Genetic Immunotherapy for Cancer

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ABSTRACT

Genetic immunization refers to treatment strategies where gene transfer methods are used to generate immune responses against cancer. Our growing knowledge of the mechanisms regulating the initiation and maintenance of cytotoxic immune responses has provided the rationale for the design of several genetic immunization strategies. Tumor cells have been gene-modified to express immune stimulatory genes and are then administered as tumor vaccines, in an attempt to overcome tumor cell ignorance by the immune system. With the description of well-characterized tumor antigens, multiple strategies have been proposed mainly aimed at optimal tumor antigen presentation by antigen-presenting cells (APC). Among APC, the dendritic cells have been recognized as the most powerful cells in this class, and have become the target for introducing tumor antigen genes to initiate antitumor immune responses. The detailed knowledge of how the immune system can be activated to specifically recognize tumor antigens, and the mechanisms involved in the control of this immune response, provide the basis for modern genetic immunization strategies for cancer treatment. The Oncologist 2000;5:87-98

INTRODUCTION

Despite the immune system, cancers arise in humans. Little if any evidence of immune response against cancer can be detected in most patients. New insights in understanding the biology of the immune system and the description of powerful ways to stimulate it allow hope for successful anticancer immunotherapy. In this review, we will discuss the rationale for attempting to activate the immune system to fight cancer, and will focus on strategies based on the transfer of genetic material to stimulate an immune response (genetic immunization).

Anticancer Immune Responses and T Cell Immunity

The experience with allogeneic bone marrow transplantation (BMT) in patients with chronic myelogenous leukemia (CML) provides the most direct evidence of the existence of a cellular (lymphocyte-mediated) antitumoral immune response. Allogeneic BMT between twins (exact immune systems) provides higher relapse rates than allogeneic BMT between HLA-matched non-twins (similar immune systems). If the graft of an HLA-matched non-twin is depleted of T lymphocytes (no immune effectors), relapse rates increase. If the T lymphocytes from the donor (immune effectors) are given to an allogeneic BMT recipient with a leukemia relapse, a secondary remission can be achieved [1]. Therefore, cellular immunity mediated by donor T lymphocytes is critical for optimal control of CML following allogeneic BMT and is powerful enough to induce durable regressions. Furthermore, a graft-versus-tumor effect against solid tumors, including lymphomas and renal cell carcinomas, can be generated following minidose allogeneic BMT. Other examples of the benefit of immune responses in cancer are the low but remarkable responses to interleukin 2 (IL-2) and interferon alpha (IFN-α) in melanomas and kidney cancers.

The attempts to generate a protective antibody-mediated humoral response have generally failed to provide a clinical benefit in human cancers. The mechanism of action of the two FDA-approved antitumor antibodies for human use, trastuzumab (Herceptin) and rituximab (Rituxan), is thought to be a receptor-specific signal transduction effect, as opposed to an immune-based cytotoxic effect, which makes them a target-specific “drug” rather than an immune treatment. The allogeneic BMT and the cytokine-based strategies described above are based on the stimulation of polyclonal antitumor cellular immune responses. The lim-
ited applicability of these strategies resides in their lack of antigen specificity, leading to poor antitumor responses and a high frequency of toxic effects, including the generation of graft-versus-host disease following allogeneic BMT, and the severe flu-like symptoms associated with systemic cytokine administration (IL-2, IFN-α). A better understanding of the mechanism of antigen presentation and recognition by the immune system, and the recent description of well-characterized tumor antigens in many cancers, may enable the generation of tumor antigen-specific protective immune responses with less systemic toxicity.

Antigen Presentation to the Immune System

The form of an antigen able to stimulate a cellular immune response is a short peptide presented by major histocompatibility complex (MHC) molecules. There are two types of MHC molecules, known as class I and class II.

MHC class I molecules are expressed on the surface of the great majority of the cells in the body. In humans, they correspond to HLA-A, -B and -C molecules. Their crystal structure has been described, which resembles a baseball glove with a closed groove where the antigenic peptide (or epitope) resides [2]. The peptide is only 8 to 10 amino acids (aa), and is tightly bound to the MHC class I molecule through two anchor residues (usually the first or second aa at each extreme of the peptide) [3]. These peptides are derived from any intracellular protein. There is a constant degradation of intracellular proteins in a cytoplasmic complex known as the proteasome, and the resulting short peptides are sampled by the TAP transporters, which transfer them to the endoplasmic reticulum (ER). In the ER, the peptides bind to nascent MHC class I molecules depending on the aa located in the anchor positions, and are then presented on the cell surface (Fig. 1). This random sampling of intracellular proteins and MHC-restricted surface presentation of these peptides serve as a mechanism for showing the environment what is happening inside the cell.

The crystal structure of MHC class II molecules has a similar disposition, but the groove is open at both ends, allowing the presentation of longer peptides (usually 15-34 aa long) [4]. In humans, they correspond to HLA-D molecules. MHC class II molecules have a restricted tissue expression, mainly on “professional” antigen-presenting cells (APC) including B cells, macrophages and dendritic cells. The source of their antigenic epitopes is also different, since they mainly derive from proteins taken up from the exterior of the cell by endocytic vesicles, as opposed to intracellular proteins (Fig. 2). This is so because their primary role is to initiate an immune response, mainly to an outside aggression to the host (although there are exceptions to this rule). This is clear with bacterial or parasitic infections, but an immune response can also be initiated against proteins released by damaged cells undergoing apoptotic death and taken up by the professional APC (a process known as cross-priming) [5].

Tumor Antigens

Tumor cells have a wide range of genetic mutations and dysregulated gene expression. Since these genes lead to the production of abnormal, normal but overproduced or newly expressed proteins, the proteasome will sample and present them on the tumor cell surface in MHC class I molecules. Additionally, when these cells die, their proteins can be taken up by APC and cross-presented in MHC class II molecules, initiating an immune response. During the past 10 years, an increasing number of tumor-derived antigenic proteins have been fully characterized. Thierry Boon and collaborators originally described the MAGE-1 antigen in malignant melanomas [6]. They had previously established cytotoxic T cell clones derived from patients with malignant melanoma that were reactive against melanomas of the same HLA type, suggesting the presence of a shared antigen. To identify the gene coding for this common antigen, they cut

Figure 1. MHC class I presentation of endogenous epitopes to CD8+ cytotoxic T lymphocytes.

Figure 2. MHC class II presentation of exogenous epitopes to CD4+ helper T lymphocytes.
the genes expressed in these melanomas, transfected them separately in small pieces into cells with the same HLA type, and determined which transfected cells containing a small portion of cDNA of the melanoma were killed by the cytoxic T cell clones. This allowed them to narrow down which part of the melanoma genome had the antigenic gene, and then sequenced it.

Since then, several classes of tumor antigens have been described. The MAGE family of genes (MAGE 1, 2 and 3, BAGE, GAGE, RAGE) belongs to a class of antigens that can be considered truly tumor-specific [7]. They are expressed in a low percentage of a variety of tumors, but the only nontumoral tissue that expresses them are the germ cells in the testes. Since germ cells do not express MHC molecules, they cannot present MAGE epitopes to the immune system. Therefore, the first time that MAGE epitopes are presented by MHC molecules to the immune system is on the surface of tumor cells.

Another class of tumor antigen is the lineage-specific antigen, most of them described in melanomas. When neuroectodermic cells differentiate into melanocytes, they start expressing several new proteins which are then generally turned off in resting mature melanocytes. Melanomas have increased or abnormal expression of these melanocyte-lineage proteins, and several epitopes derived from them have been shown to be the target of most antimelanoma cytotoxic T cell clones. These include MART-1/Melan-A, gp100, tyrosinase and TRP-1 and -2 among others [7]. However, this poses a conceptual question: how can the immune system be activated against melanocyte-lineage self-antigens to which it has been extensively exposed and has failed to reject before? A T cell response against these antigens would really represent an autoimmune response, a situation that the immune system can react has changed the existing paradigms in immunology. The distinction between self and nonself has become less important. Instead, the immune system seems to be able to differentiate the expression of the same antigen in different environments, and then decide if it is appropriate to react to it. The focus has shifted from the origin of the antigen (self or nonself) to how it is presented to the immune system (in a stimulatory or inhibitory way). This concept was postulated by Polly Matzinger as the “Danger Hypothesis” [11]. When an antigen is presented in a danger environment, it stimulates an immune response. If the same antigen is presented in a nondanger environment, it induces immune tolerance. During the past years the “Danger Hypothesis” has been tested in several systems, and seems to closely fit how the immune system behaves.

MHC/peptide complexes by themselves do not initiate immune responses. An immune response follows a two-signal pattern [12], one corresponding to the MHC/peptide complex (signal 1) and another to a series of costimulatory molecules (signal 2). Since antigen presentation is the deciding element between response or tolerance, a key player is the APC, which has the greatest concentration of MHC/peptide and costimulatory molecules. When an antigen is presented by non-APC cells (in our case tumor cells), the antigen is presented without costimulation (first signal without second signal), therefore not perceived as dangerous by the immune system. If the same antigen is presented by APCs, both first and second signals are present, and the immune system senses danger to that antigen. The amount of first and second signal on the surface of an APC increases when these cells are exposed to bacterial products, infected with virus, or recognized by activated CD4+ T cells, all of which are obvious “danger” situations (Fig. 3).
Cells of the Immune System

APC

These cells have the ability to initiate an immune response by presenting an antigen in an immune stimulatory environment. Cells specialized in antigen presentation are B cells, macrophages and dendritic cells. B cells tend to skew the immune system to a humoral response, which is very effective in clearing particulate pathogens (bacteria and other extracellular microorganisms), but has a limited ability to fight intracellular pathogens (virus) or cells with abnormal behavior (tumor cells) [13]. Monocytes and macrophages have the adequate machinery, with the ability to take up apoptotic material from the extracellular compartment, process and present antigenic epitopes in MHC complexes (signal 1) together with costimulatory molecules (signal 2), and produce stimulatory cytokines. However, these cells have evolved to be responsible for the removal of cellular debris, and their antigen expression is not intended to be perceived as dangerous. In fact, foreign antigen presentation by macrophages has been shown to induce tolerance [14]. The role of initiating a cellular immune response has been left to the dendritic cells, a closely related cell type. Dendritic cells have higher levels of MHC, costimulatory and adhesion molecule expression than macrophages and B cells [15, 16]. They reside in very small numbers in any peripheral tissue, where they are specialized in antigen capture (Langerhans’ cells are dendritic cells that reside in the skin, a good example of the sentinel role of dendritic cells in peripheral tissues). Antigen capture leads to a series of changes in the dendritic cell phenotype, leading to their next role, antigen presentation to the immune system. They gain the ability to migrate through the lymphatics into T cell areas of lymphoid organs (lymph nodes, spleen), and they lose the capacity to capture antigen [15, 16]. The amount of MHC (10 to 100 times higher MHC density in dendritic cells compared to activated macrophages or B cells) [13], costimulatory and adhesion molecule surface expression increases, making them ideally suited for recognition by T cells. Selective activation of naïve T cells is further enhanced by the secretion of chemokines specific for naïve T cells. This process is called dendritic cell maturation, and can be triggered by several “danger” signals, which include bacterial products (lipopolysaccharides [LPS], Staphylococcal Aureus strain Cowan I [SAC], CpG oligonucleotide sequences), viral infections, or MHC class II-dependent antigen recognition by CD4 T cells leading to CD40-receptor stimulation (Fig. 4) [17-19].

In recent years, methods to produce large quantities of dendritic cells ex vivo have been described. Hematopoietic stem cells can be differentiated to dendritic cells in vitro by culturing in stem cell factor, GM-CSF and tumor necrosis factor-alpha (TNF-α) [20]. Dendritic cells can also be differentiated from CD14+ monocytes obtained from peripheral blood when cultured for one week in GM-CSF and IL-4 [21]. Similar cytokine combinations generate dendritic cells in other animal species (primate and nonprimate), allowing extensive studies of their biology [22]. Dendritic cells have also been obtained by gradient centrifugation of peripheral blood, where they only constitute 0.5% of the peripheral blood mononuclear cells [23]. The number of dendritic cells in peripheral blood can be increased by the systemic administration of cytokines like Flt-3-ligand [24]. As happens in vivo, dendritic cells generated by these methods first go through a phase of immaturity, when they are most capable of endocytosing particulate elements, which is optimal for antigen capture. In vitro-generated dendritic cells can be differentiated to
full maturity, a state associated with optimal antigen presentation but loss of antigen capture ability, using similar differentiation (danger) signals as in vivo: bacterial products (LPS, SAC, CpG), CD40 ligation (by activated CD4+ T cells, activating anti-CD40 antibodies, or recombinant CD40-ligand), by TNF-\(\alpha\) and by a macrophage-conditioned media [17, 25]. The new insight on the in vitro generation and maturation of dendritic cells has important implications in tumor-bearing subjects, since immunosuppressive tumor-derived molecules that inhibit dendritic cell maturation and function in vivo have been described. Tumor-bearing animals and patients with cancer have been shown to have dendritic cells with suboptimal antigen-presenting function due to tumor-produced vascular endothelial growth factor, IL-10 and prostaglandins. When dendritic cells from these same tumor-bearing hosts are differentiated in vitro by GM-CSF-containing media, their dendritic cells can be rescued and become as efficient antigen presenters as dendritic cells generated from cancer-free subjects [26], thereby bypassing one of the powerful immunosuppressive effects of tumors. This provides a strong rationale for the use of in vitro-generated dendritic cells in cancer immunotherapy.

**CD4+ T Cells**

Their primary role is to help in the activation of CD8+ T cells specific for a certain antigen, and are commonly known as T helper cells. This definition underestimates the real role of CD4+ T cells, since in many situations they are indispensable (as opposed to just helpful) for the initiation of CD8+ T cell-mediated cytotoxic immune responses. CD4+ T cells recognize long peptides presented by MHC class II molecules. Since MHC class II expression is mainly restricted to “professional” APC, it prevents the immune system from being overtly activated against self-antigens. When a CD4+ T cell recognizes its specific antigen on the surface of an APC, it binds to the MHC class II/peptide with its T cell receptor (TCR), and creates a complex known as the “immunological synapse,” composed of several MHC molecules, adhesion molecules (for example, ICAM-1) and costimulatory molecules. If the binding affinity, peptide characteristics and number of MHC/peptide molecules are optimal, the CD4+ T cell will be activated. This in turn activates the APC through the coupling of the CD40-ligand (on the CD4+ T cell) and the CD40-receptor (on the APC) [18, 19, 27], or a similar interaction between TRANCE (on the CD4+ T cell) and the TRANCE receptor, also known as RANK (on the APC) [28]. APC activation increases the ability to activate naïve CD8+ T cells by upregulating MHC and costimulatory molecule expression in the APC and increasing cytokine production, therefore initiating the effector arm of the immune response (Fig. 5). CD4+ helper T cell function is further shaped by the production of certain activating (type 1) or inhibitory (type 2) cytokines [29]. The APC also produces the same type of cytokines, which will create an environment that will either facilitate or inhibit CD8+ T cell activation and the eventual lysis of target cells. Type 1 cytokines are associated with the generation of a cytotoxic response and include IFN-\(\gamma\), IL-2 and TNF-\(\alpha\) when produced by helper T cells, and the same cytokines plus IL-12 when produced by APC. Type 2 cytokines have the opposite effect, that is, downregulation of cytotoxic responses and, therefore, induction of cellular immune tolerance. On the other hand, they upregulate humoral (antibody-mediated) responses. Type 2 cytokines include IL-4, IL-5, IL-10 and transforming growth factor-beta. CD4+ T cell activation is also shaped by costimulatory B7 molecules, which are usually recognized by CD28, a molecule that generates a downstream proactivating response. However, once the T cell is activated, it upregulates surface expression of cytotoxic T lymphocyte antigen 4 (CTLA-4), an inhibitory receptor with four times greater affinity for B7 molecules [30]. Another control mechanism to avoid immune overstimulation is the expression of the pro-apoptotic molecule Fas-ligand on activated CD4+ T cells. This enables them to induce apoptotic death in cells with Fas-receptor on their surface, most frequently activated lymphocytes [31].

**CD8+ T Cells**

These are the primary effector cells of the immune system, commonly known as cytotoxic T cells. Their principal role is to recognize MHC class I/peptide complexes on the surface of any cell and induce cytotoxic death. Peptides recognized by CD8+ T cells are shorter than those recognized by CD4+ T cells. Initial naïve CD8+ T cell activation and proliferation requires the recognition of its specific antigen. 

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**Figure 5. CD4+ T helper cell activation and helper function.**
(signal 1) on the surface of an activated APC, together with B7 costimulation (signal 2), adhesion molecules and a stimulatory, type 1 cytokine environment. If this naïve CD8+ T cell only recognizes an MHC/antigen complex in the absence of adequate second signal (costimulation), the CD8+ T cell will become anergized, as commonly happens when tumor antigens are expressed by tumor cells or resting normal cells. This situation changes after CD8+ T cell activation. When an activated CD8+ T cell finds its specific MHC/peptide complex on the surface of a target cell, it triggers the killing mechanism without requiring the presence of signal 2 (therefore, it can happen when antigen is presented by tumor cells). The cytotoxic activity of CD8+ T cells is mediated by the release of Perforin, which forms a pore in the membrane of the target cell, and release of Granzyme B, which enters the target cell through the Perforin pore and triggers the activation of the caspase cascade (a series of intracellular serine proteases) leading to apoptotic death (Fig. 6). Since this is a powerful attack system, stringent regulatory mechanisms exist. If no stimulatory cytokines are present, the activated CD8+ T cells die of deprivation [32], and if too much antigen is recognized repetitively, the activated CD8+ T cells upregulate both Fas receptor (a TNF-superfamily molecule) and Fas-ligand, leading to fratricidal (killing of similar activated CD8+ T cells) or suicidal apoptotic death, mediated by the same caspase cascade system [31]. CD8+ T cells can also produce polarized type 1 or type 2 cytokines, and noncytotoxic cytokine-producing CD8+ T cells have been described.

Natural Killer (NK) Cells

These cells belong to the innate immune system. Their killer activity is inhibited by the recognition of MHC molecules on the cell surface. This protects normal host cells from NK attack. However, when cells have decreased or are absent MHC expression, or if cells have a foreign MHC molecule, they are cleared by NK cells. NK cells have also been shown to be activated by dendritic cells (Fig. 7) [33], and produce both type 1 and type 2 cytokines, therefore providing a bridge between antigen-nonspecific (innate) and antigen-specific (adaptive) immune systems. The defined relations between NK and antigen-specific T cell responses are not fully understood at this time, and are further complicated by the ability of NK cells to lyse APC due to the overruling of the protective effect of self-MHC expression by concomitant high levels of costimulatory B7 molecule expression [34].

Natural Killer T (NKT) Cells

More recently, cells that express both a TCR and an NK1.1 receptor have been described [35]. Like NK cells, they also seem to provide a bridge between innate and adaptive immune systems. They can be activated by dendritic cells and have a strong ability to produce type 1 and type 2 cytokines in a highly polarized fashion in a short time frame relative to other cell types (Fig. 7). They seem to act in a more simplified fashion, like a primitive immune system, since they recognize a limited array of antigens presented by the MHC-like nonpolymorphic CD1 molecules. CD1 is highly expressed on the surface of dendritic cells, and it binds to an unusual set of epitopes, mainly of lipid origin. The range of antigen presented by CD1 is not as wide as MHC class I and II presentation. Also, in many cases, the TCR genes used by NKT cells are restricted to a certain V gene (Vα14 in mice, Vα24 in humans) [35]. Their exact relationship to antigen-specific responses is unclear, but they may be important in the initiation step of an immune response and type 1/type 2 polarization [35].

Figure 6. CD8+ T cytotoxic cell activation, proliferation and cytotoxic function.

Figure 7. Interaction between dendritic cells and the adaptive and innate cellular immune system.
Genetic Immunization Strategies

Tumor Vaccines

Gene transfer techniques have been used to convert tumor cells into better antigen presenters. Several groups inserted immune stimulatory genes into tumor cells in an attempt to vaccinate against the parental tumor. A central hypothesis is that tumor cells already expressed signal 1 (the antigen), but lack signal 2 (costimulation) or the production of immune stimulatory cytokines. Therefore, the genes of costimulatory factors and several cytokines have been inserted into tumor cells with slightly different rationales. For example, tumor expression of IL-2 was thought to provide the tumor antigens and a cytokine able to directly stimulate CD8+ T cells, therefore bypassing the need for CD4+ cells [36]. Another strategy is the introduction of the costimulatory molecule B7 into tumor cells, therefore providing signal 2 and converting the tumor cells into their own APC [37]. In a serial testing of these tumor vaccines, Dranoff et al. noted that the introduction of the gene for GM-CSF was superior to the insertion of other genes into tumor cells [38]. Mechanistic analysis of the immunological effect of GM-CSF produced by tumor cells revealed a surprise: the tumor vaccines did not present their own antigens. GM-CSF production by the tumor vaccines attracted a greater number of host APC, with antigen taken up from the dying tumor vaccines and cross-presented to the host’s immune system (cross-priming) [39]. Several tumor vaccines produced by the insertion of immune stimulatory genes into autologous tumor cells are currently in clinical trials [40]. Limitations include the requirement of the generation of a stably transfected tumor cell line from each patient, which is feasible in only some tumors like melanomas, but more difficult in common epithelial tumors like breast, lung or colon carcinomas. Alternative strategies include the transfection of allogeneic tumor cell lines hopefully containing common antigens, or the transfection of easily obtainable skin fibroblasts admixed with tumor cells. The initial studies have demonstrated that genetically modified tumor vaccines are safe, and there is early evidence of immune activation, most frequently demonstrated by the development of a delayed-type hypersensitivity reaction to the autologous tumor cells [40].

DNA Immunization

The description and full characterization of genes coding for tumor antigens enable the use of these genes to immunize against cancer. When naked DNA (a plasmid) is injected into muscle or the skin, it can passively enter local cells (myocytes or fibroblasts) and use the host cell’s machinery to produce the protein coded in its gene. This is a powerful method to immunize against microbial and viral antigens, and is able to stimulate both an antibody and a cellular response to the proteins coded by that gene [41]. The mechanism of this immune stimulation has also been proven to be through cross-priming [42]. The transfected cells, myocytes or fibroblasts, produce and secrete the protein, which is taken up by local or circulating host APC, which then process and present the antigenic epitopes on the APC surface, together with MHC molecules, after migrating to lymphoid organs, thereby initiating the immune response (Fig. 8).

The nature of the naked DNA injected has an effect on the ability of the inserted gene to stimulate an immune response. The vector backbone used to carry the foreign gene in this approach is derived from bacterial DNA, and has been shown to contain immunostimulatory nucleotide sequences (unmethylated cytidine phosphate guanosine—CpG-motifs) that have the ability to induce a type 1 immune response in humans [43]. Therefore, these bacterial-specific DNA sequences serve as a danger signal for the immune system to attract and mature more APC. Other clever strategies to increase the immune stimulatory capacity of the naked DNA injected include the coexpression of GM-CSF or chemokines known to attract dendritic cells, or the coexpression of immune stimulatory molecules like CD40-L or IL-12.

Several animal models have shown that this approach is able to generate tumor-protective responses [8, 9]. Its advantages are the relative simplicity of highly purified naked DNA production for clinical use and the fact that DNA is an “off-the-shelf” reagent not needing costly and complicated in vitro culture systems. This approach has been proven to immunize humans against intracellular infections like malaria [44], and clinical trials attempting to generate immune responses to a variety of antigens in human subjects are under way [45].

Figure 8. Mechanism of immune stimulation by gene-modified tumor vaccines (A) and naked DNA intramuscular injection (B).
Dendritic Cells

Dendritic cells have emerged as the key cell type responsible for initiating and controlling cellular immune responses (Fig. 7). They are the best equipped and most powerful APC, the only cell type able to stimulate naïve T cells [16]. The strategies described so far have also shown that dendritic cells are central to the immune-stimulating effects, which clearly substantiates the great interest in utilizing dendritic cells to initiate antitumor immune responses. This has been enabled by the description of methods for in vitro generation of dendritic cells and several methods of antigen loading (Fig. 9).

In animal models, dendritic cells have been pulsed with antigenic peptide epitopes derived from model tumor antigens, and have been shown to induce antitumor responses [46]. This strategy also generates clinically relevant responses in human subjects with low-grade lymphomas, multiple myelomas, advanced malignant melanomas and prostate cancers [23, 47, 48]. The main caveat for the wide use of this approach in clinical settings is that the immunogenic epitopes derived from each tumor antigen in the patient’s own HLA type have to be previously defined. For common HLA types, like HLA-A*0201 (40%-50% of Caucasians), it may be feasible for antigens expressed in a high proportion of tumors if there is a single or a limited number of previously described immunodominant epitopes, but this is not often the case. Other caveats include that the currently described epitopes are only codominant epitopes, but this is not often the case. Other caveats include that the currently described epitopes are only presented by MHC class I molecules, therefore potentially being suboptimal in immune stimulation due to lack of CD4+ T cell activation. An attempt to overcome this limitation is the inclusion of a keyhole limpet hemocyanin-derived “helper” epitope to provide nonspecific T helper cell activation [48].

To overcome these shortcomings, several alternative strategies to load tumor antigens onto dendritic cells have been developed (Fig. 8). The whole tumor antigen protein (purified from tumor lysates) can be fed to dendritic cells, which will endocytose it, process and present antigenic epitopes through the exogenous MHC class II pathway, as well as in MHC class I molecules. This can also be achieved if apoptotic cells are cocultured with dendritic cells, allowing them to macropinocytose the apoptotic cells and cross-present their epitopes in a physiological way. Clinical trials using these promising approaches are under way [47]. Dendritic cells have also been fused to tumor cells using cell fusion techniques similar to the ones employed for many years to generate hybridomas [49]. This would enable the use of the antigen-presenting capacity of the dendritic cells by the tumor cell. The dendritic cell/tumor cell fusions have been able to generate protective responses in mice [49]. Use in humans is likely to be limited by the need to generate autologous tumor cell lines for each patient, which would have to be completely free of nontumor-derived cells before dendritic cell fusion (due to the danger of inducing autoimmunity). In addition, the need for determination of the stability of the fused population and inactivation of contaminating or poorly fused tumor cells in the vaccine are other limitations.

In an attempt to generate a persistent endogenous production of antigenic epitopes, the cDNA or mRNA coding for antigenic proteins has been introduced into dendritic cells. Gilboa et al. have demonstrated that tumor-derived RNA can be introduced into dendritic cells and used to generate tumor-specific cellular responses [50]. This strategy, although technically challenging, has the benefit of not requiring a defined tumor antigen, since it can use uncharacterized mRNA transcribed in the tumor cells. A strategy more widely tested is the transfection of dendritic cells with well-described foreign genes [9, 10, 51-56]. In a serial testing of different methods to introduce foreign genes into dendritic cells, replication-incompetent adenoviral vectors proved to be by far the most efficient [51]. Viral vectors used in this approach have been modified by the deletion of one or several of the genes that enable self-replication, and that space is used to introduce a tumor antigen gene under the control of a promoter with the capacity to always be “on.” Therefore, when the replication-defective virus infects a dendritic cell, it will use the dendritic cell’s machinery to transcribe its genes (as viruses do efficiently), but it will not be able to generate infective viral progeny due to its inability to replicate itself. The inserted tumor antigen gene will be translated into a protein in the dendritic cell cytoplasm, and from there, continuously be presented on the cell surface in MHC class I and II molecules (Fig. 10). The ability to stimulate an MHC class I and II-restricted response by gene-modified dendritic cells has been demonstrated both in animal models and human in vitro systems [10, 52-55].
The use of dendritic cell approaches to stimulate antitumor responses has several current limitations for its effective translation to the clinic. The most important is the requirement of expensive in vitro culture techniques to generate autologous dendritic cells from the blood of each patient, which has to be performed under good manufacturing practice conditions and assured to be reproducible and contamination-free. Unanswered questions regarding the clinical use of dendritic cells include the optimal source of dendritic cells (in vitro differentiated from CD14+ peripheral blood monocyte/dendritic cell precursors, from CD34+ pluripotential hematopoietic precursors, directly purified by gradient centrifugation or expanded in vivo by Flt-3-L administration); the method of antigen loading (Fig. 9); and the source of tumor antigen. A final limitation is the current lack of full understanding of the optimal immunization strategy. Critical factors need to be defined, like the number of dendritic cells, optimal route of vaccination, number of vaccinations, and level of antigen expression. The paradigm of “more is better” may not be applicable to dendritic cell-based immunizations, since dendritic cells have also been shown to be critical in the generation of immune tolerance [25].

**Control Mechanisms**

While great progress in understanding how to stimulate the immune system has been made, successful antitumor immunization also requires thorough knowledge of the mechanisms that the immune system uses to maintain homeostasis. In fact, the immune system is usually maintained under powerful inhibitory signals, and even when an immune response has been generated, a great deal of effort is spent in limiting this immune response and returning to the baseline status.

**T Cell Anergy**

When T cells recognize their specific antigen (first signal) in the absence of costimulation (second signal), they produce lower levels of cytokines and do not proliferate [58], remaining in a dormant state. However, they are not deleted from the T cell pool, and adequate antigen presentation can still rescue them, induce a proliferation response and effectively break tolerance.

**Immune Deviation**

When an antigen is presented in an immune stimulatory way, it can induce a cytotoxic response, mediated by type 1 cytokines. However, several factors can induce a deviation from a cytotoxic type 1 to a tolerant type 2 response. They include antigen dose, multiple antigen exposure, the cytokine milieu in which the antigen is recognized, and poorly characterized non-MHC genes [59, 60]. If the immune response is skewed to a type 2 response, the antigen-specific T cells are no longer able to kill their targets, and instead potentiate the generation of an antibody response, which is more suited for protection against extracellular pathogens [29].

**Clonal Deletion: Cytokine Deprivation and Activation-Induced Cell Death**

A T cell that recognizes its antigen in the context of MHC molecules and proper costimulation becomes activated, a state where IL-2 is produced and T cells actively proliferate. This increases the number of T cells specific for that antigen several-fold in a short time, requiring certain space in lymphoid organs. It is then critical to allow the maintenance of only the number of T cells that are needed to effectively respond to the dangerous antigen. If the source of antigen ceases, then the availability of IL-2 decreases, and these T cells die of cytokine deprivation [32]. However, if the antigen persists, the activated T cells are repeatedly exposed to that antigen and the immune system eventually recognizes that it may no longer be in its best interest to further respond to that antigen. This is mediated by a process known as “activation-induced cell death.”

**TCR-Specific Strategies**

Another recent development is the cloning and characterization of tumor-specific TCR. Introduction of the antigen-specific TCR gene into T cells of a cancer patient would allow the creation of a large pool of T cells with TCR specific for a certain tumor antigen [57]. The gene-modified T cells can be expanded in vitro, activated and infused back to the patient. This strategy has proven to be more technically challenging than tumor antigen-based strategies, mainly due to a less complete understanding of the biology of the TCR, and the difficulty in stably gene-modifying lymphocytes.

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*Figure 10. Antigen processing and presentation by dendritic cells transduced with replication-incompetent adenoviral vectors.*
or AICD, where the same stimulus that leads to T cell activation (the antigen/MHC complex) induces the upregulation of the proapoptotic molecules, Fas-receptor, and Fas-ligand, on the surface of the activated T cell population, which leads to fratricidal and suicidal clearance of those cells (Fig. 11) [31].

**Negative Costimulation (CTLA-4)**

Signal 2, provided by the costimulatory molecules B7.1 (CD80) and B7.2 (CD86) on the APC, is recognized by CD28 in both CD4+ and CD8+ T cells. When these T cells become activated, they upregulate the cytotoxic T lymphocyte antigen 4 (CTLA-4 or CD152), which recognizes B7 molecules with a higher avidity than CD28 and has an opposite immunological effect [30]. Cross-linking of CTLA-4 by B7 inhibits T cell activation, IL-2 production and T cell proliferation by directly binding to the TCR complex and inhibiting TCR signal transduction [30], therefore also serving as a mechanism for downregulating antigen-specific T cell responses (Fig. 11).

**CONCLUSION**

Effective cancer therapy requires a tightly controlled target-specific weapon. The immune system is such a weapon, which continuously protects us from the environment, but fails to protect subjects with cancers from endogenous tumor development. Understanding the mechanisms that activate and limit tumor antigen-specific responses in animal models, preclinical human systems and cancer patients will allow the rational development of cancer immunotherapy.

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