l’infection. Les progrès récents dans la compréhension de la fonction des molécules costimulatoires laissent supposer que les molécules costimulatoires qui participent aux réponses immunitaires initiales diffèrent de celles utilisées en cas de réponses après un rappel. Ces nouveaux développements ont des conséquences importantes pour la conception de vaccins thérapeutiques. Dans la présente analyse, les auteurs traitent de la fonction des molécules costimulatoires des lymphocytes T dans l’activation du système immunitaire et de leur potentiel pour améliorer l’efficacité des vaccins thérapeutiques.

T CELLS AND IMMUNITY TO VIRUSES

Although antibodies can be effective in neutralizing extracellular viruses, CD8+ T cells are important in killing viraly infected cells and CD4+ T cells are critical in providing help for both antibody-mediated and CD8+ T cell-mediated responses. The initial T cell response to pathogens is dependent on the activation of APCs. Figure 1 summarizes the current view of how this process occurs. DCs are thought to be the critical APCs for the initiation of T cell responses (1,2). DCs are a diverse group of APCs scattered throughout the skin and tissues. In their resting state, DCs express receptors...
suitable for pathogen uptake, as well as receptors capable of sensing infection. Once activated by exposure to pathogens or inflammatory stimuli, DCs undergo a maturation process that involves their migration to the draining lymph node and increased cell surface expression of molecules involved in T cell activation.

DCs and other APCs express a family of receptors known as the Toll-like receptors (TLRs) (3). These receptors are pattern recognition receptors that bind conserved features of pathogens. There are at least 10 such receptors and their diversity is increased by heterodimerization (Figure 2). For example, TLR4 is required for the response to bacterial lipopolysaccharide. TLR3 recognizes double stranded ribonucleic acid (dsRNA) associated with viral infections and TLR9 recognizes demethylated CpG motifs that are enriched in bacterial DNA. Once triggered by these pathogen associated molecular patterns, TLRs induce a response in the APC leading to new gene transcription and the induction of molecules involved in triggering inflammation and immunity. This activation process renders DCs competent to activate naïve T cells in the lymphoid organs. Naïve T cells, via their clonally distributed antigen-specific T cell receptors, recognize peptide-MHC complexes presented on the activated DCs. At the same time, T cells need to recognize so-called 'costimulatory molecules' in order to be activated. Antigen presentation in the lymphoid organs results in clonal expansion leading to a population of activated 'effector' T cells (Figure 1). These effector T cells then home back to the site of infection, where they carry out their functions in eliminating or containing the infection. The finding that the initiation of the T cell response requires the presence of costimulatory molecules induced by microbial infection explains in part how the immune system can respond vigorously to an infection while avoiding recognition of self-tissues.

WHAT IS A T CELL COSTIMULATORY MOLECULE?

T cells require two signals for activation: an antigen specific signal and a second 'costimulatory' signal. The concept of two signals for lymphocyte activation goes back 30 years (4). The
The current model is derived from experiments in the late 1980s that showed that although an initial transient response might be observed if T cells were triggered only through their antigen-specific receptors, they went on to die or become unresponsive. However, if a signal was given through both the antigen-specific T cell receptor and an additional cell surface receptor known as CD28, then the T cells made high levels of cytokines required for T cell proliferation and also upregulated survival factors that prevented programmed cell death (Figure 3) (5).

Figure 4 shows the important interactions during initial T cell activation by a DC that has been exposed to a pathogen. Peptides derived from degradation of the infecting pathogen are brought to the DC surface via binding to the MHC proteins (also known as human leukocyte antigen [HLA] proteins in humans) inside the cell. In addition, B7 molecules present on activated DCs bind to CD28 molecules on the T cell surface.

An extensive body of evidence supports the idea that the binding of the T cell surface receptor CD28 to its ligand, B7.1 or B7.2, allows initial T cell expansion and short term survival (6). Thus, it is now widely accepted that the binding of B7.1 or B7.2 on pathogen-activated APCs to CD28 on naïve T cells provides the first costimulatory signal for initiation of T cell mediated immunity. However, in recent years the picture has become more complicated. We now know that following initial contact there is further activation in both the APC and the T cell, resulting in the appearance of new receptor-ligand pairs on the interacting cells (7). Some of these molecules are illustrated in Figure 4. One of the challenges facing immunologists is to understand the specific roles of this large array of immune stimulatory molecules. An emerging idea is that while CD28 is important for initial T cell activation, other costimulatory molecules may be important in sustaining responses, diversifying the response or controlling different aspects of the response, such as initial versus recall responses, memory versus effector function, or differentiation into different kinds of effector cells.

Two major families of proteins have received a lot of attention in the field of T cell costimulation. The CD28 family (8) and the tumor necrosis factor receptor (TNFR) family (9). As discussed above, the T cell surface receptor CD28 provides a critical signal for the initiation of T cell responses (6). Mice lacking CD28 have very poor T cell responses in general, although they can respond to some pathogens, such as lymphocytic choriomeningitis virus (10,11). CD28 is part of a family of related receptors, which are summarized in Table 1. Two of the family members, CD28 and inducible costimulator (ICOS), are stimulatory, whereas the two other family members, PD-1 and cytotoxic T lymphocyte associated antigen-4 (CTLA-4), inhibit T cell activation. CD28 is expressed on resting T cells whereas the other members of the family are expressed only after T cell activation. CD28 and ICOS bind to different ligands. ICOS binds to a B7-related protein known as ICOS ligand or B7-related protein 1 (B7RP1). ICOS is important in enhancing the ability of T cells to make cytokines (12). Mice lacking ICOS make very small germinal centers and have a greatly diminished antibody class switch, resulting in a predominantly Immunoglobulin M (IgM) response (13-15). ICOS knockout (+) mice show decreased immune responses in several infectious models,
TABLE 1
Costimulatory molecules in T cell activation

<table>
<thead>
<tr>
<th>CD28 superfamily:</th>
<th></th>
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<tbody>
<tr>
<td>Receptor</td>
<td>Expression</td>
<td>Ligands (expression)</td>
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<tr>
<td>---</td>
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</tr>
<tr>
<td>CD28</td>
<td>Constitutive T cells</td>
<td>B7.1 (CD80) B7.2 (CD86) Activated APC</td>
</tr>
<tr>
<td>ICOS</td>
<td>Activated T cells Higher expression on Th2 cells</td>
<td>B7RP1 also known as LICOS, ICOSL, on activated APC and some nonlymphoid tissues</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Activated T cells</td>
<td>B7.1, B7.2</td>
</tr>
<tr>
<td>PD-1</td>
<td>Activated T and B cells</td>
<td>PD-L1, PD-L2 Lymphoid and non-lymphoid tissues</td>
</tr>
<tr>
<td>TNFR superfamily:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-1BB (CD137)</td>
<td>Activated CD4+ and CD8+ T cells Activated dendritic cells Activated NK cells</td>
<td>4-1BBL (activated APC)</td>
</tr>
<tr>
<td>OX40 (CD134)</td>
<td>Activated CD4+ T cells</td>
<td>OX40L (Activated T, B, DC, vascular endothelial cells)</td>
</tr>
<tr>
<td>CD40</td>
<td>B cells, dendritic cells and macrophages</td>
<td>CD40L (activated T cells)</td>
</tr>
</tbody>
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APC Antigen presenting cell; B7RP1 B7-Related protein 1; CTLA-4 Cytotoxic lymphocyte associated antigen-4; DC Dendritic cell; ICOS Inducible costimulator; CD CD40 B cells, dendritic cells CD40L (activated T cells) B cell and dendritic cell activation

including viral and bacterial infections (16-18). However, defects in immune responses in ICOS−/− mice are not as severe as those in CD28−/− mice (17). CD28 and the inhibitory homologue CTLA-4, both bind to the same ligands, B7.1 (CD80) and B7.2 (CD86). Initially, T cells express only CD28, so the effect of binding B7.1 and B7.2 is an increase in the immune response. However, with time T cells start to express CTLA-4, which has a 20-fold higher affinity for B7.1 and B7.2 than does CD28, with the net effect that CTLA-4 limits the amount of T cell activation (19). Mice lacking CTLA-4 develop a progressive lymphoproliferative disorder, consistent with the role of CTLA-4 in limiting T cell activation (20,21). Mice lacking the other inhibitory member of the family, PD-1, develop a late onset lupus-like disease and arthritis, which is consistent with an immune inhibitory function (22). However, in vitro studies with the ligands for PD-1 have sometimes shown inhibition and sometimes stimulation of T cell responses, suggesting perhaps that there is another stimulatory receptor for these ligands. The ligands for PD-1, PD-L1 and PD-L2, are present in both lymphoid and non-lymphoid tissues. It has been proposed that inhibition by PD-1 in the tissues may increase the signal threshold required for an immune response (8). Blockade of CTLA-4's inhibitory signal is being tested as an immune stimulatory regimen in cancer trials (23). However, a caveat of this approach is that it is also likely to increase the risk of autoimmunity and, as such, is unlikely to gain widespread support as a means of controlling chronic infectious diseases.

In addition to the focus on the CD28 gene family, several members of the TNFR family have come to prominence in terms of T cell-mediated immunity (9,24,25). The TNFR family is a family of receptors involved in the regulation of cell life and death. This family can be broadly divided into two groups. The first group, including Fas and TNFR1, have death domains in their cytoplasmic tails and link extracellular signals to cell death pathways. A second group, typified by TNFRIII, lacks death domains and directly binds adaptor proteins called TNFR associated factors (TRAFs). TRAFs link extracellular signals through TNFR family members to cell survival and differentiation pathways (26). There are more than 27 members in the TNFR family, a small subset of which are highlighted here. The importance of TNFRs and their ligands in the immune response is exemplified by the finding that viruses create soluble homologues of these receptors to subvert their function (27,28). CD40, a member of the TNFR family, is expressed on DCs and B cells, and is highly important in the regulation of APCs and B cell function. Upon initial T cell activation, T cells upregulate CD40 ligand (CD40L), which can bind to CD40 on DCs to enhance the expression of costimulatory molecules, including B7.1, B7.2, 4-1BB ligand (4-1BBL) and OX40 ligand (OX40L). In addition, Interleukin 12 (IL-12), a cytokine important in regulating interferon gamma (IFN-γ) and T helper 1 (Th1) cell responses, is upregulated by CD40 signaling in DCs (29). Upon activation, T cells also upregulate the TNFR family members OX40 and 4-1BB, receptors thought to be important in sustaining T cell responses. 4-1BB is inducible on both CD4+ and CD8+ T cells upon activation and is capable of providing a CD28-independent costimulatory signal to CD28− T cells (30,31). OX40 is primarily expressed on activated CD4+ T cells and appears to only work in conjunction with a CD28 signal (32). Mice lacking OX40 or its ligand have impaired secondary CD4+ T cell proliferative responses (33-36). 4-1BBL−/− mice show decreased secondary CD8 responses to viruses, with no detectable defect in CD8+ T cell responses to virus (37-39). However, in vitro analysis indicates a role for 4-1BB on both CD4+ and CD8+ T cells (40). The finding that 4-1BBL can stimulate CD28−/− T cells is relevant to the human immune response because, in contrast to mice, humans accumulate CD28−/CD8+ T cells with age (41,42).
Recent evidence from our laboratory shows that 4-1BBL can provide a costimulatory signal to the human CD28– T cells, leading to cytokine production, cell survival and the upregulation of molecules associated with cellular cytotoxicity (43). Memory CD8+ T cells specific for chronic viral pathogens (Hepatitis C Virus [HCV], HIV, Cytomegalovirus, Epstein-Barr virus) are found among the CD28– T cell population in human blood (44,45). The proportion of T cells lacking CD28 is increased in people with chronic viral infections or other persistent conditions such as multiple myeloma (44-49). The observation that 4-1BBL can stimulate CD28– T cells makes it an attractive candidate for boosting immunity in chronic viral infection, where the lack of CD28 on the memory cells will make this population of cells insensitive to B7 stimulation. As will be discussed below, stimulatory anti-4-1BB antibodies have been tested in vivo in mouse models. Provision of anti-4-1BB can systemically increase both anticancer and antiviral immunity (50-52).

MHC TETRAMERS CAN BE USED TO FOLLOW CD8 T CELL EXPANSION DURING VIRAL INFECTIONS

In the last few years there has been a revolution in our ability to follow the response of T cells to infection without the need to culture the T cells. This was made possible by the development of soluble MHC-peptide tetramers linked to fluorescent tags that allow one to follow cells of a particular antigen-MHC specificity using a flow cytometer (53). Using this approach, researchers can monitor human blood samples for the presence of T cells of particular specificities. This has allowed investigators to analyze T cells in the blood of infected patients without the need to first expand the specific T cells by restimulation in culture. The resulting new data revealed that past experiments, in which one needed to restimulate and expand T cells in culture to detect particular specificities, led investigators to greatly underestimate the number of viral-specific T cells produced during acute viral infection. Use of this approach in mouse models and with human blood has revealed a number of important insights about the extent and timing of T cell activation in vivo during acute and chronic infections (54).

Figure 6 describes the structure and application of MHC tetramers. The tetravalent nature of the MHC-peptide complexes allows them to bind in a stable fashion to T cells specific for the MHC-peptide complex making up the tetramer, so the reagents can be used as antibody-like reagents to detect
populations of epitope-specific T cells. In the example shown, it can be seen that seven days following infection with influenza A, 7% of all CD8+ T cells in the spleen of a C57BL/6 mouse are specific for the major T cell epitope of the influenza nucleoprotein NP366-374. This approach can also be combined with the technique of intracellular cytokine staining (55), allowing researchers to determine whether cytokines are being produced by the activated virus-specific CD8+ T cells. The use of MHC tetramer technology by the scientific community has been accelerated by the creation of a National Institutes of Health-supported tetramer facility at Emory University in the United States. This facility is open to the scientific community via an online application process (www.niaid.nih.gov/reposit/tetramer/index.html).

MHC tetramers have been used to follow the acute response to infection in several mouse infectious disease models. The initial expansion of CD8+ T cells in response to infection with influenza virus, lymphocytic choriomeningitis virus or the bacterium *Listeria monocytogenes* is very rapid, with the maximum number of antigen-specific CD8+ T cells observed in the spleen or lymph node by day 7 or 8 after infection (56-58). This is followed by a period of contraction in the number of T cells in the lymphoid compartment, thought to be due to their migration from the lymphoid compartments to the tissues, as well as to programmed cell death of the effector cells after they have carried out their functions at the site of infection. As predicted by classical immunology, a proportion of ‘memory’ cells remain behind after this contraction process (59) and approximately three weeks after the influenza infection of mice about 1% to 2% of the CD8+ T cells in the spleen are still specific for the major influenza nucleoprotein (NP) epitope. Upon subsequent challenge, the response occurs about two days earlier than the primary response and is of higher magnitude due to the presence of the expanded memory cell population that was not present on first exposure to the pathogen. The kinetics of the primary response to infection do not appear to be dependent on the infectious dose, but rather appear to be preprogrammed (60). Once a T cell is engaged and receives its antigen-MHC and costimulatory signal the cells undergo a series of rapid divisions that are not dependent on the continued presence of the antigen, resulting in rapid expansion of a clone of T cells capable of recognizing infected cells (61-64). The decline of this population also seems to be preprogrammed, and is independent of the disappearance of the pathogen (65). This may be important in chronic infections because it limits the pathological damage that a sustained immune response might entail.

The ability to monitor viral-specific responses directly in blood samples using MHC tetramers, combined with sensitive methods for detecting which cytokines are produced, has important implications for monitoring vaccine trials. It is now possible to closely monitor CD8+ T cell responses using MHC tetramers and intracellular cytokine staining to determine the correlates of protective immunity. For technical reasons, the tools to follow CD4+ T cell responses in the same manner have lagged behind the CD8+ T cell specific reagents; however, this is currently an area of intense activity.

**ROLE OF COSTIMULATORY MOLECULES CD28 AND 4-1BB DURING ACUTE VIRAL INFECTION IN VIVO**

The use of MHC tetramer technology combined with mouse models lacking particular costimulatory molecules allows one to assess the importance of particular ligand-receptor interactions in the immune response. Figure 7 shows the impact of the removal of CD28 or 4-1BB on the numbers of CD8+ T cells specific for the immunodominant influenza epitope NP366-374 in C57BL/6 mice (38). Wildtype mice infected intraperitoneally with influenza A X31 show a rapid expansion of influenza-specific CD8+ T cells in the spleen, peaking at day 7 after primary infection at 7% of total CD8+ T cells. This is followed by a rapid decline of influenza-specific CD8+ T cells in the spleen between days 7 and 21, and then a more gradual loss of these cells over time. Upon subsequent challenge, the response involves about twice as many T cells as the primary response and occurs with slightly enhanced kinetics. However, if mice lack CD28 there is a very poor initial expansion of the T cells and, as a consequence, a very poor secondary response. By contrast, mice lacking 4-1BB show little defect in the primary response but fail to show an enhancement of CD8+ T cell expansion upon secondary challenge. Testing the killing function of the T cells in these mice shows that the ability to kill target cells coated with viral peptides is proportional to the number of tetramer-binding T cells detected (38).

Further work has shown that the systemic administration of stimulatory anti-4-1BB antibodies can correct the defect in CD28+ mice when a single dose is provided during the primary influenza infection. This results in full restoration of the memory T cell response, suggesting that for CD8+ T cells, CD28 is only required for initial T cell activation and not for recall responses. Conversely, a single dose of anti-4-1BB antibody
Corrects the defect in the CD8+ T cell recall response in 4-1BBL+/− mice only when added at the time of viral challenge, arguing that the 4-1BB costimulatory signal is more important during recall responses (unpublished data). Why the immune system switches from CD28 to 4-1BB as a costimulatory molecule during primary versus secondary responses is not clear. Interestingly, administration of anti-4-1BB (100μg) during viral challenge results in a 2-fold increase in the recall response to influenza virus, even in wild type mice. The physiological levels of 4-1BBL in mice appear to be very low, as the ligands are difficult to detect except after extensive stimulation of the cells in vitro (31). This is consistent with the finding that the provision of extra 4-1BBL or stimulatory anti-4-1BB antibodies is immune stimulatory even in immunocompetent mice (52,66). These findings suggest that 4-1BBL, rather than B7, may be the better choice of immune stimulatory agents to use in an immunotherapeutic regimen for antiviral immunity.

HOW CAN KNOWLEDGE OF COSTIMULATORY MOLECULES BE APPLIED TO THERAPEUTIC VACCINATION?

With chronic viral diseases such as HIV there may well have been a vigorous initial immune response to the pathogen, but the combination of viral escape variants and viral interference with the immune system results in the establishment of a chronic infection. One model for applying a therapeutic vaccine to the problem of HIV is to first use anti-viral therapy to reduce viral load as much as possible. This would be followed by the interruption of anti-viral therapy and provision of HIV antigens together with costimulatory molecules, in the hope that the enhanced immune response could eradicate the residual virus and prevent further erosion of the immune system. Again, 4-1BBL is an attractive candidate because of the evidence that it is important in recall anti-viral responses.

A key issue becomes how to deliver the antigen and costimulatory molecules. As has been demonstrated in mouse models of cancer or acute viral infection, systemic administration of stimulatory antibodies against receptors involved in immune triggering is one possible approach. However, the need to produce large amounts of protein product for therapy could be prohibitively expensive. On the other hand, the recent success of a recombinant protein therapy for arthritis (soluble TNFR) suggests that such approaches might be feasible (67). An alternative approach that may be cheaper to administer would be to use recombinant replication-defective viruses containing both viral epitopes and the genes encoding costimulatory molecules as a therapeutic vaccine. Replication-defective adenoviruses, modified canary pox, and other viral vectors are being developed for immunotherapy (68-70). Recombinant replication-defective adenoviruses are particularly attractive as there are two regions of their genome that can readily accommodate foreign DNA so that one could independently incorporate both antigens and costimulatory molecules. Furthermore, multiple serotypes of a virus should allow for more than one immunization.

Another approach being considered for HIV therapy is known as adoptive immunotherapy. In this approach, patient lymphocytes are obtained by leukopheresis and stimulated in vitro before reinfusion into the same patient. In a recent example, Levine and colleagues (71) used anti-CD3 (a component of the T cell receptor) and anti-CD28 coated beads to stimulate HIV patient T cells in a nonspecific way. The T cells were then infused back into the patients and their CD4+ T cell counts were found to improve, at least temporarily (71). The advantage of this approach is that the stimulatory agents are not delivered systemically, reducing toxicity concerns. This approach, using antigen-specific activation together with costimulatory molecules, such as 4-1BBL, could be used to generate useful T cells for reinfusion into patients. This approach could also use a replication-defective viral delivery vector containing HIV epitopes and costimulatory molecules delivered to the patient’s APCs ex vivo. These would be used to activate the patient’s T cells, and the activated T cells are subsequently reinfused, again avoiding systemic delivery of the virus.

CONCLUSIONS

Many challenges remain in the development of therapeutic vaccines for chronic viral infections. However, great strides have been made over the last few years in our understanding of T cell activation and in our ability to precisely follow T cell responses during infection. These advances suggest that we will see a number of new therapeutic vaccine approaches developed over the next few years.

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CANVAC: Our research in therapeutic vaccines for anti-viral immunity is being conducted under the auspices of the CANVAC, the Canadian Vaccines and Immunotherapeutics Network of the Networks of Centres of Excellence program. CANVAC is an interactive group of more than 47 Canadian academics working with private sector biotechnology companies, government and nongovernmental partners toward the common goal of rational vaccine design for chronic viral diseases and cancer. Recognizing that the same immunological principles could be applied to cancer as to chronic viral diseases, CANVAC currently focuses on the development of vaccines for HIV, HCV as well as prostate cancer. By bringing together expertise in vectors, antigens, costimulatory molecules and immune monitoring, as well as expertise in vaccine preparedness, epidemiology, ethics, clinical trials and regulatory issues, CANVAC hopes to tackle the broad number of issues required to bring new vaccines to the clinic (www.canvacc.org).

REFERENCES


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