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Steven T. Rosen, M.D., Series Editor
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Tumor Immunology and Cancer Vaccines

edited by
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TUMOR IMMUNOLOGY AND CANCER VACCINES

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PREFACE

It all started with an observation. Edward Jenner, an English physician, observed that milkmaids who contracted cowpox were rarely victims of smallpox epidemic, a disease that inflicted a heavy toll on humankind with an estimate of 500 million victims worldwide. In 1796, Jenner inoculated the extracted fluid from blisters on the hand of a milkmaid who was infected with cowpox into the arm an 8 year old peasant boy. After the boy recovered from a mild illness caused by this inoculation, Jenner exposed him to smallpox and to his delight the boy did not develop the disease. He published his work in 1798 in three publications titled "Vaccination Against smallpox", where the term vaccination is derived from the Latin word "vacca" meaning cow. Jenner was recognized to be the father of modern immunology, and his work marked the commencement of a new dawn in medicine that led to the 1979 declaration by the World Health Organization (WHO) of the global eradication of smallpox. By the beginning of the 20th century, vaccines for typhoid fever, rabies, polio, plaque and diphtherias were in use, and nowadays we are equipped with effective vaccines against more than 20 infectious diseases such as meningitis, rubella, whooping cough, rabies, and hepatitis B among others.

It is indisputable that the immune system plays a role in the natural history of cancer. This theory is supported in animal models by the fact that tumors develop earlier and more frequently in nude mice than in mice with normal immune systems. In humans, the principal evidence comes from many facts including that many 'immunocompromized' cancer patients have higher incidences of a number of tumor types, including those of the lung, colon, kidney and pancreas, as well as malignant

melanoma; immune response modifiers have been shown to be effective in treating tumors and in some anecdotes; tumors are known to regress spontaneously; and increased patient survival correlates with the presence of T cells (or tumor infiltrating lymphocytes, TIL) in a variety of tumors such as melanoma, neuroblastoma, and breast, bladder, colon, prostate, ovary, and rectal cancers. This indicates that tumors are amenable for immune recognition, and hence, are able to present antigens that are recognized by the immune cells. These antigens are called tumor antigens. Therefore, it is concluded that tumors develop due to the failure of the immune system to recognize and reject cancer, this is called "Tumor immune escape"; we now understand some of the factors that lead to tumor immune escape which will be discussed along with the principle of tumor antigens in the chapters of this book.

Advances in both immunology and molecular biology in the past decade have led to the identification and characterization of these tumor antigens. That in turn led to the revival of immunotherapy as the fourth modality of treatment of cancer. This treatment can be highly specific and an effective therapy based on the ability to develop tumor-specific antigen directed vaccines. The concept of Immunotherapy for cancer is over one hundred years old. The first reported "Cancer Vaccine" trial was by W.B. Coley in 1894. Coley's toxin's, as it was called, was not so much a vaccine as a non-specific immuno-stimulant. He used thirteen different preparations of bacterial extracts, between 1892 and 1936, to treat patients with a variety of malignancies with surprising success. He and others, including investigators at Mayo Clinic, reported over 50% durable responses in patient populations where 10-15% survival was historically expected. About the same time, in the early 1900's, Paul Ehrlich proposed the concept of "Immune Surveillance". Ehrlich suggested that tumors present unique antigens that could be recognized by the immune system, leading to continuous identification and removal of transformed cells. It was another fifty years before his theory could be proven. In the 1950's, when inbred mouse strains became available, Ehrlich's theory was tested and proved the immunogenicity of tumors. The tumor antigens were subsequently identified.

The new era of biotechnology is helping us rapidly progress in our efforts to identify tumor antigens, compare their immunogenecity, and then design effective delivery system to present the most powerful antigens to the immune system. With the completion of the human genome project, new technologies such as microarray analysis and proteomics have been added to our repertoire and have proved useful in identifying antigens that produce the best immune response; a pivotal requisite to the success of a cancer vaccine. Such a success is also dependent on how the antigen is delivered to the patient, the vehicle used along with the choice of adjuvant and cytokines. This wealthy "vaccine basket" provides researchers with tremendous choices when planning clinical trials and emphasizes the need to compare different strategies of vaccine design and delivery according to its efficacy in combating cancer in clinical trials.

In lieu of the tremendous amount of knowledge in areas of tumor immunology and cancer vaccines, we recognized the need to provide researchers and clinicians alike with a comprehensive up-to-date book on tumor immunology and cancer vaccines.

The first section of the book includes in depth analysis of basic tumor immunology, both cellular and humoral. This section explains mechanisms of antigen presentation, as well as the molecular reasons why tumors evade the immune system. The second section includes six chapters encompassing different vaccine strategies with emphasis on their preclinical development and current clinical data. How to enhance the immune response to cancer vaccines is the question tackled by the third section of this book. It documents preclinical and clinical developments in cytokine therapy, peptide vaccines and adoptive cellular immunotherapy. Finally, the last section of the book emphasizes the different issues regarding clinical trials design and application in addition to the latest advances in immune monitoring.

Tumor Immunology and Cancer Vaccines is the fruit of tremendous cooperation between our knowledgeable and devoted authors and the commitment and foresight of our publisher. We worked hard to make this book an effective resource, which we hope will translate to discoveries in the field of tumor immunology and more effective treatments of patients with cancer.

I. BASIC TUMOR IMMUNOLOGY

1. ANTIGEN PROCESSING AND PRESENTATION

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In the ongoing search for effective and reliable immune-based approaches to cancer therapy, much of the work is focused on T lymphocytes as effectors. $CD8^+$ T lymphocytes (T_{CD8+}) are of particular interest as they combine specificity and lethality at a level that no current chemotherapeutic or radiation regimen can match. One can only marvel at the effectiveness with which these cells are able to clear an acute respiratory tract infection, leaving the involved tissues intact—the precise goal of cancer therapy. $CD4^+$ T lymphocytes (T_{CD4+}), relatively specific, but generally less cytotoxic than T_{CD8+}, can also mediate potent anti-tumor effects in certain settings. While a great deal has been learned about how T_{CD4+} and T_{CD8+} responses are induced and sustained, further exploration will be necessary if the full potential of these populations is to be harnessed. One aspect worthy of closer inspection is that of antigen processing and presentation—the various intracellular steps that prepare antigen for T cell recognition. It is intuitive that greater understanding and controlled manipulation of these events, which usher in the adaptive response, could have profound influence on the final character of the anti-tumor immunity that is engendered.

1. INTRODUCTION

This chapter will review fundamental aspects of antigen processing and presentation with special emphasis on how they pertain to tumor-specific immunity. Three points must be made at the outset. First, there is no intent to evaluate the relative efficacy of various therapeutic strategies that have been based on principles of antigen processing and presentation. Only a handful of possible permutations have been tested at this

point and, in any event, outcomes will certainly be different depending upon the experimental model or clinical situation. Second, there is minimal segregation of findings in animal models (usually mouse) and humans. Most of the fundamental cell biology is similar even though decades of experimentation and practical application have made it clear that success in mouse models does not ensure success in patients. Finally, the topic of tumor antigen processing and presentation is now sufficiently large that a comprehensive review in a single chapter is not possible. While an attempt has been made to cover a large amount of conceptual territory, space does not allow for all of the relevant work to be mentioned here.

2. THE BASIS FOR T CELL RECOGNITION: FRAGMENTS OF ANTIGEN DISPLAYED AT THE CELL SURFACE BY SPECIALIZED "PRESENTING" MOLECULES

2.1. Peptide Binding

While B cells and their antibody products recognize antigens in their native forms, T cells respond to pieces of antigens held at the cell surface by various "presenting molecules" and generated by a variety of intracellular, and even extracellular processes known collectively as antigen processing. Class I molecules are made up of a heavy chain encoded within the major histocompatibility complex (MHC) and a noncovalently associated light chain, β2-microglobulin. Class I heterodimers bind peptides that are generally 8–11 amino acids in length and present them to T_{CD8+} whose most appreciated response is killing of the peptide-presenting cell. Class II molecules, comprised of α and β chains, both encoded within the MHC, generally bind peptides 11–17 amino acids in length, and present them to T_{CD4+} which respond by elaborating factors that guide and potentiate both B cell and T_{CD8+} responses.[1] The variation in lengths of peptide bound by class I and class II molecules is due to distinct structural differences in the peptide-binding grooves (1). The binding grooves of class I molecules are closed at both ends, with the consequence that a peptide must be a specific length in order to be bound. In contrast, class II binding grooves are open at both ends so that quite large peptides have the capability of binding. Despite this, relatively short peptides are usually isolated from class II molecules, presumably due to the exposure of any extended portions to intracellular and extracellular proteases. As might be surmised from several different crystal structures (2), peptides that directly interact with the binding groove of both class I and class II molecules are resistant to proteolysis, as are the presenting molecules themselves (3–7). Many readers may know that a key feature of class I and II molecules is their tremendous polymorphism, with hundreds of versions of each encoded by many different loci within the MHC existent in the human population. Greatest variation is in the residues that line the peptide-binding grooves, leading to distinct peptide-binding specificities and, thus, differences among individuals in the parts of any antigen that are responded to. This variation is a powerful strategy for a population to counteract the rapid replication and mutation rates that many

[1]CD4 molecules bind to conserved regions of class II molecules and CD8 molecules bind to conserved regions of class I molecules, in both cases participating in activation.

microbes are capable of, but constitutes a major impediment for tissue transplantation and immune-based cancer therapy since both applications may require individually-tailored therapies. The basis for binding specificity is a series of pockets in the floor of any peptide-binding groove into which side chains of the peptide extend. Some of these pockets provide anchoring points that are quite stringent in terms of the side chains that are acceptable, while others are much more permissive. Thus, only specific segments within a protein, with appropriate amino acids properly spaced apart, are able to bind any particular MHC molecule. Those side chains that do not participate in binding to the groove are available for interaction with the T cell receptor. As mentioned at the outset, recognition of peptides by T cell receptors can be highly specific and sensitive. Single amino acid changes in a peptide, including residues that do not directly contact the T cell receptor and even simple phosphorylation of a peptide, can profoundly influence T cell recognition (8–10). In terms of sensitivity, relatively few copies of a particular peptide are required for full T cell activation—on the order of tens to hundreds (11–13). This can be derived from an amount of antigen that cannot be detected using standard biochemical methods (14). Both specificity and sensitivity are highly variable among different T cell clones (15), being determined by both intrinsic factors, such as receptor sequence and density, and extrinsic factors such as the balance of stimulatory and suppressive cytokines. These factors will obviously vary dependent upon the tissue(s) where the antigen is expressed.

From the standpoint of peptide presentation, targets of T cell-mediated tumor immunotherapy can be divided into three broad categories: foreign, mutated self, and nonmutated self epitopes. Examples of the first category (foreign) are epitopes from the growing number of viruses that establish persistent infections and induce transformation, such as the papillomaviruses and herpesviruses. Within the second group are the proteins altered by point mutations, deletions or chromosomal translocation, which are incidentally or coincidentally connected with transformation. All of these can result in new peptide sequences that have the ability to bind to an MHC class I or class II molecule and potentially elicit a response. An emphasis must be placed on the words *can* and *potentially*. Such mutations do not guarantee the generation of a neo-epitope that can bind to an MHC molecule and binding does not guarantee T cell stimulation. At least with respect to peptide binding, some level of prediction is possible. Algorithms, based upon known epitopes, have been developed for many mouse and human MHC molecules, such that one can query an open reading frame for the presence of segments with a high likelihood of binding (16, 17). Nonmutated peptides could be of potential interest if they are: 1) derived from antigens, such as carcinoembryonic antigen, that are expressed at low levels or not at all in the adult, but highly expressed in the cancerous cell, 2) expressed by a differentiated (specialized) cell type, such as the melanocyte, that is expendable, 3) expressed by a fraction of a particular cell type, expendable or not, such immunoglobulins, the product of B cell lymphomas, that can provide unique T cell epitopes from the hypervariable regions (18, 19), or 4) altered by cellular processes that have gone awry as a result of transformation. An example of this would be phosphorylation due to aberrant kinase

activity, as recently suggested by the formation of antigens within papillary thyroid carcinomas (20).

2.2. Epitope Identification

Several different approaches can be used for the identification of class I- and class II-restricted epitopes in proteins of the three classes—foreign, nonmutated self and mutated self. The course taken is dictated by what is available. In the best circumstance, the target protein has been identified, a T cell clone or line specific for that protein is in hand, and the presenting molecule has been identified through antibody blocking or transfection experiments. In the past, mapping under these circumstances involved progressive fragmenting of the protein, either genetically or biochemically, or identification of relevant regions with known sequence variants, until synthetic peptides could be used for precise mapping of the key residues. This is the general approach that was taken to identify mouse epitopes within the transforming (T) antigen of SV-40 (21, 22). Alternatively, with fairly small antigens, overlapping synthetic peptides covering the entire open reading frame have been used, as in the cases of the E6 and E7 oncogenic proteins of papillomavirus (23). With the identification of many peptide-binding motifs, more often than not, one now fragments the protein "electronically" by utilizing the algorithms mentioned above, and then testing a set of synthetic peptides that score the highest according to the algorithm for the ability to stimulate the T cell line/clone. The approach is still fairly imprecise and the immunodominant epitopes within a protein may not be those that score highest by any algorithm.

Often a protein is merely suspected of being a viable target for immunotherapy and a tumor-specific T cell population may or may not be in hand. In this case, the protein can be analyzed for MHC-binding segments (in humans, this is usually the prevalent HLA-A2 molecule), and then high-scoring synthetic peptides are tested for the ability to stimulate a tumor-specific T cell response or to activate tumor-associated T cells (24). In yet another scenario which is quite common, a tumor-specific T cell line or clone has been generated but the target protein is unknown, in which case algorithms are of no value. When Boon and colleagues were confronted with this situation over a decade ago with the P815 murine tumor cell line, their approach for identifying the tumor antigen and, ultimately, the epitope, involved systematic transfer of DNA from the immunogenic tumor cells to non-immunogenic tumor cells, and eventual identification of the open reading frame coding for a protein that activated the tumor-specific T cells (25, 26). Fortunately, progress has replaced this labor-intensive approach with a more straightforward, though still technically challenging, method. The current approach, several years old by now, entails detergent lysis of large numbers of the tumor cells, optional purification of the class I or class II molecule which is known to present the epitope, and separation of eluted peptides by HPLC (27–29). These pools are then tested for the ability to stimulate the T cell line/clone. Reactive pools are analyzed by electrospray ionization tandem mass spectrometry which allows for the isolation and sequencing of individual peptides. Synthetic versions of each peptide within a reactive fraction

can then be tested with the T cell line/clone and databases can be searched to identify the parent protein. In the event that no candidate is identified with bioinformatics, a degenerate oligonucleotide pool can be used to fish out the gene that encodes the protein. Far fewer class II-restricted tumor associated epitopes have been identified for two reasons, the first being that less effort has been expended for reasons discussed below. The second is that in cases where the protein that contains the epitope is not known (most cases), it is technically more challenging to identify these epitopes (30). This can be attributed to the open ended groove of class II molecules, such that a class II epitope does not constitute a discrete peptide species, as is usually the case with class I, but a set of "nested" peptides, all containing the same core epitope sequence. Thus, the "signal" will be distributed in many fractions following HPLC purification, causing significant dilution. The challenge is greater when the tumor cell does not express class II, which may often be the case (30), and must stimulate T_{CD4+} cells via a cross-presentation mechanism that is discussed below.

It is important to keep in mind when taking any of these approaches, particularly when attempting to identify class I-restricted epitopes, that not all epitopes are derived from the conventional open reading frame. Alternative splicing, unconventional translation initiation, and translational frameshifting can all generate unpredicted peptide sequences that might contain T cell epitopes (31). The extent to which such epitopes contribute to the overall T cell response remains to be seen but several anti-tumor responses to such epitopes have already been documented (32–35).

Identification of the epitope facilitates a number of therapeutic approaches, as discussed below. A step some have taken to enhance epitope-based strategies is the "redesign" of the natural sequence through amino acid substitutions (36–38). Changes can enhance anchoring into the binding groove, a factor that can contribute to immunodominance (39), and/or improve contact with the T cell receptor. The key is that the alterations must preserve reactivity on the part of at least some participating T cell clones with the wild-type sequence.

2.3. Other Presenting Molecules

In addition to the "classical" class I and class II molecules, there are other presenting molecules that are less well understood, termed non-classical class I molecules or class Ib genes. In humans these include CD1, the neonatal Fc receptor for IgG, HLA-G, HLA-E, the MHC class-I chain-related gene A, and Hfe (40). Thus far, there is limited information on the presentation of tumor antigens by these molecules. NK/T cells express a highly restricted set of T cell receptors and respond to lipids and glycolipids presented by CD1d molecules (41). They have caught the attention of many due to the strong influence they can have on tumor-specific immune responses (42). However, identifying the naturally-presented molecules is extremely challenging and it will likely be several years before the basis for their participation in anti-tumor responses is understood. Interestingly, the involvement of some of these non-classical MHC molecules may be detrimental to the anti-tumor response. HLA-G, for example, is expressed by trophoblastic cells of the developing embryo

and is thought to inhibit maternal immune responses to the semi-allogeneic-fetus (40). Over expression of HLA-G has been noted in breast cancers where it may interfere with immune responses to the tumor (43). Similar concerns have been raised for melanoma where expression of HLA-G prevents tumor killing by natural killer (NK) cells (44).

2.4. The Generation of Antigenic Fragments: A Brief Overview of Antigen Processing

The two major subcellular sites of proteolysis within the cell are the cytosol and the endolysosomal compartment. In general, MHC class I molecules bind peptides that derive from cytosolic proteolysis while MHC class II molecules acquire peptides that have been generated by endosomal and lysosomal proteases. This division of labor is dictated by properties of the MHC molecules themselves and the proteins with which they transiently associate. Essentially, all antigen processing pathways represent a dovetailing of fundamental "housekeeping" processes, such as proteolysis and protein trafficking, with specialized processes, such as peptide loading and β2-microglobulin/class I association. Modulation or elimination of the specialized processes is a viable means of immune evasion, as discussed at length below, but substantial alteration of the more fundamental aspects of antigen processing may not be compatible with cell viability. A second general point concerning antigen processing is that the systems are always in action. In uninfected and nontransformed cells, peptides derived from self proteins are constantly produced and presented, although at a lower level than would be the case for many infections where products of the innate immune response cause upregulation of the class I and class II systems at several points.

2.5. Activation of Naïve T Cells: "Professional" Antigen Presentation and T Cell Help

The activation of the very small numbers of naïve class I- and class II-restricted T cells that are specific for any particular epitope requires presentation by so-called "professional" antigen-presenting cells, essentially, those that can supplement the primary MHC/peptide signals with a strong second activation signal (termed co-stimulation) in the form of surface CD80 (B7.1) and CD86 (B7.2) molecules, ligands for CD28 molecules on the surface of T cells. Naïve T cells that receive the primary signal without co-stimulation (secondary signal) are inclined to enter a state of unresponsiveness (anergy) or die, a mechanism for the induction of peripheral T cell tolerance (45). The major, if not exclusive, professional APC for activation of naïve T cells is the dendritic cell (DC). These bone marrow-derived cells are highly mobile, carrying antigens they have acquired in the tissues, via a process termed cross-presentation, to the regional lymph nodes, where the opportunity for T cell activation is maximal (46–48). The bases for cross-presentation is not fully understood, but likely involves the uptake of dead or dying cells, debris from dead cells, and/or the transfer of antigenic peptides via heat shock proteins which, like MHC class I and class II, bind proteins in their linear, processed, forms (49, 50). In order for

DCs to carry out this function, it must itself undergo activation (commonly termed "maturation") in which it is converted to a cell with reduced antigen uptake, optimized antigen processing and presentation functions through changes that include upregulation of MHC and co-stimulatory molecules, and lymphoid homing capability (51, 52). DC activation is triggered by the receipt of signals such as TNF-alpha and type I interferons that are produced in the tissues as a result of innate responses to molecules indicating the presence of "danger" and/or "stranger" signals (53, 54). Examples of such cues are double-stranded RNA (a hallmark of many viral infections), formylated peptides, and terminal mannose groups on glycoproteins (both hallmarks of bacterial infection). A persisting question is whether danger/stranger cues from cancer cells are sufficiently robust to activate the DC. While there may be some elements of this due, for example, to necrosis or inappropriate cytokine/chemokine production, most cancers in their advanced stages simply do not evoke the intense innate immune responses that acute viral or bacterial infections do. Thus, there is a strong possibility that, despite the presence of unique epitopes within a particular tumor cell, a lack of sufficient co-stimulation will result in the unresponsiveness or death of tumor-specific T cells. Therefore, vaccines for cancer, like vaccines for any infectious organism, must be formulated in a way that facilitates presentation on activated APC. An open question in T cell activation that may be particularly relevant for tumor-specific immunity, is whether the spectrum of epitopes presented by the professional APC via vaccination or cross-presentation is similar to the spectrum of epitopes presented by the tumor cell itself. Indeed, there is good reason to suspect that this will not always be the case.

It must be kept in mind, however, that other cell types, including tumor cells, can take on a professional APC phenotype in an inflammatory environment. The processing capabilities of, and the peptide display by such cells may be distinct from DCs. Of note, professional APCs, be they DCs or tumor cells, may themselves serve as targets for the cytolytic T cells that they have activated, providing a potential negative feedback mechanism that might limit the scope of the response (55).

An important function of T_{CD4+} is the potentiation of both B cell and T_{CD8+} responses. Recent studies have shown that T_{CD4+} participation during a primary response is critical for the development of durable T_{CD8+} memory (56–58). A key molecular interaction in the generation of "help" for T_{CD8+}, is the binding of CD40 and CD40 ligand (CD40L). Abundant evidence shows that one mechanism for signal delivery is indirect, in which activated T_{CD4+} expressing CD40L "back signal" or "license" the CD40-expressing APC which then presents peptide and the co-stimulatory signal to CD40L-expressing T_{CD8+} (59–61). This provides one means of overcoming the need for the APC, the rare antigen-specific T_{CD4+}, and the rare antigen-specific T_{CD8+} to be simultaneously conjugated to one another. More recently, it has been shown that activated T_{CD8+} can express CD40, allowing for direct CD40:CD40L signaling between antigen-specific T_{CD4+} and T_{CD8+} after each has seen antigen (62). This is the same mechanism for delivery of help to antigen-specific B cells. It seems likely that the ratio of direct and indirect help to T_{CD8+} will vary depending upon the antigens and nature of the challenge.

Many current tumor vaccines have been designed with the concepts of cross-presentation and CD40 ligation in mind. One strategy that has been tested in many experimental and clinical systems involves pulsing of autologous in vitro-expanded and activated DCs with synthetic peptide epitopes from tumor-specific, tumor-associated antigens (63, 64) or the antigens themselves (18, 19). Uptake of peptide by DCs can be enhanced by targeting to the gp96 heat shock protein receptor (65–69) and of whole protein by targeting to the DC Fc receptor in the case of lymphoma-produced antibody or synthetic antigen-antibody complexes (70, 71), the mannose receptor via mannosylation of the protein (72), or by conjugating the protein to a membrane-crossing protein such as HIV TAT (73). Alternatively DCs have been transfected with DNA or RNA (74–76), or transduced with viral vectors, encoding the tumor antigen (77–80). It can be argued that the most appropriate targets for an cancer in an individual may not have been identified and/or that the most effective anti-tumor response will be directed at many different tumor specific/associated targets. Thus, in a number of experimental and clinical settings, DCs have been pulsed with whole tumor cell lysate (81–83) or with apoptotic tumor cells (76, 82, 84–86), allowing for the presentation of many different proteins expressed by the tumor cell. Alternatively, cross-presentation, would not be necessary if the tumor cell itself can naturally serve as a professional antigen-presenting cell. One might assume this to be the case with B cell lymphomas and myeloid leukemias but both appear to be weak antigen presenting cells (87–90). In the absence of natural APC capacity, many groups have converted the tumor cell to a professional APC by transfection/transduction with the genes encoding co-stimulatory molecules (91–94), or fusion of tumor cells with DCs (86, 95–98).

Rather than bypassing cross-presentation, one can seek to maximize the process. One such approach involves transfection or infection of in vitro-expanded tumor cells to allow for expression of DC-attracting cytokines such as GM CSF prior to reintroduction (92, 99, 100). An interesting variation of this, is the transfection of tumor cells (murine melanoma) with a modified GM CSF gene that results in expression of the cytokine at the cell surface, with the intent of maximizing direct interaction between the tumor cell and the professional, GM CSF-receptor-expressing APC (101). For accessible tumors, such as melanoma, GM CSF-expressing viruses can be directly injected into the tumor in situ (102, 103). Another strategy involves immunization with the tumor antigen, Flt-3 ligand (a DC growth factor), and CpG-containing DNA, which activates DC via the Toll-like receptor 7 (104, 105). One intriguing method involves the decoration of in vitro-expanded (leukemia and lymphoma) tumor cells with alpha-galactose, and returning these modified cells to the patient, taking advantage of the naturally-existing anti-alpha-galactose antibodies that will mediate opsonization by professional APCs (106). Lastly, is the use of a heterobifunctional monoclonal antibody intended to connect the tumor cell with the APC (107). In the example cited, one binding site of the antibody is specific for the HER-2/Neu protooncogene product, and the other, for Fc-gamma receptor 1, expressed on the surface of myeloid cells. The effect is intended to be two-fold: antibody-dependent cellular cytotoxicity (ADCC) against

the tumor cells and uptake by professional APC for class I- and class II-restricted presentation.

Strategies have also been attempted to facilitate the delivery of costimulatory signals to T_{CD4+}. Administration of anti-CD40 during immunization (80), or transduction of peptide-pulsed DCs with the CD40L gene (108) have been reported to enhance tumor-specific immunity.

3. MHC CLASS I-RESTRICTED PROCESSING AND PRESENTATION

3.1. Fundamentals[2]

MHC class I molecules are standard type I glycoproteins which are translocated into the endoplasmic reticulum (ER) during their translation. Prior to acquiring peptides of the correct length and sequence, class I molecules are retained within the ER by chaperonins that, in essence, view empty class I molecules as incompletely folded. In cells where peptide supply is chronically limited, surface class I levels are generally reduced. In terms of proteolytic capacity, the ER appears to be limited to trimming of peptides at the amino terminus (123–125). Thus, the cytosol with its rich proteolytic activity, bears the prime responsibility for generating class I-binding peptides, particularly the correct C-termini. The most notable cytosolic protease is the proteasome, a huge catalytic protein complex made up of a central barrel that is sealed at both ends by complex cap structures. Substrates are degraded to peptides 3–22 amino acids in length (126) within the barrel by three different proteases whose destructive capacity is insulated from the cytosol by the caps that regulate which proteins enter the inner chamber (127, 128). The best known means of qualifying a protein for degradation via the proteasome is through ubiquitinylation. In this case, the 76 amino acid-long ubiquitin polypeptide chain is attached to available lysine residues of the targeted protein via an isopeptide bond (129–132). Ubiquitin molecules can themselves be ubiquitinated at their own lysine residues. Once the target protein is decorated with at least four ubiquitin moieties, the proteasome cap engages the substrate which is guided to the interior where the active sites of the three distinct proteases reside[3]. Ubiquitin molecules are removed for reuse during this process. While ubiquitinylation appears to be the most common means of targeting a protein for destruction, it is not the sole means. For example, ornithine decarboxylase is targeted for destruction via association with a molecule termed antizyme (133) and the cyclin-dependent kinase inhibitor $P21^{Cip1}$ can apparently direct its own degradation via association with a subunit of the proteasome barrel (134)[4]. With respect to antigen processing, even a relatively large epitope-bearing polypeptide with no lysine residues can nonetheless be efficiently processed (135) although the targeting mechanism is presently unknown.

[2]Many additional reviews on the topic of MHC class I-restricted antigen processing and presentation are available (109–122).

[3]Until recently, ubiquitinylation was considered to have the single effect of targeting proteins for degradation. It is now clear that unbranched ubiquitinylation can have powerful regulatory effects upon proteins, such as alteration in activation state or subcellular location.

[4]How such association results in degradation is presently unknown.

Several pieces of evidence implicate the proteasome in class I-restricted antigen processing. First, various inhibitors of the proteasome block the production of many different epitopes. Indeed, such inhibitors reduce the expression of many class I allomorphs at the cell surface, presumably due to limited peptide supply and the retention of class I in the ER. Second, there are actually two different "flavors" of proteasomal catalytic subunits: *constituitive* and *interferon-inducible*. Proteasomes comprised of these inducible subunits (so called "immunoproteasomes") are upregulated by the same innate cues that activate APCs and appear to skew generation of peptides towards those that are likely to bind to class I molecules (136). Thus, most peptides bound by human class I molecules feature a basic or hydrophobic residue at the C-terminus and immunoproteasomes demonstrate enhanced production of peptides with such C-termini. In addition to the substitution of catalytic subunits, interferon gamma also induces substitution of the constituitive 19S cap with the PA28 cap, which has been implicated in enhanced production of class I-restricted epitopes (137, 138). One such epitope derived from a melanoma-associated protein is presentable only when proteasomes possess the PA28 cap (139). As with cross-presentation, there is a concern about epitopes that are presented at different phases of the response. During the induction of anti-tumor immunity, immunoproteasomes may dominate while, during the effector phase, in the absence of frank danger/stranger signals, constituitive proteasomes may be the major producers of epitopes within tumor cells. Some overlap in epitope production by constitutive and immunoproteasomes will be critical if performing therapeutic vaccination against a cancer that does not generate frank danger signals. Experimental evidence demonstrates such an overlap, but the PA28-dependent melanoma epitopes, and others like it, may not be appropriate targets for T_{CD8+}-mediated immunotherapy.

While proteasomes appear to be the main engine for cytosolic proteolysis and class I-restricted antigen processing, there is mounting evidence for the participation of other cytosolic proteases such as leucine aminopeptidase (140), thimet oligopeptidase (141), purine-sensitive aminopeptidase (142), bleomycin hydrolase (142) and tripeptidyl peptidase II (143–145). The activity of these proteases suggests that they act upon products of the proteasome that require additional trimming to meet class I binding requirements. The question of whether these or other proteases can act in parallel with (replace) the proteasome is open. Indeed, there are some epitopes whose presentation is *enhanced* by the addition of proteasome inhibitors. Such observations are compatible with the notion of competition, in the case of some epitopes, between epitope-generating proteases and an epitope-destroying proteasome. However, the picture is complicated by the fact that none of the current proteasome inhibitors completely shuts down the proteasome. Thus, it has been suggested that they are better termed proteasome "modifiers", rather than inhibitors (146–148). Therefore, an equally plausible model is that modified proteasomes are more efficient at producing certain epitopes. Tripeptidyl peptidase II (TPPII) has been suspected of being able to substitute for the proteasome because it is markedly upregulated when cell lines are treated chronically with proteasome inhibitor (149). A recent publication supports this notion (145), but much more work is needed before a

full appreciation for the level of reciprocity can be attained. One suspects that reciprocity, if existent, will be limited. Compared to the proteasome, TPP II is relatively simple from a structural standpoint, and its capabilities are probably far more limited.

The efficiency with which epitopes are produced from various proteins varies widely (12, 150) and reasons for this have been of interest to investigators for many years. Over a decade ago, it was proposed by Townsend and colleagues that the turnover rate of a protein determines the efficiency with which a given epitope is produced (151). For the most part, this idea has been upheld by several (151–156), but not all (157) groups, who have shown that modifications of an antigen that decrease its half-life, increase the efficiency with which a contained epitope is presented. This model has been refined by Yewdell and colleagues and articulated as the "DRiP" (for "defective ribosomal products") hypothesis which proposes that epitopes are mainly derived from nascent proteins that are not produced correctly due to errors during transcription, splicing, translation and/or folding (158) and consequently targeted for rapid destruction—a notion that has recently received experimental support from the same (159) and another (160) group. Thus, one might think of engineering an antigen so that every copy will fail quality control and be targeted for rapid degradation. If the epitope has been defined, and maximizing epitope expression is the goal, then simply expressing the epitope alone from a "minigene" construct, thereby sidestepping issues of processing efficiency altogether, and even attaching a signal sequence to the C-terminus (sidestepping TAP transport issues) are options that many have investigated for cancer immunotherapy (161–168). However, it is important to consider the possibility that maximal epitope production may not induce an optimal T cell response. In fact, it has been demonstrated that stimulation of T cells with low levels of epitope preferentially expands T cells with high avidity MHC/peptide receptors that provide a strong protective effect while stimulation with high levels of epitope produces a T cell population with a lower average avidity that is not protective (169)[5]. In addition, priming mice with a minigene construct can result in the expansion of T cells, a large portion of which have no detectable effector function (13, 171). Finally, "drippiness" does not appear to be the only parameter that influences the efficiency of processing. Primary sequence can be a very important parameter (172–174), due at least in part to the obvious effect that it has on cleavage efficiencies of proteases.

Once generated, peptides must be transported into the lumen of the endoplasmic reticulum in order to have a chance of binding to by nascent class I molecules. This is *not* the function of the translocon, the pore through which glycoproteins such as MHC class I molecules are conveyed during their syntheses. Rather, there is a separate transporter termed TAP (transporter of antigenic peptides) whose sole job appears to be transfer of potential class I ligands into the lumen of the ER. TAP has both length and sequence requirements that are necessarily broader than those of class I, since TAP

[5]In these experiments stimulation of T cells was performed in vitro. Recent experiments involving the priming of mice with dendritic cells pulsed with varying amounts of synthetic peptide suggest that avidity selection in vivo may be more restricted (170).

must supply peptides to a wide variety of different class I molecules. Experiments with isolated microsomes suggest that efficiency of transport is highest for peptides that are 8–16 amino acids in length (110, 175), comfortably encompassing class I length requirements. In addition, evidence suggests that TAP performs a filtering function in selecting for transport those peptides with C-termini that match class I-binding preferences—hydrophobic for mouse, hydrophobic and basic for human. Presently, there is no definitive evidence for a physical connection between the proteasome and TAP. Therefore, it is not known how products of the proteasome and other proteases are conveyed to TAP, though it is commonly speculated that some of the many cytosolic chaperonins may play a role here. Connections are clearer on the other side of the ER membrane as TAP is physically attached to nascent class I molecules via a specialized chaperonin termed tapasin that allows class I molecules to have an immediate opportunity to sample the spectrum of peptides produced in the cytosol (120, 176).

One of the attractive aspects of the class I processing pathway in terms of cancer immunotherapy is its potential to present epitopes from any type of protein produced by the cell, whether it be cytosolic, nuclear, mitochondrial, expressed at the plasma membrane or secreted. It is easy enough to see how cytosolic proteins, nuclear proteins, and even mitochondrial proteins derived from the host genome can enter the pathway via delivery to the proteasome since they all reside at one time or another within the cytosol. Processing of secreted and cell surface proteins that never have a natural cytosolic phase is less intuitive. Not long ago, two complementary possibilities were considered: 1) A small fraction of the mRNA is inappropriately translated on free ribosomes rather than translocon-associated ribosomes, resulting in delivery of some protein to the cytosol where, not being in the appropriate environment to fold properly, it is targeted for rapid turnover and delivery to the class I processing pathway. 2) Proteases resident within the ER degrade proteins that fail quality control, with some fraction loaded onto class I molecules prior to complete digestion. While there is evidence that both of these mechanisms may contribute to the generation of epitopes from glycoproteins (177), a recently-deduced pathway appears to explain most cases of presentation for this category of antigen. Following a quality control failure, such proteins are directed to the cytosol, via the translocon and delivered to the proteasome for TAP-dependent presentation (178, 179). These considerations point to potential strategies for enhancing tumor-specific immune responses. Accordingly, in priming of a response to such an antigen, one might consider genetically modifying the protein so that it is delivered directly to the cytosol, through removal of the signal sequence that targets the protein for translocation into the ER. However, the pathway from the ER to cytosol appears to be quite efficient and, indeed, for reasons that are unclear, the processing of antigen is qualitatively different when the antigen originates from the ER vs. the cytosol (180). Thus, in modifying an antigen, one may alter the processing, generating a peptide profile that does not match that of the actual tumor cell. As with the proteasome/immunoproteasome question, the extent to which this should be a problem remains to be seen.

One final area must be discussed before turning to the role of this pathway in anti-tumor immunity—the presentation of exogenous (extracellular) antigen. Because the processing pathway for most antigens begins in the cytosol of the cell with digestion by the proteasome or other proteases, nascent antigen (synthesized within the presenting cell) is considered to be the prime source of processing substrate. However, cross-presentation, discussed above as critically important in most, if not all cases of T_{CD8+} priming, involves the uptake of antigen by DCs. How does antigen then gain access to the the cytosol? Two potentially complementary mechanisms have been proposed. First, professional APCs appear to have somewhat "porous" endocytic vesicles, allowing delivery of internalized material to the cytosol (116, 181–183). Perhaps mediators of cross-priming, heat shock proteins being likely candidates, are transferred to the cytosol following uptake with high efficiency. Second, there is evidence for TAP-independent acquisition of peptides by mature class I molecules within the endosome (184). Many details of both alternative pathways remain to be elucidated, and their relative contribution to cross-presentation is also unclear.

3.2. "Escape" of Tumors from Class I-Restricted Recognition

A great deal of effort from many laboratories has focused upon the expression level of molecules that play a part in class I restricted antigen processing and presentation with the idea that reduced expression in cancer implies active evasion of immune recognition. As pointed out in recent reviews (185, 186), care must be taken in making this conclusion since the evidence is indirect. One would need to demonstrate the generation of an active immune response (something from which to escape)—as opposed to the onset of tolerance - during the earliest stages of tumorigenesis, nearly impossible in the clinical setting. As mentioned above, the processing and presentation system is a dovetailing of fundamental and accessory cellular functions. In this light, it is not surprising that major disturbances in fundamental functions such as proteasome activity, ubiquitinylation, and protein trafficking have not been noted. In contrast, defects in essentially all of the accessory functions have been noted. Several categories can be delineated: 1) *Mutation of the antigen*. The most straightforward means of evading recognition is mutation of the tumor antigen-encoding gene in such a way that the antigen is no longer expressed, as documented in the melanoma system (187) or so that the epitope is no longer presented. This could be achieved by mutation of anchor residues, resulting in loss of binding, or mutation of T cell receptor contact residues. Consequences of the latter type of mutation can be complex. The simplest outcome is complete loss of recognition by every participating T cell, although there is the possibility for activation of an entirely new fraction of the $CD8^+$ T cell population that recognizes the mutated peptide. However, another consequence of altering the peptide ligand, is partial or complete antagonism in which case the T cell can be driven to an altered state of activation or even anergy. One might argue that, among the set of participating T cell receptors, a change could lead to all four permutations (loss of recognition, partial antagonism, antagonism, and continued agonism) and that those T cells continuing to receive an agonizing signal would remain effective. However, the possibility must be considered that the

antagonized population produces factors that inhibit activity of agonized cells. Space does not permit continued discussion of this topic, but interested readers are directed to detailed reviews (188–191). An additional antigen-based evasion strategy is mutation of a residue flanking the epitope so that the epitope can no longer be presented, as in the case of a p53 variant (192). While it appears that the class I-restricted processing machinery can extract epitopes from most contexts (193), proximal and distal sequence can clearly affect the efficiency of this extraction and can, in some cases, ablate it (135, 172–174). In the case of the p53 variant, evidence suggested that the extraepitopic mutation prevented generation of the proper C-terminus of the epitope by the proteasome (192), but another potential mechanism is the introduction or enhancement of a proteolytic cleavage site within the epitope, so that it is destroyed, rather than generated (174); 2) *Alteration of class I.* Many groups have noted reduced class I expression in several types of tumors including human head and neck squamous cell carcinoma (194), colorectal carcinoma (195), melanoma (196), and breast cancer (197). Recent observations of this reduction have been observed with freshly obtained tissue where class I expression has been compared with adjacent normal tissue. This is more credible than assessing class I levels in tumor cells that have been in culture for extended periods of time. Reduction in class I can be specific for a particular allomorph, leaving open the possibility of continued recognition via other class I molecules, or can affect all six loci in which case there is likely to be a defect in regulation that includes other components of the processing pathway, as discussed further below; 3) *Alteration of β2 microglobulin*. Due to stringent structural constraints, loss of β2 microglobulin will effectively eliminate expression of all class I molecules. Mutations of β2 microglobulin in several different tumor types have been noted. (195, 198–200), although one comprehensive study concludes that it is not commonly found in tumor cells with total loss of class I (201); 4) *Alteration of the Proteasome*. Subtle changes in proteasome function can also provide a means for diminished epitope production. In the case of an HLA-A2-restricted epitope within tyrosinase-related protein 2 (TRP2), expression is possible only when the PA28 cap structure is expressed (139). Loss of PA28 could therefore provide a means for immune evasion, although it must be noted that production of another TRP2 epitope is diminished by interferon treatment, suggesting that it is more efficiently produced by the constitutive proteasome. 5) *Alteration of TAP.* Soon after the role of TAP was defined, several groups investigated the possibility that in some tumor cells TAP is downregulated as a possible means of immune evasion. Indeed, many different tumor cell lines and primary isolates have reduced TAP expression (202–212) which may correlate with malignancy (208, 209, 212). Of course, with sufficient reduction in TAP expression, many class I molecules will be downregulated due to lack of ligand and consequent retention in the endoplasmic reticulum. Unresponsiveness of TAP to interferon γ has been observed in a renal carcinoma line (213), but most cases of TAP downregulation are reversible with interferon treatment, indicating that the defect is at the regulatory level. In several cases, upregulation of TAP through gene transfer has been observed to enhance immunogenicity. However, increased TAP expression may not be altogether desirable. TAP is a member of the APC

(ATP-binding cassette) family of transporters which also includes the multi-drug resistance (MDR) transporter. The MDR protein, located at the plasma membrane, is often upregulated on cancer cells in response to chemotherapy, thereby thwarting the impact of these agents. Evidently, despite its location, TAP has some degree of MDR-like activity, as its increased expression has been correlated with resistance to chemotherapy (214, 215)[6]. Thus, local or systemic treatment with interferon may enhance the immunogenicity of the cancer but may also increase its drug resistance; 5) *Alteration of Tapasin*. Given the role of tapasin in mediating the exchange of peptide between TAP and class I, defects in this protein could also be a means of universally limiting peptide presentation. Thus, it is not surprising that reduced tapasin expression has been observed in several different tumor cell types and that expression can be upregulated by cytokine treatment (216). 6) *Multiple Defects*. It is evident that in many cases, reduced class I expression is due to defects in regulation that can be reversed with cytokine treatment. Since class I, TAP, tapasin and immunoproteasome subunit expression are all coordinately regulated, it could be predicted that there are many reports of tumor cells with reduced expression of many of the components of the class I processing pathway (89, 198, 199, 203, 206, 207, 209, 217–224).

The extreme sensitivity of $CD8^+$ T cells has been discussed. Thus, it might be predicted that even substantial loss of class I expression via any of the mechanisms previously discussed, would not allow for immune evasion. This is at odds with reports that reversal of low class I expression via cytokine treatment enhances immunogenicity (225–227). However, such reports are balanced by other work indicating little impact of TAP or class I downmodulation (210, 228). Indeed, in some cases, an *increase* in immunogenicity has been correlated with reduced expression/function (229, 230). This latter outcome is apparently due to the elimination of natural killer (NK) cell recognition, which is based, in part, upon loss of class I expression. Only time will tell how significant these various defects in the processing pathway impact cancer progression and the extent to which attempts to reverse the defects have a therapeutic impact. There is greater certainty about evasion tactics when it comes to viruses. The number of viruses shown to encode proteins that interfere specifically with the class I-restricted antigen processing pathway continues to grow. Strategies include interference with proteasome function, occlusion of the TAP transporter, and destruction of class I molecules (231, 232). Most notorious are the herpesviruses, including the cancer-associated Epstein-Barr virus (233), Kaposi's sarcoma-associated herpesvirus gammaherpesvirus (234), and cytomegalovirus (235). Oncogenic papillomaviruses (236) and adenovirus (237) have also been demonstrated to attack, in specific fashion, the class I antigen processing pathway. Thus, in many cases the advantage of having clear targets for immunotherapy in the case of virus-induced cancers may be more than offset by specific and highly effective downmodulation of such targets. Further, full appreciation of immune evasion needs to take into account other factors, such

[6]Transport of a chemotherapeutic compound from the cytosol to the ER (away from the nucleus) is probably nearly as effective as transport to the extracellular space.

as the elaboration of IL 10, (a suppressor of cell-mediated responses), by the tumor cell (198, 238).

4. MHC CLASS II-RESTRICTED PROCESSING AND PRESENTATION

4.1. Fundamentals[7]

There are several ways that MHC class II-restricted processing and presentation differ from their class I counterparts. First, peptides are mainly acquired, not in the endoplasmic reticulum, but within the endocytic compartment of the cell. In large part this can be explained by the co-assembly of class II molecules with the invariant chain (Ii) (244, 249). Ii influences the fate of class II molecules at several levels. If nascent class II heterodimers do not complex with Ii, they are reminiscent of empty nascent class I molecules in that they are largely retained in the ER. Complexing with Ii results in the peptide binding groove being occupied by a part of Ii termed "Clip" (for "Class II-associated invariant chain peptide") preventing other peptides from binding (250), and the complex being targeted to the endosomal compartment by a specific sequence within the cytoplasmic tail of Ii (251). Unlike class II, Ii is highly susceptible to endosomal proteases and, once the class II-Ii complex reaches the endolysosomal compartment, it is catabolized until only the Clip segment remains. Clip is then exchanged for linear segments made available for binding within the endocytic compartment through unfolding and/or degradation. For many class II allomorphs, the Clip/peptide exchange is facilitated by an endosomal-resident heterodimer termed HLA-DM (H-2M in the mouse), which mediates the exchange of lower affinity peptides for higher affinity peptides and also preserves the integrity of empty class II molecules which would otherwise unfold and aggregate in the harsh environment of the late endosome (252). Evidence suggests that some class II molecules rely heavily upon DM action for peptide exchange while others do not (253). The basis for variable DM dependence is still under investigation. It is nevertheless clear that the actions of DM strongly influence the profile of peptides that are presented at the cell surface, thereby playing a major role in determining epitope hierarchies within CD4 responses (254).

A second major distinction is the class II peptide binding groove. While it is similar to class I molecules in having pockets that define binding specificity, the class II groove is open at both ends, meaning that peptides of essentially any length can be bound as long as they are in a linear form (255). Thus, in essence, unfolding of the antigen is the only processing step that is required for class II-restricted presentation (256). This could be accomplished through the reduction of disulfide bonds by enzymes such as the recently discovered endosome-resident disulfide isomerase termed "GILT" (for "gamma-interferon-inducible lysosomal thiol reductase") (257). Alternatively, some antigens may unfold on their own in response to endosomal acidification. This is the case with many viral proteins that mediate fusion through acid-triggered conformational changes (258–260). Certain bacterial toxins also undergo acid-mediated

[7]Many reviews on the fundamental aspects of MHC class II processing and presentation are available (239–248).

conformational changes that may constitute a processing step for some epitopes (261, 262). Other epitopes may be embedded in structurally stable regions of the antigen, in which case, proteolysis is likely to be a mandatory processing step. Lack of a strict length requirement, and therefore no dependence upon precise proteolysis, may be one of the reasons why the number of class II-restricted epitopes in the average protein usually exceeds the number of class I-restricted epitopes.

Two additional points concerning fundamental aspects of the class II processing pathway need to be made. First, because peptide binding capability is gained within the endosome, most bound peptides are likely derived from proteins taken up by the cell. Such antigens are presented via the "exogenous" presentation pathway. However, some peptides are derived from proteins that the cell itself has synthesized, and are presented by the "endogenous" pathway (263–270). This is easily understood for some cellular proteins, such as those that are transient or permanent residents of the endosolysosomal compartment. However, some peptides are derived from proteins whose subcellular locations (the cytosol or nucleus, for example) do not predict access to class II loading compartments. It is not known whether such antigens are delivered whole or in fragments to the endosome, and what the intracellular transport pathways are. There is some evidence to support a role for autophagy, a mechanism that delivers cytosolic contents to the lysosome, in the presentation of one endogenous protein (271), and there are also reports that the proteasome can play a role in class II-restricted antigen processing (272, 273). This is an important issue for tumor-specific immunity since endogenously presented tumor-specific antigens will ensure that activated CD4+ T cells interact directly with tumor cells that express class II. An even better situation may be when an epitope can be presented *only* from the endogenous source, meaning that the CD4+ T cell response will be focused exclusively on the tumor cell, and not include other class II-expressing cells that have taken up cell debris. There are at least two examples of such endogenous-only presentation. Influenza neuraminidase contains an epitope presented by the (mouse) H2-IE^{d} class II molecule that is presented by a B cell lymphoma only when antigen-presenting cells are pulsed with infectious virus, rather than uv-inactivated virus (264). The same is true for a class II-restricted epitope contained within a class I molecule (274). The epitope is not presented when the antigen is provided as a recombinant protein, but is presented when the APC is transfected with a plasmid encoding the class I molecule. The basis for this processing phenotype, in either case, is not understood. One possibility is that some epitopes are rapidly degraded following uptake of the antigen prior to gaining a class II loading compartment. Endogenous sources of antigen, in contrast, might be delivered directly to a class II loading compartment where the competition between class II molecules and proteases is more level. It remains to be seen how widespread this type of presentation is, but tumor immunotherapists might do well to keep a sharp eye out for it.

A frequently overlooked aspect of class II molecules is their relatively efficient internalization from the cell surface, allowing for additional rounds of presentation by these recycled molecules. While nascent class II molecules appear to load epitope

in a late endosomal compartment in a process that is DM dependent and requires Ii expression (for delivery to the late endosome), presentation by recycling class II molecules requires neither DM nor Ii (275–277). The details of how and where recycling class II molecules lose old cargo and acquire new cargo are not known.

Another key feature of class II molecules is their expression pattern. Whereas most cells in an individual express class I molecules, constitutive expression of class II is reserved for a small fraction of the total: DCs, macrophages and B cells. Many cell types can be induced to express class II molecules through exposure to Interferon-γ as naturally occurs at sites of inflammation. Such restricted expression creates even greater problems on the class II side for tumor immunologists since it is generally considered that most tumor cells do not constitutively express class II and, as already mentioned, sites of tumor growth may not feature a robust inflammatory milieu that could induce class II expression. The exceptions, however, are numerous. Many different tumor types have been shown, at least on occasion, to express class II including glioma (278), adenocarcinoma (279), melanoma (280), colorectal cancer (281), transitional cell carcinoma of the bladder (282), esophageal carcinoma (283) thyroid carcinoma (284) and non-small cell lung carcinoma (285). Class II-expressing melanoma cells, at least, have been observed to have antigen-presenting capabilities (286). Three other limitations of class II-restricted anti-tumor responses are worth pointing out. First, as indicated in the introduction, $CD4^+$ T cells are less cytotoxic than $CD8^+$ T cells. Second, while the class I-restricted pathway is open to any type of protein, the class II loading pathway is restricted to those proteins that can end up, in one form or another, in the endosome. This is clearly not every protein made by the cell. Unfortunately, the general rules for protein trafficking have not yet been sufficiently developed to allow a prediction of which proteins fit in this category. Finally, if one is resigned to some kind of vaccine strategy being a necessary component of anti-tumor immunity, the participation of $CD4^+$ T cells is probably essential for full development of the $CD8^+$ population, but the specificity of this population need not be for a tumor-specific antigen. $CD4^+$ T cells specific for the vaccine vector will be able to provide ample help in most situations or the need for $CD4^+$ T cell participation can be bypassed through CD40 cross-linking (80). Given these considerations, it is not surprising that fewer efforts have been made to investigate the potential of class II-restricted responses in tumor immunotherapy. Nevertheless, there is increasing interest in this arm of the immune response due to evidence in animal models that $CD4^+$ T cells can have anti-tumor effects even when the tumor does not express class II. The basis for this is speculated to be the cross-presentation of tumor antigens by macrophages that are in-turn activated by the release of cytokines from the $CD4^+$ T cells. The tumor cells would then be exposed to toxic factors released by the activated macrophage and/or $CD4^+$ T cells (30). For this scenario to work, it is critical that the reaction be kept local or that the tumor cells be more sensitive to these mediators than the normal cells in the vicinity of the tumor. The tissue damage due to activated macrophages can be extensive.

4.2. Vaccine Strategies

Not many groups have focused upon vaccine strategies intended to optimize class II-specific responses to tumor antigens. One successful approach in the mouse has been transfection of tumor cells with class II- and B7-encoding genes to create a complete professional antigen-presenting tumor cell. This allowed for direct presentation of tumor antigens via the endogenous presentation pathway vs. cross-presentation of exogenous antigen (287–293). Likewise, pulsing of activated (class II-positive) B cells with melanoma lysate was shown to elicit $CD4^+$ responses that protected against subsequent melanoma challenge (294). In another approach, the tumor antigen, specifically the E7 oncoprotein of human papillomavirus, was genetically modified by addition of an endosomal sorting signal, thus driving the antigen into class II loading compartments. The engineered protein, delivered by a vaccinia-based vaccine, provided substantial protection from challenge with an E7-expressing tumor (295).

4.3. Evasion

There are some notable examples of altered antigen presentation capabilities that may represent evasion from $CD4^+$ T cell recognition. An interesting example of antigenic variation comes from the cloning of Class II resticted tumor antigens in human melanoma. In one case, the antigen contained a mutation in the coding sequence that was not the T cell binding epitope, but rather changed the intracellular localization of the protein and therefore the constellation of epitopes generated. Thus, the mutation changed the processing of the protein, liberated a peptide that was not mutant yet immunogenic (296). Given this mechanism, searching for tumor antigens based on mutated sequence and TCR binding is limiting. With respect to alterations in the processing machinery, one interesting observation is the expression of the HLA-DR class II molecule on only a minority of human small cell lung carcinomas and its marked reduction on infiltrating leukocytes, and in regional lymph nodes, suggested to be due to release of inhibitory soluble factors from the tumor (297). Such a mechanism would be effective in reducing both direct and cross-presentation. Expression levels of many key components of the class II presentation pathway including class II, Ii, and DM are regulated by a transcription factor termed CIITA (298). For example, mouse tumor cell lines can be divided into three groups: constitutive expression of CIITA (and class II), interferon γ-inducible expression of CIITA, and absent/noninducible CIITA (299). This last group points to a potential means of escape, but only from direct presentation. A relatively old finding that is still interesting to consider is the tumor cell release of a protease, cathepsin L, that prevents presentation through "over-processing" of the antigen. Since cathepsin L is active only at low pH, the proposed mechanism is uptake of both antigen and protease by and destruction within the endosomal compartment of the cross-presenting APC (300) Finally, a recent study has demonstrated that the uniformly low GILT thiol reductase levels in a panel of class II^+ human melanoma cell lines

results in presentation of antigen that is quite distinct from that of professional, GILT-positive APCs (301), suggesting that T cells activated by the professional APCs will not necessarily be specific for the tumor cells themselves. This is reminiscent of the constitutive proteasome vs. immunoproteasome issue raised previously, and suggests an advantage to direct recognition of tumor cells by $CD4^+$ vs. the nonspecific effects mediated by cross-presentation outlined above.

5. CONCLUDING REMARKS

The concept of anti-tumor immunity is over a century old while our general understanding of MHC class I- and class II-restricted antigen processing and presentation is much younger. As the new principles have been applied to the old problem, a measure of progress can be appreciated. However, many important details of processing and presentation remain unknown, and the technologies for identifying and exploiting viable T cell targets on an individualized basis are truly in their infancies and limited by the lack of basic knowledge. Therefore, we are far from having enough information to determine whether or not immunotherapy will be a standard approach to cancer. Based upon the rate of recent progress, the upcoming years should provide many opportunities for applying new concepts in antigen processing and presentation to experimental models and, ultimately, patients.

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2. ANTIGEN RECOGNITION AND T-CELL BIOLOGY

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1. INTRODUCTION

Both humoral and cellular anti-tumor immunity have been described which can mediate tumor regression in animal models and cancer patients. However, recent studies have focused on T cell based anti-tumor immunity. It is well established that tumor reactive T cells can be isolated and expanded *ex vivo* from the peripheral blood, lymph nodes, spleens, and tumor lesions of tumor bearing animals and humans. Adoptive transfer of tumor reactive T cells can protect mice from subsequent tumor challenge and treat established tumors in mice and humans. Agents such as interleukin-2 (IL-2) or immunization with cancer vaccines can enhance anti-tumor T cell immunity resulting in a favorable impact on disease progression. These observations have led to the belief that T cells are a major component of the anti-tumor immune response and that enhanced T cell anti-tumor immunity would lead to tumor rejection.

Although recent clinical trials have found evidence of increased anti-tumor reactivity in the blood of vaccinated patients, relatively few clinical responses have been observed. Furthermore, many of the patients that have objective clinical responses have no detectable T cells in their blood reactive with the tumor vaccine. Based on these observations, it is clear that the mere presence of large numbers of tumor reactive T cells in blood, spleen, and lymph nodes or in the tumor lesions themselves is generally not sufficient to halt the progression of local and metastatic cancer. Factors such as T cell avidity, T cell receptor (TCR) affinity, immune suppression and others

must play key roles in determining the therapeutic potential of a T cell. This chapter will review critical factors that influence T cell recognition of tumor antigens, the TCR repertoire that is available to target tumor antigens, and strategies which have been developed to manipulate the T cell repertoire to more effectively target tumor antigens.

2. DIVERSITY OF THE T CELL REPERTOIRE AGAINST TUMOR ANTIGENS

Antigen recognition by T cells is major histocompatibility complex (MHC) restricted meaning that T cells exhibit specificity for both the antigens and major histocompatibility complex (MHC) alleles expressed by target cells (1). T cell specificity is due to the expression of a unique TCR on the surface of each T cell (2–4). The ligand recognized by the TCR is a complex of consisting of an antigenic peptide bound to an MHC molecule (5, 6). Considerable effort has been devoted to identifying the antigens recognized by tumor reactive T cells and to characterize their TCR genes. This section will focus on the nature of human tumor associated antigens and the diversity of the anti-tumor immune response as represented by their TCR variable (V) gene repertoire.

2.1. Targets of Tumor Reactive T Cells

Over the last decade, a major effort has been devoted to identifying and cloning T cell antigens expressed by human tumor cells. The goal of this effort was to use these tumor antigens to vaccinate cancer patients to boost the immune response against their tumor. To date, several dozen genes have been identified that encode T cell epitopes expressed by human tumors (7–9). These tumor associated antigens (TAA) fall into five categories based on their expression and recognition pattern by T cells. One group of antigens are expressed exclusively by cells of the melanocyte lineage and are called melanoma/melanocyte differentiation antigens (10, 11). These melanoma/melanocyte differentiation antigens, which include MART-1, gp100, and tyrosinase, are considered to be shared TAA since they are expressed by the vast majority of melanomas tested (12–17). A second group of antigens called cancer/testis antigens are expressed by normal testis and a variety of human cancers including melanoma, breast, bladder, colon, lung, head and neck, gastric, ovarian, neuroblastoma, and prostate cancer cells (7–9, 18). Although cancer/testis these antigens are expressed by many different types of humans cancers, only a small fraction of tumors usually express these antigens (19–26). A third group of antigens are expressed by virus induced tumor cells (7–9). Antigens such as EBNA-3 from Epstein Barr virus (EBV) induced lymphomas and the E6 and E7 genes from human papilloma virus 16 (HPV 16) induced cervical cancers contain epitopes that can be recognized by human T cells (27–29). A fourth group of antigens have aberrant expression in tumors relative to normal tissues (8, 9). Antigens such as Her-2/neu and p53 fall into this category since they are highly overexpressed by tumor cells relative to normal tissues (30–36). A fifth group of antigens contain mutations that affect the sequence of the epitope (8, 9). Mutations in the β-catenin and CDK4 genes (as well as others)

create mutations that alter the normal sequence of the T cell epitope (29, 37, 38). Therefore, many common tumors express antigens that can be targeted by human T cells.

Human tumor reactive T cells have been isolated that recognize at least 100 epitopes encoded by these genes and can be restricted by HLA A, B, C, or DR alleles (7–9). For practical reasons, the antigens considered to be most relevant clinically are shared antigens (antigens that are expressed by most tumors of a given histology) that are restricted by HLA-A2 (17, 39–41). HLA-A2 is the predominant MHC class I allele expressed in the US with roughly 50% of all Caucasians being HLA-$A2^+$(42). The rationale for targeting shared HLA-A2 restricted antigens is that any treatment that targets shared HLA-A2 restricted antigens could treat a larger proportion of cancer patients than treatments targeting antigens with limited expression or restricted by other HLA-molecules.

2.2. TCR Diversity Among Tumor Reactive T Cells

Since T cell specificity and reactivity is mediated by the TCR, it has been proposed that tumor reactive T cells can be isolated and expanded for patient treatment based on their TCR expression (43–45). Isolating and expanding tumor reactive T cells based solely on their TCR would require that there is limited TCR V gene usage among T cells reactive with a given target epitope. The problem has been identifying TCRs commonly expressed by tumor reactive T cells. TCRs are heterodimers composed of an α and β chain with each T cell expressing a unique receptor which is responsible for the T cells reactivity and specificity (46). The ligand recognized by the TCR consists of a short antigenic peptide fragment bound to an MHC molecule (5, 47). Given the number of possible antigenic peptides and the allelic variation of the MHC molecules, the number of potential ligands for the TCR is extensive. As a result, it is estimated that up to 10^{15} distinct TCRs are possible (48). This tremendous TCR diversity is due in part to the structure of the TCR. Each TCR chain consists of a variable (V) segment, a joining (J) segment, and a constant (C) region with the β chain also containing a diversity (D) region. Germline rearrangements occurring within the TCR α and β loci during T cell development, randomly join different V-J or V-D-J regions into a single transcriptional unit, leading to some of the observed TCR diversity. However, the majority of the TCR diversity is the result of the random insertion or deletion of nucleotides at the junctions between the V and J segments for the α chain, and between the V and D and the D and J segments for the β chain. It is these V-J and V-D-J junctions of the α and β chains respectively, which encode the putative third complementarity determining region (CDR3), which is critical for antigen recognition (49, 50).

The diversity of TCR V genes used by tumor reactive T cell clones has been examined. While initial reports suggested that there was a limited TCR repertoire used by tumor reactive T cells (44, 51–55), we and others have failed to find evidence of restricted TCR V gene usage (56–63). A detailed analysis of the TCR V genes used by MART-1:27–35 and gp100:209–217 (unpublished) reactive T cells has been performed. At least 19 of the 46 functional TCR Vβ genes can been used the

MART-1:27–35 reactive T cell clones (56, 59, 61, 64–67). Similarly, we have found that at least 16 of the 46 functional TCR Vβ genes can be used by gp100:209–217 reactive T cell clones (unpublished). Furthermore, no homology has been found within the CDR3 regions of the TCR β chains of MART-1:27–35 or gp100:209–217 reactive T cell clones. These observations suggest that there is likely to be considerable TCR diversity among tumor reactive T cells.

The consequences of a diverse TCR repertoire has both positive and negative implications for the immunotherapy of cancer patients. For cancer vaccine development, a diverse TCR repertoire means a higher likelihood that any given patient has the genetic capacity to possess antigen reactive T cells that can recognize their tumor. However, a broad TCR repertoire limits the possibility of using the expression of individual TCR V genes as a marker for isolating and expanding T cells reactive with a given antigen for patient treatment.

2.3. Effect of TCR V Gene Usage on Tumor Antigen Recognition

A broad TCR V gene repertoire and diversity among the CDR3 regions of tumor reactive T cells implies that each T cell might recognize the same peptide/MHC complex in slightly different ways. Alternatively, the genetics of the immune system may be allow different TCR V genes and CDR3 sequences to recognize the same peptide MHC complex in the same way. The pattern of antigen reactivity of three different MART-1:27–35 reactive T cell clones was analyzed using a panel of homologous peptides derived from human self antigens and human pathogens (68). Each T cell clone displayed a distinct pattern of reactivity against this panel of peptides. Diversity in the fine structure recognition of a larger panel of MART-1 reactive T cell clones was also reported using alanine substitutes MART-1 peptides (69, 70). We can conclude that the diversity in the genetics of the TCR of MART-1:27–35 reactive T cell clones leads to functional diversity which influences the way T cells recognize tumor cells.

In a similar analysis of gp100:209–217 reactive T cell clones, we used alanine substituted peptides to examine the pattern of antigen recognition. In some cases we find that different T cells bearing distinct TCRs recognize antigen differently (Figure 1). However, other T cells bearing distinct TCRs can recognize antigen in the same manner (Figure 1). These observations further the notion that the genetics of the TCR can indeed influence the recognition pattern of tumor antigens. It is also clear that the genetics of the TCR permits redundancy in the way T cells recognize antigen. The consequence of TCR diversity is to help ensure that T cells with the relevant reactivity are present in an individuals T cell repertoire while redundancy ensures that the individual has multiple opportunities to generate those relevant T cells. Therefore, TCR diversity and redundancy within the anti-tumor immune response is ultimately a benefit for the cancer patient.

3. FACTORS THAT INFLUENCE T CELL RECOGNITION OF TUMOR CELLS

In the previous section, we established that T cells exist in tumor bearing hosts which have a diverse repertoire of TCRs capable of recognizing each tumor antigen

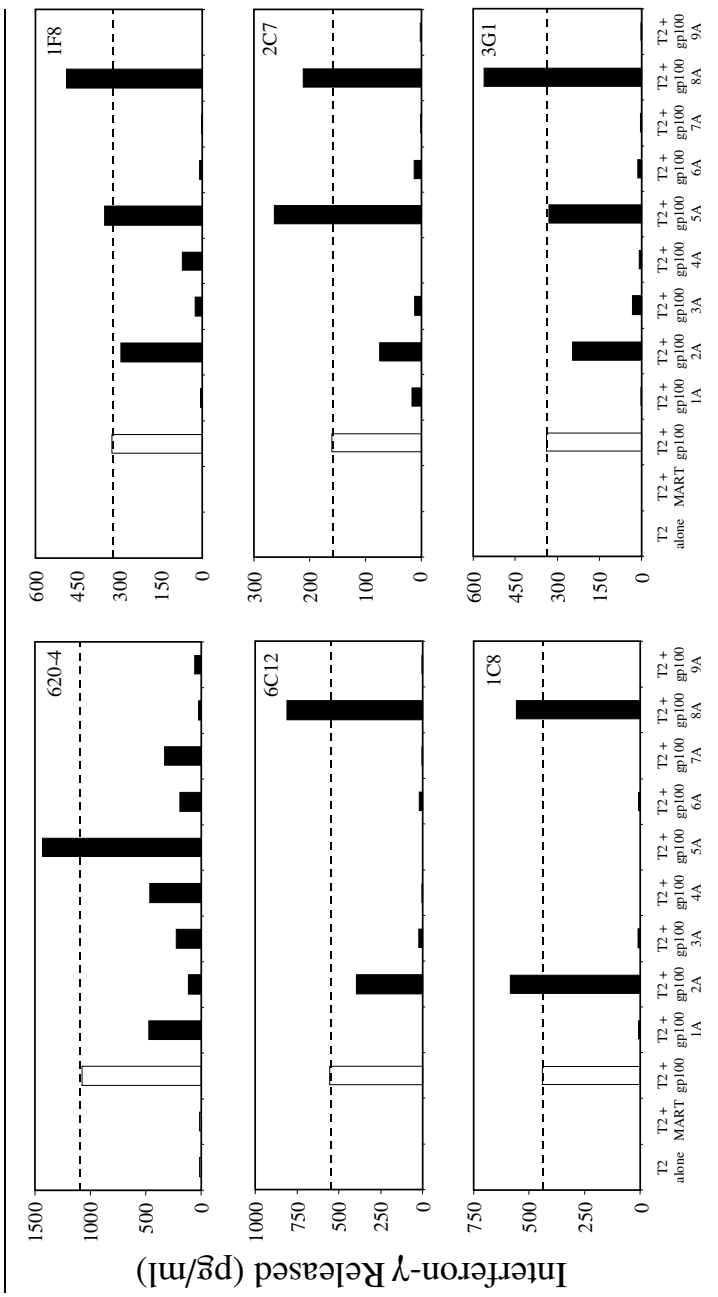


Figure 1. Antigen Recognition Pattern of gp100:209–217 Reactive T Cell Clones. The influence of the genetics of the TCR V gene usage on antigen recognition was determined by stimulating gp100:209–217 reactive T cell clones with T2 cells loaded with a panel of alanine substituted peptides. Six different T cell clones isolated from different patients were evaluated in this assay. The amount of interferon-γ released was measured by ELISA. The open bars define the amount of interferon-γ released by each T cell clone when stimulated with the native peptide (represented by the dashed lines). The filled bars represent the amount of interferon-γ released when stimulated with each alanine substituted peptide.

in multiple ways. However, in the face of this large TCR diversity, the influence of genetics of the TCR is clearly not sufficient to ensure that each patient has potent anti-tumor effector T cells. In this section, we will focus on other factors that influence tumor cell recognition. We will also discuss the role the TCR plays in tumor cell recognition.

3.1. T Cell Avidity and Tumor Cell Recognition

In general, T cells are extremely sensitive to activation by antigen. Using extremely sensitive assays, it has been reported that one TCR/peptide/MHC interaction can lead to activation of a T cell as measured by Ca^{+2} mobilization, three interactions lead to target cell lysis, and ten interactions lead to full activation as measured by T cell proliferation (71). However, routine measurements of T cell function such as cytokine secretion or cytolysis require far more antigen to elicit a detectable T cell response. In these assays, a high avidity T cell requires far less antigen (<1 nM peptide loaded on an APC) to activate the T cell than a moderate (1–100 nM peptide loaded on an APC) or low (>100 nM peptide loaded on an APC) T cell (reviewed by 72). The correlation between the relative avidity of polyclonal T cell cultures and target cell recognition was first reported among HIV gp160 reactive T cells (73). In this study, it was shown that immunization with high doses of antigen led to expansion of T cells with low avidity whereas immunization with low doses of antigen led to expansion of T cells with high avidity (73). It was subsequently shown that T cells with high avidity undergo apoptotic cell death when exposed to high levels of antigen (74). These studies indicate that the quality of the immune response is important and that the amount of antigen encountered by a T cell can influence the immune response to that antigen.

Other groups have reported similar results with mouse tumor models. Zeh et al. (75) reported that high avidity tyrosinase related protein 2 or p15E reactive T cells were more effective at eliminating B16 lung metastases than low avidity T cells. It was also shown that high avidity T cells reactive with the HLA-A2 binding tyrosinase:368–376 peptide induced vitiligo in HLA-A2 transgenic mice (76). Therefore, T cell avidity does correlate with target cell destruction in mouse tumor models.

A correlation between T cell avidity and tumor cell recognition has also been observed among human T cells. Dudley et al. (77) found that individual T cell clones reactive with the gp100:209–217 epitope could vary by several logs in their relative avidity. Furthermore, there was a clear correlation between T cell avidity and tumor cell recognition. Taken together, these mouse and human results indicate that the relative sensitivity of a T cell to antigen influences its ability to recognize tumors. Furthermore, it suggests that high avidity T cells are required for efficient anti-tumor immunity.

While it intuitively makes sense that highly avid T cells would be better able to recognize tumor cells than T cells with lower avidity, there are reports of T cells which do not follow these avidity rules. In the search for new tumor antigen targets, many groups have successfully sensitized T cells to recognized peptides synthesized

from candidate tumor antigens based on MHC binding motifs (reviewed by 78). However, some of these antigens elicit high avidity T cells which recognize peptide loaded targets but not tumor cells. One possible explanation for these results is that the antigenic peptide is not processed and presented on the surface of tumor cell. One epitope which remains quite controversial is the 369–376 peptide from Her-2/neu. Immunologic monitoring of two clinical trials could detect T cells reactive with peptide loaded cells but not tumor cells in vaccinated patients (79,80). In fact, one group isolated several high avidity Her-2/neu:369–376 reactive T cell clones from those that failed to recognize Her-2/neu$^+$ tumor cells leading them to conclude that the Her-2/neu:369–376 epitope is not presented on the surface of Her-2/neu$^+$ tumor cells (79). At face value, this interpretation seems reasonable except that several other groups have reported isolating Her-2/neu:369–376 reactive T cells capable of recognizing Her-2/neu$^+$ tumor cells (81–85). Therefore, even though Her-2/neu:369–376 reactive T cells have the genetic capacity and sufficient avidity to recognize Her-2/neu$^+$ tumor cells, it appears that factors other than the TCR appear to preclude them from being potent anti-tumor effectors.

3.2. Relationship Between T Cell Avidity and TCR Affinity

It has been postulated that T cells with high avidity for antigen would express TCRs with higher affinity for their ligand (45, 86, 87). Intuitively, this makes sense given that the current models of T cell activation state that the stability of the TCR/peptide/MHC complex is an essential component of T cell activation. Experimentally, it has been also shown that T cells which stain brightly with peptide/MHC tetramers have higher avidity than more dimly staining T cells (87–89). It has been argued that the greater the level of tetramer staining, the higher affinity of the TCR (87, 90). However, others find no correlation between tetramer binding and T cell avidity (91, 92). Furthermore, it has been reported in TCR transgenic mice that both high and low avidity T cells exist bearing the same TCR suggesting that T cell avidity is not always a valid predictor of TCR affinity (93).

We have several lines of evidence which supports the notion that T cell avidity is not necessarily predictive of TCR affinity. First, we have identified human T cell clones reactive with gp100:209–217 which express identical TCRs. Some of these "sister" T cell clones can have dramatically different relative avidities which translates into differences in tumor cell recognition (Figure 2). These observations are not confined to melanoma T cells since "sister" Her-2/neu:369–377 reactive T cell clones have been isolated with different reactivities against the same Her-2/neu expressing target cells (data not shown). These results are in agreement with the TCR transgenic studies that indicate that T cell bearing the same TCR can have varied avidity.

Second, our TCR gene transfer studies have shown that T cell avidity does not necessarily correlate with TCR affinity since cells with poor avidity can recognize tumors while in other cases highly avid cells can't (94, 95). We have used TCR transduced Jurkat cells (which lack CD8 expression) as a readout for peptide and tumor cell recognition (94). One gp100:209–217 reactive T cell clone (R6C12) has

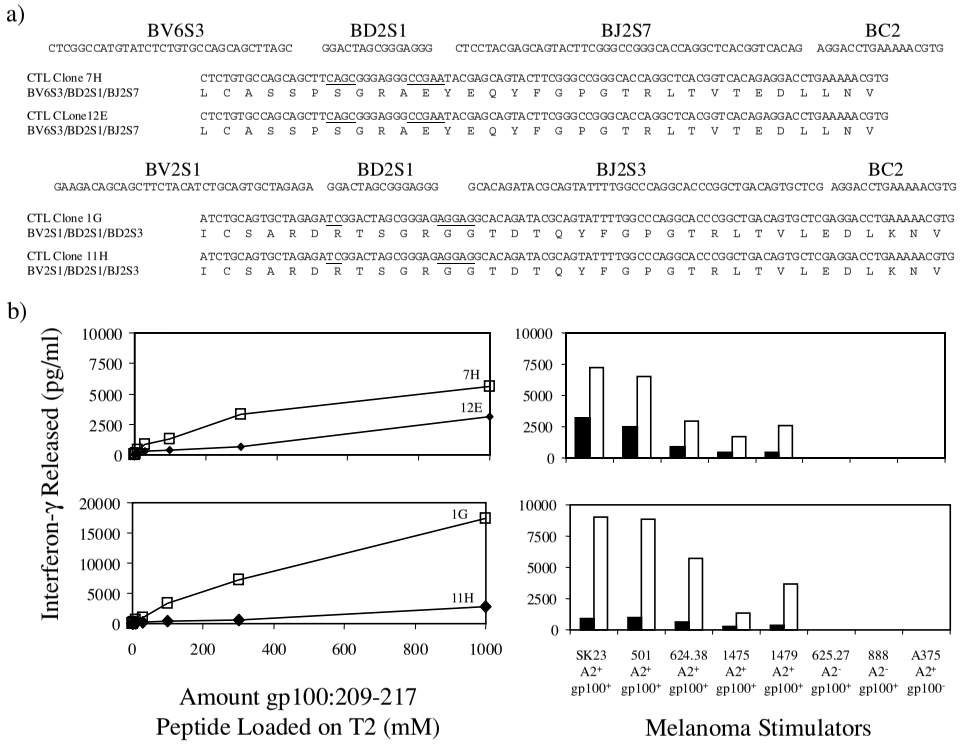


Figure 2. Differences in Relative Avidity and Tumor Cell Recognition by gp100:209–217 Reactive "Sister" T Cell Clones. T cell clones expressing identical TCR genes were evaluated for their ability to recognize peptide loaded targets and tumor cells. a) The V/D/J regions from two pairs of gp100:209–217 reactive T cell clones and their corresponding germline segments are shown. The underlined bases represent nucleotides not present in the germline sequences that were inserted during the V/D/J recombination events that gave rise to these TCR chains. Clones 7H and 12E and clones 1G and 11H have identical sequences indicating these clones were derived from the same T cell and are "sister" clones. b) The relative avidity of each T cell clone was measured by stimulating gp100:209–217 reactive T cell clones with T2 cells loaded with decreasing amounts of gp100:209–217 peptide and the amount of interferon-γ released was measured by ELISA. The reactivity of each T cell clone with tumor cells was measured by tumor cell lines that differ in their expression of HLA-A2 and gp100. The open and filled symbols in the line graphs correspond to the open and filled bars respectively in the bar graphs.

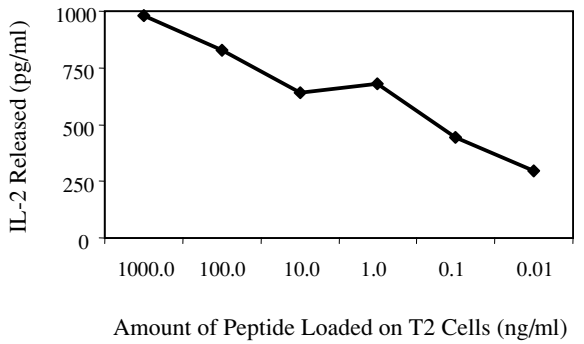


Figure 3. Relative Avidity of R6C12 TCR Transduced Jurkat Cells. The relative avidity of R6C12 TCR transduced Jurkat cells was measured by stimulating the cells in a 1:1 ratio with T2 cells loaded with various concentrations gp100:209–217 peptide. The amount of interleukin-2 released was measured by ELISA.

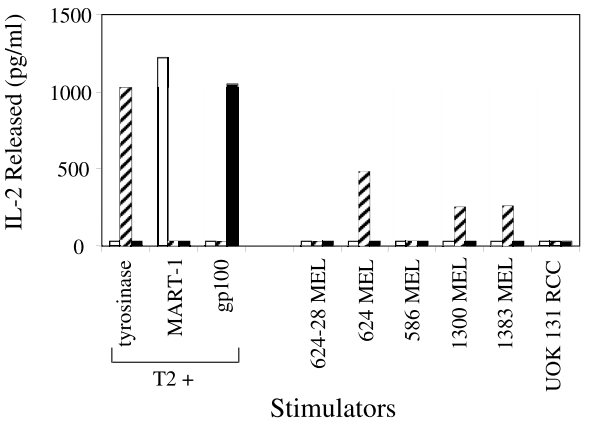


Figure 4. Tumor Cell Recognition by R6C12 TCR Transduced Jurkat Cells. The ability of R6C12 TCR transduced Jurkat cells (black bars) to recognize the physiologic levels of antigen presented by tumor cells was measured by stimulating the cells in a 1:1 ratio with a panel of tumor cells and the amount of interleukin-2 released was measured by ELISA. The tumor cell panel included HLA-A2$^+$ gp100$^+$ (624 MEL, 1300 MEL, and 1383 MEL) melanoma cells, HLA-A2$^-$ gp100$^+$ (624-28 MEL and 586 MEL) melanoma cells, and an HLA-A2$^+$ gp100$^-$ renal cell carcinoma cells. TCR transduced Jurkat cells expressing a high affinity (TIL 1383I, hatched bars) or low affinity (TIL 5, open bars) TCR were included as control for tumor cell recognition.

been shown to have extremely high avidity and can recognize HLA-A2$^+$ gp100 positive tumor cells (96, 97). Transfer of the R6C12 TCR to PBL-derived T cells results in high avidity T cell cultures (98). However, Jurkat cells expressing the R6C12 TCR can have high avidity for antigen (Figure 3) yet fail to recognize tumor cells (Figure 4) indicating R6C12 expresses a low affinity TCR. R6C12 cells also fail to bind HLA-A2 tetramers loaded with the native gp100:209–217 peptide but could

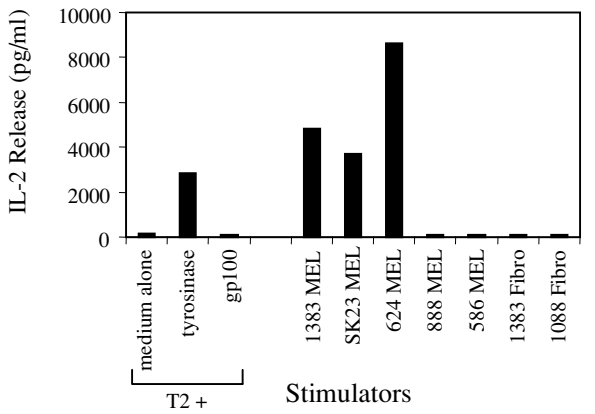


Figure 5. Antigen Recognition by TIL 1383I TCR Transduced 58 $\alpha^-\beta^-$ Cells. The ability of TIL 1383I TCR transduced 58 $\alpha^-\beta^-$ cells to recognize HLA-A2$^+$tyrosinase$^+$ targets was measured by stimulating the cells in a 1:1 ratio with a peptide loaded T2 cells and a panel of tumor cells and the amount of interleukin-2 released was measured by ELISA. Peptides used to load T2 cells were tyrosinase: 368–376 or gp100:209–217. The tumor cell panel included HLA-A2$^+$ tyrosinase$^+$ (624 MEL, SK23 MEL, and 1383 MEL) melanoma cells, HLA-A2$^-$ tyrosinase$^+$ (624–28 MEL and 586 MEL) melanoma cells, an HLA-A2$^+$ tyrosinase$^-$ fibroblast line (1383 Fibro), and an HLA-A2$^+$ tyrosinase$^-$ renal cells.

weakly bind HLA-A2 tetramers loaded with a modified gp100:209–217 peptide substituted with a methionine at position 2 which enhances binding to HLA-A2 (99). This binding was easily inhibited by anti-CD8 mAb supporting the notion that despite the high avidity of CTL clone R6C12, its TCR has relatively low affinity for antigen.

In other studies, we recently described an HLA-A2 restricted, tyrosinase:368–376 reactive CD4$^+$ T cell with intermediate to low avidity since it requires between 10 and 100 nM peptide to elicit cytokine production yet can efficiently recognize HLA-A2$^+$ tyrosinase$^+$ tumor cells (95,100). The TCR from this T cell clone was transferred to 58 $\alpha^-\beta^-$ mouse hybridoma cells which lack human CD8 expression. The resulting transductants were capable of recognizing HLA-A2$^+$ tyrosinase$^+$ tumor cells (Figure 5) despite requiring greater than 100 ng/ml of peptide to stimulate the cells to secrete IL-2 (Figure 6). Despite the low avidity of the parent T cell and the transduced cells expressing its TCR, the CD8 independent tumor cell recognition indicated that the TCR alone had sufficient affinity to transduce the necessary signals for activation of the T cell. These TCR gene transfer studies led us to conclude that T cell avidity does not necessarily predict the affinity of the TCR. Furthermore, it appears that T cells can modulate their avidity independent of the affinity of their TCR.

3.3. Factors Which Influence T Cell Function

As we have discussed, T cells have the capacity to alter their responsiveness to antigen stimulation by factors independent of the TCR. Tumor bearing hosts appear to be

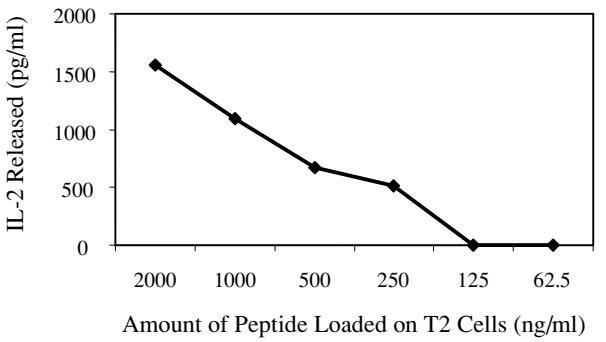
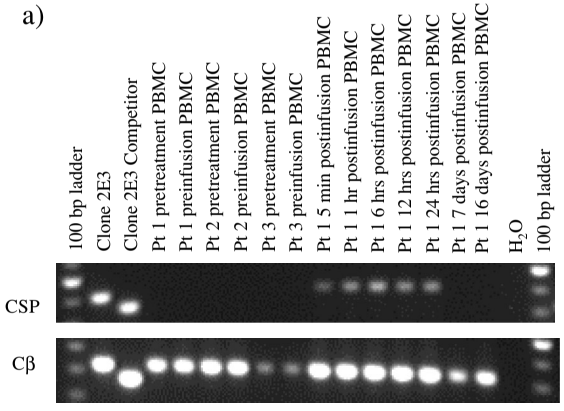


Figure 6. Relative Avidity of TIL 1383I TCR Transduced 58 $\alpha^-\beta^-$ Cells. The relative avidity of TIL 1383I TCR transduced 58 $\alpha^-\beta^-$ cells was measured by stimulating the cells in a 1:1 ratio with T2 cells loaded with various concentrations the tyrosinase: 368–376 peptide. The amount of interleukin-2 released was measured by ELISA.

particularly susceptible to suppression of their T cell responses (101). We recently reported the results from a clinical trial where high avidity T cell T cell clones reactive with gp100:209–217 were infused into melanoma patients with stage IV disease (96). Using T cell clone specific PCR primers, we monitored each patients blood for the presence of the infused clone at various times post-infusion. A detailed analysis of one patient revealed that despite the presence of the infused T cell clone at significant levels in the blood (0.5%–6.9%) (Figure 7), its functional reactivity could only be detected in the patients blood at one hour post-infusion when the frequency of the clone was 6.9%, and only when the stimulators used were T2 cells loaded with the modified gp100:209–217 2M peptide (Figure 7) and not the native gp100:209–217 peptide. These observations suggest that these suppressive effects on T cell function can occur rapidly *in vivo*, even to T cells with high avidity for antigen *in vitro*.

Several mechanisms have described which can alter T cell function in cancer patients. Mizoguchi et al. (102) reported that mice bearing MCA 38 colon carcinoma tumors had reduced expression of CD3gζ chain expression on their surface. It was also found that tumor bearing mice had reduced levels of the tyrosine kinases $p56^{lck}$ and $p59^{fyn}$ (102) and the transcription factor NFκB (103). Subsequently, NK cells have also been shown to have decreased surface CD3gζ chain expression in tumor bearing hosts (104, 105). These signaling defects have been confirmed by others in several mouse tumor models and in patients with colorectal carcinoma, renal cell cancer, head and neck cancers, and other malignancies (106–110). Given that CD3gζ chain, $p56^{lck}$ and $p59^{fyn}$ are required for TCR-mediated signaling to occur (111), decreased expression of these molecules in tumor bearing hosts will result in impairment of T cell immunity. It was recently reported that the levels of L-arginine in the cell culture medium could regulate CD3gζ chain expression (112) and that the enzyme arginase I produced by macrophages may regulate the levels of L-arginine



b)

PBMC Sample	Percent clone 2E3
Pre infusion	<0.01
5 min post infusion	0.50 ± 0.11
1 hr post infusion	6.87 ± 1.11
6 hrs post infusion	2.34 ± 0.28
12 hrs post infusion	2.21 ± 0.71
24 hrs post infusion	1.21 ± 0.27
7 days post infusion	<0.01
16 days post infusion	<0.01

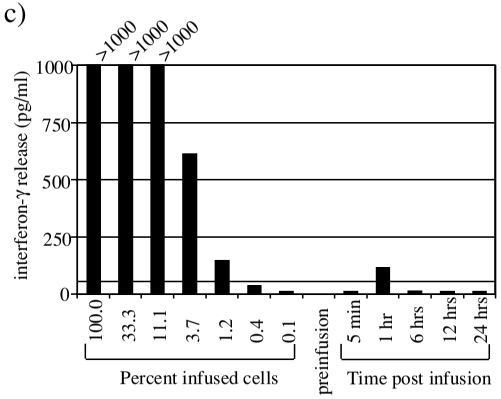


Figure 7. Frequency and Function of CTL clones After Adoptive Transfer in Patients. High avidity gp100:209–217 reactive T cell clones were isolated an used to treat patients with metastatic melanoma. a) The presence of T cell clone designated 2E3 was detected in the blood of the patient by RT-PCR using T cell clone specific PCR primers. b) The frequency of this clone in the patients blood was measured at various time points post-infusion using a competitive RT-PCR assay. c) The anti-gp100:209–217 reactivity in the blood was measured at various time points post-infusion by culturing PBL-derived T cells with T2 cells loaded with the gp100:209–217 2M peptide. The amount of interferon-γ released was measured by ELISA. A standard curve was established to approximate the sensitivity of the assay by diluting the infused T cell clone into the patients PBMC.

in cancer patients (113). Other metabolic pathways also appear to regulate T cell function including oxidative stress from hydrogen peroxide released by cells of the monocyte/macrophage lineage (114) and the levels of tryptophan and its metabolites as a consequence of indoleamine 2,3-dioxygenase expression by macrophages (115, 116). Therefore, the influence of tumors on the physiology of the host can regulate the immune response to their malignancies.

It has also been reported that cell surface molecules such as the CD8 coreceptor can regulate T cell avidity. The CD8 coreceptor, as previously discussed, plays a critical role in the activation of $CD8^+$ T cells by binding to the $\alpha 3$ domain of MHC class I and recruiting the kinase $p56^{lck}$ to the CD3 complex {119}. Although CD8 exists on the membrane of T cells as an $\alpha\beta$ heterodimer (117, 118) a CD8 $\alpha\alpha$ homodimer form has been recently described (120). Transfection studies have shown that the CD8 $\alpha\beta$ heterodimer has higher affinity for MHC class I and $p56^{lck}$ and more efficiently mediates T cell activation than the CD8 $\alpha\alpha$ homodimer (120). It has been reported that the ratio of CD8 $\alpha\beta$ to CD8 $\alpha\alpha$ as well as the ability for CD8 $\alpha\beta$ to colocalize with the TCR to lipid rafts can have a profound impact on T cell avidity (93). Therefore, the expression of the CD8 coreceptor and other cell surface molecules can play essential roles in determining the relative avidity of a T cell.

4. STRATEGIES TO ACTIVATE TUMOR REACTIVE T CELLS

Considerable effort has been devoted to using cancer vaccines to activate and expand the T cell repertoire for the benefit of cancer patients. In this section, several strategies have been used to improve the immunogenicity of tumor antigens by altering the amino acid sequence of the peptide epitope to enhance its binding to the MHC and the TCR. In this section, examples of how antigenic peptides have been altered to improve their immunogenicity will be discussed with a special emphasis on what effects these modifications have on the T cell repertoire.

4.1. Enhanced Immunogenicity by Enhanced HLA Binding

Tumor antigen based clinical trials have had relatively few objective clinical responses (122–127). In addition, many cancer vaccine trials show little evidence of anti-tumor immunity in the peripheral blood of patients following vaccination (125, 128). In an effort to enhance the immunogenicity of known antigenic peptides, the effect of modifying their amino acid sequence has been evaluated. It has been shown that substituting the amino acids at anchor positions in the antigenic peptide will lead to enhanced peptide/MHC binding (reviewed by 68). Most importantly, some of these substitutions can enhance the immunogenicity of an otherwise weakly immunogenic peptide both *in vitro* and *in vivo* (129–132). The best example of the influence of peptide modifications designed to increase binding to MHC has on the immunogenicity of a weakly antigenic peptide are the substituted gp100:209–217 peptides. Substituting a methionine for the native threonine at position 2 enhances binding of this peptide to HLA-A2 9-fold (129). More importantly, this M substitution enhances

the immunogenicity of the peptide *in vitro* (129) and *in vivo* (122) with the resulting T cells having the capacity to recognize tumor cells.

Modifications at MHC anchor residues to weakly immunogenic peptides can have other desirable effects. There are examples of weakly immunogenic peptides where the MHC anchor modifications result in other benefits such as enhancing a peptides stability in solution. An example of a peptide with enhanced stability is the HLA-A2 restricted NY-ESO-1:155–163 peptide (reviewed by 68). A substitution of valine for cysteine at position 9 in the peptide not only enhances binding to HLA-A2, it prevents disulfide bridge formation thus eliminating dimerization of the peptide in solution (131). Similarly, a substitution of a serine or alanine for the cysteine at position 2 of the HLA-A1 restricted tyrosinase:243–251 decreases the amount of peptide required to elicit T cell responses *in vitro* by two to three logs (133). These modifications to otherwise poorly immunogenic peptides can activate populations of T cells that would otherwise be refractory to stimulation. This simple approach of modifying the MHC binding residues of weakly antigenic peptides represents a powerful strategy for activating strong T cell responses that would otherwise be unresponsive.

4.2. Enhanced Immunogenicity by Altered TCR Contact Residues

It has been shown that immunization with xenogeneic proteins can lead to enhanced immunity to the native protein. The genes encoding the human or rodent homologs of several tumor antigens have been used to vaccinate mice (76, 78, 134, 135). In these studies, the xenogeneic antigens routinely result in greater immune responses and these immune responses can lead to effective anti-tumor immunity. It was speculated that differences in the amino acid sequence between the xenogeneic antigen and the target antigen resulted in heteroclitic peptides (peptide analogs susbtituted at positions other than MHC contact residues with increased potency) that are capable of inducing both effector and helper T cell responses. This hypothesis was directly tested using by modifying a peptide from the H-2L^d restricted tumor antigen AH-1 (136). Substituting an alanine for a valine at position 5 increased the binding to the TCR while having no impact on binding to the H-2L^d. This substitution increased the ability of the AH-1 peptide to elicit CTL responses that protect mice from challenges with AH-1 expressing tumors (136). These animal studies indicated that modifications to TCR contact residues can enhance the immunogenicity of antigenic peptides.

These studies with peptides modified at their TCR binding residues have been extended to human studies (137–139). The best example of a peptide modified at a TCR contact residue is the 605–613 peptide epitope from carcinoembryonic antigen (CEA). Substituting an aspartic acid for the asparagine at position 6, enhances the capacity of this peptide to elicit CEA reactive T cells that can recognize processed CEA antigen presented by tumor cells (137). Furthermore, clinical responses have been reported in colon cancer patients vaccinated with dendritic cells loaded with this heteroclitic CEA peptide (140). Based on these promising results, other groups have evaluated modified peptides and identified heteroclitic peptides from several tumor

antigens (68, 141, 142). These modified peptide represent a promising approach for vaccinating cancer patients with otherwise weakly immunogenic peptides.

4.3. Influence of Peptide Modifications on the TCR Repertoire

Despite the enhanced ability of modified peptides to elicit strong anti-tumor immune responses, these peptides have generally failed to induce effective anti-tumor immunity leading to tumor regressions in most patients (122,124). One potential reason may be due to the impact of the peptide modifications on the T cell repertoire (143). It has been shown that amino acid substitutions in antigenic peptides at the TCR contact residues can influence TCR binding resulting in changes in the TCR repertoire. TCR transgenic models where mice that express only one of the transgenes (α or β) were vaccinated with the native moth cytochrome C (MCC) peptides or peptides containing nonconservative amino acid substitutions at the TCR contact residues and MCC reactive T cell hybridomas were isolated (144). If a positively charged amino acid was present in a TCR contact residue of the immunizing peptide, negatively charged amino acids were found in the corresponding CDR3 regions of the nontransgenic TCR chains (144). Therefore, the amino acid sequence of the CDR3 regions of the nontransgenic TCR chains of MCC reactive T cells were influenced by the sequence of the immunizing peptide. Given these observations, it is important to consider the potential effects on the T cell repertoire when evaluating heteroclitic peptides since the impact on the TCR repertoire may lead to undesirable consequences.

Changes in the TCR repertoire due to vaccination are not confined to peptides modified at their TCR contact residues. Even though the anchor residues point downward into the groove of MHC class I molecules, we found that vaccinating patients with the gp100:209–217 peptide containing a methionine instead of a threonine at position 2 led to changes in the TCR repertoire (143). The best evidence for this altered TCR repertoire came from the isolation of clones reactive with the modified peptide but not the native peptide or tumor cells. In fact, we successfully isolated tumor reactive T cell clones from one patient prior to vaccination with the modified gp100 peptide. After vaccination, the only T cell clones we could isolate reacted only with the modified peptide or could recognize the native peptide but had no tumor reactivity. These observations indicate that even changes in the antigenic peptide which do not face the TCR can impact on the TCR repertoire.

5. SUMMARY

Despite the wealth of information that has been acquired regarding the way T cells recognize their targets, we are left with far more questions than answers regarding how to manipulate the immune response to better treat cancer patients. Clearly, most patients have a broad repertoire of T cells capable of recognizing their tumor cells. Despite the presence of these tumor reactive T cells and our ability to increase their frequency though vaccination or adoptive transfer, patients still progress. From the T cell side, defects in T cell signaling may account for much of our failure to achieve significant numbers of objective clinical responses. In spite of these negatives,

the horizon does remain bright for T cell based immune therapy of cancer. The periodic objective clinical response tells us that immune therapy can work. Now that we know that cancer patients have the capacity to mount immune responses against their tumors, current and future investigations with agents which alter T cell function combined with vaccination or adoptive T cell transfer may help tip the balance towards effective immune therapies.

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3. MECHANISMS OF TUMOR EVASION

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1. INTRODUCTION

1.1. Escaping the Immune Response: A Historic Perspective

The clinical experiments of William Coley in the 1890's [1] demonstrating a therapeutic effect of the "Coley Toxins" in some patients, and the animal models of Prehn and Main [2] in the 1950's demonstrating the existence of tumor specific antigens, established an era of active research in immunotherapy as a treatment for cancer. Several studies demonstrated that tumors arising from oncogenic viruses could induce a protective immune response during the early phases of tumor development. [3–4]. However, results in some animal tumor models [5] and especially in patients with cancer, failed to demonstrate the presence of a protective immune response to the progressively growing tumor. Hersh and Oppenheim [6] instead demonstrated that Hodgkins disease (HD) patients had a decreased delayed type hypersensitivity (DTH) response to PPD and DNBC (di-nitrochlorobenzene) and a diminished in vitro response to mitogen stimulation, which persisted even in patients who had achieved a complete clinical response to chemotherapy [7]. Furthermore, Hellstrom and colleagues [8] showed a decreased cellular immune response, but a marked increase in serum immunoglobulins in patients with melanoma. Similarly observations in patients with renal cell carcinoma, prostate and bladder cancer [9], lung cancer [10] and breast cancer [11], suggested that tumors might impair the immune response. However the clinical relevance of these findings or the mechanisms causing them remained unclear.

Table 1. Mechanisms of tumor evasion

Target	Major Changes
Changes in tumor cells	Selection of tumor cells resistant to apoptosis Changes in the expression of HLA Absence of co-stimulatory molecules
Alterations in antigen presenting cells	Arrested maturation of DC Selective increase in DC2
Dysfunction of effector cells	Induction of regulatory T cells Increased apoptosis of T effector cells Alteration in T cell signal transduction

A renewed enthusiasm for immunotherapy started in the 1980's with the cloning and production of pharmaceutical grade cytokines and the isolation and purification of tumor associated antigens. However the results of clinical trials in patients failed to reproduce the undisputable therapeutic benefit shown in animal models, bringing forth the need to understand how tumors escape the immune response. Various mechanisms of tumor escape have been identified ranging from the loss of HLA markers in tumor cells making them difficult to recognize by T cells, to the gradual deterioration of the immune response with the progressive growth of the tumor. Here we will discuss some of the most recent concepts on how tumor cells may escape and/or inhibit the normal function of the immune system (Table 1).

2. CHANGES IN TUMOR CELLS

2.1. Selection of Resistant Tumor Cells

The concept of "immune surveillance" proposed by Jones and Burnet [12] in the 1970's suggested that the immune system was vigilant to destroy any malignant cells before they developed into a clinically relevant tumor. However, aside from the demonstration of the existence of natural killer cells there was little proof or understanding of how this mechanism worked. In the early 1990's work by Schreiber and colleagues [13; 14] demonstrated that early tumor growth is comprised mostly of transformed cells that undergo apoptosis when they bind IFNγ and chemokines produced by cells of the innate immune response including natural killer cells, $\gamma\delta$ T lymphocytes and macrophages. This effectively eliminates most of the tumor cells, however it also selects for a minority of malignant cells that have mutations or alterations that make them resistant to an immune induced apoptosis. The absence of one or more chains of the IFNγ receptor, or mutations in the tyrosine kinases associated with this receptor (Jak 1, Jak2 or Stat 1), prevent the triggering of the apoptosis cascade making these cells resistant to the immune surveillance mechanism. These resistant clones then develop into tumors of clinical significance unimpeded by the immune response. Therefore, the innate immune response may eliminate most transformed cells during the early stages of tumor growth, however it may also result in the selection of a resistant population of malignant cells, a process that was coined by Schreiber as cancer immunoediting [15]. Alternatively, Khong and Restifo [16]

suggested that tumors are not rejected during early stages of tumor growth because they do not cause significant tissue damage and therefore fail to send "danger signals" that could activate the immune response, a concept presented by Matzinger [17]as a means for certain normal tissues of causing immune tolerance.

2.2. Decreased HLA Antigen and Co-stimulatory Signal Expression

HLA Class I Antigen Expression

The continued growth of tumor leads to tissue destruction and the generation of "danger signals," which may trigger an adaptive immune response. The activation of tumor associated antigen (TAA)-specific T lymphocytes occurs through the recognition of two combined signals by the T cell i) peptides, derived from TAA, presented by self-HLA class I molecules (i.e. HLA class I antigen-TAA peptide complex) and ii) co-stimulatory signals such as B7.1 (CD80) or B7.2 (CD86) [18] [Fig. 1A]. This recognition results in the development of effector cytotoxic T lymphocytes (CTL) that recognize and lyse tumor cells presenting the relevant HLA class I antigen-TAA peptide complex [Fig. 1B] Therefore, tumor cells can evade hosts' immune response by being poor stimulators of T cells or being poor targets for effector CTL. Specifically, malignant cells may posses abnormalities in the expression of molecules required for effective T cell recognition, such as HLA class I antigens, costimulatory molecules and/or the TAA itself [16, 19, 20].

In the case of HLA class I antigens, a large body of evidence indicates that malignant transformation is associated with abnormalities in HLA class I antigen expression [19]. Analysis of cell lines in long term culture, through a combination of binding and immunochemical assays, has identified distinct defects in the expression of HLA class I antigens in tumor cells [19, 21] [Fig. 2]. These defects do not represent artifacts of *in vitro* cell culture, since they have also been identified in surgically removed tumors by immunohistochemical (IHC) staining with monoclonal antibodies (mAb). In fact, with the exception of liver carcinoma [22–24] and leukemia [25], IHC staining of a large number of surgically removed malignant lesions with mAb to monomorphic determinants of HLA class I antigens has identified abnormalities in the HLA class I antigen expression in 16% to 50% of all malignant lesions analyzed [19, 21, 26] [Fig. 3].

The reason(s) for differences in the frequency of HLA class I defects is (are) not known. They are likely to reflect the time length between onset of tumor and diagnosis, since a long interval gives tumor cells more chances to mutate in the genes involved in HLA class I antigen expression and allows mutated cells to over-grow cells without abnormalities in their HLA class I phenotype in the presence of T cell selective pressure [27–29], as it will be discussed later. Figure 3 summarizes data for tumors for which at least 70 lesions have been analyzed. HLA class I antigen downregulation or loss has also been described in other tumor types. However, the number of lesions that have been analyzed is too low for one to draw definitive conclusions. These types of tumors include stomach [30], pancreatic [31], bladder [32], germ cell [33] and basal cell [34] carcinomas. It is noteworthy that HLA

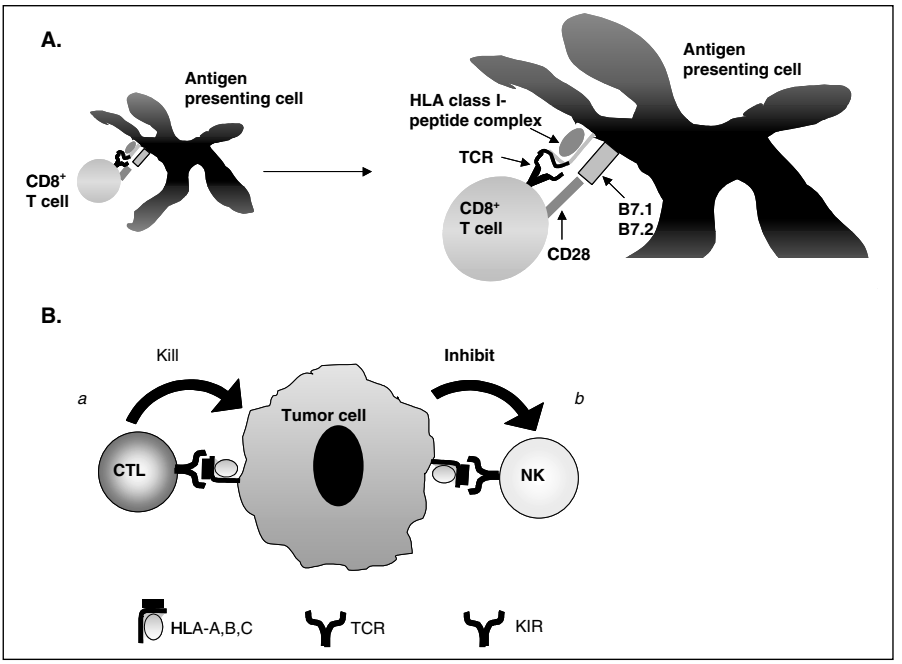

Figure 1. (A) The activation of specific T lymphocytes occurs through the recognition of two combined signals. The first signal is specific, requiring T cell receptor (TCR) recognition and binding to specific HLA class I antigen-peptide complexes presented by an antigen presenting cell. The second signal is nonspecific, resulting from the binding of B7.1 (CD80) or B7.2 (CD86) ligands on the antigen-presenting cell with its receptor, CD28, on the T cell. If both signals are provided, the T cell will proliferate and secrete cytokines. **(B)** CTL recognition of target cells occurs through the interaction of T cell receptor (TCR) with HLA class I antigens complexed to peptides generated by the antigen processing machinery. The trimeric HLA class I-β_2m-peptide complex plays a major role in the interactions between target cells and (*a*) activation of peptide-specific CTL through TCR; (*b*) inhibition of T cell subpopulations through inhibitory receptors KIR.

class I antigen loss or downregulation does not occur in all types of malignancies. In leukemia, defects in HLA class I antigen expression in malignant cells have been only occasionally identified. This finding is not likely to reflect a lack of genetic instability in leukemic cells, since like solid tumor cells, leukemic cells harbor many genetic and/or epigenetic alterations in their DNA [35]. Furthermore, in view of the role of immunoselection in the generation of malignant cell populations with HLA class I defects [27–29], lack of immune responses against leukemic cells is unlikely to be the mechanism. This possibility is supported by the higher frequency of HLA class I antigen abnormalities in sporadic diffuse large cell lymphoma than in immunodeficient and transplant-related lymphomas [36]. Therefore, it is likely that the lack of defects in HLA class I antigen expression identified in leukemia reflects the time interval between the onset of leukemia and its diagnosis, which is likely to be shorter than that of solid tumors. A short time interval between the onset of

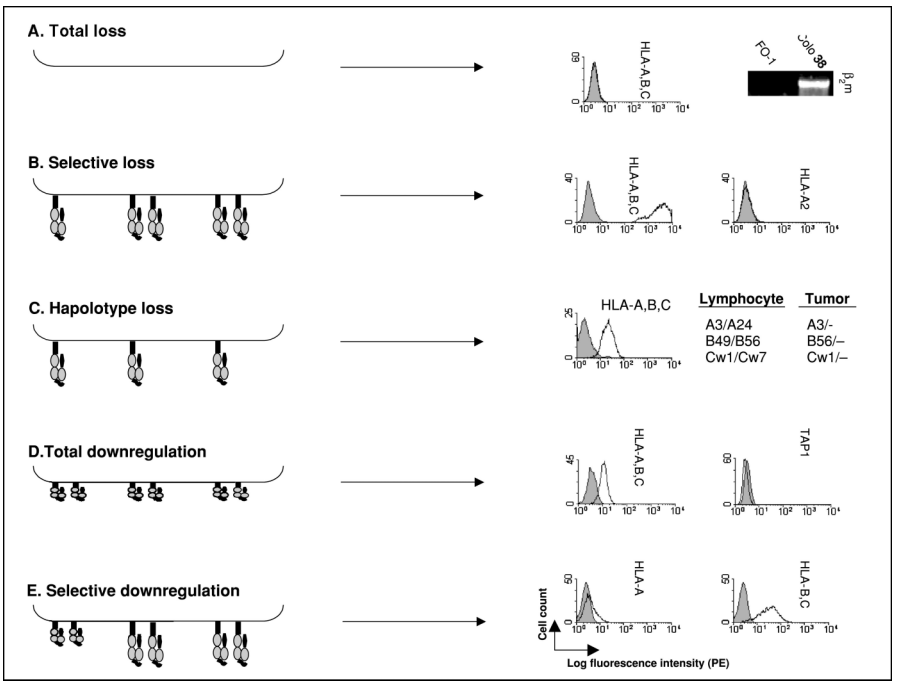

Figure 2. Abnormal HLA class I antigen phenotypes identified in malignant cells. Several molecular mechanisms can (A) Total loss of the gene products of the HLA-A, B and C loci can be caused by mutations in the β_2m gene; (B) Selective loss of one HLA class I allospecificity can be caused by loss of the gene(s) which encode the lost HLA class I allele(s) or by mutations which inhibit their transcription or translation; (C) Total loss of all HLA class I antigens encoded in one haplotype, which can be caused by LOH in of chromosome 6; (D) Total downregulation of all HLA class I antigens expressed by a cell, which can be caused by downregulation or loss of expression of the antigen processing machinery components and (E) selective downregulation of the gene products of one HLA class I locus, which can be cause be alterations in HLA class I antigen transcriptional factors.

leukemia and diagnosis may not allow sufficient time for cells to acquire mutations in the gene(s) involved in HLA class I antigen expression and for selective pressure to facilitate the expansion of malignant cells with HLA class I abnormalities. In the case of liver carcinoma, normal hepatocytes, which do not express or express very low HLA class I antigen levels [22], acquire the expression of these antigens during malignant transformation. The results obtained with liver carcinoma cell lines suggest that HLA class I antigen upregulation may result from the induction of antigen processing machinery components by cytokines secreted by immune cells infiltrating malignant lesions [22].

Abnormalities in HLA class I antigen expression in malignant lesions appear to have clinical significance, since they are associated with histopathological characteristics of the lesions and/or with clinical parameters in several malignant diseases [19, 37–43]. However, depending on the tumor type, HLA class I antigen defects can be associated

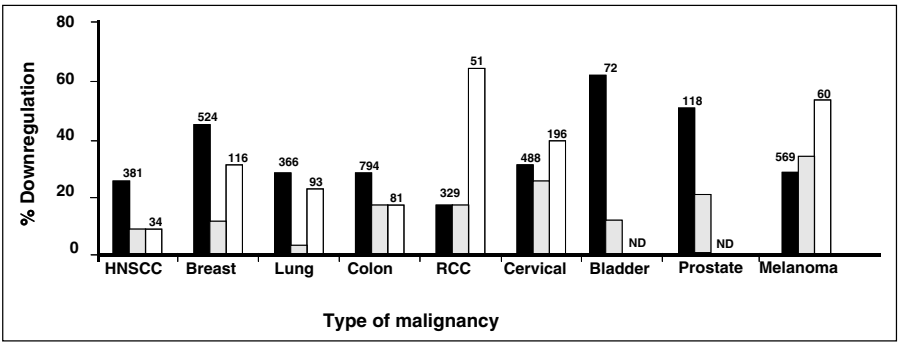

Figure 3. Frequency of HLA class I antigen and TAP1 downregulation in malignant lesions of different embryological origin. The most common types of solid tumors for which more than 70 or 30 lesions have been analyzed for HLA class I antigen or TAP1 expression, respectively, are shown. (■) Indicates total HLA class I antigen downregulation; (■) indicates selective HLA class I allospecificity loss; and (□) indicates TAP1 downregulation. Figures indicate the number of lesions analyzed. ND: not determined. Data has been adapted from

directly (head and neck squamous cell (HSCC), breast, small cell lung, prostate, bladder and cervical carcinoma and cutaneous melanoma), inversely correlated (uveal melanoma and colon carcinoma) or not associated (pulmonary adenocarcinoma, squamous cell carcinoma of the uterine cervix, cutaneous squamous cell carcinoma, large cell and large immunoblastic lymphoma and non-small cell lung carcinoma) with disease progression and/or poor clinical outcomes [36, 44–49]. The reasons for these discrepancies are not known but may reflect differences in the characteristics of the patient population, the methods of analysis and/or the system used to score HLA class I antigen expression. In addition, these findings may be attributed to differences in types of immune response elicited by tumors of different tissue or differences in routes of metastasis. An example is represented by the opposite association of HLA class I antigen downregulation with the clinical outcome in cutaneous and uveal melanomas [45]. HLA class I antigen downregulation is associated with a poor prognosis in cutaneous melanoma, where CTL are believed to control the metastatic tumor spread via the lymphatics [45]. In contrast, HLA class I antigen downregulation is associated with a favorable clinical outcome in uveal melanoma, where NK cells, which tend to kill tumor cells with a low HLA class I antigen expression [50, 51], have been suggested to limit metastasis via the blood.

The potential role of HLA class I antigen abnormalities in the clinical course of malignant disease has stimulated the characterization of the molecular mechanisms responsible for HLA class I antigen abnormalities. Through the effort of a number of investigators, characterization of cell lines originated from malignant lesions with HLA class I abnormalities has shown that distinct molecular mechanisms underlie the abnormal HLA class I phenotypes of tumor cells [Fig. 2]. The frequency of complete HLA class I antigen loss has been found to be between about 15% in primary cutaneous melanoma lesions and 50% in primary prostate carcinoma lesions [19].

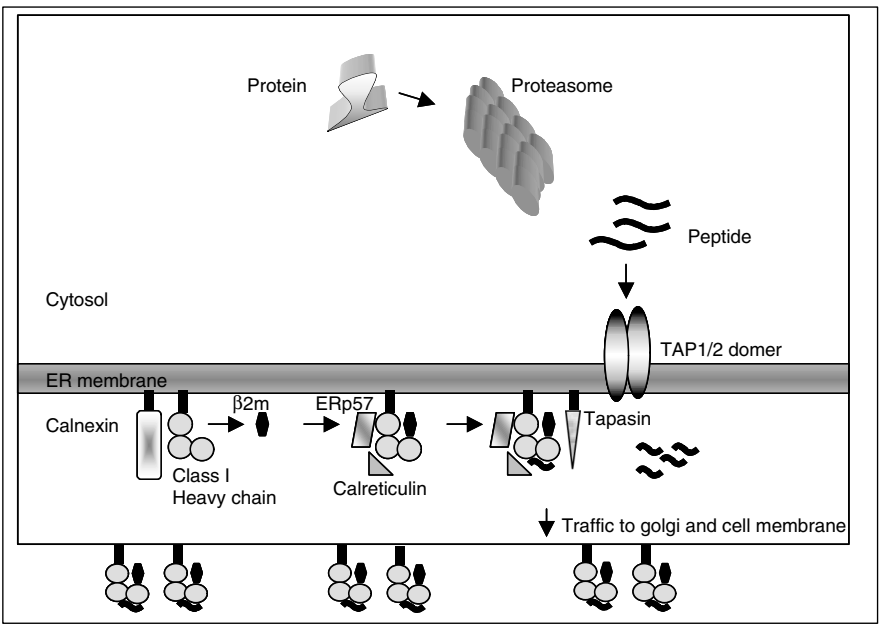

Figure 4. (A) Generation and interaction of HLA class I antigen-peptide. Intracellular protein antigens, which are mostly endogenous, are marked for ubiquitination within the cytosol and subsequently degraded into peptides by proteasomal cleavage. The constitutive proteasome subunits delta, MB1 and Z and the interferon-γ inducible immunosubunits LMP2, LMP7 and LMP10 are responsible for the catalytic activity of the proteasome. Once generated, peptides are transported into the endoplasmic reticulum through the dimeric transporter associated with antigen processing, TAP1 and TAP2. TAP is responsible for both qualitative and quantitative peptide translocation. Nascent, HLA class I antigen heavy chains are synthesized in the ER and associate with the chaperone immunoglobulin heavy chain binding protein (BiP), a universal ER chaperone involved in the translation and insertion of proteins into the ER. Following insertion into the ER, the HLA class I heavy chain associates with the chaperone calnexin and the thiol-dependant reductase ERp57. Calnexin dissociation is followed by HLA class I heavy chain association with β_2m, tapasin and the chaperone calreticulin. Calnexin, calreticulin and ERp57 play a role in folding of the HLA class I heavy chain. Subsequently, tapasin brings the HLA class I heavy chain, β_2m, chaperone complex into association with TAP and plays a role in both quantitative and qualitative peptide selection. The trimeric HLA class β_2m-peptide complex is then transported to the cell membrane.

The frequency of this phenotype varies significantly between different malignancies. As indicated above, it is likely that these differences reflect the time length between onset of tumor and diagnosis. Complete HLA class I antigen loss can be caused by defects in β_2-microglobulin (β_2m) which is required for the formation of the HLA class I heavy chain-β_2m-peptide complex and its transport to the cell membrane [20], epigenetic changes in the DNA or alterations in the antigen processing machinery components [52–56] [Fig. 2A]. The latter play a crucial role in the assembly of functional HLA class I antigen-peptide complexes and in their expression on the cell membrane [57] [Fig. 4]. Inactivation of the β_2m genes completely abrogates HLA class I antigen expression at the cell surface and has marked effects on peptide presentation. β_2m defects result from two events: loss of one copy of the $\beta_2 m$ gene

at chromosome #15, which carries the $\beta_2 m$ gene in humans [58], and mutations in the other copy the $\beta_2 m$ gene which inhibits its transcription in a few cases and its translation in most cases. It is not known which of these two events occurs first in malignant cells. The mutations identified thus far in β_2m genes range from large to single nucleotide deletions; in most cases they inhibit the translation of β_2m mRNA [20, 59–62]. Although the mutations are distributed randomly in β_2m genes, a mutation hotspot has been suggested to be located in the CT repeat region in exon 1 of the β_2m gene. Mutations in this region have been identified in more than 75% of tumor cells with total HLA class I antigen loss [63] and have been found to parallel the mutator phenotype in tumor cells [64], reflecting the increased genetic instability of this region during malignant transformation of cells [64]. It is noteworthy, that for some tumors such as head and neck squamous cell, laryngeal, breast, colorectal, renal and bladder carcinoma, β_2m gene mutations are not responsible for complete HLA class I antigen loss [52–56, 65]. These observations suggest that genetic mutations in the β_2m gene may not be the predominant molecular mechanism underlying total HLA class I antigen loss and suggest that other mechanisms may be involved in total HLA class I antigen loss. In this regard, post-transcriptional regulation of the β_2m gene expression has been suggested as a possible mechanism for total HLA class I antigen loss [52–56, 65]. In addition, epigenetic changes that cause total HLA class I antigen loss have been observed. Hypermethylation of three HLA class I antigen loci has been observed in neoplastic cells to selectively switch off HLA class I antigen gene expression. The characteristics of these tumors are the significant reduction in or complete absence of mRNA from the heavy chain gene and normal expression of β_2m and antigen processing machinery components. DNA hypermethylation has been implicated as a major mechanism for transcriptional inactivation of HLA class I antigen genes in esophageal squamous cell carcinomas and is also responsible for the total HLA class I antigen loss in melanoma [53, 66, 67].

Selective HLA class I allospecificity loss, e.g. HLA-A2 loss, is caused by loss of the gene(s) encoding the lost HLA class I heavy chain(s) or by mutations which inhibit their transcription or translation [63] [Fig. 2B]. It is noteworthy, that selective HLA class I antigen loss results from only one mutational event in a heterozygous allelic background. This may explain why, in most malignancies, the frequency of selective HLA class I antigen losses is higher than that of total HLA class I antigen losses [19]. As in the case of the β_2m gene, the mutations found in HLA class I heavy chains range from large deletions to single base deletions [68–72]. The mutations appear to occur randomly. Whether a mutation hotspot in the genes encoding HLA class I heavy chains exists remains to be determined.

Loss of one HLA class I haplotype, e.g. HLA-A24, -B56, -Cw7, appears to be frequently caused by loss of segments of the short arm of chromosome 6 where HLA class I genes reside [73], however in some instances it can be caused by the loss of specific transcription factors that specifically bind to HLA-A or HLA-B promoters [74] [Fig. 2C]. This phenotype is often identified by HLA class I genotyping and LOH analysis of chromosome 6. LOH at chromosome 6 appears to represent a frequent mechanism contributing to selective HLA haplotype loss in tumors [75].

This finding may reflect the frequent genetic recombination events at the human *MHC* located at chromosome 6p21.3, which carries the highest density of genes among all gene loci in human chromosomes [76].

Total HLA class I downregulation can be caused by multiple mechanisms. First, transcriptional activity of HLA class I heavy chain genes can be suppressed by the presence of silencer located at the distal promoter [77] or by epigenetic mechanisms such as hypermethylation and/or altered chromatin structure of the HLA class I heavy chain gene promoters [66, 78, 79]. Second, the restoration or enhancement of HLA class I antigen expression in malignant cells by IFN-γ suggests that altered regulation of non-mutated genes may play a part in defects in HLA class I antigen expression [80]. Lastly, the level of HLA class I antigens expressed on cells can be reduced by downregulation or loss of antigen processing machinery components [37] [Fig. 2D]. Defects in antigen processing machinery components may effect the generation of peptides from antigens, their transport into the endoplasmic reticulum (ER), their loading on HLA class I antigens and/or the repertoire of peptides presented by HLA class I antigens. It is noteworthy that in the majority of cases, antigen processing machinery component loss or downregulation can be corrected by treating cells with cytokines, e.g. IFN-γ, indicating that these abnormalities are usually caused by regulatory and not structural defects [19, 81]. This mechanism may explain why the frequency of downregulation of one or multiple antigen processing machinery components in malignant lesions is high, in spite of the codominant expression of the two genes encoding each antigen processing machinery component. An alternative, although not exclusive, mechanism is represented by the downregulation, by IL-10, of antigen processing machinery components, which leads to reduced HLA class I antigen cell surface expression [82]. This finding may be of clinical relevance,since a large number of human tumors secrete IL-10 [83]. Therefore, these patients, at variance with those with structural defects in HLA class I antigen-encoding genes, are likely to benefit by combining T cell-based immunotherapy with administration of IFN-γ and/or anti-IL-10 antibodies.

Information in the literature regarding antigen processing machinery component expression in various types of malignancies is scanty. Only a few components have been analyzed and only in a limited number of lesions. It is also noteworthy to point out that no information is available as to what constitutes normal or abnormal expression profiles of antigen processing machinery components in cells, since to the best of our knowledge no study has quantitated the level of antigen processing machinery component expression in normal cells of different embryological origin. The paucity of the available information reflects the limited or lack of availability of antibodies and methodology to quantitate antigen processing machinery components. Therefore, one must exercise caution in interpreting studies that analyze antigen processing machinery component expression in malignant cells, since the phenotype of the normal counterparts is not known in many cases. Among the antigen processing machinery components, TAP1 has been most extensively investigated. TAP1 downregulation and/or loss has been found in HNSCC, in carcinomas of the breast, small cell lung (SCLC), colon, kidney, cervix and prostate and in cutaneous

melanoma with a frequency ranging from 10–84% [37, 84] [Fig. 3]. A few studies have investigated TAP2 expression in malignant cells and the frequency of TAP2 downregulation tends to correlate with that of TAP1 [19]. TAP1 downregulation or loss is likely to be caused by abnormalities in regulatory mechanisms, since in some instances they can be corrected by *in vitro* administration of cytokines, such as IFN-γ and TNF-α, and is accompanied by an increase in HLA class I antigen expression [85, 86]. The increase in HLA class I antigen expression following induction of TAP1 expression is correlated with an increased susceptibility to TAA-specific CTL lysis, in most but not all cases [87–89]. In addition, it is expected that the frequency of TAP downregulation is higher than that of total HLA class I antigen losses, due to the distinct mechanisms underlying these two phenotypes. While two mutational events are required for total HLA class I antigen loss, TAP downregulation appears to be primarily due to abnormalities in regulatory mechanisms. To the best of our knowledge, structural defects in TAP1 as a result of mutations have been observed only in two human tumor cell lines [90, 91].

Only recently has tapasin expression been analyzed in a few types of tumors. Abnormalities in tapasin expression can lead to reduced HLA class I antigen expression, alterations in the repertoire of peptides presented by HLA class I antigens and resistance of malignant cells to CTL [57]. Heterogeneous and reduced levels of tapasin mRNA has been observed in HNSCC, SCLC, hepatoma, RCC, colon carcinoma, pancreatic carcinoma, neuroblastoma and cutaneous melanoma cell lines [92, 93]. In the majority of cases, *in vitro* incubation of cells with cytokines such as IFN-α, IFN-γ, TNF-α and IL-4 has resulted in tapasin transcriptional upregulation [92, 93]. However, in the melanoma cell line COPA159 we have identified a single-base deletion at position 684 in exon 3 of the *tapasin* gene resulting in a reading frameshift of the mRNA with a subsequent introduction of a premature stop codon at positions 698–700. This cell line demonstrates reduced HLA class I antigen expression, which can be restored upon transfection with the wild-type *tapasin* allele [Chang et al., unpublished data]. To a limited extent, tapasin expression has been investigated in surgically removed malignant lesions. In these studies tapasin has been found to be downregulated in both RCC and HNSCC lesions [50, 94, 95]. In the latter malignancy, this downregulation is associated with poor prognosis [95]. If this is a cause-effect relationship, it is likely to reflect the reduced susceptibility of tumor cells to CTL-mediated lysis because of HLA class I antigen downregulation and alterations in the HLA class I antigen peptide repertoire in cells with reduced tapasin expression.

Selective downregulation of the gene products of one HLA class I locus can be caused by alterations in the transcription factors for genes encoding HLA class I heavy chains [96, 97] [Fig. 2E]. However, there is limited information regarding selective downregulation of the gene products of one HLA class I locus, since the expression of some HLA class I allospecificities in malignant lesions has not been assessed because of the lack of appropriate probes.

The major role played by the HLA class I-TAA peptide complex in the recognition of tumor cells by CTL can be further illustrated by the association found between abnormalities in the expression and/or function of antigen processing machinery

components and poor clinical course of the disease in some malignancies [19]. This association most likely reflects the importance of these components in the generation of functional HLA class I-TAA peptide complexes. Notably, TAP1 downregulation has been reported to associate with tumor staging and reduction in patients' survival in breast carcinoma, SCLC, cervical cancer and cutaneous melanoma [19]. An increased frequency of TAP1 downregulation in metastatic lesions when compared to primary lesions has also been reported in breast carcinoma, cervical carcinoma and cutaneous melanoma [19]. Most recently, the role of tapasin in the clinical course of malignant diseases has been suggested by Ogino et al. who reported that tapasin downregulation in conjunction with HLA class I antigen downregulation was associated with reduced survival in patients with maxillary sinus squamous cell carcinoma [94]. It remains to be determined whether this finding applies to other types of tumors. Nevertheless, all of these findings are likely to reflect the crucial role of TAP1 and tapasin in the generation of HLA class I antigen-TAA peptide complexes and suggest that alterations in the repertoire of peptides presented by HLA class I antigens may provide an alternate route of immune escape for malignant cells. This possibility highlights the need to monitor specific HLA class I antigen-TAA derived peptide complex expression in malignant lesions. To this end, we have begun to develop probes capable of recognizing allospecific HLA class I antigen-TAA derived peptide complex expression on malignant cells (manuscript in preparation).

Generation of cells with HLA class I antigen defects results from mutations in the gene(s) which are involved in the expression of HLA class I antigens. It is likely that these mutations occur randomly due to increased epigenetic changes and genomic instability in the early stages of tumor development [63]. In general the frequency of HLA class I antigen defects in metastatic lesions is higher than that in primary and premalignant lesions [19]. It is also noteworthy to point out that especially in malignant cells isolated from patients with advanced disease the presence of multiple defects affecting different antigen processing machinery components and HLA class I subunits appears to be the rule more than the exception [63]. Moreover, an increase frequency of HLA class I antigen loss variants have been found in recurrent metastatic lesions in patients who had experienced clinical responses following T cell-based immunotherapy [28]. Therefore, one important question to ask is which mechanism(s) play(s) a role in the expansion of cells with HLA class I defects in malignant lesions. In view of the continuous exposure of tumor cells to the host's immune response [21, 15], one might ask whether immune selective pressure plays a major role in the expansion of cells with HLA class I antigen defects so that they become the major population in a lesion. One can envision two possible scenarios: (i) if immune selective pressure plays a major role, then tumor cells with HLA class I defects expand because of escape from host's immune response which targets tumor cells without HLA class I antigen defects; (ii) if on the other hand, immune selective pressure does not play a role, then the expansion of tumor cells with HLA class I antigen defects is independent of the development of an immune response in the host. The available evidence derived from studies in animal model systems and in patients treated with T cell-based immunotherapy argues in favor of a major role

played by immune selective pressure in the generation of malignant lesions with HLA class I antigen defects [27–29]. From a practical viewpoint, the possible role played by immune selective pressure in the generation of malignant lesions with HLA class I antigen defects suggests that the use of T cell-based immunotherapy for the treatment of malignant diseases may only be successful in a limited number of cases.

HLA class I antigen downregulation may provide malignant cells with a mechanism to escape CTL recognition and destruction. This possibility has raised the question of why HLA class I antigen downregulation does not increase the sensitivity of malignant cells to NK cell-mediated cytotoxicity. The latter phenomenon has been convincingly shown in mice where MHC class I downregulation is correlated with increased target cells' susceptibility to NK cells (missing-self hypothesis). The mechanisms by which NK cells recognize and kill target cells have been poorly understood only until recently [98, 99]. NK cell recognition and killing mechanisms are now believed to be governed by a balance between activating and inhibitory signals received by the NK cells. These signals are generated by specific target cell ligand-NK cell receptor interactions. To date, there is evidence that the non-classical HLA class I antigens HLA-E, F, G may serve as inhibitory NK cell ligands [100], while the MHC class I related chain A and B (MICA and MICB) [98, 99] and the UL16-binding protein 1, 2 and 3 (ULBP1, ULBP2 and ULBP3) [98, 99] may act as activating NK cell ligands. In this regard, tumors can express stress induced ligands MICA and MICB, [52, 101–103], which inhibit NK cytotoxic function and IFN-γ production when released in soluble form [104].

Co-stimulatory Molecule Expression

To achieve activation, T cells require a minimum of two signals provided by antigen and co-stimulatory membrane proteins such as CD80 and CD86 [105]. Stimulation of T cells in the absence of costimulatory signals leads to anergy of T cells and eventually to T cell apoptosis [18]. A decreased expression of costimulatory signals CD80 (B7-1), CD86 (B7-2) and CD45 has been demonstrated in B cell malignancies, lung and colon cancer, making them not only poor stimulators of a T cell response, but also potential inducers of T cell apoptosis [106–110]. In concert with these observations, in vitro experiments showed that transfection of tumor cells with the CD80 and CD86 genes, increased their immunogenicity. Although this led to the rejection of B7 transfected tumor in murine models, it did not always lead to the regression of the non-transfected malignant cells [111]. Tumor cells can also evade recognition by T cells by decreasing the expression of tumor antigens through mechanisms that remain unclear, but appear to be independent of HLA expression. The loss of gp100, MART 1 and tyrosinase in melanoma have been associated with tumor progression and resistance to immunotherapy [112, 113].

3. CHANGES IN CELL MEDIATED IMMUNE RESPONSE IN CANCER

During the early 1980's North and colleagues [114–117] developed animal models where they carefully studied T cell function during progressive tumor growth.

An initial protective T cell response could be readily demonstrated during the first days after tumor implantation, followed by a rapid decline in the response with the appearance of Ly1+ suppressor T cells. This suppressor function could be transferred into naive animals and was eliminated with low doses of cyclophosphamide, re-establishing a therapeutic anti-tumor response. These findings provided an insight into a dynamic interaction between the tumor and the immune system that could be manipulated to the benefit of the host. An alternative explanation to the presence of suppressor cells came from studies on the function of cytokines produced by CD4+ helper clones. Mossman and colleagues classified T helper cells according to the type of cytokines they produced and the response elicited. Th1 cells mainly produced IL2, IFNγ, and TNFα, promoting cellular responses, while Th2 cells mainly secreted IL4, IL13 and IL10, promoting antibody production [118; 119]. It was therefore possible that the progressive growth of tumor induced a loss of Th1 activity and an increased Th2 function, leading to a diminished cellular response and an enhanced antibody production. Most of these concepts remained as interesting research observations, but with minor relevance in the treatment of patients.

The advent of immunotherapy in the 1980's using the adoptive transfer of tumor-infiltrating lymphocytes (TIL) revealed to a greater extent the degree of T cell dysfunction in patients with cancer. In vitro testing of freshly isolated TIL demonstrated that these cells had a markedly decreased proliferation when stimulated with mitogens or tumor cells and had a significantly diminished clonogenic potential [120–122]. This T cell dysfunction was however not limited to TIL cells, but was also seen in peripheral blood T cells or splenic T lymphocytes in tumor bearing mice. Furthermore this cellular dysfunction appeared to have a major detrimental effect on the therapeutic success of immunotherapy. Loeffler and colleagues [123] studying an immunotherapy model of adoptive transfer of T lymphocytes, demonstrated that T cells from mice bearing tumors for >21 days had a markedly diminished anti-tumor effect when used to treat tumor-bearing recipients. In contrast, T cells from mice bearing tumors for <14 days had a high therapeutic efficacy when transferred into tumor bearing recipients. In vitro tests demonstrated a diminished cytotoxic activity in the T cells from long-term tumor bearing mice, which could in part be explained by a diminished expression of the perforin gene [124]. Sondak et al. [125] also confirmed the diminished cytotoxic function of T cells from tumor bearing mice, which was more significant in mice bearing visceral metastases as compared to those with subcutaneous tumors. Therefore animal models not only reproduced the T cell dysfunction seen in cancer patients, but also suggested that these alterations could have an important impact on the outcome of cancer immunotherapy. These observations sparked an increased research effort to elucidate the mechanisms of tumor escape that started in the 1990's and continues today.

Major advances in understanding the fundamental mechanisms of antigen processing and presentation, costimulatory signals and T cell activation, as well as the molecular basis for T cell signal transduction, provided important tools to start exploring the intricate interactions between tumors and the immune system.

3.1. Changes in Antigen Presenting Cells

Antigen presenting cells (APC) in the form of macrophages or dendritic cells (DC) process and present antigens to T lymphocytes. Gabrilovich and colleagues [126, 127] first described a selective increase in the number of immature myeloid DC in the circulation of tumor bearing mice and cancer patients. Surgical removal of the tumor resulted in a decrease in the immature DC cells and a recovery of T cell responses. In tumor-bearing mice, the immature myeloid cells are represented by a population of Gr-1, CD11b and MHC class I positive cells. Gr-1 (+) cells do not impair T cell responses to mitogens such as Con A, but completely block T cell responses *in vitro* and *in vivo* to peptides presented by MHC class I. Therefore, immature DC preferentially inhibit CD8-mediated antigen-specific T cell responses [128]. The increased immature DC cells appear to be result of VEGF produced by tumor cells, which arrests DC maturation by suppressing the activation of the transcription factor NFκB. In fact, there is a high degree of association between increased serum levels of VEGF and a high numbers of immature DC in patients with gastric, lung and head and neck cancer [128]. In addition to arresting dendritic cell maturation, tumors can also induce a selective increase in the number of DC2 cells or regulatory dendritic cells, which can induce T cell anergy [129]. Stromal derived factor–1 (SDF-1) produced by ovarian carcinoma cells selectively recruits plasmacytoid dendritic cells and modulates their function [130]. These in turn appear to preferentially activate regulatory T lymphocytes that express CD25.

Tumors may also impair the cytotoxic function of macrophages by blocking nitric oxide production. Nitric oxide is an important component of the cytotoxic mechanism displayed by macrophages, endothelial cells and neurons. Several studies have found that macrophages from patients with cancer or tumor bearing mice have a decreased production of nitric oxide when compared to normal individuals. However, these studies did not find a decreased expression of iNOS, suggesting that other mechanisms, such as the depletion of the nitric oxide substrate, arginine, may be the mechanism for the inhibition in nitric oxide production[131, 132].

3.2. Induction of Regulatory T Cells

The recently described subset of regulatory T cells comprises a subset of mostly CD4+, CD25+ T cells, which constitute approximately 5–10% of the total CD4+ cells and appear to control key aspects of tolerance to self antigens [133]. Depletion of this T cell subset can induce an autoimmune response against endocrine organs in mice. Patients with melanoma, colon and head and neck cancer have an increased percentage of regulatory T cells (CD4+/CD25+) in their circulation. The depletion of these cells in tumor bearing mice increases the response to tumor associate antigen [134]. However, depletion of regulatory T cells alone is not enough to treat established tumors in mice [135].

3.3. Apoptosis of Effector T Cells

Elimination of T cells responding to autologous antigens through the binding of Fas ligand (FasL) to the Fas receptor is a well-established mechanism for the induction of

apoptosis and tolerance to normal tissue antigens. A high expression of FasL has been reported in tumor cells from lung carcinoma, melanoma, colon carcinoma and liver carcinoma [136–138]. Therefore tumors that express Fas-ligand, or shed Fas-ligand into the serum could induce apoptosis in T cells infiltrating the site of tumor or in circulating T cells, effectively escaping the effector arm of the immune response [139]. Several reports have recently suggested an increased percentage in apoptosis of T cells in the peripheral blood of patients with head and neck cancer [140]. Tumor cells have also been shown to lose the expression of Fas, developing resistance to apoptosis induced by FasL expressed by effector cells of the immune system.

3.4. Changes in T Cell Signal Transduction

In the mid 1980's major advances in T cell biology provided the basis to understand the molecular events that lead to T cell activation. Among these were the elucidation of the elements that form the T cell antigen receptor (TCR) and the mechanisms of T cell signal transduction after antigen stimulation [141–143]. Briefly two polymorphic chains, the α and β chains confer antigen specificity to the T cell and form the antigen-binding site. These are covalently linked to the CD3 complex formed by the invariant chains $\gamma\delta\varepsilon$ and ζ The latter forms homodimers $\zeta\zeta$ (CD3ζ) or heterodimers ($\zeta\eta$). Two Src family members of tyrosine kinases are critical in the signal transduction of this structure, namely $p56^{lck}$ that is associated with CD4 or CD8, and $p59^{fyn}$, associated with CD3ζ. The binding of antigen to the $\alpha\beta$ TCR complex triggers the mobilization of calcium from intracellular stores and the hydrolysis of IP3 to IP2 freeing high-energy phosphates used by kinases in the phosphorylation of several signal transduction proteins. In parallel, HLA molecules and CD80 and CD86 bind to their receptors, activating various tyrosine kinases including $p59^{fyn}$ and ZAP-70, which activate nuclear transcription factors such as NFκB that translocate into the nucleus and activate or repress various genes [144, 145].

Major advances were also made in understanding the molecular changes that accompany T cell unresponsiveness or anergy. Quill and colleagues [146, 147] and Jenkins and Schwartz [148] demonstrated that T cells stimulated by antigens presented on fixed antigen presenting cells (APC) were anergic, i.e., unresponsive to repeated antigenic stimuli and unable to produce IL2. Furthermore, stimulation of T cells with streptococcus superantigen produced a state of T cell anergy and resulted in a decreased expression of $p56^{lck}$ and $p59^{fyn}$[149, 150]. Anergic T cells also had several molecular changes including the inability to phosphorylate p21 Ras [151] and a decreased ability to activate nuclear transcription factors NFκB and AP-1, important in regulating cytokine production [152].

In the early 1990's Mizoguchi and colleagues [153] studying the dysfunctional T cells from long-term tumor bearing mice demonstrated a marked decrease in the expression of CD3ζ chain, $p56^{lck}$ and $p59^{fyn}$ tyrosine kinases. These changes were accompanied by a decreased tyrosine kinase phosphorylation and a diminished Ca++ flux. These findings provided for the first time a molecular basis to explain T cell dysfunction in cancer patients. Li [154] and Ghosh [155] later showed that T cells from some patients with renal cell carcinoma and from long-term tumor bearing mice were unable to translocate NFκBp65 nuclear transcription factor, resulting in a

Table 2. Most frequent T cell signal transduction abnormalities reported in cancer patients

Target	Effect
CD3ζ	Decreased expression
$p56^{lck}$	Decreased expression
JAK-3	Decreased expression
Calcium signaling	Decreased mobilization
NFκBp65	Inability to translocate
IL2 production	Decreased production

predominance of NFκBp50/50 homodimer known to act as a repressor of the IFNγ gene [156]. In fact, cytokine production during the progressive growth of tumors in mice demonstrated a Th1 response (IL2 and IFNγ) early after tumor implantation, followed by an increased production of Th2 cytokines (IL4 and IL10) after three weeks [157].

Results in cancer patients confirmed the initial observations in murine models. T cells and NK cells from approximately half of the patients with renal cell carcinoma, colon carcinoma, ovarian carcinoma, gastric cancer, breast cancer, prostate cancer, Hodgkins disease, acute myelocytic leukemia and other tumors showed a decreased expression of CD3ζ chain and a decreased in vitro response to antigens or mitogens [158–162]. In addition, T cells from renal cell carcinoma patients also had a diminished ability to translocate NFκBp65. However, changes in signal transduction molecules were not limited to those associated with the T cell receptor (Table 2). Kolenko and colleagues demonstrated that Jak-3, a tyrosine kinase associated with the γ chain, a common element to IL2, IL4, IL7 and IL15 cytokine receptors, was also decreased in T cells from renal cell carcinoma patients [163]. Initial work in colon carcinoma [164] and renal cell carcinoma [165] suggested that patients with more advanced stages of the disease had a higher frequency of T cell signal transduction alterations. However, in cervical carcinoma [166] some patients with carcinoma *in situ* already showed a diminished expression of CD3ζ, suggesting that T cell signal transduction alterations could occur early in the disease and were not an exclusive characteristic of advanced stages of cancer. Other reports have also suggested an association between the expression of CD3ζ and survival. Patients with muetastatic melanoma (Stage IV) treated with IL2 + anti-CD3 monoclonal antibody and patients with head and neck cancer that had normal levels of CD3ζ chain at the initiation of treatment had a significantly longer survival compared to those who had undetectable levels [167, 168].

The expression of CD3 ζ changes with treatment. Patients with non-Hodgkins lymphoma and patients with Hodgkins disease [169, 170] who responded to chemotherapy showed a re-expression of normal levels of ζ chain, which decreased again in patients who had a recurrence of the disease. Limited data from clinical trials in ovarian carcinoma, melanoma, renal cell carcinoma and colon carcinoma showed that patients receiving IL2 based therapies could recover CD3ζ expression

[171]. However, this did not always coincide with a full recovery of T cell function since tyrosine kinase activity was not always fully restored.

3.5. Mechanisms Leading to Alterations in T Cell Signal Transduction

Otsuji et al. [172] and Kono et al. [173] demonstrated in a series of elegant in vitro experiments that H_2O_2 from macrophages induced the loss of CD3ζ chain in naive T cells, a phenomenon that could be blocked by the depletion of macrophages or the addition of oxygen radical scavengers. A similar effect was seen with H_2O_2 produced by neutrophils in patients with pancreatic and breast cancer [174]. Other macrophage products also appear to be able to alter the expression of T cell signal transduction proteins. Kolenko and colleagues [175] showed that PGE2 in combination with substances that increase cAMP can diminish the expression of Jak-3 in naive T lymphocytes, effectively blocking signal transduction through the IL2 receptor. A second mechanism leading to loss of CD3ζ chain was found while studying Fas-FasL induced T cell apoptosis [176, 177]. T cells undergoing apoptosis lose the expression of CD3ζ as one of the early changes in this process. Gangliosides expressed on the membrane of different tumors have also been shown to be powerful immunosuppressors of T cells. Uzzo et al. showed that gangliosides from renal cell carcinoma cells can suppress nuclear transcription factor NFκBp65 in T cells and induce apoptosis [178–180]. Therefore the diminished expression of CD3ζ chain seen in cancer patients could in part be explained by an increased frequency of apoptotic cells in peripheral blood.

3.6. Modulation of T Cell Function and CD3ζ Expression by Amino-Acid Availability

Recent observations have demonstrated the important role of amino-acids in regulating T cell function. Among these tryptophan and arginine appear to play an important role in cancer. Dunn and colleagues demonstrated that macrophages producing Indoleamine 2, 3-dioxygenase (IDO) can deplete the essential amino-acid tryptophan and sensitizes activated T cell to apoptosis. Preliminary data in tumor bearing animals suggests that tumor cells may express IDO [181, 182]. The use of an IDO inhibitor, 1-methyl-tryptophan (1-MT) is currently being studied in animal models as a means reversing the inhibitory effects of this metabolic pathway.

Non-essential amino acids can also cause severe T cell dysfunction. Taheri et al. and Rodriguez and colleagues recently demonstrated that T cells cultured in the absence of arginine lose the expression of CD3ζ, have a decreased proliferation and a decreased production of IFNγ [183, 184]. Arginine levels can be regulated in vivo by the enzymes nitric oxide synthase (iNOS) and arginase I produced by macrophages or tumor cells. Arginase I production in macrophages is increased by Th2 cytokines and is able to deplete the extracellular levels of arginine, causing a down-regulation of CD3ζ. In addition, cells cultured in the absence of arginine show a decreased translocation of NFκBp65 similar to these observed in T cells from cancer patients. The CD3ζ down-regulation in the absence of arginine is caused by post-transcriptional mechanisms leading to a decrease in CD3ζ mRNA stability.

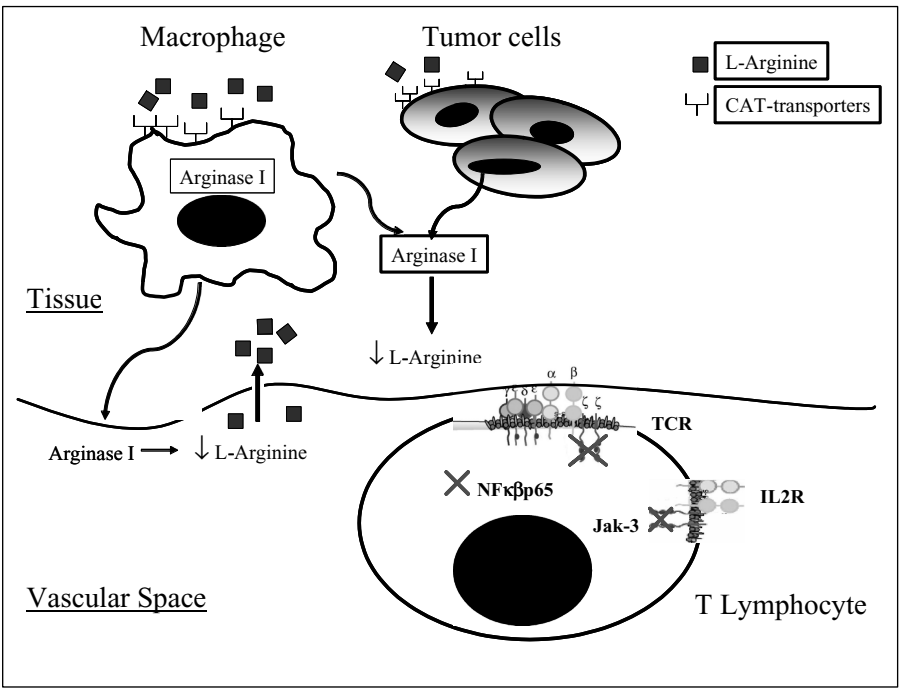

Figure 5. Regulation of T cell signal transduction can occur by limiting the availability of the amino acid arginine. Arginine is avidly taken up by activated macrophages and tumor cells through the CAT transporters. In addition, arginase produced by tumor cells or macrophages depletes arginine in the tissues (tumor microenvironment) or in the vascular space, inducing a decreased expression of CD3ζ, Jak-3 and NFκBp65 in activated T lymphocytes, effectively impairing the effector arm of the adaptive immune response.

These recent observations have led us to postulate the following scenario for the induction of T cell dysfunction in cancer [Fig. 5]. Arginase I produced by tumor cells or macrophages depletes arginine, causing the loss of CD3ζ and inhibiting cytokine production by T cells. This results in the inability of T cells to develop an anti tumor response and may impair the efficacy of immunotherapies.

4. SUMMARY

The results from in vitro immunological experiments, murine tumor models and patients with cancer clearly demonstrate that tumors have multiple mechanisms to evade the immune response. During the early stages of tumor development malignant cells can be poor stimulators, present poor targets or become resistant to the innate immune response, while at later stages, progressively growing tumors impair the adaptive immune response by blocking the maturation and function of antigen presenting cells and causing alterations in T cell signal transduction and function. Preliminary results also suggest a correlation between some of these changes

and an increased metastatic potential of the tumor cells, a diminished response to immunotherapy, and poor prognosis. Carefully coordinated basic research studies and clinical immunotherapy trials will be required to fully determine the impact on the outcome of the disease and the response to treatment. However, understanding the mechanisms used by tumor cells to evade the immune system could result in new therapeutic approaches for preventing and/or reversing these immune alterations and have the potential of improving the current results of immunotherapy trials.

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4. TUMOR ANTIGENS AND TUMOR ANTIGEN DISCOVERY

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INTRODUCTION

From the time that it was first proved that the immune system could recognize, and then react against tumor cells, identifying potential targets for the immune attack on cancer has became critically important. Decades long experience with standard cancer therapies, such as surgery, chemotherapy and radiation, has shown that cancer is not likely to be defeated by continuing to make incremental improvements in those therapies, and that new approaches must be sought. On the other hand, centuries-long experience with defeating infectious pathogens by generating effective immunity, has suggested immunotherapy and immunoprevention of cancer as new weapons against this disease. Identifying new tumor antigens (TA) has been an essential step in the progress towards the development of successful cancer immunotherapy protocols. The list of molecules that can be considered potentially good TA has grown over the last decade, and several of the best TA studied to date have been incorporated into the development of therapeutic vaccines. For a variety of reasons, most of which are well understood and can be ascribed to strict regulation that restrict and often prevent the most scientifically sound testing, the success of these vaccines has been marginal at best. Keenly aware of the problem, tumor immunologists are focused on fully understanding the useful characteristics of known tumor antigens, such as immunogenicity and safety, which will allow testing in appropriate clinical settings, as well as identifying additional antigens. With today's new technologies, researchers have the means to identify a greater number of

potential TA, and also the ability to test them to determine which of these will qualify as a true "tumor rejection antigen". In this chapter the field of TA is approached from the perspective of antigen discovery, with an emphasis on identifying TA with molecular and functional properties that have the best chance of controlling cancer and that will eventually allow their use in vaccines to prevent cancer in high risk individuals.

PROPERTIES OF A GOOD TUMOR ANTIGEN

Immune recognition of cancer in humans has been well documented [1–3], and since the landmark studies of Boon and Rosenberg [4, 5] the search for tumor antigens has been undertaken by many researchers. All of their combined effort to date has led to the discovery of TA that can be characterized in a variety of ways based on their expression patterns and the type of immune response they can elicit (for a comprehensive list see [6]. And while evidence of immune responses to these TA is plentiful, these responses do not always lead to the rejection of tumor *in vivo*. From the vaccination standpoint, an ideal TA would need to elicit an immune response that is clinically beneficial. Such an antigen is referred to as a "tumor rejection antigen". Achieving the *in vivo* immune response to a specific TA is dependent on many factors such as the vaccination protocol, the composition of the vaccine itself (i.e., adjuvants, cytokines), and the tumor that is targeted. For example, a TA that may have proven ineffective at inducing tumor rejection in one study, might prove otherwise in a different study that utilized the same TA but a different adjuvant. This explains why different studies can reach contradictory conclusions, even in animal models, about the usefulness of a particular TA [7–9].

T cells play a critical role in inhibiting tumor growth and causing the regression of cancer. Adoptive transfer of tumor-infiltrating lymphocytes along with interleukin 2 (IL-2) to melanoma patients was associated with tumor regression [10, 11] and some of the first TA to be identified were those recognized by cytotoxic T lymphocytes (CTL) [5, 12]. Because $CD8^+$ CTL are able to lyse tumor cells directly, and infiltrate and destroy tumor masses *in vivo*, much attention has been paid to TA recognized by CTL. As summarized by Gilboa [13], there are three good reasons why a TA recognized by CTL would be an effective tumor rejection antigen. (1) The major histocompatibility complex (MHC) class I processing pathway ensures that any changes in the repertoire of antigens expressed by most somatic cells would be recognized by CTL [14]. MHC class I molecules are expressed on most somatic cells, and therefore, on tumors arising from those cells. In contrast, MHC class II molecules are expressed only on a select subset of cells, and only on a few tumors, such as melanoma. (2) Studies in mice using antibody depletion or adoptive transfer of T cell subsets have shown that the $CD8^+$ CTL comprise the primary antitumor effector arm of the adaptive immune response. (3) There is a strong correlation often seen between tumor progression and loss of human leukocyte antigen (HLA) class I expression in cancer patients [15, 16], implying that in order to progress, tumors must escape the MHC class I- restricted T cell response. These observations underscore the importance tumor immunologists have put on the $CD8^+$ T cell response

against tumors, and also explains why to this point there have been more MHC class I-restricted TA identified than class II-restricted TA. However, as has been obvious in other immune responses, there is growing evidence that $CD4^+$ T cells play a major role in both initiating and maintaining an effective antitumor response [17, 18]. Several studies using animal models have demonstrated the critical role played by $CD4^+$ T cells in the priming of antitumor immunity [10, 19–21] specifically by generating a more effective $CD8^+$ CTL response. Other studies have shown that $CD4^+$ T cells are critical for maintaining $CD8^+$ T cell survival and long-term memory (reviewed in [22]). This makes the identification of MHC class II-restricted TA critical because an effective cancer vaccine would optimally include TA capable of generating both $CD4^+$ and $CD8^+$ T cell responses.

The TA expression pattern, temporal as well as tissue specific, plays a major role in determining its ultimate utility. Ideally, its expression would be induced at the very earliest stages of tumorigenesis, or even earlier, during what would be considered a pre-malignant stage. In unpublished data from our laboratory we have shown that the recently identified TA cyclin B1 [23], which is aberrantly expressed in lung cancer, is also aberrantly expressed in dysplastic lung tissue of long-term smokers. Vaccination with a TA that is expressed early in the malignant process, would allow the generation of an immune response that would not be hindered by the immunosuppressive factors known to be produced by tumors (reviewed in [24]). Fully transformed tumors are capable of secreting cytokines such as transforming growth factor-beta (TGF-β), interleukin 10 (IL-10), and vascular endothelial growth factor (VEGF), all of which can lead to both generalized and specific suppression of the immune response. Tumors are also capable of inducing tolerance in tumor-specific CTL [25]. Therefore, a TA-specific immune response would be expected to be more effective if it did not have to function in an environment susceptible to tumor-induced immunosuppression.

Where a TA is expressed is also important. Ideally, a TA would be expressed either by the majority of human cancers or the majority of cancers of the same type, and its expression would be different of that of the normal cell from which the tumors originated. In addition to the primary tumor, a specific TA must be also expressed on metastatic lesions. In the setting of immunotherapy, the primary tumor will most often be removed surgically, and a vaccine would be administered to prevent metastatic growth and recurrence of the tumor. For a similar reason, it is advantageous to have identified a TA that has a known crucial role in the oncogenic process, or is required for the continued growth of the tumor. Thus, if antigen loss variants arise that can escape the immune system, these variants will also have reduced malignant potential. Immune escape variants have been described in clinical vaccine trials that targeted melanoma-associated TA such as MAGE-3 [26] and Melan A/MART-1 [27], molecules not essential for tumor survival. In both of these cases, the presence of antigen-specific CTL was followed by the appearance of antigen-negative tumors, suggesting that immunoselection for antigen-loss variants had occurred. These studies are very good examples, and in fact proof that tumor rejection responses can be elicited in patients by vaccination, but they also illustrate the importance of selecting appropriate TA.

A good TA would also be capable of eliciting a response from a broad T cell repertoire, involving a large number of T cell clones. A TA possessing a large number of potential epitopes with high affinities for common HLA alleles has a greater chance of eliciting such a T cell response. In contrast, a tumor vaccine consisting of one or a few short peptides would severely limit the repertoire of responding T cells and encourage outgrowth of immune escape variants.

The majority of human TA described so far are self-proteins and there is evidence that some peripheral tolerance to these antigens exists and in some cases could be difficult to break [28]. While there are strategies to overcome tolerance (and ignorance) [29, 30], it might be best to choose TA that differ (through mutations or expression pattern) from the normal self protein.

TUMOR ANTIGEN DISCOVERY

Early methods used to identify TA were based on the identification of targets that were recognized by T cells of cancer patients. Tumor-infiltrating lymphocytes (TILs), as the name implies, are lymphocytes that have infiltrated into the stroma of cancer nodules [31]. Using these TILs, a strategy was developed and used successfully to identify a number of TA from melanoma (reviewed in [32]). To identify the TA recognized by TILs, cDNA libraries prepared from tumor cells were transfected into target cells that expressed the appropriate HLA. TILs that had been associated with tumor regression *in vivo*, were tested for their ability to lyse specific target cells. Some of the initial studies were performed in order to identify TA restricted by HLA-A2, since this is one of the most common HLA alleles. A cDNA library was transfected into the HLA-$A2^+$ breast cancer cell line MDA-231, and stable transfected clones were tested for their susceptibility to the HLA-A2 restricted TILs 1200 or 1235 [4, 33]. This method was later modified so that pools of cDNA were transiently transfected into an easily transfected cell line, such as the COS monkey kidney cell line or the 293 human embryonic kidney cell line, which also expressed the appropriate MHC restriction molecule [34, 35]. After screening the transfectants, subclones of the positive pools were made and this process was repeated until a single gene was identified that transferred susceptibility to TIL reactivity. Some of the earliest melanoma TA were identified using this method. Included in this group are MART-1 [4], gp100 [33], and tyrosinase [34], all of which are considered melanocyte differentiation antigens. It is worth noting that tyrosinase is also recognized by class II-restricted, $CD4^+$ TILs [36]. TRP-1 [35], p15 [37], and β-catenin [38] are three other TA originally identified using TILs.

While the previous method relies directly on a TA's ability to elicit a T cell response, the following method is based on the humoral immune response to an antigen. The approach is termed Serological Analysis of Recombinant cDNA Expression Libraries, or SEREX [39]. In this approach, a cDNA library is constructed from a fresh tumor specimen, packaged into a lambda-phage vector and expressed in *E. coli*. The recombinant proteins are transferred onto nitrocellulose membranes and

screened for recognition by high-titer IgG antibodies present in the patient's serum. Any positive clones are subcloned further to monoclonality, and the sequence of the inserted cDNA is determined.

The original strategy established by Sahin et al. [40], and summarized in [41] is as follows: (a) DNA sequencing is performed to establish identity or similarity with regard to genes in the existing data banks while also searching for possible mutational abnormalities; (b) mRNA expression patterns in normal tissues and tumors are determined through Northern blots and reverse-transcriptase polymerase chain reaction (RT-PCR) assays; (c) Immunogenicity is determined by the frequency of antibodies in a limited panel of sera from normal individuals, and patients with the same tumor type. The use of fresh tumor samples restricts the analysis to genes that are expressed by the tumor *in vivo* thus avoiding *in vitro* artifacts. There have been several modifications made to the original method to enrich the cDNA expression libraries for tumor-specific transcripts [42]. This method has an important weakness in that epitopes that undergo conformational changes when expressed in bacteria (predicted to be many), and epitopes dependent upon glycosylation (known to be many), will escape detection in this system.

Several new TA identified using SEREX are HOM-MEL-40 [43], NY-ESO-1 [44], SCP1 [45], and CT7 [46]. TA such as MAGE [5], BAGE [47], and GAGE [48], initially identified as targets of CTL, were also detected using the SEREX method. NY-ESO-1 has also subsequently been defined using T cell reactivity [49]. SEREX has been applied to a wide range of tumor types including melanoma, renal cancer, Hodgkin's disease [40], lung cancer [50]; [51], and colon cancer [52], among others. This methodology has proven to be capable of identifying a large number of TA to this point, but further testing, including testing for T cell reactivity and sequence and functional analyses, is required for each TA before it can be determined if it is a good candidate for vaccine development.

By Biochemical Methods

Enlisting the latest biochemical technologies to facilitate identification of new TA has had some notable success, but it has also proven to be very labor intensive. These methods generally involve growing large numbers of tumor cells, lysing the cells and purifying the MHC class I proteins. The peptides bound in these molecules are acid-eluted and fractionated using high-performance liquid chromatography (HPLC). Peptide fractions can be loaded onto antigen-presenting cells (APCs) and tested against known tumor-reactive T cells. Fractions capable of eliciting a T cell response are further analyzed using mass spectrometry (MS) analysis in hopes of ultimately identifying the peptide(s) driving this response [53]. These techniques can also be employed without the use of a known tumor-reactive T cell line. In this alternative approach [54], peptides are eluted from the class I MHC molecules of tumor cell lines, analyzed using MS, and specific peptides are tested for their ability to bind to class I molecules. Peptides are identified on the basis of their natural processing and presentation on tumor cells, without any information

on the immunogenicity of the peptide. Instead this approach allows for the rapid screening of large numbers of tumor-associated gene products in an effort to find naturally processed peptides presented by MHC class I molecules present on tumor cells.

With the help of a $CD4^+$ TIL line, similar biochemical methods can be used to identify MHC class II-restricted TA. Tumor cell homogenates are fractionated using various chromatographic techniques (e.g., ion-exchange, size-exclusion) with the fractions screened against the TIL line. Fractions eliciting a T cell response are further purified and characterized until a specific peptide(s) is identified. Using this method Pieper and colleagues [55] identified a class II- restricted melanoma antigen. The TA was a mutated glycolytic enzyme, triosephosphate isomerase, with the mutation causing a threonine to isoleucine conversion. This conversion created a new T cell epitope which possessed enhanced stimulatory ability of at least five logs over the wild-type peptide.

While this approach is capable of identifying new TA, and the specific class I- or Class II-restricted T cell epitopes derived from that antigen, it is limited by the technical complexities that are associated with sequencing very small amounts of peptide recovered from the tumor cell surface.

By Gene Expression Analysis

Analyzing the gene expression patterns of cancer cells can be used to reveal overexpressed gene products, including oncogenes, mutated gene products, viral genes and anything else that may be aberrantly expressed in tumor cells compared to normal tissue. The products of any of these over-expressed genes may potentially serve as ideal TA. Several different techniques can be used to analyze gene expression at the transcriptome level. These include the widely used microarrays, serial analysis of gene expression (SAGE), cDNA subtraction and representational-difference analysis (RDA). The SAGE method, which allows for the quantitative and simultaneous analysis of a large number of transcripts, was originally used to analyze pancreatic transcripts [56]. This method was later applied to analyze the global profiles of gene expression in both colorectal (CR) and pancreatic cancers [57]. In this study more than 300,000 transcripts representing about 49,000 different genes were identified from human CR epithelium, CR cancers or pancreatic cancers.

Studies combining cDNA subtraction and microarray analysis have lead to the identification of genes that are over-expressed in lung squamous cell carcinoma [58]. In this specific study seventeen genes (including four novel genes) were identified that were preferentially expressed in the lung tumor cells and several were also found to be expressed in head and neck squamous tumors. Using the same methods, this group went on to identify L552S an overexpressed, alternatively spliced isoform of XAGE-1 (a previously identified, cancer testis TA) in lung adenocarcinoma [59]. Further analysis showed that both isoforms, L552S and XAGE-1, are expressed in lung adenocarcinoma with L552S expressed in both early and late stages. Also, antibody responses to recombinant L552S protein were observed in 7 of 17 lung pleural effusion fluids of lung cancer patients suggesting that this protein is immunogenic.

RDA, a modification of a PCR-based subtractive hybridization method is used to isolate differentially expressed genes from a given cDNA population compared with another [60, 61]. This method was used to isolate the LAGE-1 [62], and a member of the MAGE family [63]. This method was also used to analyze the melanoma cell line SK-MEL-37, known to express a wide array of cancer testis (CT) antigens [46]. This analysis lead to the identification of the novel CT antigen, CT10 [64]. Two out of one hundred cancer patients tested exhibited antibody reactivity to the recombinant CT10 antigen, showing that it was immunogenic, and therefore a potential target for vaccine therapy.

Gene expression analysis represents a high throughput approach to identify genes that are highly, or aberrantly expressed in tumors as compared to normal tissue, thus a potential source of TA. The caviat inherent in this approach is that that the immunogenic potential of the products of these genes must be tested and in the case of many candidates it is not always clear how their priority would be determined.

By Reverse Immunology

This approach combines several of the methods described above, with epitope deduction through "reverse immunology", being a key step. The initial step of the approach, (as summarized in [65]), involves identifying candidate genes by gene expression analyses. This can potentially result in a large number of genes that may be overexpressed in tumor cells. This number can be reduced by only considering genes that are critical to the oncogenic process or survival of the tumor. As mentioned previously, targeting gene products that are essential to the survival of the tumor decreases the chances for the appearance of immune-escape tumor variants. Furthermore, it is also important to identify genes that are expressed early in the process of tumorigenesis, and in particular genes whose products are expressed in metastatic lesions. Once these candidate genes are selected they are put through the process of epitope deduction. This process involves several steps described below:

- *MHC binding prediction:* There are several algorithms that are publicly available for predicting binding affinities of peptides to MHC class I and class II molecules. It is much more difficult to predict binding affinities to MHC class II molecules, and therefore there are fewer programs available to do this. These algorithms are based on the assumption that each amino acid of the peptide epitope contributes independently to the overall binding, and that certain positions in the peptide are critical to its ability to bind to the MHC molecule. These critical positions are referred to as anchor residues, and the amino acids at these positions can either make a favorable or unfavorable contribution to binding of the peptide based on the interaction (attraction or repulsion) with their counterpart in the MHC molecule. In these motif-based algorithms, the occurrence of certain residues at specific positions in the peptide is used to predict the binding affinity between the

peptide and the MHC molecule (summarized in [66]. Examples of these programs can be found at: BioInformatics and Molecular Analysis section* (BIMAS), at http://bimas.dcrt.nih.gov/molbio/hlabind/ [67], and the SYFPEITHI database, at http://www.syfpeithi.de [68].

- *Antigen processing prediction:* This is based solely on algorithms such as the Prediction Algorithm for PROteasomal Cleavage (PAProC) [69, 70], and NetChop [71] which predict proteasomal cleavage, and do not take into account any other intracellular events that may also shape the peptide repertoire. For this reason, testing to confirm these predictions is very important. (PaProC can be found at http://www.paproc.de and NetChop is found at http://www.cbs.dtu.dk/services/)
- *Testing of antigen processing:* Because all of the steps involved in antigen processing have not been clearly and definitively laid out, the process of verifying that a TA can be processed by APCs, is critical. Also there are differences in cleavage patterns between immunoproteasomes found in APCs and proteasomes found in tumor cells and other non-APCs. It is therefore necessary to identify epitopes that are presented by the MHC molecules on the surface of a tumor cell. Elution of peptides from MHC molecules isolated from tumor cell lines, and their subsequent HPLC/MS analyses, is a direct approach to identify peptides that are presented by tumor cells [54].
- *Testing of MHC binding:* Several tests have been described that measure the binding affinity of peptides to MHC molecules [72, 73]. These include cellular assays using transporter associated with antigen processing (TAP)-deficient T2 hybridoma cells and ELISA-based assays using purified MHC proteins. The stability of the peptide-MHC complex is critical in determining the immunogenicity of the peptide, and therefore it is important to confirm it experimentally.
- *Analysis of the T cell repertoire:* A good TA must contain epitopes that can be recognized by T cells. Ideally, numerous epitopes will be generated through the processing of the TA for both MHC class I and class II presentation. The analysis of TA specific T cells can be done in normal donors and cancer patients by testing for: TA-specific cytotoxic function (CTL assay), specific cytokine production (ELISPOT assay), and by tetramer analysis. T cells can be tested for recognition of targets that express the MHC molecule of choice and have been loaded with the specific peptide, or against tumor cell lines that are known to express the TA being tested. After this *in vitro* screening, the next step would be to test the immunogenicity of the TA in HLA-transgenic mice.

An example of a TA identified using this approach is the catalytic subunit of the human telomerase reverse transcriptase or hTERT [74]. It is expressed on >85% of all tumors [75], but not in the vast majority of normal tissue. Telomerase expression is important in oncogenic transformation because it permits unlimited replicative potential of the cell [76, 77]. These features suggested hTERT as a good candidate for epitope deduction. Several studies have demonstrated the immunogenicity of this

TA through the generation of HLA class I-restricted CTL [74, 78–81]. This ability to elicit an anti-tumor T cell response along with several other favorable qualities possessed by hTERT have allowed this TA to be labeled a "universal TA" (reviewed in [82]. This approach has also been successful in identifying CTL epitopes in the TA PRAME [83], TRP-2 [84], along with identifying epitopes from the clonotypic TCR in cutaneous T cell lymphoma [85].

This strategy, which combines several technologies, has proven successful in identifying epitopes capable of eliciting a specific antitumor response. It exemplifies the type of approach that will be useful in identifying new targets that can be used in designing vaccines for cancer.

By Dendritic Cell-Based Methods

Dendritic cells (DC) are considered the most potent APCs because of their ability to capture and present antigens to naive T cells [86]. The method of TA discovery based on DC takes advantage of their ability to do this *in vitro*, thus being capable of priming T cells from healthy donors to tumor-derived peptides [23, 87]. Other methods have relied on the availability of T cell lines or clones from cancer patients in order to identify new TA. In the DC-based approach, using T cells from healthy, immunocompetent donors utilizes the full repertoire of T cell specificities not yet manipulated by the presence of the tumor. Peptides are prepared by acid elution from affinity purified tumor MHC-Class I molecules, and HPLC-fractionation. Individual fractions are loaded onto the DC which are used to prime individual T cell populations. T cells are cultured for several weeks, with periodic restimulation with peptide loaded DC or macrophages, before they are tested for their ability to recognize and kill the tumor from which the peptides were derived. The peptide fraction(s) that prime tumor-specific T cells are analyzed by further fractionation and sequencing using tandem mass spectrometry (MS/MS) in order to identify the specific peptide(s) that the T cells recognize. This approach has led to the identification of cyclin B1 as a TA [23].

This method can be modified to identify TA recognized by $CD4^+$ T cells. $CD4^+$ T cells from healthy donors are primed and restimulated with DC loaded with HPLC-fractionated tumor cell lysate. The individual T cell populations are then tested for their ability to proliferate against various preparations of tumor cells. In our group's experience, a good source of recall antigen is apoptotic tumor cells taken up and processed by DC or macrophages. When the priming fractions that supported proliferation to processed apoptotic tumor cells were analyzed, in addition to several fractions yielding new proteins, we found that two contained cyclin B1 (Graziano et al., unpublished observations). $CD4^+$ T cells, obtained from a healthy donor, were then primed against a recombinant form of cyclin B1. After several restimulations, these T cells were able to make IFN-γ in response to DC loaded with cyclin B1. Work is currently underway in our laboratory to further characterize the class II-restricted T cell epitopes of cyclin B1, and to better understand what role cyclin B1 plays in the oncogenic process of the tumor.

By In Silico Transcriptomics

The recent advances made in the field of genomics have provided researchers with a wealth of new information. This has led to discoveries being made not in the laboratory, but instead, on the computer. Tumor immunologists have followed suit by screening the human genome "in silico" in search of TA. In particular, the databases of "Expressed Sequence Tags" (EST) can be searched in an approach (reviewed in [88] referred to as "digital transcript profiling" or "in silico transcriptomics" [89, 90].

ESTs are partial sequences (about 300 bp) of cDNAs made from the mRNA that is extracted from a specific cell line or tissue. Databases contain a large number of ESTs from hundreds of tissues, including tumor tissues from different types and stages of cancer. With the use of specially designed informatics tools, these databases can be searched for potential TA candidates. EST sequences from selected libraries are compared in order to find genes that are specifically expressed or overexpressed in tumor tissues, and whose expression in normal tissues is either much lower or completely absent. The whole method is based on the idea that a good TA would be encoded by a gene that is either tissue-specific or tumor-associated, while being minimally expressed in normal tissue. Candidates that are selected in silico can be further tested for specific expression in tumor and normal tissue using PCR or hybridization techniques. Promising candidates can be tested further for their biological and immunological relevance.

The strengths of this approach are that it is easy and quick to implement so that existing data can be analyzed in a high-throughput manner. It is capable of identifying genes that are cancer- specific (e.g., TFF1, MDM2) and tissue-specific (e.g., prostate, colon) along with genes that are ubiquitously expressed in normal tissue (e.g., histone H1x). The weakness from a tumor immunologist's point of view is that candidate genes identified by this approach may or may not have immunological relevance.

By Identifying Viral Antigens

Certain malignancies are associated with specific viral infections, and the viral gene products expressed in these tumors are immunogenic, capable of eliciting T cell and antibody responses. Cervical cancer is associated with a chronic viral infection of epithelial cells with human papilloma virus (HPV). The majority of studies examining T cell responses to cervical cancer focus on the HPV E7 gene product as the target, since it is expressed in all disease stages [91] and is associated with tumorigenesis. Reports have identified the presence of class I-restricted, HPV-specific T cell responses after in vitro stimulation with peptides [92], or by using autologous DC to stimulate the T cell response [93] directed against peptides from the HPV E7 gene product. Other studies [91, 94] have identified class II-restricted T cell responses in cervical cancer patients, that were also specific for E7 derived peptides. $CD4^+$ T cells specific for tumor-derived peptides are likely to be important because defects in class I antigen processing or presentation are often associated with progression of cervical cancer lesions [95].

Epstein-Barr virus (EBV) was the first human virus implicated in oncogenesis. EBV infection is associated with several malignancies including Hodgkin's disease, nasopharyngeal carcinoma, lymphoproliferative disease (LPD), and Burkitt's lymphoma. All of these malignancies involve the latent state of infection, with varying patterns of gene expression associated with each disease (reviewed in [96]). In EBV-positive Hodgkin's lymphoma, only a limited number of EBV-derived antigens are expressed (EBNA-1, LMP1, LMP2 and BARF0). Of these, only two, LMP1 and LMP2, have shown the ability to elicit antigen-specific $CD8^+$ CTL, and most studies have focused on generating LMP2-specific CTL [97–99]. LMP1-specific CTL are rare in EBV-positive donors, which may be in part due to the considerable heterogeneity at its C- terminus [100–102]. Nasopharyngeal carcinoma (NPC) exhibits the same limited pattern of gene expression, and LMP2-specific CTL have been identified (at a low frequency) in NPC patients [103].

In EBV-associated LPD, all nine latency proteins are expressed (EBNAs 1, 2, 3A, 3B, 3C, LP, BARF0, LMP1, LMP2). This is the same expression pattern seen in EBV-transformed lymphoblastoid cell lines (LCL), which can be readily prepared from any donor. For these reasons, LCL can be used to generate and test EBV-specific CTL *in vitro* (summarized in [96]). While the generation and subsequent administration of EBV-specific CTL has proven to be effective in reducing viral load [104] and inducing tumor regression [105], the possibility of inducing escape mutants does exist even when using polyclonal CTL lines [106].

In Burkitt's lymphoma only two latency genes, EBNA-1 and BARF0, are expressed in tumor cells. EBNA-1 is not processed for HLA class I presentation, and therefore is not recognized by $CD8^+$ CTL [107]. BARF0 is expressed at a very low level, therefore it too is not recognized by CTL. For these reasons, CTL therapy may not be a feasible approach for Burkitt's lymphoma. However, there are reports demonstrating the existence of EBNA-1-specific $CD4^+$ CTL [108, 109], thereby allowing for the possibility that $CD4^+$ T cells may play a role in the control and therapy of Burkitt's lymphoma.

Infection with either hepatitis B (HBV) or hepatitis C virus (HCV) can lead to a chronic infection causing chronic liver disease that predisposes to the development of hepatocellular carcinoma (HCC). Most people develop both a cellular and humoral immune response to the virus [110]. Chronic HBV infection is characterized by an inefficient T-helper cell response to the hepatitis B surface antigen (HBsAg), and by a variable T-helper response to the nucleocapsid antigens, hepatitis core antigen (HBcAg), and the hepatitis e antigen (HBeAg) [111]. Recently, four HLA-A2-restricted CTL epitopes from the HBV X (HBx) protein were identified [112], and two of these peptide epitopes induced specific CTL in blood taken from patients with chronic HBV. When a specific CTL clone was adoptively transferred into nude mice that were xenografted with HCC, the tumors were eradicated. Ongoing studies are comparing different vaccine protocols in order to find out which vaccine is most effective at boosting both the T cell and antibody responses to HBV [111, 113].

An effective immune response that includes both T cells and antibodies is the best way to control acute viral infection, prevent chronic infection, and ultimately

prevent development of HCC. The HCV-infected liver is infiltrated with both $CD4^+$ and $CD8^+$ T cells. $CD8^+$ T cells recognize various epitopes of HCV, including those from the core and envelope proteins [114–116]. $CD4^+$ T cells also recognize regions from the core antigen [117, 118], and recognition of the nonstructural 3 (NS3) protein is associated with viral elimination after acute hepatitis C infection [119]. For both HBV and HCV, using vaccines to boost T cell and antibody response to these viral antigens is an effective way of preventing the development of HCC.

Viral antigens possess qualities that make them attractive candidates for vaccine therapy. They are foreign proteins capable of eliciting specific T cell and antibody mediated responses. They are expressed early, long before the onset of tumorigenesis in patients with chronic infections, and in certain cases (e.g., HBx protein) their expression is essential for tumorigenesis [120, 121]. While efforts to use vaccines in a therapeutic setting in patients with chronic disease or early cancer is of potential benefit, vaccination will most certainly be more effective in preventing the initial viral infection. The recently reported success of the HPV vaccine in preventing cervical cancer further illustrates the success that is achievable by immunizing against viral antigens [122].

CLASSIFICATION OF TUMOR ANTIGENS

While we have devoted most of this chapter to discussing TA primarily from the perspective of their discovery, their ever increasing number warrants a look at how TA have traditionally been classified. Generally TA can be placed into the five groups:

1. Cancer testis antigens: These antigens are expressed only in tumors and germ cells of the testes. This group includes a large number of MAGE antigens [5, 123, 124], GAGE [48], and the more recently discovered NY-ESO-1 [44].
2. Melanocyte differentiation antigens: This group of antigens is expressed during melanocyte differentiation, in normal melanocytes and in melanomas. Included in this group are MART-1/MelanA [33, 125], tyrosinase [136], and gp100 [127].
3. Tumor-specific mutated gene products: This group includes antigens that are products of mutated normal genes. These mutations are usually responsible for oncogenic properties of the tumor cell. CDK-4 [128], β-catenin [38], MUM-1 [129], mutated p53 [130, 131], and ras (H- and K-ras) (reviewed in [132] all belong to this group of TA.
4. Overexpressed or widely expressed self-antigens: This class of TA is encoded by genes that are widely expressed in normal tissues but are also selectively expressed on tumor cells. This group includes PRAME [133, 134], SART-1 [135], P15 [37] wild type p53 [136], MUC1 [137], cyclin B1 [23], Her2-neu [138], and CEA [139].
5. Viral antigens

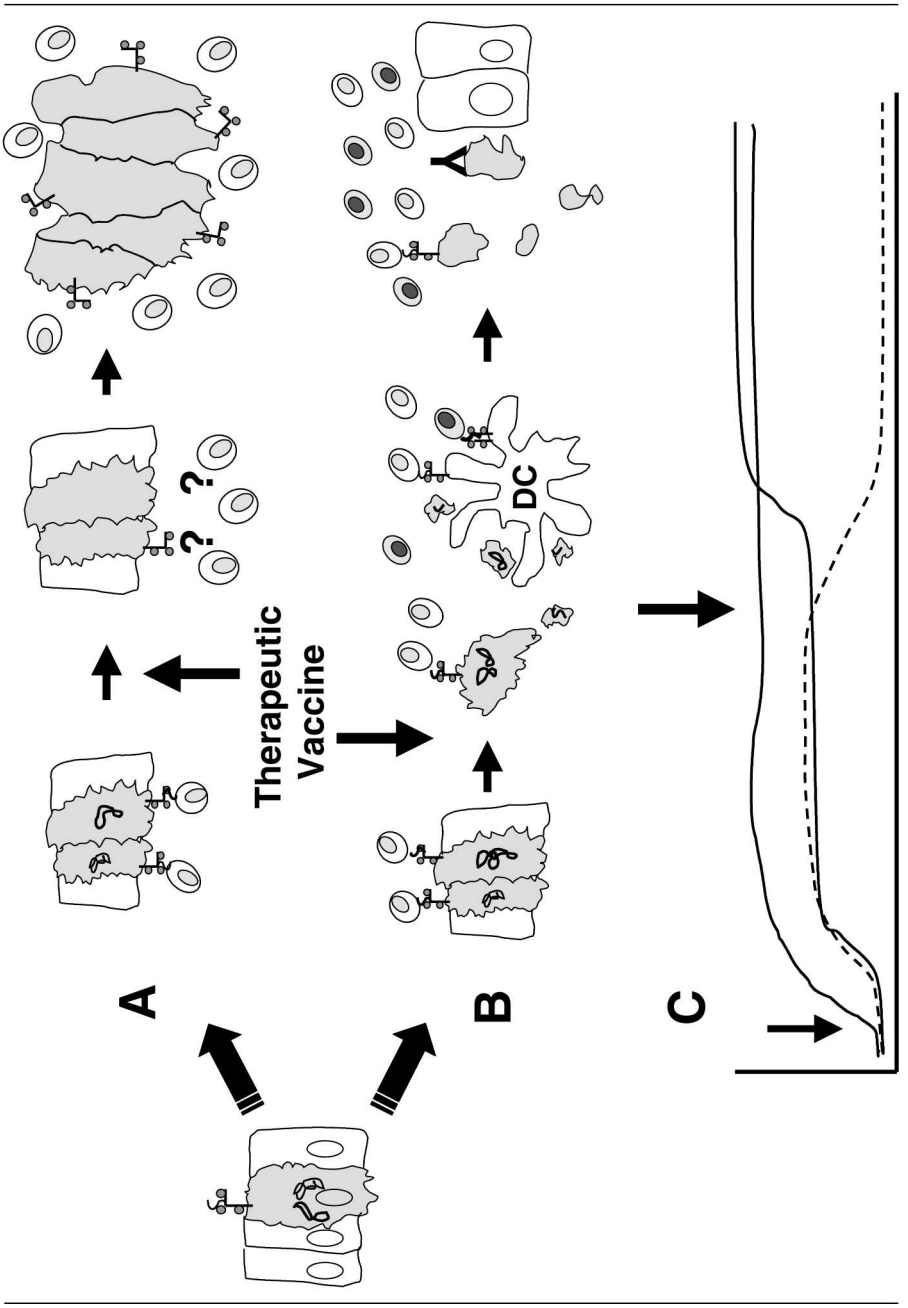
This classification is not inclusive of all the TA discovered to date. A more comprehensive list of TA, along with a list of their abbreviations can be found at: http://www.istitutotumori.mi.it [6].

CONCLUDING REMARKS

Knowledge of the existence of good TA, or the ability to identify new ones, will be essential for cancer vaccine development. With the recent advances in the field of genomics and proteomics, along with the wealth of new technologies now available, we can expect the discovery of new TA to increase dramatically in the near future. Hurdles such as emergence of tumor antigen-loss variants and overcoming tolerance or T cell anergy to some of the TA illustrate the need to identify additional TA that might allow us to solve these problems. It is also important to make the distinction between TA used in therapeutic vaccines and TA for preventive vaccines. TA that may have failed when used in therapeutic vaccines should not be overlooked when developing preventive vaccines. As stated earlier, a large tumor burden can suppress a specific immune response which, if generated in a healthy individual at high risk of developing cancer, could serve well as a prophylactic response.

Furthermore, the vast majority of known TA are MHC class I-restricted, and while the presence of tumor-specific $CD8^+$ CTL is essential for antitumor immunity, it is now obvious that $CD4^+$ T cells are critical for initiating and maintaining the immune response against tumors. Recent work by Vlad et al. has shown that $CD4^+$ T cells could be generated against tumor-specific glycopeptide epitopes [140]. This supports work done previously that showed that dramatic changes in protein glycosylation can create tumor-specific glycoproteins that are recognized by the immune system [141]. Glycopeptide epitopes possess two qualities that are coveted in a TA. First, their post-translational modification is often tumor-specific, and second, the modified epitope is capable of eliciting a $CD4^+$ T cell response.

Which qualities are most important when it comes to selecting the best TA for vaccination? The answer in part depends on whether the vaccine will be used for immunoprevention or immunotherapy. The expression pattern displayed by a TA that will be used for immunoprevention is critical. Such a TA should be expressed very early, in the premalignant stage, and its expression must be highly specific to cancerous or premalignant tissue. For example, our group has found that cyclin B1 that is overexpressed in the majority of lung tumors is also expressed on dysplastic lung tissue of long-term smokers (data not published). This suggests that cyclin B1 may be a good candidate TA for immunoprevention. Long term smokers with a genetic predisposition and/or a family history of lung cancer may be good candidates for a cyclin B1 vaccine for prevention of lung cancer. Another tumor antigen that our group has studied for many years, MUC1, is overexpressed in colon cancer and also in adenomatous polyps that are known to be precursors of colon cancer. Thus, while MUC1 has been tested as a vaccine in patients with advanced colon cancer, it is likely to be much more effective in targeting polyps and thereby preventing their progression to cancer.


A
B
C
Therapeutic Vaccine
DC
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?

The ability to elicit an effective immune response without inducing autoimmunity is another quality a TA must possess. Therefore it is critical to choose a TA whose expression is limited to tumor tissue, and/or distinct enough from the normal tissue counterpart that the responses that it elicits do not react with normal cells. This is a quality that will be difficult to predict, because an immune response that initially targets tumor tissue may ultimately damage normal cells.

The ability of a TA to elicit a response that leads to determinant spreading is also desirable. This is a process in which antigenic determinants different from, and non-cross-reactive with an inducing antigen, become subsequent targets of an ongoing immune response [142, 143]. Determinant spreading is initiated by a single epitope-specific clone that targets an immunodominant epitope. The resulting cytolytic effect leads to an inflammatory cascade at the target tissue, which allows cross-presentation of tissue debris by resident APCs. (For a complete review, see [144]). In three reports, that tested for determinant spreading in vaccinated cancer patients, those showing tumor regressions, also showed evidence of acquired determinant spreading [145–147].

Selecting the appropriate TA for vaccination is critical in determining the ultimate success or failure of the protocol. Figure 1 illustrates this point as it relates to both therapeutic and prophylactic vaccine strategies. Since lack of toxicity and general safety for many TA has been established in animal models, and the potential benefit of treating or preventing cancer outweighs the unlikely risk of autoimmunity or other complications, testing of the potential of the best defined TA in the clinic

Figure 1. The importance of a TA in generating an effective antitumor immune response. Transformation of a normal cell to a dysplastic or premalignant cell may be accompanied by the generation of multiple tumor antigens presented on MHC class I molecules that can be recognized by T cells. These tumor-specific T cells are used to identify the TA. In the first scenario (A), T cells recognize epitopes derived from the TA that is not essential for tumor progression. The tumor evades complete eradication by no longer expressing the TA. Using this antigen as a vaccine to boost tumor-specific immunity may increase the number of TA-specific T cells, but with no clinical benefit. In the second scenario (B), tumor-specific T cells identify a good TA, one which possesses several of the qualities described in the text. Its expression is critical to the growth of the tumor and it contains multiple epitopes capable of being presented by multiple class I alleles. Boosting the T cell response to this TA by vaccination could be beneficial. Damage done by the initially small number of tumor-specific T cells can lead to some tumor cell death, and the resulting debris can be captured by resident DC that process and present class I- and class II-restricted T cell epitopes. Recruitment of tumor-specific $CD4^+$T cells helps to maintain and further promote the anti-tumor response by secreting cytokines that will activate $CD8^+$ CTLs and promote isotype switching in B cells. (C) A hypothetical graph of what would be the expected long term anti-tumor immune responses based on the two types of TA. In A (dashed line), some immunity will be generated by the primary tumor and may be boosted by the vaccine. However, overgrowth of the antigen negative tumor will lead to the ineffectiveness and eventual disappearance of this immune response. In B (lower solid line), the low level immune response is maintained by the continuous presence of the tumor antigen. Boosting that response with a vaccine (thick arrow) can lead to long term immunity and tumor eradication.

The critical difference between the TA represented by these two very different scenarios A and B, might be completely erased if either TA is used for prophylactic vaccination (thin arrow). If long-term specific immunity is elicited to either antigen prior to the tumor occurrence, the few nascent tumor cells that express the TA could be destroyed before any changes in either the TA or MHC expression can take place. The upper solid line represents the anti-tumor immune response that would be expected from a prophylactic vaccine (thin arrow).

needs to continue on a larger scale and in healthier individuals than has been allowed to date.

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II. CANCER VACCINE DEVELOPMENT

5. PEPTIDE VACCINES AGAINST CANCER

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Multiple vaccine modalities have been explored in the search for an effective immunotherapy for cancer, but to date, only limited success with any one of these has been reported. Each has potential advantages and disadvantages. Peptide vaccines have turned out to be the most successful approach so far for melanoma, using either free peptides or peptides coated on dendritic cells. They have the feature of focusing the immune response on specific epitopes, of particular advantage for the many tumor antigens that are self antigens. This approach has been shown to facilitate the breaking of tolerance to self, for example in the case of Her-2/neu (1, 2). In the case of mutant molecules unique to the tumor, peptides also have the advantage of targeting only the mutant epitope that identifies the tumor cells, and avoiding other parts of the antigen that would be present in normal cells (3). Peptides are also relatively easy to modify, so that panels of variants can be studied to increase affinity for the relevant Major Histocompatibility Complex (MHC) molecules, to make the peptides more immunogenic, a process we have termed epitope enhancement (4–6). Likewise, the peptide sequence can be modified also to increase the affinity of the peptide-MHC complex for the T cell receptor. Here we will review how these peptide vaccine approaches have been used to target a variety of tumor antigens in both animal models, human *in vitro* studies, and human clinical trials. Several excellent reviews of peptide vaccines for cancer and clinical trials of these have been published recently (7–10).

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Table 1. Strategies to increase peptide vaccine efficacy

Approach	Mechanism
Inclusion of helper T cell epitopes	Induce helper T cells to activate/mature antigen presenting cells and secrete cytokines (It is not necessary to use a peptide from the same antigen as the CTL epitope.)
Incorporation of immunostimulatory molecules	Recruit professional antigen presenting cells (dendritic cells) to the site of antigen administration and skew T cells to the Th1 and CTL phenotypes
Blockade of negative regulators	Blocking negative regulatory cytokines and receptors to relieve mechanisms that dampen the response to the vaccine
Delivery of peptide on dendritic cells	Directly use professional antigen presenting cells which express high levels of co-stimulatory molecules to serve as a natural adjuvant; also to bypass the factors from the tumor that inhibit dendritic cell maturation
Epitope enhancement	Enhance binding ability of peptides to MHC without changing T cell interaction or increase affinity for T cell receptor without changing binding affinity for MHC

VACCINE STRATEGIES USING PEPTIDE VACCINES

Several approaches have been developed to immunize with peptides, as single free CTL epitope peptides are not inherently as immunogenic as larger constructs or live viral vectors. We briefly review some of the major strategies (Table 1).

Requirement for Attached or Intrinsic T Helper Epitopes

First, for induction of a CTL response, a helper epitope is necessary in addition to the CTL epitope to obtain an optimal CTL response. We originally found that for non-emulsion adjuvants, covalent linkage of helper epitope and CTL epitope is critical (11), whereas when the two peptides are physically associated in an emulsion adjuvant or other physical linkage, covalent linkage is not necessary. It is likely that this association is needed to get both epitopes into the same antigen presenting cell, so that the helper T cell can activate this presenting cell to "license" it to activate the CTL precursor (12–15). When a helper epitope is intrinsic to the peptide containing the CTL epitope, no additional helper epitope is needed (16). Even priming with just a helper epitope led to a protective CTL response against an MHC class II negative tumor (17). Accordingly, it was recently found that a longer peptide from human papillomavirus E7 protein containing both a helper and a CTL epitope was more effective at inducing a CTL response and treating an established E7-expressing tumor than the free CTL epitope, in a murine tumor model (18, 19). For these reasons, peptide vaccines should optimally be designed to contain both helper and CTL epitopes.

Incorporation of Cyotokines, Chemokines, and Costimulatory Molecules

Second, incorporation of cytokines, chemokines, costimulatory molecules, or immunostimulatory DNA sequences (CpG oligonucleotides) can enhance the response to a peptide vaccine (6, 20). An emulsion adjuvant such as incomplete

Freund's or the human grade equivalent, Montanide ISA-51, is conducive to incorporation of such agents, because they can be emulsified together with the antigen so that they are present in the same slow release depot and drain to the same draining lymph nodes (21–24). Some of these agents have been reviewed in an accompanying chapter. The most widely used are cytokines, such as GM-CSF (25, 26), which we showed increased antigen presenting cells and function in the draining lymph node (22). We and others also found synergy between GM-CSF and IL-12, which act by different mechanisms (22, 24, 26–28). In addition, we reasoned that if GM-CSF recruited dendritic cells, CD40L might mature them, and indeed, we found that the combination of GM-CSF and CD40L is synergistic (23). Costimulatory molecules can also increase the magnitude of the CTL response (29), as well as select for higher avidity CTL (30) that are more effective at clearing virus (31) or killing tumor cells (30, 32–34). IL-15 as an adjuvant in a vaccine has also been found to select for CTL with a long-lived memory phenotype that remains responsive to IL-15-induced homeostatic proliferation for at least 14 months, more than half a mouse lifetime (35). Some of these cytokines have been tested in human clinical trials (36–39). Chemokines have also served as successful adjuvants, attracting T cells to the site of the vaccine (40–42). Finally, CpG-containing immunostimulatory oligonucleotides can be used to steer the response toward Th1 cyokine production and CTL (43, 44), and can even be covalently linked to the antigen (45) to induce an even stronger CTL response.

Blockade of Negative Regulatory Pathways and Signals

Third, one can also block negative regulatory pathways that dampen the immune response. For example, IL-13 made by regulatory NKT cells dampens or partially inhibits natural tumor immunosurveillance mediated by CTL, and blockade of IL-13 with a soluble receptor construct, IL-13Rα2-Fc, or elimination of the $CD4^+$ NKT cell mediating the suppression can enhance immunsurveillance and prevent tumor recurrence (46, 47) or increase the efficacy of vaccines aimed at eliciting CTL (23). Similarly, one can block IL-10 or TGF-β made by $CD25^+$ and other immunoregulatory cells or eliminate such $CD25^+$ suppressive cells (48–50). Likewise, CTLA-4, a costimulatory receptor that paradoxically delivers an inhibitory signal to the T cell (51, 52), can be blocked to increase CTL responses to tumors (50, 53–55). Indeed, blockade of CTLA-4 and elimination of $CD25^+$ suppressor cells was synergistic in a murine tumor model (50).

Peptide Presentation on Dendritic Cells

Fourth, peptides can be coated or pulsed onto dendritic cells (DC) or other antigen presenting cells, and these used as an autologous cellular vaccine. This approach has the advantage that dendritic cells are the professional antigen presenting cells that most effectively activate naïve T cells, and that serve as "nature's adjuvant" (56). Dendritic cells as purified from bone marrow, spleen, or peripheral blood, or as differentiated in vitro from monocytes, tend to be immature, better at antigen uptake and processing than at antigen presentation. However, these cells are low in

costimulatory molecules, and negative or dull for CD83 in the human, and relatively poor at presenting antigen. When matured by various agents, the dendritic cells express CD83, lose their facility at antigen uptake, but upregulate costimulatory molecules and become much more effective at stimulating T cells. Indeed, different methods for maturing dendritic cells can affect their potency as vaccines when pulsed with antigen (57). The most effective agent for maturing dendritic cells is CD40L, which corresponds to the natural molecule on helper T cells that matures the dendritic cell when it binds to CD40 on the cell. There is some evidence that immature dendritic cells, which are low in costimulatory molecules, can actually be tolerogenic, as found in a human clinical trial (58). Therefore, most clinical protocols using dendritic cells as antigen presenting cells now employ matured DC (58, 59). Furthermore, use of autologous dendritic cells matured in vitro bypasses the problem that vascular endothelial growth factor (VEGF) and perhaps other factors made by tumors can inhibit the maturation of dendritic cells in tumor-bearing animals and patients (60–63). Thus, peptides have the advantage that they can be easily coated onto dendritic cells ex vivo, circumventing this impass to induction of vaccine responses in cancer patients.

Epitope Enhancement

Finally, the epitopes incorporated into peptide vaccines can be improved by sequence modification, a process we call epitope enhancement (4–6). Natural epitopes from cancer cells may not be optimal for binding to MHC molecules, because tumors probably lose the most immunogenic epitopes to become malignant. Many tumor antigens are self proteins, and self-tolerance is likely to delete the high avidity T cells specific for the most dominant epitopes. Thus, subdominant epitopes, to which there is less tolerance, may turn out to be the most effective tumor antigens. However, such subdominant epitopes may have suboptimal affinity for the MHC molecule, and therefore suboptimal immunogenicity. To improve immunogenicity, the sequence can be modified by epitope enhancement to increase the affinity for the MHC molecule (4–6, 64). The idea is to alter only residues interacting with the MHC molecule, so that the surface of the peptide-MHC complex recognized by the T cell receptor is unchanged, in order to induce T cells that still respond to the natural sequence present in the tumor (or virus). In practice, some alterations may affect both MHC and T cell receptor binding, so care must be taken to achieve the right balance between increased MHC affinity and T cell crossreactivity (65). This approach has been applied to viral vaccines (15, 65–69), as well as to tumor antigens, such as gp100, a melanocyte differentiation antigen (70, 71), and to p53, a tumor suppressor protein (72, 73).

An alternative related approach is to modify the amino acid residues interacting with the T-cell receptor, rather than with the MHC molecule. By this approach, one can generate peptide MHC complexes with higher affinity for a particular T cell receptor. If this receptor or ones like it predominate in the response to that epitope, then one can create a more immunogenic peptide to use as a vaccine, including epitopes from tumor antigens carcinoembryonic antigen (CEA) and p53 (74, 75).

TUMOR ANTIGEN TARGETS OF PEPTIDE VACCINES

Mutant Oncogene or Tumor Suppressor Gene Products

P53

The tumor suppressor gene p53 is mutated almost 50% of cancers of many common types (76, 77), and the mutant protein is usually overexpressed in the cancer cells. These two features make p53 a potential target for vaccine immunotherapy, since the mutation is a unique marker for the cancer cells, and the overexpression may also distinguish cancer cells from normal cells (3, 78). One caveat to the latter concept is that the overexpression appears to be due to reduced degradation, rather than increased production, and as such, might decrease the amount of mutant epitope processed and presented, rather than increase it. Nevertheless, T cells specific for wild-type p53 sequences have been found to preferentially kill tumors with mutant p53 that is overexpressed, so the overexpression can favor CTL recognition of tumor (79). Mutations in p53 have been shown to create neoantigenic epitopes in p53 which allow killing of cancer cells without harming normal cells (80). Also, mutant p53 peptides given with cytokines such as IL-12 (81) or pulsed onto dendritic cells (82) have been able to induce anti-tumor immunity and even treat established tumors in mice. Such murine CTL to mutant p53 can also lyse human tumor cells expressing the p53 mutation (83).

The disadvantage of focusing the immune response on the mutation is that there are so many different p53 mutations found that each vaccine would have to be custom-made for each tumor, and not all mutations would be in amino acid sequences that could bind to and be presented by the HLA molecules of the patient. Therefore, investigators have searched for common sequences that might be more immunogenic in cancer cells. A few of these that are presented by HLA-A2 have been described (79, 84) and some have been improved by epitope enhancement (72, 73). Indeed, in one case the wild type epitope was not presented by HLA-A2 in individuals with a mutation one residue downstream of the epitope (85), presumably because of a processing problem, but surprisingly, it was possible to reverse the defect by compensatory amino acid substitutions within the epitope itself (73). CTL to wild-type p53 epitopes have also been found to recognize peptides presented by other HLA molecules, including HLA-A24, HLA-B51, HLA-B46, and others in patients with bladder cancer (86) and head and neck cancer (87), colon cancer (88), and breast cancer (89). Human CTL raised in vitro by stimulation with peptide-pulsed dendritic cells have been found to lyse human tumor cells overexpressing p53 (88, 90, 91).

Although most of the work on p53 as a tumor antigen have focused on $CD8^+$ CTL responses, p53-specific $CD4^+$ T cell responses have also been described and been shown to be important for induction of CTL as well as potentially for their own effector function in the anti-tumor immune response (92). Overall, as either a custom peptide to uniquely target a cancer expressing a p53 mutation, or as a broader vaccine focused on overexpressed wild-type p53 sequences, p53 remains an attractive target molecule for the immunotherapy of cancer.

Ras

Ras is mutated in a number of human cancers, including about a third of colorectal cancers and a larger fraction of pancreatic cancer. Unlike p53, only a handful of mutations occur commonly, mostly in codon 12, in which a glycine residue is replaced by a valine, aspartic acid, alanine, cysteine, or arginine. Some mutations occur less commonly in codon 13 or codon 61. Codon 12 and 13 were found to fall within a 10-residue sequence that has a binding motif for the most common class I HLA molecule, HLA-A2.1 (93). The mutations actually increase the ability of the epitope to be presented compared to the wild type ras sequence, but the affinity is still relatively low. Much work has been done showing efficacy of CTL raised against mutant ras in mouse tumor models, reviewed previously (3, 94, 95). Cheever's group pioneered the idea of targeting ras as a tumor antigen in mice, defining $CD4^+$ T cell responses to the Arg 12 mutant ras (96) and the Leu 61 mutant form (97). Skipper and Stauss (98) first identified murine $CD8^+$ T cell responses to mutant ras targeting a codon 61 mutation (Lys 61), and Peace et al. mapped residues 59–67 as the best binder able to elicit specific CTL (99). Fenton et al. (100) then showed that a recombinant mutant ras protein with the Arg-12 mutation was able to elicit CTL and protect mice against challenge with tumors expressing this mutation.

Human $CD4^+$ T cell responses have also been reported to ras codon 12 and 61 mutations (101–106). Indeed, promiscuous $CD4^+$ T cell epitopes were found encompassing codon 12 and codon 61 mutations (102, 103). However, a clinical trial of peptide-pulsed PBMC induced T cell proliferative but not clinical responses in pancreatic cancer patients (107, 108).

Also, human CTL have been raised *in vitro* against some of the common codon 12 or 13 mutant peptides, from blood of patients either unimmunized or immunized with mutant ras peptides, and have been shown to lyse human tumors (106, 109, 110). On the other hand, several attempts to raise CTL to such mutant ras peptides resulted in CTL that would kill only targets pulsed with peptide, not tumor cells expressing mutant ras (111, 112). Several clinical trials have been carried out to immunize against mutant ras in cancer patients, including use of 13-residue peptides in adjuvant (106, 113) and 10-residue or 17-residue peptides pulsed onto antigen presenting cells, either peripheral blood mononuclear cells or enriched dendritic cells (110, 114). Although no clinical responses were seen in these studies, a correlation between specific cytokine response and survival was observed (114) (Carbone et al., manuscript submitted). In another study of ras peptide intradermal immunization of pancreatic cancer patients using GM-CSF as adjuvant, some clinical responses were seen, and median survival was longer in those achieving an immune response to the vaccine (115). A clinical trial of peptide-pulsed autologous CD40L-matured dendritic cells with codon 12 and codon 13 mutant ras peptides in colorectal cancer patients is underway in the National Cancer Institute (J. Janik, J. Morris, D.P. Carbone, J.A. Berzofsky, personal communication). Thus, mutant ras peptides remain a promising target for therapy of a number of types of cancer.

VHL

The von Hippel Lindau (VHL) protein was originally found to be mutated in this syndrome involving hereditary renal cell cancer, and then was also found to be mutated in sporadic renal cell carcinoma (116). The mutations occur at many positions, more like the case of p53 than that of ras. However, a number of them fall within sequences predicted to bind to HLA-A2.1, so a clinical trial is underway to examine whether peptides spanning these mutations can induce a CTL response and/or a clinical remission in HLA-$A2^+$ patients with renal cell cancer (S. Khleif and J.A. Berzofsky, personal communication).

Chromosomal Translocations

A number of types of malignancy are associated with chromosomal translocations, especially sarcomas, and chronic myelogenous leukemia (CML). In general, these chromosomal translocations have been found to contribute to the malignant phenotype by creating fusion genes at the junction of the two chromosomes that produce a new oncogene. Usually these involve genes that regulate other genes, such as transcription factors. For example, the DNA binding domain of one transcription factor may be joined to the activation domain of another transcription factor, resulting in aberrant transcription (3, 117, 118). However, the fusion protein created by the chromosomal translocation, which is responsible for the malignancy, may also be its Achilles heel. At the breakpoint junction within the fusion protein, there is a new amino acid sequence for every peptide that spans the breakpoint, that does not occur in either of the normal proteins that are the parents of the fusion. These new peptides may serve as neoantigenic determinants that can flag the tumor for recognition by the immune system, since these amino acid sequences are absent in non-malignant cells (119). Furthermore, the tumor cannot afford to lose or turn off production of the fusion gene product, because this product is also serving as an oncogene to maintain the malignant phenotype.

Bcr-Abl is a the classic example of a fusion protein, in this case formed by the t(9;22) chromosomal translocation associated with the Philadelphia chromosome of CML (95% of cases) as well as 10% of childhood and 25% of adult acute lymphocytic leukemia (ALL) (120). The tyrosine kinase activity of the fusion protein is increased compared to the normal C-Abl tyrosine kinase (121, 122) and the fusion protein has transforming activity (123), so it is likely the tumor cells cannot lose it without losing their malignant state. Cheever's group first targeted the BCR-ABL fusion protein in mice (124, 125), inducing $CD4^+$ T cell responses, and similar responses have been seen in human $CD4^+$ T cells (126). Murine CTL to the fusion protein were not able to lyse tumor cells expressing the fusion protein (127), but subsequent studies of an epitope presented by HLA-A3 showed that human CTL could lyse tumor cells (128, 129), supporting the use of BCR-ABL as a tumor vaccine antigen. Recent clinical trials of BCR-ABL breakpoint peptides as vaccines in CML patients have induced cellular immune responses but not yet achieved clinical remissions (130, 131).

PAX3-FKHR, EWS-FLI1, SSX-SYT are all examples of fusion proteins created by translocations in pediatric sarcomas, such as alveolar rhabdomyosarcoma, Ewing's sarcoma, and synovial sarcoma, respectively (3, 117). Murine CTL raised to a peptide spanning the breakpoint of the PAX3-FKHR fusion protein were able to lyse adenocarcinoam cells transfected with the full-length PAX3-FKHR DNA (118). Some of the peptides spanning the breakpoints have been found to bind to common HLA molecules, such as HLA-A3, A1 or B7 (117–119). In such cases, it has been possible to raise human CTL to these peptides that can recognize and kill human tumor cells expressing the fusion protein, for example in the case of an SSX-SYT fusion peptide of synovial sarcoma presented by HLA-B7 (119). Some of these are currently in clinical trials as therapeutic vaccines in patients.

Her-2/neu

Her-2/neu is a 185 Kd transmembrane protein in the epidermal growth factor receptor family of tyrosine kinase receptors (94, 132). Her-2/neu is overexpressed in about 30% of breast carcinomas in humans, and so has been considered a target for immunotherapy of breast cancer. Its value as a target is underlined by the success of trastuxumab (Herceptin), a monoclonal antibody to Her-2/neu licensed to treat breast cancer (133–135). As a vaccine target, both antibodies and T cell immunity have been sought. With regard to antibodies, a peptide vaccine consisting of B cell epitopes joined to a promiscuous helper T cell epitope induced antibodies in rabbits that inhibited human breast cancer cells in vitro, and substantially protected 83% of Her-2 transgenic mice from development of spontaneous breast cancers (136). With regard to T cells, Cheever, Disis and colleagues found that it was easier to break tolerance to murine Her-2 in mice with short peptides as vaccines than with the whole protein (2). Thus, this may be an ideal case for a peptide vaccine. Clinical trials of Her-2 peptides in humans were successful in inducing $CD4^+$ T cell proliferative responses that correlated with delayed type hypersensitivity (DTH) responses (137, 138). A later study using peptides that contained both helper epitopes and CTL epitopes presented by HLA-A2, with GM-CSF as an adjuvant, induced both $CD4^+$ T cell proliferative responses and $CD8^+$ HLA-A2-restricted CTL in patients previously treated for breast, ovarian, or lung cancer that overexpressed Her-2, with no evident or minimal residual disease (139). T cells induced by the vaccine lysed human tumor cells expressing Her-2 and persisted for more than a year in some patients. However, some CTL induced by peptides from Her-2 have been found not to recognize Her-2-expressing tumors (140). Also, short peptides are less likely to induce antibodies that may have clinical benefit like that of trastuxumab. Nevertheless, Her-2/neu remains a promising target for cancer vaccines.

MUC1

MUC1 is a mucin-family glycoprotein that is overexpressed as well as underglycosylated on many types of cancers of epithelial origin compared to normal epithelial cells (9, 141, 142). It contains multiple copies of a 20-residue repeat sequence. Early studies of human CTL responses to MUC1 revealed a surprising lack of HLA

restriction that was thought to be related to the multivalency of the repeat structure (141). In addition, conventional HLA-restricted epitopes have been found (143). MUC1 peptides pulsed on dendritic cells were more effective than the same peptides in adjuvant at eliciting CTL and inducing tumor rejection in both wild-type mice and mice transgenic for MUC1, indicating an ability to break tolerance (144). Several clinical trials of peptide vaccines have succeeded in eliciting $CD4^+$ or $CD8^+$ T cell responses and specific antibodies, and antibodies raised to MUC1 peptides conjugated to keyhole limpet hemocyanin (KLH) as carrier in patients can mediate antibody-dependent cellular cytotoxicity against tumor cells in vitro (145), but so far no consistent clinical responses except for stabilization of disease has been observed (146–152).

CEA

Carcinoembryonic antigen (CEA) is an embryonic antigen not normally expressed substantially in the adult, but overexpressed in most colorectal, gastric, breast, pancreatic, and non-small cell lung cancers. An immunodominant CTL epitope has been identified presented by HLA-A2.1, called CAP-1, and by application of epitope enhancement, an improved epitope has been shown to have higher affinity to the most frequently used T cell receptors (74, 153, 154). Several early clinical trials used the CAP-1 peptide as a vaccine either in adjuvant (Detox) or pulsed onto autologous dendritic cells. T cell responses have been observed, but no consistent clinical responses were seen, although there was one case of a partial remission in a thyroid carcinoma patient treated with peptide-pulsed dendritic cells (155–157). However, a more recent clinical trial of the epitope-enhanced CAP-1 peptide CEA605-613 pulsed onto dendritic cells that had been expanded with Flt3 ligand produced clear tumor regressions in two colorectal cancer patients, of 12 HLA-$A2^+$ patients with colorectal or non-small-cell lung cancer studied (158). Another CEA peptide presented by HLA-A2 (CEA691) has been improved by epitope enhancement (159), and immunzation with autologous dendritic cells pulsed with an HLA-A24-presented peptide of CEA (CEA652) has been associated with stable disease in two of ten patients (160). Also, a promiscuous T helper epitope of CEA (residues 653–667), presented by HLA-DR4, DR7, and DR9, has been identified (161), overlapping the latter HLA-A24-presented CTL epitope. Therefore, much recent progress has been made toward developing peptide vaccines against CEA that can impact the clinical course of cancers expressing this antigen.

Melanocyte Differentiation Antigens

As melanoma is one of the tumors most associated with spontaneous remissions thought to be immune-mediated, it has been a major focus of cancer vaccines and immunotherapy (162). Using tumor infiltrating lymphocytes as probes, several shared tumor antigens have been identified, many of which turned out to be melanocyte differentiation antigens, such as Mart-1/Melan A, gp100, tyrosinase, and tyrosinase-related protein (TRP)-2 (162–164). Patients treated with vaccines or adoptively transferred T cells specific for these antigens often develop vitiligo, or

depigmentation in patches of skin, confirming the crossreactivity of the T cells for normal melanocytes and melanoma cells (163). Indeed, there is a correlation between vitiligo and clinical remissions, in that cases of vitiligo have been seen only in patients showing some clinical response to the therapy (164–166). This side effect, largely cosmetic, has been considered an acceptable trade-off if one can successfully treat a largely fatal malignancy. One of the most effective vaccine approaches has been found to be synthetic peptides corresponding to HLA-A2-binding segments of these antigens. In a phase I trial of MART-1 peptide in incomplete Freund's adjuvant, 10 of 22 patients developed interferon-γ responses specific for the peptide and these responses correlated with prolonged relapse-free survival (167). A modification of this peptide has been described to increase stability in plasma (168).

Epitope enhancement has been applied to make a more effective vaccine of gp100 peptide 209–217, by substituting a methionine at position 2 in the peptide sequence (70). In a clinical trial comparing the wild type and enhanced peptide, the enhanced peptide was much more effective at eliciting a $CD8^+$ T cell response (71). When given with IL-2, this peptide was also the most effective at producing clinical remissions, significantly more than in the case of treatment with IL-2 alone. This promising finding has led to a phase III clinical trial in progress. Interestingly, however, the use of IL-2 that increased clinical responses reduced the CTL activity detectable in peripheral blood, suggesting sequestering at the tumor site (169). In that regard, it was found that when melanoma patients were vaccinated with a mixture of four peptides from gp100 and tyrosinase restricted by HLA-A1, A2, and A3, with GM-CSF in Montanide ISA-51 adjuvant, CTL could be detected in 5/5 patients in lymph nodes draining the immunization site, but in only 2/5 patients in the peripheral blood (170). In addition, a MART-1 peptide vaccine in two types of emulsion adjuvants increased the number of antigen-specific T cells ex vivo, but did not convert them to an active effector state seen after virus infection, suggesting the need for additional vaccine components to activate the T cells induced (171). In this regard, responses to the gp100-209-217 (2M) peptide could be increased by immunization of melanoma patients with the peptide in Montanide ISA-51 adjuvant when recombinant human IL-12 was administered at the same sites (172). In a trial of $CD34^+$ progenitor-cell derived dendritic cells pulsed with 4 different melanoma peptides presented by HLA-A2.1, response to more than two of these peptides correlated with lack of disease progression (173). Thus, melanocyte differentiation antigen peptides remain one of the most promising types of tumor antigen for vaccine therapy.

Tumor-Testis Antigens (MAGE, etc.)

Some of the first T-cell tumor antigens mapped were found by cloning genes using T lymphocytes as a probe (174–177). The first one, MAGE, found in melanoma, turned out to be the prototype of a class of tumor antigens called tumor-testis antigens because they were found primarily in tumors, but also in one normal tissue, testis. These proteins contain segments presented by HLA-A1 or A2 and peptides from these antigens binding HLA molecules have been used in cancer vaccine clinical trials for melanoma, either in adjuvant or pulsed on dendritic cells, resulting in some

clinical tumor regressions and/or lack of progression (173, 178–183). Peptides pulsed on to dendritic cells have also induced $CD4^+$ Th1 cell responses to MAGE peptides in melanoma patients (184).

Viral Tumor Antigens: HPV 16-E6 and E7

The most foreign tumor antigens are ones that are actually encoded by a viral genome rather than the human genome. Although several virally-induced tumors are known, such as B cell lymphomas related to Epstein-Barr virus (EBV) and adult T-cell leukemia/lymphoma associated with HTLV-I, the best characterized antigenically is cervical carcinoma associated with human papillomavirus (HPV). Over 95% of human cervical cancer is now believed to be caused by human papillomavirus, primarily types 16 and 18 (185). The premalignant dysplastic cells and carcinoma in situ, as well as invasive cancer, all express two oncogene products encoded by the virus, E6 and E7. E6 contributes to the malignancy by binding to and facilitating the degradation of the tumor suppressor protein p53 (186), while E7 contributes by binding and inactivating another tumor suppressor protein, Rb (187). Together they have been shown to be sufficient to transform cells (188), and so they must be retained to maintain the malignant phenotype. Much work has been done to target these with vaccines, but they have not proven to be the most immunogenic proteins. Nevertheless, a dominant epitope of E7 presented by a murine class I MHC molecule has been shown to be an effective vaccine against murine tumors expressing E7 (189). Epitopes binding to human HLA-A2 and other HLA molecules have been mapped, and human CTL raised (190). These have been the subject of a number of clinical trials of peptide vaccines. Initial clinical trials with HPV-16 E7 peptide vaccines were not very successful at inducing CTL or clinical responses (191–193), but a more recent one using two peptides from E7 (residues 12–20 and 86-93) showed increased CTL activity to E7 in 62% of individuals tested and some viral clearance from cervical scrapings in two thirds (194). It is not clear why such a foreign tumor antigen as a viral antigen should be so weakly immunogenic. Nevertheless, if these problems can be overcome, E6 and E7 remain strong choices for cancer vaccines because they are so completely foreign that at least tolerance does not need to be broken, and no autoreactivity should be induced.

CONCLUSIONS

Much recent progress has been made in the identification of new tumor antigens and their epitopes that may serve as potential cancer vaccines. Many of these have been studied as synthetic peptide vaccines corresponding to epitopes identified as presented by particular common human class I HLA molecules, especially HLA-A2.1, the most common human class I molecule. Although a number of strategies for immunization against cancer have been investigated, no strategy targeting a specific antigen has yet proven consistently more clinically effective than synthetic peptides. Peptides have the advantage that they can be easily modified by epitope enhancement to improve binding to the MHC molecule or the T cell receptor, and they can be combined with cytokines, chemokines, and costimulatory molecules to increase

vaccine potency and steer the responses toward a desired phenotype, such as CTL or Th1 cells. Peptides can also be coated onto dendritic cells, the ultimate professional antigen presenting cell, to bypass any defect in antigen-presenting cell function or maturation related to the presence of the cancer. Thus, as both research tools and as potential clinical vaccines, synthetic peptides remain at the forefront of research in the vaccine immunotherapy of cancer.

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6. DNA VACCINATION IN IMMUNOTHERAPY OF CANCER

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INTRODUCTION

Nucleic acid immunization has garnered much attention as a promising approach for cancer therapeutic development. This innovative vaccine strategy uses non live, non replicating, non spreading DNA formulations which utilize the host's cellular machinery for expression of proteins (antigens). Such novel delivered and expressed antigens become recognized by the host immune response and induce specific T and B cell responses against the gene encoded proteins. The foundational basis for DNA vaccines originated from the observation that delivery of gene sequences in vivo could lead to their expression [reviewed in (1)]. In the 1950s and 1960s experiments aimed at understanding the fundamental nature of the basis for cancer delivered as either nucleic acid or proteins to animals and followed tumor development. Tumor development segregated with nucleic acids and tumor bearing animals could seroconvert to tumor antigens, establishing the ability of nucleic acid transfer to drive protein expression and activate the immune response. In the 1980s the understanding that the immune response was a nemesis for gene therapy antigen delivery started to impact vector studies. Wolff et al. reported that activity of reporter genes could be detected for up to two months without a delivery system (2). Many investigators were focusing on if such proteins implemented for such gene therapy experiments could be employed to express antigens to stimulate the immune system. A study

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published by Tang et al. utilizing DNA coated gold microprojectiles to transfect cells *in vivo* reported the generation of an antibody response against the DNA encoded proteins (3). The study utilized a delivery system called a gene gun as this group had doubts that the efficiency of direct injection was likely substantial enough to deliver enough antigen to produce a significant immune response. However, papers appeared from Ulmer and Wang almost coincidently (4, 5) describing different formulations for IM delivery inducing responses against true human pathogens, influenza and HIV, followed rapidly by a paper from Fryan on influenza. These papers demonstrated that not just antibodies could be induced by this technology, but that cellular response as well as cytokine profiles could also be altered. In addition, protection from animal challenge could also be achieved.

Among the most intriguing and yet promising aspect of DNA vaccines is the feasibility of manipulation towards benevolent immune responses, juxtaposed with the remarkable ability to activate both arms of the immune system (4, 5). These unique attributes are a result of direct transfection of encoding plasmids into cells *in vivo*, whereby direct presentation on MHC Class I can materialize. As will be discussed later, this very principle has been exploited to specifically amplify the cellular arm of the immune response. The other beneficial aspect is its ability to engineer responses robust enough to often break tolerance against nonimmunogenic tumor antigens. This is particularly important when dealing with antigens exhibiting minimal immunogenic properties. In view of these findings, we review the potential utility of applying DNA vaccines as both a therapeutic and prophylactic approach against cancer and other forms of neoplastic growth. The requirement of potent T-cell activation rather than antibody activation as an essential criterion for tumor rejection validates such a use for efficacious treatment for tumor immunotherapy (6).

ESSENTIAL ROLE OF DENDRITIC CELLS IN TUMOR IMMUNOGENICITY: OPTIMIZING TO TARGET DC'S

In the body's immune system, cells need to process and present antigenic peptides to lymphocytes in order to stimulate antigen specific immune response. Thus, antigen must be processed and presented to T lymphocytes by antigen presenting cells (APCs) (7). Antigen presentation and recognition is a complex biological process that involves many interactions between antigen presenting cells and T cells. There are four primary components that are critical in the professional APCs' ability to present the antigen to T cells and activate them for appropriate immune responses. These components are MHC-antigen complexes, costimulatory molecules (primarily CD80 and CD86), intracellular adhesion molecules, and soluble cytokines. Naive T cells circulate through the body across lymph nodes and secondary lymphoid organs such as the spleen. Their migration is mediated among other factors by intercellular adhesion molecules and cytokines. As the T cells travel, they bind to and dissociate from various antigen presenting cells (APCs). Their movement is guided by chemokines but binding to cells is mediated through adhesion molecules. When a naive T cell binds to an APC expressing relevant MHC:peptide complex, the T cell

up regulates costimulatory molecules such as CD40L which further activates the APC increasing expression of the T cell costimulatory molecules CD86/CD80. These costimulatory signals bind to CD28 on the T cells inducing increased levels of high affinity IL-2 receptor. Only when this T cell receives a strong enough costimulatory signal through CD80/CD86-CD28 interaction does the T cell make soluble IL-2, which then binds to the receptors and drives the now-armed effector T cell to activate and proliferate.

Much speculation on the immunogenicity of tumor antigens has concentrated on the efficiency of antigen presentation from APCs to T-cells. Specifically, the roles of Dendritic cells (DCs) and the essential antigen specific clonal expansion have been thoroughly investigated. When compared with other "professional" antigen presenting cells (APCs), dendritic cells are preferentially advantageous due to their exclusive ability to activate naïve T-cells within the secondary lymphoid organs. Structurally, dendritic cells express elevated quantities of co-stimulatory molecules including CD80 and CD86, and large amounts of peptide-MHC complexes allowing potent T lymphocyte activation and differentiation (Reviewed in 8).

The role of DC's in regulating tumor antigenicity have been extensively documented. In fact, tumors have developed immune evasive attributes to prevent DC maturation and prevent the eventual antigen specific T-cell generation. For instance, some tumors secrete substantial amounts of Vascular Endothelial Growth Factors (VEGF) which promote not only angiogenesis but also retards the maturation of antigen captured DC's (9) and this effect appears to operate through a direct suppression of NF-κB (10). Furthermore, continuous infusion of VEGF into mice leads to a dramatic inhibition of dendritic cell development, associated with an increase in the production of B cells and immature Gr-1(+) myeloid cells (11). In addition to VEGF, some tumor cells secrete IL-10, which directly prevents the maturation of dendritic cells (12, 13). This effect dramatically diminishes the antigen specific activation of both CD4 and CD8 T cells through the attenuation of DC's, as *ex vivo* generation of bone marrow-derived DC eradicates this effect (14). These results suggest a paramount theme; tumors suppress the maturation of DC's to impede the functional stimulation of T cells. Unfortunately for the development of anti tumor immunity, this effect is essential for proficient activation of tumor specific T cells.

Essential Role of DCs in DNA-based Immunizations

In addition to anti-tumor immunity, the development of DNA vaccine-based immune responses also requires the activation and and perhaps in part direct transfection of dendritic cells. Condon et al. demonstrated that gene gun delivery of reporter gene plasmids to the skin resulted in expression of the reporter genes in cells exhibiting dendritic cell-like morphology localized within the local draining lymph nodes (15). Others have proposed that secretion of expressed antigens from somatic cells or their destruction (i.e. muscle cells and keratinocytes) may provide the path for dendritic cells to take up antigen and present through the exogenous MHC class II pathway (16). In fact purification of APCs from immunized mice will stimulate naïve T cells in an antigen specific manner, suggesting that uptake

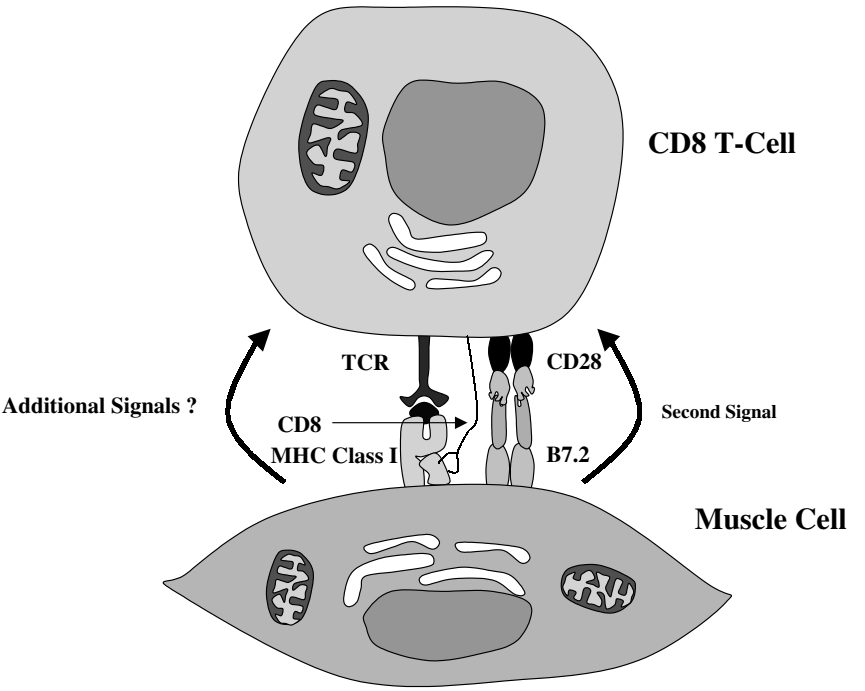

Figure 1. Muscle Cells as Antigen Presenting Cells (APCs). A schematic diagram of the *in vivo* expression of B7 surface molecules on muscle cells. The direct transfection of B7 activates the secondary signal by signaling through CD28 to activate MHC mediated presentation and activation of T-cells. This coimunization model induced potent antigen specific CTL activation with muscle cells functioning as APCs.

of antigen by APCs is important for DNA immunization based immune activation. Additionally, IM injection of plasmids expressing EGFP results in co-localization of EGFP expressing cells and APC markers CD80 and CD86 in the draining LN's (17). These results suggest that 1) APC activation and migration to the regional lymph node is essential for immune activation following plasmid immunization, and 2) APCs can take up antigens through direct transfection and/or exogenous phagocytosis. Therefore, vaccine strategies targeting the enhanced uptake of antigens by DCs or increased chemotaxic migration of DCs to the site of antigen expression may provide increased vaccine potency and increased therapeutic potential.

Enhancing DC-directed Antigen Uptake

In addition to powerful signaling, dendritic cells often function as scavenger cells by engulfing and processing apoptotic bodies. Specifically, immature DCs uptake dead cells or apoptotic bodies via surface receptors $\alpha V\beta 5$ integrin and CD36 (18–20). This uptake promotes a process termed cross-priming whereby exogenous antigens are processed and presented through the endogenous MHC class I pathway (Figure 1).

Specifically, the engulfment of these bodies with either tumor or viral antigens by dendritic cells provokes the activation of MHC class I restricted CD8+ CTLs (20, 21). Both dendritic cells and macrophages have been shown to present apoptotic engulfed antigens but the latter is much less potent at activating naïve T-cells, which is a vital step in the generation of adaptive immunity (21, 22). Furthermore, several studies also demonstrate that there is a quantitative dependency on apoptotic bodies by dendritic cells in inducing the secretion of pro-inflammatory cytokines TNF-α and IL-1β both *in vitro* and *in vivo* (20, 23). Accordingly, an optimum strategy to develop potent vaccines would include the activation of dendritic cells and the packaging of immunogens in apoptotic bodies facilitating cross priming and broader cellular immunity. In a recent study, a novel strategy was used whereby immunogen constructs were coimmunized with an apoptosis inducing receptor Fas. This coimmmunization resulted in significant augmentation of antigen specific immune responses as measured by enhanced CTLs and Th1 cytokines including INF-γ and IL-12 (24) (Figure 2). There is other evidence to suggest that apoptosis signals that aliquot ample time for immunogen expression will provide adjuvant properties. A more recent study implemented mutant caspases to decrease apoptotic efficiency to increase the time of immunogen expression prior to the apoptotic event, while still delivering apoptosis-mediated antigens to dendritic cells (25). This specific adjuvant raised both CD4 and CD8 responses, indicating that antigen uptake by DCs presented peptides into both the endogenous and exogenous pathways.

While apoptosis mediated delivery has provided an insight to the possibility of cross priming, others have utilized directly secreted antigens to target dendritic cells. This raises the prospect that exogenous antigens may function in generating MHC Class I-restricted responses by directly entering the cytosol in a DNA vaccine model through the endogenous cross-priming process (26–28). It has been previously suggested that antigens can be expressed by transplanted cells, which maintain the ability to induce CTLs through the direct transfer of antigens to host's antigen presenting cells (29, 30). Various tumor studies directed at ascertaining the precise functions of somatic MHC class I molecules have determined that bone marrow-derived antigen-presenting cells play the dominant role of presenting these somatic-based antigens (31). Additionally, other exogenous antigens such as bacteria are internalized and processed for presentation by MHC class I molecules (32, 33). These reports unequivocally imply extracellular uptake of antigens is a prominent pathway implemented by the immune system to generate CTLs. In this regard, dendritic cells express surface cell receptors for Fc regions of antibodies called FcγR's, which enhances the uptake of antigen-antibody complexes and leads to presentation on MHC class II molecules (34). These receptors also assist in the activation and maturation of dendritic cells and regulate efficient presentation of exogenous antigens (35). Hence introduction of exogenous antigens through FcγR-mediated internalization into the cytoplasm may help effectively prime antigens to activate MHC Class I-restricted CTLs (36). Exogenous antigen endocytosis could then employ the endogenous TAP dependent antigen processing pathway and in theory present peptides on MHC Class I molecules (37). This strategy was directly tested in a DNA vaccine model by fusing

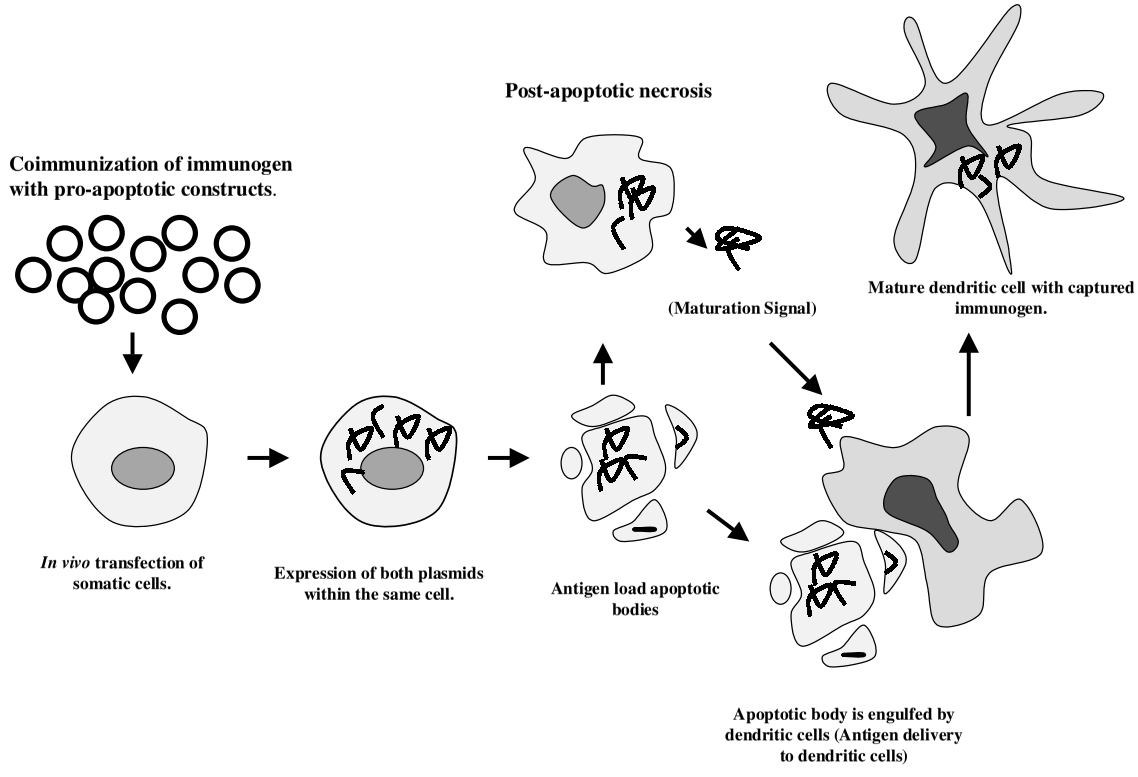

Figure 2. Apoptosis-mediated Delivery of DNA Vaccines. The following model describes a possible mechanism by which antigen loaded apoptotic bodies deliver and induce maturation of dendritic cells. As shown, potent and rapid induction of cell death generates apoptotic bodies loaded with antigens to specifically deliver to antigen presenting cells. Late apoptosis or necrotic bodies may provide essential signals need to induce the maturation.

the hepatitis B virus (HBV) e antigen and the Fc portion of an IgG1 antibody (38). The antigen-Fc immunogen was secreted by somatic cells and taken up effectively by dendritic cells resulting in stimulation of both CD4+ and CD8+ T cells *in vivo*. The adjuvant effectively augmented the secretion of proinflammatory cytokines including INF-γ and IL-2 as well as enhancing CTL and lymphocyte proliferative responses (38).

Another promising DC targeting immune modulator is the family of chaperones called the Heat Shock Proteins (HSPs). The initial experiments that elucidated the immunocapability of HSPs were from purification experiments from antigenically distinct sarcoma cells (39, 40). In fact, it was later ascertained that this 96 kDa glycoprotein was not itself immunogenic, but became immunogenic in circumstances when it was conjugated with peptides. Overall comparison of immunogenicity with other HSP family members including Hsp70 and Hsp90 suggest that immunogenicity is associated with two vital factors, the associated ATPase activity and the association of HSP with peptides (41). The ATPase activity likely determines the ability of the chaperone complex to transfer peptide to acceptor molecules and this association with peptides is the rationale for the autologous nature of these complexes (42). These chaperones also possess intrinsic inflammatory qualities, including among many properties the maturation and activation of DCs (43), direct cross-priming abilities (44–46), and release of NO from APCs (47). The endocytosis and eventual presentation of antigens is thought to be a consequence of the universal targeting of all HSPs by its receptor CD91, a natural ligand for alpha 2-macroglobulin (48, 49). The post uptake processing implements the endogenous pathway, which partially explains the cross-priming effect of these proteins. The significance of HSP in tumor-specific and non-vaccine related circumstances has been examined. Specifically, immunotherapy of cancers with HSPs purified from tumors or reconstituted *in vitro* from tumor cell cultures when administered as vaccines also regressed the growth of tumors (50–52).

The copious immunogenic attributes of HSP suggest these complexes may function as useful adjuvants for DNA vaccines. Specifically, the HSP70 of Mycobacterium tuberculosis was fused to the Human HPV-16 E7 antigen generating a chimeric DNA vaccine (53). The E7-HSP70 DNA vaccine induced significantly enhanced levels of Th1 mediated responses including a ratio of 435:14 (E7-HSP70 to E7) of E7 specific INF-γ spot-forming CD8+ T cells via ELISPOT assays. Additionally, data from this group suggested that the eradication of pre-existing tumors and the resulting immune response was via CD4+ independent mechanisms, implying that cross-priming was crucial for this effect. Where the T cell help for this cross priming event was supplied is unclear.

Another member of the HSP family that has shown to augment the potency of DNA vaccines is calreticulin (54). The idea of calreticulin as an immune modulator was based on previous findings that calreticulin in conjugation with tumor peptides stimulates potent peptide specific CD8+ T cell responses (55). Like the other HSP members, calreticulin also implements the CD91-dependent pathway for APC uptake, making cross-priming another presumable immunogenic outcome from use

of this molecule (49). Additionally, calreticulin and its fragment vasostatin also operate as inhibitors of angiogensis (56, 57). Accordingly, when calreticulin was fused to HPV-16 E7 antigen as a DNA vaccine, a potent, anti-tumor effect was provoked; the resulting response was attributed to both the enhanced immunogenicity against E7 and the generation of anti-angiogenesis.

Amplifying DCs at the Site of Expression

In addition to the enhanced uptake of antigens, an amplification of DC quantity is likely advantageous for enhanced antigen delivery to and presentation within the regional lymph nodes. Compensating for DC paucity can be ameliorated through DNA vaccine-mediated engineering of immune responses. Among various strategies, Flt3 (Fms-like tyrosine kinase 3) ligand (FL) has been utilized to stimulate the activation and amplification of dendritic cells (58). Functionally, FL treatments in mice significantly increase dendritic cell population in many areas including the bone marrow, gastro-intestinal lymphoid tissue (GALT), liver, lymph nodes, lung, peripheral blood, peritoneal cavity, spleen, and thymus (59). FL has revealed tumor suppressive attributes in mice partly by the generation of large number of dendritic cells (60). Accordingly, recent work indicates enhanced frequency of CD8+ T cells when FL was fused to the human papillomavirus-16 E7 antigen (58). The response was CD4+ independent and maximum effect was observed when the antigen and FL were fused together. Most importantly, 100% of mice vaccinated with FL-E7 were protected when challenged with TC-1, a tumor cell line derived from C57BL/6 mice cotransformed with HPV-16 E6 and E7 and c-Ha-ras oncogenes (58).

Other methods have concentrated on the direct chemotatic migration of DCs to the site of injection. One report specifically coimmunized M-CSF and resulted in enhanced levels of CD8+ T cell dependent responses. This effect was in direct correlation with elevated DC migration to the site of injection with an increase in the Beta-Chemokine MIP-1β (61). Others have reported that coimmunization with GM-CSF augments the migration of immature DCs to the injection site. The maximal migration occurred between days 3–5 post injection and was not positive for CD80 or CD40 (62).

CONVERTING MUSCLE CELLS INTO APCS

The generation of immune responses via DNA vaccines requires the delivery and/or presentation of immunogens to professional antigen presenting cells (APCs). It has been proposed that a significant target for *in vivo* transfection of plasmids are also the muscle cells themselves and their transfer and/or presentation of immunogens may be a vital tool in the development of immune responses (Reviewed in 63). One of the consequences suggested as an issue for muscle delivery of plasmid vectors is that less effective antigen presentation will occur as the muscle cells fail to express the costimulatory molecules (i.e. CD80, CD86, B7RP1) necessary to send a second signal. One approach to this limitation may be to codeliver antigen expressing plasmids with costimulatory molecule expressing plasmids (64–66). In theory the coexpression of CD86 on muscles that express a high ratio of MHC class I and can

not a highly invasive approach which exploits the host's own immune system to generate intrinsic anti-tumor defenses. In addition, it can be easily combined with other approaches thus is attractive to patients and physicians. Additionally, side effects associated with conventional chemotherapy and radiotherapy are nonexistent with immunotherapies. To this end, several specific TAAs have become targets for DNA vaccine development (reviewed in 79, 80).

DNA Vaccines Against Melanoma

An imposing barrier for any immune therapy approach is the potential for immune tolerance to tumor antigens. It has been suggested that the unique presentation of tumor specific antigens in the context of DNA vaccines may facilitate breaching of this potential immunological barrier. Among the numerous TAAs that have been identified for melanoma, several have been studied in the context of DNA vaccines. Specifically, an early experiment conducted by Weber et al. targeted the gp75/ tyrosinase-related protein-1 as an antigen in a DNA vaccine model. This specific TAA is well tolerated as a vaccine antigen although it is difficult to develop strong cellular immunity against. However, mice primed with the human gp75 and boosted with murine gp75 appear to be able to break tolerance and developed immunity and tumor protection in a mouse challenge model. This immune response was dependent on both the induction of CD4+ T cells and NK cells (81). Another innovative strategy used minigenes as specific targeted immunogenes, by fusing distinct dominant class I epitopes from gp100 and TRP-2 into the vaccine candidate. In addition, the ubiquitin gene was fused on the 5′ end of the vaccine and was delivered by oral gavage using an attenuated strain of *Salmonella typhimurium* as carrier. The enhanced effect was concomitant with increased INF-γ production and specific lysis of tumor cells by activated CD8 T cells. The effect is thought to be a consequence of increased processing and targeting of the antigen into the MHC Class I presentation pathway (82). Several studies have tested plasmid vaccines against different models of melanoma. Some of these studies induced destruction of pigment cells as a possible correlate of destruction of melanoma *in vivo*. However, the early results appear to just be scratching the surface. It is likely that more potent DNA vaccines incorporating molecular adjuvants or in prime boost protocols will be more effective than these early approaches.

DNA Vaccines Against Colon Cancer

Human CEA is a 180-kDa glycoprotein expressed in elevated levels in 90% of gastrointestinal malignancies, including colon, rectal, stomach, and pancreatic tumors, 70% of lung cancers, and 50% of breast cancers (83, 84). CEA is also found in human fetal digestive organ tissue, hence the name carcinoembryonic antigen (85). It has been discovered that CEA is expressed in normal adult colon epithelium as well, albeit at far lower levels (86, 87). Sequencing of CEA shows that it is associated with the human immunoglobulin gene superfamily and that it may be involved in the metastasizing of tumor cells (85).

The immune response to nucleic acid vaccination using a CEA DNA construct was characterized in a murine model. The CEA insert was cloned into a vector containing the cytomegalovirus (CMV) early promoter/enhancer and injected intramuscularly. CEA specific humoral and cellular responses were detected in the immunized mice. These responses were comparable to the immune response generated by rV-CEA (86). The CEA DNA vaccine was also characterized in a canine model, where sera obtained from dogs injected intramuscularly with the construct demonstrated an increase in antibody levels (88). Cellular immune responses quantified using the lymphoblast transformation (LBT) assay also revealed proliferation of CEA-specific lymphocytes. Therefore a CEA nucleic acid vaccine was able to induce both arms of the immune responses (88). CEA DNA vaccines are currently being investigated in humans, but as yet there is little data presented for guidance.

DNA Vaccines Against Prostate Cancer

Prostate cancer is the most common form of cancer and the second most common cause of cancer related death in American men (89). The appearance of prostate cancer is much more common in men over the age of fifty (90). Three of the most widely used treatments are surgical excision of the prostate and seminal vesicles, external bean irradiation, and androgen deprivation. However, conventional therapies lose their efficacy once the tumor has metastasized, which is the case in more than half of initial diagnoses (91, 92).

PSA is a serine protease and a human glandular kallikrein gene product of 240 amino acids, which is secreted by both normal and transformed epithelial cells of the prostate gland (93, 94). Because cancer cells secrete much higher levels of the antigen, PSA level is a particularly reliable and effective diagnostic indicator of the presence of prostate cancer (95). PSA is also found in normal prostate epithelial tissue and its expression is highly specific (96).

The immune responses induced by a DNA vaccine encoding for human PSA has been investigated in a murine model (96). The vaccine construct was constructed by cloning a gene for PSA into expression vectors under control of a CMV promoter. Following the injection of the PSA DNA construct (pCPSA), various assays were performed to measure both the humoral and cellular immune responses of the mice. PSA-specific immune responses induced *in vivo* by immunization were characterized by enzyme-linked immunosorbent assay (ELISA), T helper proliferation cytotoxic T lymphocyte (CTL), and flow cytometry assays. Strong and persistent antibody responses were observed against PSA for at least 180 days following immunization. In addition, a significant T helper cell proliferation was observed against PSA protein. Immunization with pCPSA also induced MHC Class I CD8+ T cell-restricted cytotoxic T lymphocyte response against tumor cell targets expressing PSA. The induction of PSA-specific humoral and cellular immune responses following injection with pCPSA was also observed in rhesus macaques (97). These responses were achieved in either female or male animals. As the PSA construct was human in design, and human and rhesus construct are 98% identical these results support that the DNA vaccines could break tolerance in this model. This is a rare demonstration

of this ability in a non human primate. In addition to cellular immunity, strong antibody responses were also observed. Such antibody responses may also be valuable in a clinical setting. Recently, PSMA based DNA vaccines have entered the clinic for initial evaluation. The results of these studies are pending but will likely provide important information about targeting prostate disease using DNA technology.

DNA Vaccines Against Cervical Cancer

Human Papillomavirus (HPV) 16 associated proteins including E6 and E7 are some of the most common proteins in cervical cancers and are ubiquitious expressed within these cells (98,99). However, DNA based vaccine targeting these proteins seem to elicit minimal immune responses and may necessitate potent adjuvants to provide efficacious tumor protection. A DNA vaccine based HPV E6 vaccine in mice was able to provide anti-tumor activity when adjuvanted with IL-12 into the skin. This specific study implemented exclusively the amino terminal which of E6, which lacks the transforming property (100). One of the early E7 vaccines employed mutational variants within the zinc-binding motifs that led to rapid degradation. Ironically, this specific vaccine exhibited stronger E7-specific CTLs (101). In a similar fashion, several other studies have targeted the processing of HPV vaccines to specific compartments to enhance potency. An early study by T.C. Wu and colleagues fused the E7 antigen with the lysosomal-associated membrane protein (LAMP-1), which directs processing of E7 antigens into the MHC class II pathway for presentation (102). When compared to the E7 antigen alone, the LAMP-1 mediated targeting enhanced antigen specific CD4+ helper T cells, greater antigen specific E7 CTL activity, and antibody responses (103). On the contrary, similar manipulation has been implemented to direct proteins into the MHC class I presentation pathway. Specifically, the HSV-1 structural protein VP22, which exhibits an intercellular trafficking property (104), was directly fused to the E7 antigen to perhaps increase presentation productivity (105). Incredibly, this specific adjuvant stimulated a 50-fold increase in the overall quantity of E7-specific CD8+ T cells (105). Similarly, fusion of E7 to gamma-tubulin, a target for the centrosomal compartment which possesses proteasomes, led to a dramatic increase in the quantity of E7-specific CD8+ T cells. This effect was dependent on the proteasome, as mice deficient in TAP-1 failed to develop such an enhancement (106).

A more recent report within the clinics also suggests that immunization through DNA can also therapeutically attenuate the growth of neoplastic cells in humans. These studies specifically encapsulated DNA plasmids encoding HLA-A2-restricted epitopes of the HPV E7 antigen within biodegradable polymer microparticles. Early work suggests no adverse side effects, while enhancing immune responses when implementing this specific therapy (107). These results are very exciting as the doses of DNA used in these studies are very low. In addition to the demonstration of immune response induction these investigators noted a regression in precancerous phenotype in this cervical progression model. While spontaneous regressions are noted in this model, the rate of regression gives hope that this regression was a result of the vaccine. However, strong conformation of these results will await a

clinical study that includes a placebo control to firm up these important observations. However, the results remain highly exciting and may mark a turning point in the application of this technology to cancer therapy of Papillomavirus infection.

DNA Vaccines Against Breast Cancer

The *erbB-2/neu* proto-oncogene is a member of the EGFR family that dimerizes to activate *trans* phosphorylation to activate signal transduction and is also overexpressed in 15–40% of all human breast cancers (108–110). Accordingly, Chen et al. generated DNA constructs expressing the full length *neu*, the extracellular domain, and the extracellular-transmembrane domains. The latter two mutants were created to avoid potential transformation, and all three were immunized and challenged with Tg1-1 cell line, which was garnered from a FVB/N *neu*–transgenic mouse. The authors report protection when challenged with this specific cell line and this effect was augmented with IL-2 as an adjuvant, and was antibody independent (111). Others have implemented innovative strategies by mutating domains responsible for kinase activity and adding leader sequences to redirect towards antigen processing and have generated similar results (112). Importantly, the prophylactic attributes of this vaccine was demonstrated when it was shown to prevent spontaneous formation of tumors in FVB/N *neu*-transgenic mice when administered in conjunction with IL-12 (113). One concern for clinical evaluation of this approach is that neu is expressed in many other tissues besides breast cancer, including lining of the brain and in heart tissue, at low levels. The consequences of this expression for DNA immune therapy is at this time unknown but must be considered in clinical trial design.

Conclusion

The recent progress of immunotherapy for treatments against cancer can largely be attributed to a greater overall understanding of the immune system. Identification of processing pathways and targeting receptors has allowed the development of novel adjuvants in augmenting the overall potency of these vaccines. In addition, the growing lists of TAAs provide copious targets to develop immunity against tumor formation. Furthermore, therapies can also target factors that are essential for the survival and propagation of tumors. For instance, a recent study targeted the receptor of the angiogenesis factor VEGF. The authors immunized mice against vascular-endothelial growth factor receptor 2 (FLK-1) through an oral vaccine and targeted proliferating endothelial cells in the tumor vasculature. Protection was observed from numerous cell types including melanoma, colon carcinoma, and lung carcinoma (114). Accordingly, this combination of basic immunology and TAA isolation is providing an auspicious path for immunotherapies against cancer. All together, these promising results also emphasize the potential of DNA Vaccines as therapies against cancer. The particular advantages of DNA in manufacturing, lack of replication based pathogenesis, specificity for the tumor target, lack of vector immunity allowing for routine reimmunization are all properties of ideal immunization strategies for cancer

immune therapies. The challenges as we go forward will be to take these collection of positive attributes and add additional immune potency to the mix. At that time it is likey that DNA vaccines will take their place at the center of programs for tumor immunotherapy.

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7. ANTIBODY INDUCING POLYVALENT CANCER VACCINES

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1. THE RATIONALE FOR ANTIBODY-INDUCING CANCER VACCINES AGAINST MULTIPLE CELL SURFACE ANTIGENS

Antibodies Eliminate Tumor Cells Invivo

Preclinical models demonstrate that passively administered or actively induced antibodies against cancer cell surface antigens can prevent tumor recurrence in rodents [reviewed in 1, 2]. The syngeneic murine tumor models involving EL4 lymphoma are particularly informative in terms of trial design [2]. EL4 lymphoma naturally expresses GD2 ganglioside which is recognized by monoclonal antibody (mAb) 3F8. Vaccines containing GD2 covalently conjugated to KLH and mixed with immunological adjuvant QS21 are optimal for vaccination against GD2. Relatively higher levels of mAb 3F8 administered two or four days after intravenous tumor challenge or moderate titers induced by vaccine that were present by day four after tumor challenge were able to eradicate disease in most mice. If mAb administration was deferred until day seven or ten after IV challenge, little or no benefit could be demonstrated. If the number of cells in the EL4 challenge was decreased, giving a longer window of opportunity, the vaccinations could be initiated after tumor challenge and good protection seen [2]. These results are consistent with the need to initiate immunization

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with vaccines inducing antibodies in the adjuvant setting, when the targets are circulating tumor cells and micrometastases.

Comparable benefit is also seen when we use a subcutaneous foot-pad tumor challenge model which more closely mirrors the clinical setting. Vaccination or mAb administration after amputation of the foot-pad tumor results in cure of 60–80% of mice while 90–100% of control mice developed progressive disease. There are comparable syngeneic models demonstrating the anti-tumor efficacy of mAbs or vaccines against other glycolipids (GD3, GM3), mucin antigens (Tn, TF and MUC1) and a protein antigen (gp75). These experiments share one thing in common, benefit is seen primarily in minimal disease settings, comparable to the adjuvant setting in the clinic. With regard to clinical settings, naturally acquired and vaccine induced antibodies against cancer cell surface antigens have correlated with improved prognosis in several different clinical trials [reviewed in 3–6]. Also, an increasing number of clinical trials with passively administered monoclonal antibodies (mAb) against cell surface antigens have demonstrated clinical efficacy.

Mechanisms of Tumor Elimination

Cancer antigens expressed at the cell surface are generally glycolipids or glycoproteins. Immunization against the carbohydrate components generally results exclusively in an antibody response [see 7, 8 for dissenting views], primarily an IgM antibody response. These IgM antibodies are known to induce complement dependent cytotoxicity (CDC), inflammation, and phagocytosis of tumor cells by the reticulo-endothelial system (opsonization)[reviewed in 10]. Protein antigens generally induce primarily IgG antibody responses which can also induce complement activation (with regard to IgG depending on the subclass, IgG1 and IgG3 being optimal in humans), and these same complement mediated effector mechanisms. IgG antibodies of these subclasses are also known to induce antibody dependent cell mediated cytotoxicity (ADCC). Antibodies are ideally suited for eradication of free tumor cells and micrometastases. This is the role of antibodies against most infectious diseases and they have accomplished this against cancer cells as described above in a variety of preclinical models. In adjuvant immunization trials, the primary targets are individual tumor cells or early micrometastases which may persist for long periods after apparent resection of all residual tumor [11–13]. After surgery and completion of chemotherapy is the ideal time for immune intervention, and in particular for administration of cancer vaccines aimed at instructing the immune system to identify and kill these few remaining cancer cells. If antibodies of sufficient titer can be induced against tumor antigens to eliminate tumor cells from the blood and lymphatic systems, and to eradicate micrometastases (making establishment of new metastases no longer possible) this would dramatically change our approach to treating the cancer patient. Aggressive local therapies, including surgery, radiation therapy and intralesional treatments might result in long term control of even metastatic cancers.

Polyvalent Vaccines

The basis for emphasis on polyvalent vaccines is tumor cell heterogeneity, heterogeneity of the human immune response and the correlation between overall antibody titer against tumor cells and effector mechanisms such as opsonization, CDC or ADCC. For example, using a series of 14 melanoma and sarcoma cell lines and mAbs against 3 gangliosides, we have shown that significant cell surface reactivity analyzed by flow cytometry and CDC was detected against 2–8 of the cell lines using any single mAb. This increased to all 14 of the cell lines when the 3 mAbs were pooled. The median percent CDC increased 4 fold with the pool of mAbs compared to the best single mAb [14]. Comparable findings have been generated more recently using ten SCLC cell lines and mAbs against 4 cell surface antigens (9).

2. SELECTION OF CELL SURFACE ANTIGENS AS TARGETS FOR ANTIBODY MEDIATED ATTACK AGAINST CANCER

Cell Surface Cancer Antigens: The MSKCC Experience

We have screened a variety of malignancies and normal tissues with a series of 40 mAbs against 25 antigens which were potential target antigens for immunotherapy [18–21]. Results for the twelve defined antigens expressed strongly in 50% or more of biopsy specimens of breast, ovary and prostate cancer, melanoma, sarcoma and SCLC are shown as examples in Table 1. The 10 excluded antigens (including CEA and HER2/neu) were expressed in 0–2 of the 5–10 specimens.

Our results are consistent with those from other centers with one exception, we did not find increased levels of GD2 or GD3 in small cell lung cancer (SCLC). There is a striking similarity in expression of these 15 antigens among tumors of similar embryologic background (i.e. epithelial versus neuroectodermal). Epithelial cancers (breast, ovary, prostate colon, etc) but not cancers of neuroectodermal origin (melanomas, sarcomas, neuroblastomas) expressed MUC1, Tn, sTn, TF, globo H and Le^y while only the neuroectodermal cancers expressed GD2 and GD3. SCLC shared some characteristics of each and in addition expressed fucosyl GM1 and long chains of poly-α2,8-sialic acid which were not expressed in tumors of either background.

Gangliosides GM2, GD2, GD3 and Fucosyl GM1

Gangliosides are sialic acid containing glycolipids that are expressed at the cell surface with their lipid (ceramide) moiety incorporated into the cell surface lipid bilayer. Most gangliosides considered as potential targets for cancer therapy are expressed primarily in tissues and tumors of neuroectodermal origin. This is true for the melanoma, sarcoma and neuroblastoma antigens GM2, GD2 and GD3, and the small cell lung cancer antigen, fucosyl GM1. The structures of these antigens are shown in Figure 1. Surprisingly, however, GM2 has also recently been identified in a number of epithelial cancers [18, 22, 23] and at the luminal surfaces of a variety of normal epithelial tissues.

Table 1. Proportion of Tumor Specimens with 50% or more of Cells Positive by Immunohistology

	Cell Surface Target Antigens and mAbs														
	sTn	Tn	TF	GloboH	sLe[a]	Le^Y	GM2	GD2	GD3	FUC GM1	Polysialic acid	KSA	MUC1	PSMA	CA125 MUC16
Tumor	CC49	IE3	49H.8	MBr1	19.9	BR96	696	3F8	R24	F12	735	GA733	HMFG-2	Cyt351	OC125
Breast	*5/10*	*5/10*	*6/10*	*4/5*	*5/10*	*7/10*	*5/5*	*0/5*	*0/5*	0/5	0/6	5/7	5/7	0/7	
Ovary	*4/5*	*1/5*	*5/5*	*3/5*	*0/5*	*5/5*	*5/5*	*0/5*	*0/5*	*0/5*	0/5	5/15	5/9	0/15	53/62
Prostate	*6/11*	*10/11*	*10/11*	*2/11*	*1/5*	*4/11*	*11/11*	*0/5*	*0/5*	*0/5*	*0/5*	9/9	5/9	6/9	0/5
Melanoma	0/5	0/5	0/5	0/10	0/5	0/5	10/10	6/10	8/10	0/10	0/6	0/5	0/5	0/5	0/4
Sarcoma	0/5	0/5	0/5	0/5	0/5	0/5	8/9	5/9	4/9	0/0	1/5	0/5	0/5	0/5	
Small cell lung cancer	0/5	0/5	0/5	4/6	3/5	2/5	6/6	0/6	0/6	4/6	6/6	5/5	1/5	0/5	0/2

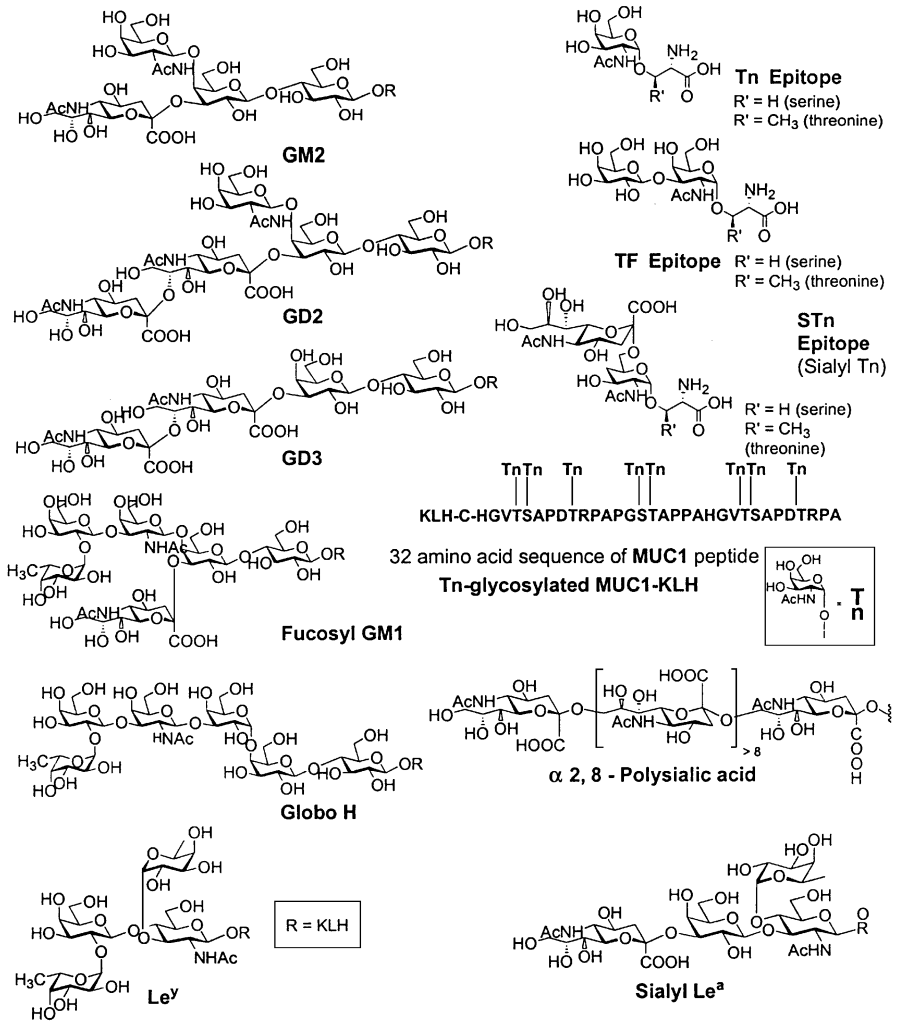

Figure 1. Antigens For Antibody Including Polyvalent Cancer Vaccines

Neutral Glycolipids Lewis^y and Globo H

Lewis^y (Le^y) and Globo H antigens are found at the cell surface of epithelial cancers primarily expressed as glycolipids attached to the lipid bilayer through their ceramide, but they are also O-linked via -OH groups of serine or threonine to mucins and N-linked via the NH_2 group of asparagine in other proteins [21, 24]. Whether

expressed as glycolipids or glycoproteins, the immune response against these antigens is predominantly against the carbohydrate moiety. The expression of Le^y and Globo H on various types of cancer cells has been well documented [24–27]. They are expressed in lesser amounts on a variety of normal tissues, again at the lumen border of ducts and in secretions as described for TF and sTn [15, 17–19]. Monoclonal antibodies against each have shown good localization to human cancers in vivo [28, 29]. The structures of these antigens in their glycolipid form are shown in Figure 1.

TF, Tn and sTn Antigens

Mucins are major cell surface antigens in breast cancers and a variety of other epithelial cancers. They are primarily large extracellular molecules made up of multiple copies of serine and threonine rich tandem repeats [30–33]. Though mucins (including carbohydrate and peptide epitopes) are also expressed on some normal tissues they have proved to be excellent targets for anti-cancer attack for two reasons: 1) Expression on normal tissues is largely restricted to the ductal border of secretory cells [31–33], a site largely inaccessible to the immune system. Cancer cells, on the other hand, have no patent ducts and so accumulate mucins. 2) Peptide backbones of cancer mucins are not fully glycosylated and glycosylation that does occur is not complete. Glycosylation of cancer mucins with mono- or di-saccharides such as Thomsen-Friedenreich antigen (TF), Tn and sialylated Tn (sTn) O-linked to serines or threonines is especially common [34, 35]. Expression of these mono- and disaccharides correlates with a more aggressive phenotype and a more ominous prognosis [36, 37]. TF (Galβ1-3GalNAcα-O-serine/threonine), Tn (GalNAcα1-O-serine/threonine) and sTn (NANAα2-GalNAcα1-O-serine/threonine) are expressed in 50–80% of various epithelial cancers [38–40]. The structures of TF, Tn and sTn linked to serine/threonin are shown in Figure 1. STn trimer (cluster) is the epitope recognized by monoclonal antibody B72.3, and TF and sTn are closely associated with the clustered epitope recognized by monoclonal antibody CC49 [41]. Clinical trials of radiolabeled CC49 administered IP in patients with breast cancer [42] and ovarian cancer [43] at this center and elsewhere have shown excellent targeting. TF has also been used successfully as a target for cancer imaging [44]. TF, Tn and sTn are expressed to a lesser extent on a variety of normal tissues, where they are expressed predominately as occasional monomers at luminal surfaces [19, 45]. Immunohistology performed with mAbs identifying these trimers (clusters) react strongly with a variety of epithelial cancers but only minimally, or not at all, with normal tissues, suggesting that focusing on the trimers of Tn, sTn and TF further increases the tumor specificity of the immune response. Immunization with TF and Tn has been shown to protect mice from subsequent challenge with syngeneic cancer cell lines expressing these antigens [46, 47]. Hence both active and passive immunotherapy trials have identified TF, Tn and sTn antigens as uniquely effective targets for cancer targeting and immunotherapy.

Polysialic Acid

The "embryonic" form of neural cell adhesion molecule (N-CAM) is expressed on the cell surface of embryonic tissues, occasional neuroendocrine cells and a variety

of neuroendocrine tumors including SCLC, neuroblastomas and carcinoids [48, 49]. Embryonic N-CAM undergoes a series of post-translational modifications, with the acquisition of $\propto$ 2,8-1inked sialic acid residues as long 20–100 residue polysialic acid chains (see Figure 1). Several monoclonal antibodies, including mAb 735 and NP-4, recognize these long polysialic acid chains [50] and have allowed characterization of this potential antigen in both normal and malignant tissue. Zhang et al. has demonstrated that 6 of 6 SCLC tumor specimens were reactive by immunohistochemistry using mAb 735, and 5 of 6 tested SCLC tumor specimens were positive using mAb NP-4 [18]. This confirms previous results of Komminoth et al. [49] and suggests that polysialic acid may serve as a useful target for immune attack against SCLC. Polysialic acid is also expressed in occasional cells in the gray matter of the brain, bronchial epithelia and pneumocytes, epithelia of the colon, stomach, and pancreas, and capillary endothelial cells and ganglion neurons in the colon. The reactivity of these antibodies in epithelia is restricted to the luminal surfaces of glandular tissues, where access to the immune system is restricted. Two to five percent of normal donors have high levels of antibody against polysialic acid as a consequence of exposure to bacteria such as *Neisseria meningitidis* group B (MenB) and *Escherichia coli* K1 that also express polysialic acid. This has not been associated with any signs of autoimmunity. Consequently, vaccines against polysialic acid are being tested to combat these diseases, however polysialic acid has proved to be poorly immunogenic.

Mucin MUC1

The peptide backbones of tumor mucins may also be targets for immune attack. Mucin 1 (MUC1) is a major mucin in breast cancers and is also expressed in a variety of other epithelial cancers. It contains a large extracellular component made up of multiple copies of a 20 amino acid tandem repeat, and a cytoplasmic tail [30–33]. Though mucins (including carbohydrate and peptide epitopes) are also expressed on some normal tissues they have proved to be excellent targets for anticancer attack for the same reasons noted for sTn, TF and Tn. Glycosylation of cancer mucins with mono- or di-saccharides such as Tn, sTn or TF O-linked to serines or threonines instead of larger, more complex carbohydrates is especially common permitting better access for antibodies to the mucin backbone. For this reason, mucin peptide specific monoclonal antibodies such as DF3 and BR2729 (against MUC1) show specificity for cancer though the amino acid sequence is apparently the same in mucins of normal cells [30, 32, 33].

Furthermore, MUC1 epitopes are known to be immunogenic in humans as a consequence of demonstrable serum antibodies in occasional patients with breast and other carcinomas [51, 52]. The APDTRPA domain of the MUC1 tandem repeat is particularly immunogenic and is recognized by a variety of immune sera, monoclonal antibodies and cytotoxic T cells obtained from patients with breast or pancreatic cancer [52–54]. Immunization against MUC1 has protected mice and rats from tumor challenge with syngeneic breast cancers expressing human MUC1 [55, 56]. Other epithelial cancer mucins such as MUC2 and MUC5AC differ from MUC1 in that they do not have transmembrane domains and so are not cell surface antigens though they are secreted and form prominent components of the glycocalyx

that surrounds epithelial cancer cells. The structure of MUC1 fully glycosylated with Tn is summarized in Figure 1.

KSA

Human adenocarcinoma associated antigen (KSA), also called epithelial glycoprotein (EGP) and EpCAM, is a 40 kDa glycoprotein associated with the cell surface of most adenocarcinomas and with the corresponding normal tissues (once again at secretory borders) [57, 58]. It has been recognized by a series of mAbs (17–1A, KS1/4, H99, GA733). Treatment with 17–1A has resulted in occasional clinical responses of advanced carcinomas [59] without toxicity and when administered in the adjuvant setting to patients with Dukes C colon cancer, has prolonged disease free and overall survival compared to randomized controls [60]. Toxicity due to antibody access to and reaction with normal tissues was not seen.

PSMA

Prostate specific membrane antigen (PSMA) is a 100 kDa integral, type II membrane protein with acidic dipeptides activity which is highly expressed in primary and metastatic prostate cancer, and to a lesser extent in normal prostate tissue [61]. PSMA expression increases with disease progression. Recently PSMA has also been detected in tumor vascular endothelium from a variety of cancers as well as at much lower levels in some normal tissues, including duodenal mucosa and some proximal renal tubules. The relevance of PSMA as a target is emphasized by successful targeting of prostate cancer with ProstaScint, an ^{111}In-labelled anti-PSMA mAb that has been licensed by FDA for this purpose.

CA125

CA125 is a mullerian duct differentiation antigen expressed in some normal secretory tissues but overexpressed in ovarian cancer and some other cancers. It has been used as a serum marker for monitoring patients with ovarian cancer since it was first identified in 1981 using a murine monoclonal antibody. It has recently been identified as a mucin (MUC16) with high seronine, threonine and proline content and many (probably >60) partially conserved tandem repeats (156aa each) at the N-terminal region. The C-terminus contains a possible transmembrane region and a potential tyrosine phosphorylation site [62–64].

3. OTHER POTENTIAL ANTIGENS AND VACCINES INDUCING T-CELL IMMUNITY

Antigens not listed in Tables 1 and 2 are not as abundantly expressed, nor are they expressed with the same high frequency on cancers from different patients as are the antigens described above. In addition, antigens such as the cancer-testis antigens and p53 are not cell surface antigens, which may restrict the relevant immune response to a T-cell response. This enormously complicates the analysis of immunogenicity in vaccine trials and attempts at active intervention for the following reasons:

1) Ideally, autologous cancer cells are required for testing and these are rarely available as cell lines or in frozen samples in sufficient quantities for a thorough analysis of the immune response and its specificity (see Chapter 20).
2) In vitro sensitization has in the past generally been required for demonstration of T-cell responses against tumor antigens and this adds significant risk of artifactual results and complicates the quantification of immune responses (see Chapter 20).
3) Augmentation of T-cell responses by vaccination is more difficult to induce than augmentation of B-cell responses and has yet to be clearly achieved and confirmed in a majority of vaccinated patients against any tumor antigen.
4) Vaccine design depends on the immune response desired. There are hundreds of available approaches or combinations of approaches to inducing T-cell immunity. These include immunization with peptides or proteins with various adjuvants, dendritic cells pulsed with or transduced to express particular antigens, viruses or bacteria transduced to express antigens, and DNA or RNA vaccines. In each case these vaccines could include approaches to augmenting cytokine or second signal induction. The range of options for augmenting T-cell immunity against cancer is daunting. Unlike the picture with vaccines designed to induce an antibody response where there is one best approach (conjugate vaccines as described below), it remains unclear which is the optimal approach for induction of T-cell immunity (see Chapters 7, 8 and 10–17).
5) It is unclear whether augmentation of CTLs or helper T-cells is the desired goal for vaccines inducing T-cells against cancer.
6) It is not clear which antigens should be selected as targets for T-cell attack against cancer, as no T-cell immune responses have been correlated with a more favorable prognosis as is true for antibody responses against glycolipids (GM2) and mucins (sTn) [3–6].
7) Tumor cells can and frequently do fail to express relevant antigens in the context of MHC as a consequence of MHC loss or problems in antigen processing (proteosomes, TAP), or they may suppress the T-cell response or become resistant to it (by production of IL10, TGFβ, VEGF, Fas-ligand, HLA-G or Bcl-2) [reviewed in 65, 66].

Given these uncertainties, selection of a single vaccine approach for inducing optimal T-cell immunity is difficult now and will remain so for some years to come. Consequently, we have focused on antibody inducing polyvalent vaccines targeting the cell surface antigens listed in Table 2.

4. IMMUNOGENICITY OF THESE CELL SURFACE ANTIGENS IN CANCER PATIENTS

Selection of KLH Conjugate Plus QS-21 Vaccines

We have explored a variety of approaches for increasing the antibody response against carbohydrate and peptide cancer antigens, including the use of different immunological adjuvants [4, 67–72], adhering the antigens to bacteria or polystyrene beads, chemical modification of gangliosides to make them more immunogenic [73–76]

Table 2. Cancer cell-surface targets for vaccine construction

Tumor	Antigens*
Melanoma	GM2, GD2, GD3
Neuroblastoma	GM2, GD2, GD3, polysialic acid
Sarcoma	GM2, GD2, GD3
Small-cell lung cancer	GM2, fucosyl GM1, polysialic acid, globo H, sialyl Le^a, KSA
Breast	GM2, globo H, Le^y, TF, Tn, sTn, MUC1, KSA
Prostate	GM2, Tn, sTn, TF, Le^y, MUC1, KSA, PSMA
Ovary	GM2, globo H, sTn, TF, Le^y, MUC1, KSA, CA125 (MUC16)

* Antigens present on at least 50% of cancer cells in at least 50% of biopsy specimens

and conjugation to various immunogenic carrier proteins [67, 77]. The conclusion from these studies is that the use of a carrier protein plus an immunological adjuvant is the optimal approach. The optimal immunological adjuvant in each case was one or more purified saponin fractions (QS-21 or GPI-0100) obtained from the bark of Quillaja saponaria [72, 78]. The optimal carrier protein was in each case keyhole limpet hemocyanin (KLH). This approach (covalent attachment of the carbohydrate or peptide antigen to KLH and administration mixed with QS-21 or GPI-0100) has proved optimal for antibody induction in mice and cancer patients for most of the antigens in Figure 1 and Table 2.

Additional Variables

Two additional variables have proved critical for increasing antibody titers, the method of conjugation and the epitope ratio of antigen molecules per KLH molecule. The optimal conjugation approached has varied with the antigen. Gangliosides are best conjugated using ozone cleavage of the ceramide double bond and introducing an aldehyde group followed by coupling to aminolysyl groups of KLH by reductive amination. This approach was not as effective for conjugation of Le^y, or Globo H to KLH where an M2C2H linker arm has proved most efficient [84, 89] or for Tn(c), sTn(c), TF(c) or MUC1 where an MBS linker group was optimal [67]. We have demonstrated that covalent conjugation of antigen (ganglioside GD3) to KLH is required, simply mixing the two is of little benefit [77]. Based on our experience with GM2 and GD3 conjugate vaccines, it is our impression that within the restrictions imposed by current conjugation methods, higher epitope ratios result in higher immunogenicity. Consequently considerable effort is devoted to maximizing this ratio with each vaccine.

We have also performed a series of Phase I dosing trials to determine the impact of dose of conjugate on antibody response in vaccinated patients, and a series of experiments in the mouse to determine the impact of treatments designed to decrease suppressor cell reactivity in mice. The lowest dose of antigen in the KLH conjugates resulting in optimal antibody titers for each antigen is 10 μg for the glycolipids and polysialic acid, and 1 or 3 μg for the mucin antigens. Decreasing suppressor cell

activity with low dose cyclophosphamide or anti-CTLA4 mAb had no impact on antibody titers [110].

Ganglioside Vaccines

We have been refining our ability to induce antibodies against GM2 in melanoma patients for fifteen years, since it was first demonstrated that patients immunized with irradiated melanoma cells occasionally produced antibodies against GM2, and that vaccines containing purified GM2 could be more immunogenic than vaccines containing tumor cells expressing GM2 [79]. Initially GM2 adherent to BCG was selected as optimal, inducing IgM antibodies in 85% of patients. Though these antibodies and monoclonal antibodies against GM2 were only able to kill 25% of melanoma cell lines by CDC, patients with natural or vaccine-induced antibodies had significantly longer disease free and overall survival [4]. This was the basis for a randomized trial comparing immunization with BCG to immunization with GM2/BCG in 122 patients with AJCC Stage 3 melanoma [5]. While the difference was not statistically significant, the GM2/BCG treated patients had a 12% improvement in survival and 15% improvement in disease free survival compared to the BCG patients after a minimum follow-up of 70 months. The IgM antibodies had a median titer of 1/160 and were short lived (8–12 weeks). IgG antibody induction was rare. We explored a variety of approaches to further improve this antibody response [77]. The use of GM2 conjugated to KLH and mixed with immunological adjuvant QS-21 was consistently optimal, inducing higher titer IgM antibodies (median titer 1/640–1/1280) in all patients and IgG antibodies in most patients (see Table 3). Reactivity against GM2 positive melanoma cells and complement mediated lysis was seen in over 90% of patients, and the antibody duration was 3–6 months after each vaccination [68, 70, 71]. Antibody titers have been maintained for over three years by administration of repeated booster immunizations at 3–4 month intervals. Antibody titers could not be further increased by pretreatment with a low dose of cyclophosphamide (300 mg/M^2) to decrease suppressor cell reactivity (see Figure 2). As with the other carbohydrate antigen vaccines described below, no evidence of T-cell immunity detected by delayed type hypersensitivity skin test reactivity (DTH) against GM2 was found.

This GM2-KLH plus QS-21 vaccine has been tested in a Phase III randomized trial in melanoma patients in this country compared to high dose interferon alpha. The trial was stopped because after a median followup of 16 months, patients receiving interferon had a significantly longer disease free and over all survival. Longer follow-up will be required to determine the long term impact, but the results to date indicate that induction of antibodies against GM2 in Stage III melanoma patients is not associated with demonstrable benefit [80]. This may be because while essentially all melanomas express some GM2, only a minority express enough GM2 to permit cell lysis with mAbs or immune sera. This is further basis for polyvalent vaccines.

Fucosyl GM1, like GM2 is highly immunogenic. Essentially all patients vaccinated with fucosyl GM1-KLH plus QS-21 produced IgM antibodies and most produced

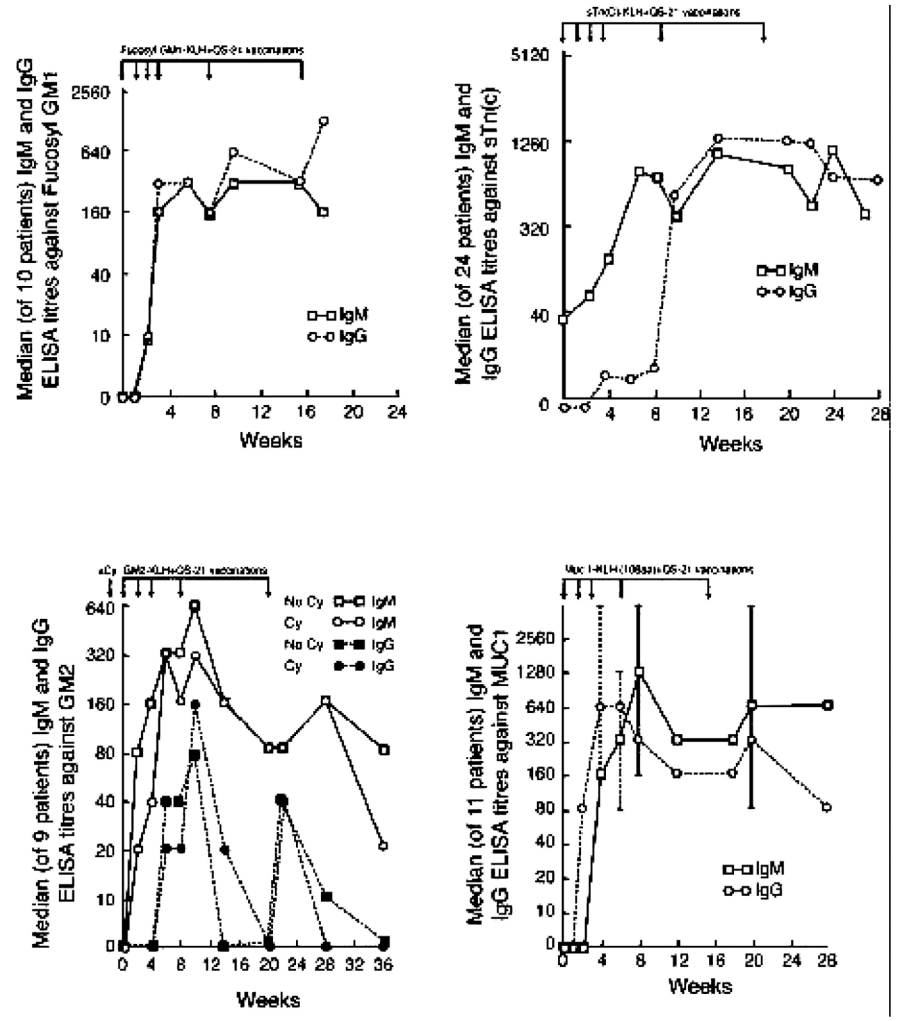

Figure 2. Median sequential ELISA IgG and IgM antibody titers induced in groups of cancer patients after vaccination with KLH-antigen conjugate vaccines plus QS-21. Some patients receiving the GM2 vaccine were treated with 300 mg/M^2 cyclophosphamide before the first vaccination.

IgG antibodies (see Figure 2) against fucosyl GM1 that also reacted with the SCLC cell surface by FACS and CDC [81].

Trials of GD2 and GD3 conjugated to KLH in melanoma patients induced only low (GD2) or no (GD3) antibodies reactive with the immunizing ganglioside or antigen positive melanoma cells. GD2 and GD3 are clearly less immunogenic than GM2. Based on early work from Hakomori and colleagues [82], we have demonstrated that conversion of these two gangliosides to lactones by treatment with acid

Table 3. Summary of Median Serological Results in Patients Vaccinated with Monovalent Vaccines Against Carbohydrates

	ELISA				FACS				Median IA	CDC	
Antigen	Total # of pts	% pts pos	IgM Pre/post	IgG* Pre/post	IgG Subclass	% pts pos	IgM Pre/post	IgG Pre/Post	post	%pts pos	Pre/post
GM2	12	100	0/640	0/320	IgG1 + 3	90	11/65	10/41	++	90	2/44
GD2L	12	80	0/320	0/160		60	10/38	11/11		50	0/30
GD3L	12	70	0/40	0/160		50	9/30	10/30	+	40	2/54
FucGM1	18	100	0/320	0/320	IgG1	90	10/84	11/33		90	9/73
Globo H	30	90	0/640	0/40	IgG1 + 3	75	10/41	10/13	++	55	4/36
Lewis Y	18	60	0/80	0		30	7/23	10/12	+	40	3/26
PolySA	6	100	0/640	0/20		80	10/48	10/12		0	—
Tn(c)	15	100	0/1280	0/1280		60	10/44	10/10	+	0	—
STn(c)	27	100	0/1280	0/160	IgG3	90	10/85	10/8	+	0	—
TF(c)	15	60	0/320	0/10		60	11/41	10/25	+	0	—
Muc1	45	90	0/1280	0/5120	IgG1 + 3	75	11/51	11/25	+	0	—

*0 = titer less than 1/10 — = not detected in any patient blank = not tested

after conjugation to KLH resulted in more immunogenic vaccines. Increased antibody titers against the native gangliosides and against tumor cells were induced in the majority of patients [111] (see results in Table 3).

Le^y and Globo H Vaccines

The development of Le^y and Globo H vaccines was previously limited by the lack of sufficient quantities of antigen for vaccine construction and testing. Over the last ten years, Dr. Samuel Danishefsky in our group has successfully synthesized both antigens [83–85]. We have immunized groups of mice with Globo H-ceramide plus or minus adjuvants QS-21 and Salmonella minnesota mutant R595, and with Globo H covalently attached to KLH or BSA plus immunological adjuvants QS-21 or GPI-0100. The highest antibody titers against both synthetic antigen and MCF7 cells expressing Globo H were induced by the Globo H-KLH plus QS-21 (or GPI-0100) vaccine [84, 86]. The antibody titer induced against synthetic Globo H was 1/120,000 by ELISA, the titer induced against MCF7 was 1/320, and potent complement mediated cytotoxicity was seen as well. Le^y-BSA and Le^y-KLH vaccines have also been tested in the mouse. High titer antibody responses have resulted against the synthetic epitope of Le^y and against tumor cells expressing Le^y in the majority of mice immunized [87]. Based on these results, clinical trials with Globo H-KLH plus QS-21 and Le^y-KLH plus QS21 have been initiated in patients with breast, prostate or ovary cancer. The results are summarized in Table 3. Antibodies against the purified antigens and against tumor cells expressing these antigens were induced in most patients immunized with globo H and occasional patients immunized with Le^y [88–90].

TF, Tn and sTn Vaccines

Patients with various epithelial cancers have been immunized with unclustered TF-KLH and sTn-KLH vaccines plus various adjuvants [91, 92]. High titer IgM and IgG antibodies against TF and sTn antigens resulted. In our hands the majority of the reactivity was against antigenic epitopes present in the vaccine which were not present on naturally expressed mucins (porcine or ovine submaxillary mucins (PSM or OSM)) or tumor cells [91, 93]. Based on previous studies with Tn antigen, Kurosaka and Nakada et al. hypothesized that MLS102, a monoclonal antibody against sTn, might preferentially recognize clusters ((c)) of sTn [94, 95]. Studies with monoclonal antibody B72.3 and with sera raised against TF-KLH and sTn-KLH conjugate vaccines in mice and in patients resulted in the same conclusion [41, 91, 93]. The availability of synthetic TF, Tn and sTn clusters consisting of 3 epitopes covalently linked to 3 consecutive serines or threonines has permitted proof of this hypothesis. In both direct tests and inhibition assays, B72.3 recognized sTn clusters exclusively, and sera from mice immunized with sTn (c)-KLH reacted strongly with both natural mucins and tumor cells expressing sTn [94 95]. Based on this background, we initiated trials with the TF(c)-KLH, Tn(c)-KLH and sTn(c)-KLH conjugate vaccines in patients with breast cancer. ELISA results for patients vaccinated with sTn(c)-KLH are summarized in Figure 2, and for all 3 antigens in Table 3. Antibodies of high titer and specificity, including against OSM or PSM and cancer cells expressing TF, Tn or sTn, were induced for the first time in our experience. Based on these results, we plan to include clustered Tn, sTn and TF in the polyvalent vaccines against epithelial cancers.

Several trials with TF, Tn and sTn vaccines have been reported from other centers, and a large multicenter Phase III trial with an sTn vaccine is currently in progress. Georg Springer's pioneering trials in breast cancer patients with vaccines containing TF and Tn purified from natural sources and mixed with typhoid vaccine (as adjuvant) began in the mid 1970s [34, 96, 97]. DTH and IgM responses against the immunizing antigens and prolonged survival compared to historical controls were reported. MacLean immunized ten ovarian cancer patients with synthetic TF conjugated to KLH plus immunological adjuvant Detox (monophosphoryl Lipid A plus BCG cell wall skeletons) and described augmentation of IgG and IgM antibodies against synthetic TF in 9 of 10 patients [98]. Lower levels of antibody reactivity against TF from natural sources were detected in some of these cases. MacLean has also immunized patients with breast and other adenocarcinomas with sTn-KLH plus immunological adjuvant Detox [6, 98, 99]. Induction of IgM and IgG antibodies against synthetic and natural sources of sTn was seen in essentially all patients and this response was further increased by pretreatment of patients with a low dose of cyclophosphamide. Reactivity of these sera with natural mucins and tumor cells despite the use of an unclustered sTn vaccine is probably explained by the several fold higher sTn/KLH epitope ratio achieved in the MacLean vaccine compared to our previous unclustered vaccine. Survival appeared to be improved overall compared to historical controls and patients who responded with high antibody titers survived longer than those with lower titers. Reactivity with breast cancer cells, including

complement dependent cytotoxicity, was described. This is the basis for an ongoing multicenter Phase III randomized trial of the sTn-KLH plus Detox vaccine versus no treatment in breast cancer patients with limited disease.

Polysialic acid Vaccines

Initial attempts at preparing a vaccine against polysialic acid for use in military recruits who are at risk of group B meningococcus infection were unsuccessful. We also have completed analysis of a trial with polysialic acid conjugated to KLH plus QS-21 and found that no antibody response could be induced. Consequently, we tested a second polysialic acid vaccine that had been modified (N-propionylated) to increase its immunogenicity in collaboration with Dr. Harold Jennings who pioneered the use of N-propionylation for this purpose [100]. This induced an antibody response against unmodified polysialic acid in five of six patients immunized (see Table 3). These vaccine induced antibodies also reacted with small cell lung cancer cells (and were cytotoxic for antigen positive bacteria) [67]. This N-propionylated polysialic acid vaccine is suitable for inclusion in our polyvalent vaccine against SCLC.

MUC1 Vaccines

We have immunized mice with MUC1-KLH, plus QS-21, and seen induction of consistent high titer IgM and IgG antibodies against MUC1 and human cell lines expressing MUC1, as well as protection from a syngeneic mouse breast cancer expressing human MUC1 as a consequence of gene transduction [56]. Mice were also immunized with vaccines containing MUC1 peptides (Tn glycosylated or not) with $1^{1/2}$ or 5 tandem repeats (32 or 106 amino acids) conjugated to KLH by one of three methods or not, and mixed with QS-21 or BCG. MUC1 containing 32 amino acids, glycosylated with Tn epitopes O-linked at serines or threonines or not, conjugated to KLH and mixed with QS-21 induced the highest titer antibodies. Based on these studies in the mouse, we initiated and completed trials with these MUC1-KLH plus QS-21 vaccines in breast cancer patients who were free of detectable breast cancer after resection of all known disease. No patient had detectable MUC1 serological reactivity by ELISA or FACS prior to immunization. ELISA results are shown in Figure 2 and summarized in Table 3. As was true in the mouse, the 32 amino acid MUC 1 peptide conjugate, glycosylated or not, was optimal. Inhibition assays were performed to better understand this serologic response [54]. Much of the IgM response and nearly all of the IgG response were against the immune dominant epitope, APDTRPA, preferentially with RPA at the terminal position.

Since MUC1 is a peptide, T-Lymphocyte responses against MUC1 would be expected. We have been unable to consistently demonstrate T-Lymphocyte proliferation, interferon-γ or IL4 release against MUC1 by ELISPOT or CTL assays, or positive DTH responses, after vaccination with MUC1. We have especially focused on proliferation and ELISPOT assays in the MUC1 trials. Patients were leukophoresed pre and post vaccination, providing ample lymphocytes for our studies. While occasional assays gave positive results, these were not positive on subsequent repeats or in a pattern with other assays that suggested impact of the immunizations. After 2 years

of steady endeavor there has been no clear evidence of augmented T-cell reactivity against MUC1 peptides of various lengths, or in HLA A2 positive patients against heteroclytic MUC1 peptides with single amino acid changes that increased binding to HLA A2 [101, 102].

Several trials with MUC1 vaccines at other centers have been reported. Trials by Goydos *et al.* [103] and Reddish *et al.* [104] showed augmentation of CTL and/or proliferation after vaccination (with MUC1 peptides mixed with BCG or immunological adjuvant Detox) in a single blood specimen in occasional patients but assays were not repeated to confirm reproducibility. A trial was reported by Karanikas *et al.* [105] with a MUC1-mannan fusion peptide and again occasional positive CTL or proliferation responses were seen but these were not repeated and were not both positive in the same patients. These three trials basically confirm our conclusion that vaccine induced augmentation of T-cell reactivity against MUC1 has yet to be convincingly demonstrated. The Karanikas report also described augmentation of antibody titers against the immunizing synthetic MUC1 peptide in 13 of 25 patients, but no tests against cancer cells or natural sources of MUC1 were described.

KSA Vaccines

Antibodies against KSA have been described in patients following vaccination with anti-idiotypic antibodies [106] and some patients vaccinated with KSA of baculovirus origin (Herlyn, D. and Spitler, L. in separate studies, personal communications). KSA is clearly potentially immunogenic in humans and these antibodies have not resulted in detectable toxicity.

PSMA Vaccines

To date, the only reports of vaccines containing PSMA involve peptides pulsed onto autologous dendritic cells [107, 108]. In these small studies, indications of immunogenicity were observed, and no obvious toxicity was described. Partial clinical responses were induced is some patients which were durable after one year. These trials indicate that PSMA is an appropriate choice of antigen for this patient population, even in the presence of metastatic disease. These studies are applicable only to HLA A2 positive patients and employed a technique involving *ex vivo* expansion and manipulation of dendritic cells, making them impractical for wide spread application. Antibody induction was not tested.

CA125 Vaccines

Because CA125 has only recently been sequenced, no trials with CA125 vaccines have been conducted to date. However, forty-two ovarian cancer patients have been immunized with the murine monoclonal anti-idiotype (ACA125), which imitates an epitope on CA125 [109]. While HAMA and anti-idiotype antibodies were induced in the majority of patients, reactivity of post immunization sera with tumor cells was induced in only occasional patients. There was no evidence of autoimmunity in these patients.

5. GLYCOLIPIDS AND GLOBULAR GLYCOPROTEINS ARE MORE EFFECTIVE TARGETS FOR CDC THAN MUCINS

One of several effector mechanisms thought to contribute to tumor cell death is complement dependent cytotoxicity (CDC). Review of the serological analysis of the series of clinical trials described above has suggested that the six vaccines containing different glycolipids induced antibodies mediating CDC whereas the four vaccines containing carbohydrate or peptide epitopes carried by mucin molecules induced antibodies that were not capable of mediating CDC. We explored whether this dichotomy was a result of properties of the induced antibodies (ie. class and effector functions), the different target cells used, or the nature of the target antigens. We compared the cell surface reactivity (assayed by FACS), complement-fixing ability (using the immune adherence [IA] assay) and the CDC activity of a panel of monoclonal antibodies and immune sera from these trials on the same two tumor cell lines. Antibodies against glycolipids GM2, globo H and Le^y, protein KSA and mucin antigens Tn, sTn, TF and MUC1 all reacted with these antigens expressed on tumor cells and all fixed complement. CDC, however, was mediated by mAbs or immune sera against the glycolipids and a globular protein (KSA), but not by mAbs or sera against the mucin antigens (112). Recently we have noted that immune sera and mAbs against polysialic acid are also unable to induce CDC (see Table 3). Like MUC1, antibodies against polysialic acid attach far from the cell surface (as shown in Figure 3). In the case of MUC1, this is because of the rigid carbohydrate collar that characterizes mucins. The same effect probably results as a consequence of the negative charge of both the sialic acid rich cancer cell surface and polysialic acid.

It must be emphasized that although we showed that mucins are poor targets for complement-mediated lysis of tumor cells, studies have shown that induction of antibodies against either glycolipid or mucin antigens results in protection from tumor recurrence in several different preclinical mouse models [reviewed in 2]. Also, antibodies against either glycolipid or mucin epitopes correlate with a more favorable prognosis in patients [3–6, 97]. It does not appear that the inability of antibodies against mucin antigens to induce complement-mediated lysis is necessarily detrimental to the anti-tumor response. Consequently, complement-mediated inflammation, opsonization and antibody dependent cellular cytotoxicity but not CDC are likely mechanisms for the prolonged survival seen in the preclinical experiments targeting mucin antigens and suggested in the clinical trials with passively administered and actively induced antibodies against mucin antigens. With regard to bacterial infections, this is supported by the severe consequences of hereditary deficiency states involving either the classical or alternate complement pathways and the comparatively trivial consequences to deficiencies of the complement membrane attack complex [109].

6. SUMMARY

The great majority of cancer patients can initially be rendered free of detectable disease by surgery and/or chemotherapy. Adjuvant chemotherapy or radiation therapy are generally only minimally beneficial, so there is real need for additional

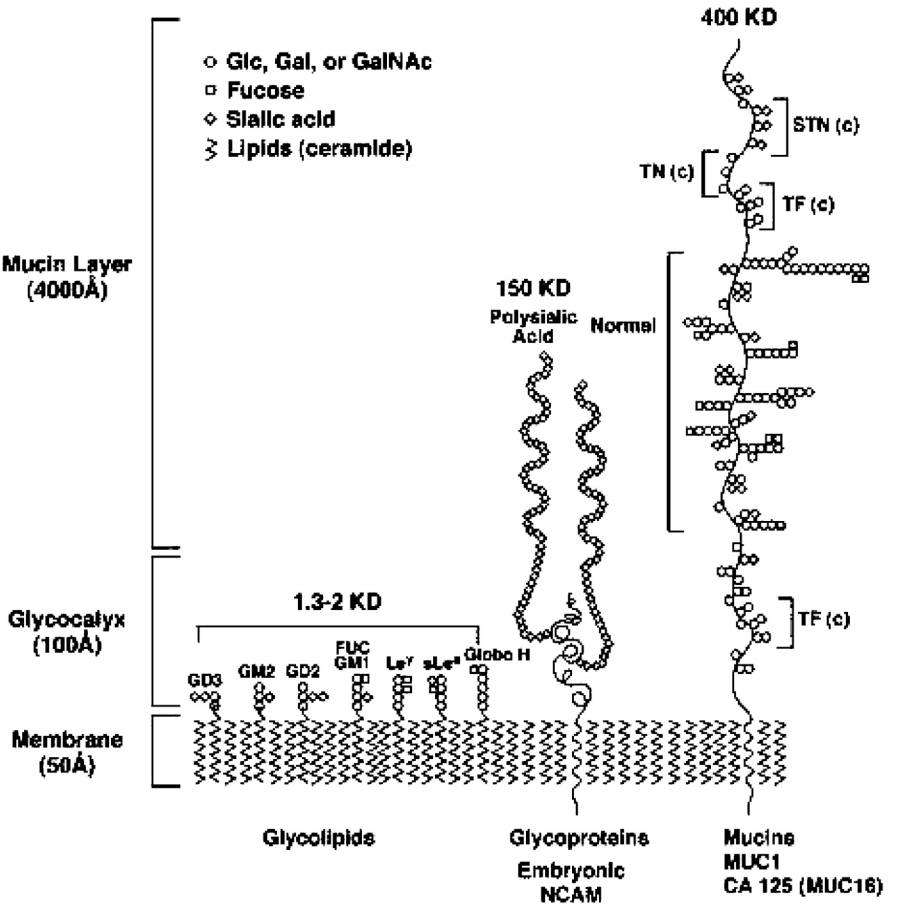

Figure 3. Glycolipids and glycoproteins expressed abundantly at the cancer cell surface. Note the intimate association with the cell surface of glycolipids and the more distant association of mucins and polysialic acid.

methods of eliminating residual circulating cancer cells and micrometastases. This is the ideal setting for treatment with a cancer vaccine. The immune response induced is critically dependent on the antigenic epitope and vaccine design. For antibody induction there is one best vaccine design, conjugation of the antigen to an immunogenic protein such as KLH and the use of a potent adjuvant such as the saponins QS-21 and GPI-0100. This approach alone induced strong antibody responses against the glycolipids GM2, fucosyl GM1 and globo H and the mucin backbone MUC1, and cancer cells expressing these antigens. Other antigens required additional modifications to augment relevant immunogenicity. GD2 and GD3 lactones and N-propionylated polysialic acid were significantly more effective

at inducing antibodies against tumor cells than the unmodified antigens. Tn, sTn and TF trimers (clusters) were significantly more effective than the monomers at inducing antibodies reactive with the cancer cell surface. The optimal approach for Le^y, KSA, PSMA, and CA125 (MUC16) remains to be determined.

Antibodies are ideally suited for eradicating pathogens from the bloodstream and from early tissue invasion. Passively administered and vaccine induced antibodies have accomplished this, eliminating circulating tumor cells and systemic or intraperitoneal micrometastases in a variety of preclinical models, so antibody-inducing vaccines offer real promise in the adjuvant setting. Polyvalent vaccines will probably be required due to tumor cell heterogeneity, heterogeneity of the human immune response and the correlation between overall antibody titer against tumor cells and antibody effector mechanisms. Over the next several years, Phase II clinical trials designed to determine the clinical impact of polyvalent conjugate vaccines will be initiated in the adjuvant setting in patients with SCLC and several epithelial cancers.

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8. DENDRITIC CELL-BASED VACCINES FOR CANCER THERAPY

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Dendritic cells (DCs) were initially described in 1973 by Ralph Steinman, who observed in mouse spleen a subpopulation of cells with a striking dendritic morphology. DCs originate from the bone marrow, and their precursors migrate via the bloodstream to almost all organs of the body, where they reside as immature cells with high phagocytic capacity (1). They acquire antigens (Ag) in peripheral tissues and migrate to lymphoid organs where they present processed peptides to naive T cells and initiate the immune response. During this process, DCs lose their Ag-capturing/processing capacity as they differentiate into mature, fully stimulatory, antigen-presenting cells (2). In addition, DCs interact with B lymphocytes to enhance B cell expansion and antibody production (3), as well as with natural killer (NK) cells to augment cytolytic activity and interferon-γ (IFN-γ) production (4).

The past 20 years have witnessed a dramatic expansion in the understanding of the relationship between DCs and the cellular immune response. DCs appear to be central to the regulation, maturation, and maintenance of a cellular immune response to cancer. Encouraging results from vaccination studies in animal models and the development of protocols to generate sufficient numbers of human DCs for clinical application have led to the first early-phase clinical trials of DCs for the treatment of cancer in patients. These studies have established the safety and feasibility of this approach and have produced some encouraging evidence of therapeutic efficacy.

This chapter will focus on mouse and human DCs, their generation, as well as selected strategies being pursued to harness their potent antigen-stimulating activity

for their use in clinical trials and murine experimental models, and finally highlighting issues for future trial design.

EXPERIMENTAL ANIMAL MODELS

Generation of Murine DCs

Although monocyte-derived DCs are the most commonly used type of human DCs, they are only rarely prepared from mice largely because the yield is small (1×10^5/mouse), and there are other easier methods available to produce murine DCs.

Mouse bone marrow is a major source of DCs when cultivated with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4. Most published reports of mouse DCs use cells derived from BALB/c or C57Bl/6 mice. However, there is no reason not to use mice of other genetic backgrounds for certain applications. In general, mice 8–12 weeks old yield sufficient numbers of DCs, precursor and progenitor cells to be easily manipulated. The technique described by Inaba et al. (5) has been modified over the years. Once generated, DCs are collected on day 5 or 6, and enriched by 14.5% (w/v) metrizamide density gradient separation. Further purification can be achieved by FACS sort on CD11b and CD11c, or by using CD11c-coated paramagnetic beads. Typical DC yields are approximately 5×10^6 cells/mouse. Further maturation of DCs can be achieved by LPS or TNF-α treatment.

Progenitor $CD34^+$ cells obtained from mouse bone marrow will differentiate into DCs when cultured with GM-CSF plus the ligand for the receptor tyrosine kinase fms-like tyrosine kinase 3 (Flt-3L) (6). $CD34^+$ cells may be obtained from mouse marrow by depletion of lineage+, nonadherent cells followed by sorting. These cells will differentiate into $CD11c^+CD11b^+$ and $CD11b^-$/dull$CD11c^+$ subsets upon culture with GM-CSF, TNF-α and CSF (7), which may be sorted by FACS on day 6 for independent culture. Addition of stem cell factor (SCF) or Flt-3L to bone marrow cultures increase the yield of DCs ultimately derived, especially when used in combination.

DC-Based Vaccination in Animal Tumor Models

A growing number of studies have reported the successful use of DCs for inducing antitumor immune responses in animals. Most of these experiments have involved in vitro isolation of DCs, followed by pulsing of DCs with different forms of tumor antigen and injection of the antigen-loaded DCs into syngeneic animals as a cancer vaccine. Tumor development was induced by injection of established tumor cell lines of various tissue origins. Following interaction with tumor cells or selected tumor Ags, DCs were effective as prophylactic tumor vaccines against subsequent challenge with the same tumor. Initial approaches using DCs loaded with tumor lysates, tumor antigen-derived peptides, soluble protein tumor antigen expressed by a B cell lymphoma, synthetic class I-MHC-restricted peptides, RNA, DNA and whole protein have all been demonstrated to generate tumor-specific immune responses

and antitumor activity against subsequent tumor challenges, and even therapeutic efficacy was reported, leading to the induction of regression of preexisting tumors [(8–15); and reviewed in (16)]. Such immunologic and anti-tumor effects depend on additional critical factors, e.g., the route of DC administration. Using vaccination with tyrosinase-related protein-2-derived peptide-loaded, Indium-111-labeled DC vaccination in a fully syngeneic B16 melanoma tumor model, Eggert et al. (17) observed a delay in tumor growth, improved survival, as well as increased antitumor cytotoxic T-cell reactivity after s.c. delivery compared to i.v. delivery. In contrast, a pilot clinical trial, performed by Fong et al. (18), using Ag-pulsed DCs as a tumor vaccine in patients with metastatic prostate cancer suggest that activated DCs can prime T cell immunity regardless of route of administration. However, these investigators reported that the "quality" of this response and induction of Ag-specific Abs might be affected by the route of administration.

With these studies providing the "proof-of-principle" for Ag-pulsed DC vaccination against cancer with respect to route of administration, recent investigations have focused on discovering more effective methods of delivering tumor Ags to DCs. One strategy has been to use recombinant viruses as a highly efficient means of introducing genes into DCs. Mouse tumor models have extensively been used to test the in vivo therapeutic efficacy of DCs transduced with viral vectors that encode different cytokine cDNAs such as GM-CSF, IL-12, or Flt-3L, immunomodulatory molecules like B7-1, ICAM-1, or LFA-3, or cDNAs encoding for model antigens or TAAs. Studies have addressed the question whether DCs genetically engineered are capable of eliciting antitumor immunity in vivo. Adenovirus-mediated gene delivery into DCs has been studied extensively in murine models since 1997. A number of reports have shown DCs to possess enhanced antitumor properties after adenoviral transfer of therapeutic transgenes. For example, mice immunized with Trp2 gene-transduced DCs were capable of inducing protection against mouse melanoma-induced lung metastasis (19). Another report showed that DCs infected with adenoviral vectors encoding endogenous TAA expressed by the murine melanoma line B16 could elicit antitumor immunity in this poorly immunogenic tumor model (20). DCs overexpressing IL-12 as a result of adenoviral-mediated IL-12 gene transfer have been found to induce antitumor immunity when injected directly into tumors (21). Because of the emerging evidence that chemokines play an important role in the priming of naïve T cells by DCs, introduction of chemokine genes into DCs are now being reported as well. For example, a recent study showed that immunization with DCs adenovirally cotransfected with gp100 and lymphotactin, a C chemokine that specifically regulates the migration of T cells and NK cells, could enhance protective and therapeutic antitumor response more effectively in a B16 melanoma model (22). Our group has studied direct administration of DCs genetically modified to express secondary lymphoid tissue chemokine (SLC) into growing B16 melanoma. SLC, a CC chemokine found in high endothelial venules and within the T-cell zones of both spleen and lymph nodes, is capable of recruiting both DCs and naïve T cells via the CCR7 receptor found on both cell types (23, 24). We

reported that intratumoral injections of SLC-expressing DCs could result in tumor growth inhibition with a substantial, sustained influx of T cells within the mass (25, 26).

A long–puzzling phenomenon was the improved efficacy of DNA vaccines containing unmethylated cytidine-phosphate-guanosine (CpG) dinucleotide (CpG-ODN) motifs that are common in bacterial DNA but not in mammalian DNA. Synthetic oligodeoxynucleotides containing CpG-ODN in specific sequence contexts mimic the immunostimulatory qualities of bacterial DNA (27). These agents can activate an "innate" immune response by activating monocytes, NK cells, DCs, and B-cells in an independent manner. The effects of CpG on DCs include increased DC migration to lymph nodes, enhanced activation of $CD8^+$ T cells, and protective CTL responses against both viral and tumor antigens (28). In vivo, CpG act also as an effective adjuvant for a tumor vaccine consisting of DCs cocultured with irradiated tumor cells, which provide a substantial increase in both prophylactic and therapeutic activity in several murine tumor models (29, 30). Clinical vaccination trials that use CpG-ODN as immunologic adjuvants are currently underway.

Utilization of exosomes, small vesicles of endosomal origin, might be another attractive approach in cancer immunotherapy, combining the anti-tumor activity of DCs with the advantages of a cell-free vehicle. Both DCs and tumor can secrete exosomes constitutively (31, 32). Tumor-derived exosomes contain whole native cytosolic and/or endosomal tumor antigens and constitutive heat shock proteins (hsps). Exosomes transfer tumor antigen to DCs and induce peptide-specific, MHC class I-restricted cross presentation to T cell clones and, in vitro, tumor-specific CTL responses in patient's lymphocytes. Exosomes produced by DCs, display a discrete set of proteins involved in antigen presentation, in particular MHC class I and II molecules, but also costimulatory molecules (CD86), and are selectively enriched in molecules potentially involved in effector cell targeting, such CD11b, lactadherin, and CD9 molecules (31). Isolation of exosomes is usually carried out by differential centrifugation, followed by floatation on sucrose density gradients to collect the exosomes (31, 33, 34). Zitvogel at al. (31) have shown that not only do DCs trigger T cell responses through direct cell-cell contacts, but exosomes secreted by DCs can also stimulate T cells. In this study, exosomes secreted by bone marrow-derived DCs (BM-DCs), which were challenged with tumor-derived peptides, activated CTLs, causing the eradication of established tumors. The mechanism of action of exosomes in vivo is poorly understood. Exosomes could stimulate T cells directly, through the MHC-peptide complexes they harbor, or they could be captured by other professional APC, which could then use peptide-loaded MHC molecules, Ags, or peptides present in exosomes to stimulate T cells. When compared with the tumor peptide-loaded DCs, DC-derived exosomes showed higher efficiency in eliciting tumor regression of the established p815 mastocytoma and TS/A mammary adenocarcinoma. Moreover, in several mouse tumor models, Wolfers et al. (33) showed that immunization of mice with DCs loaded with tumor-derived exosomes resulted in tumor prevention as well as the regression of established lesions. The antitumor immunity was mediated by $CD8^+$ T cells, because their depletion in vivo

inhibited the anti-tumor effect of exosomes. These results suggest that exosomes derived from tumor cells or DCs provide another promising avenue for the development of DC-based cancer vaccines. A clinical trial has recently been launched, aimed at vaccinating patients with metastatic melanoma and inoperable lung cancer with autologous DC-derived exosomes pulsed with MAGE-3 MHC class I- and II-associated peptides.

Fusion of tumor cells with DCs is a powerful new technology to increase tumor vaccine immunogenicity, and as been explored as a means to potentially endow DCs with the full complement of TAAs expressed by the tumor cell. In this strategy, DCs can be loaded with tumor antigens by simply fusing them to tumor cells. These cell-fusion studies have shown effective responses against both primary tumors and secondary metastases, and stimulation of both $CD4^+$ and $CD8^+$ T cells as well as NK cell anti-tumor responses (35–39). The commonly used procedure for the preparation of DC-tumor fusion cells has used polyethylene-glycol (PEG), a classical fusogenic agent that is widely used to produce B or T cell hybridomas. This procedure can be time and labor intensive because after fusion, 7–14 days of culture are usually required for selection and expansion. Among a number of means for achieving cell-cell fusion, electro-fusion seems to be particularly attractive. A recent study has compared the therapeutic efficiency of PEG versus electric pulse-mediated fusion protocols in a poorly immunogenic and it demonstrated metastatic murine mammary carcinoma cell line, and it demonstrated that electro-fusion is as efficient as PEG mediated fusion in generating an immunogen capable of inducing protective anti-tumor immunity (40). Therefore, both techniques seem to be promising for clinical application. However, in a more recent study, the further optimization of the electro-fusion parameters resulted in superior activity compared to chemical fusion (41).

Another approach that may supersede the need for ex vivo expansion and manipulation of DCs, is the administration of the cytokine Flt-3L. Treatment of mice bearing certain immunologic tumors with Flt-3L has been shown to result in tumor regression (42, 43). In an acute myelogenous leukemia (AML) model, Pawlowski et al. (44) have also shown that a significant protection against AML challenge in naïve or bone marrow-transplanted mice was provided by either in vitro tumor-lysate-pulsed DCs or in vivo Flt-3L-generated DCs, but only when initiated prior to AML challenge.

Despite the immunologically privileged status of the brain, numerous pre-clinical studies of DC-based immunotherapy for malignant brain tumors since 1997 have demonstrated that immunotherapy may be feasible and efficacious in both protection and treatment models for intracranial models of glioma and melanoma (Table 1). Numerous strategies of loading DCs with tumor antigens have been investigated including pulsing with tumor lysate (45–51), acid-eluted peptides (52), synthetic or virally transfected peptides (48, 53, 54), whole tumor cDNA (55–57), DC-tumor fusions (45, 50), RNA (46, 58), apoptotic tumor (59), and irradiated tumor cells (60). While all utilized immature, bone-marrow derived DCs, the number and characterization of the DCs used, the frequency and technique of vaccination, and

Table 1. Pre-clinical trials of DC-Based Immunotherapy for Malignant Brain Tumors

Tumor	Type	DC Characteristics	Form Antigen	Route	# DC	Frequency	*In vitro* Response	*In vivo* Response	Ref
203 glioma; B16 melanoma	Protection and treatment	MHC I+, II+, CD80+, CD86+	SFV mediated tumor cDNA	i.p.	N.S.	7 days	specific lytic activity	CD8+ infiltration	(55)
	Treatment	MHC I+, II+, CD80+, CD86+	SFV mediated tumor cDNA	i.p.	N.S.	7 days	specific lytic activity; IFN-γ production	tumor infiltrated by CD4, CD8 and NK cells	(57)
560EGFRvIII glioma cell line	Protection	MHC I+, II+, CD80+, CD86+; RB6-, F4/80-	tumor lysate	i.p.	10^5	7 days	increased Ag-specific cytotoxicity to EGFRvIII;	increased antiEGFRvII humoral response	(48)
9L	Treatment	MHC II+, OX62+, CD3-	acid-eluted surface peptides	s.c.	5×10^5	7 days	specific lytic activity	increased CD8 infiltration	(52)
	Therapeutic	OX6+, OX42+, OX62+	apoptotic tumor cells	s.c.	5×10^5	days 0, 1, 4	N.S.	s.c. tumor regressed	(59)
	Treatment	OX6+, CD80+, OX62+, CD44+, CD86+	none	i.t.	2×10^6	day 0	N.S.	N.S.	(60)
Autologous human GBM	in vitro immune response	MHC I+, II+; CD86+	autologous tumor	N.A.	N.A.	N.A.	Increased T cell proliferation; specific tumor cytotoxicity	N.A.	(50)
Autologous human malingnant astrocytoma	in vitro immune response	CD3+, CD45+, CD80+, CD86+	autologous tumor lysate	N.A.	N.A.	N.A.	Increased T cell proliferation; specific tumor cytotoxicity	N.A.	(51)
B16 melanoma	Protection and treatment	N.S.	tumor lysate, RNA	i.p.	N.S.	7 days	specific lytic activity	increased intra-tumoral inflammatory response	(46)

	Protection	MHC I+, II+, CD80+, CD86+	MART-1	s.c.	5×10^5	7 days	cytotoxicity (no specicity control); IFN-γ, IL-4 production by ELLISPOT	No CD4 or CD8 infiltrate	(53)
	Treatment	MHC I+, II+, CD11c, CD40, CD80+, CD86+	none	i.t.	N.S.	7 days	specific lytic activity; IFN-γ production	CD8+ infiltration	(56)
C3 Sarcoma cell line	Protection and treatment	MHC I+, II+, CD86+, ICAM1+	$E7_{49-57}$ (synthetic viral peptide)	i.v.	5×10^5	7 days	N.S.	increased mononuclear infiltration	(54)
GL261	Treatment	N.S.	sonicated tumor	i.p.	N.S.	7 days	N.A.	increased CD8+ infiltrates	(47)
	Protection	N.S.	tumor RNA	s.c.	N.S.	14 days		increased CD4+ and CD8+ infiltrates	(58)
	Treatment	Cloned DC2.4; MHC I+, II+, CD86+, ICAM1+	tumor extract	i.p.	2×10^6	Days 0, 1, 3,7,10,14	increased CD4 and CD8 proliferation	DTH response	(49)
SR-B10.A glioma	Protection and treatment	CD80+	irradiated tumor cells	s.c.	3×10^5	7 days	Increased T cell proliferation; specific tumor cytotoxicity	Tumor regression; increased CD4+ and CD8+ infiltration	(45)

Abbreviations: SFV, semiliki Forest Virus; N.A., not applicable; N.S., not specified.

in vitro and *in vivo* assays utilized were highly variable. The multiplicity of techniques and tumor models makes meaningful comparisons impossible. Nonetheless, nearly all demonstrated efficacy in the models studied. Several also implicated the importance of $CD8^+$ T cells, $CD4^+$ T cells, and NK cells in anti-tumor efficacy (45, 54, 56). The single negative study was that of Yang et al. (60), which demonstrated that orthotopic 9L gliosarcoma induced apoptosis of DCs delivered directly into the tumor. The investigators implicated hyaluronan on the glioma membrane via increased nitric oxide synthase (NOSi) induced by the DC-based CD44 receptor. This effect was not eliminated by stereotactic radiosurgery of the tumor, but was abrogated by pre-treatment of DCs with anti-CD44 or N-mononomethyl-L-arginine (NMMA), or pre-treatment of 9L with hyaluronidase. This suggests that the efficacy of direct intra-tumoral injection of DCs, which has been demonstrated in other tumor models (61), may not translate to gliomas.

CLINICAL CANCER VACCINE TRIALS

Generation of Human DCs

Physiologically, human DCs constitute a rare but heterogeneous population that are phenotypically distinct from macrophages and represent only a small proportion of less than 1% of the circulating leukocyte pool. For therapeutic purposes large numbers of DCs are required. Three main types of DCs have been studied for use in clinical trials.

Monocyte-Derived DCs (Mo-DCs)

The best-studied human DCs are those derived from peripheral blood CD 14^+ monocytes, which are abundantly present in peripheral blood. Monocytes can be easily obtained from peripheral blood draws or leukapheresis by several methods, including plastic adherence of Ficoll-Hypaque (62) or Lymphoprep (63) purified peripheral blood mononuclear cells (PBMCs), followed by metrizamide gradient centrifugation. Monocytes are subsequently cultured for 5 to 7 days, in the presence of GM-CSF and IL-4. Investigators have used preferentially serum-free medium or autologous plasma (1%) instead of medium supplemented with fetal calf serum, as the latter may contain trace amounts of endotoxin, TGF-β or other factors (64). After 1 week the yield of immature DCs generated varies from about 25–50% of the starting population. Yields of $0.5–2 \times 10^6$ cells per 10 ml blood are typically obtained. A representative culture will contain 95–99% $CD1a^+CD14^-CD83^{lo/-}$ cells. If cells are cultured much beyond 8 days, they will undergo spontaneous maturation with upregulation of CD83. It is not clear whether this developmental pathway of monocytes occurs frequently in vivo, or whether this represents a highly specialized stage of monocytes, expressed only under certain conditions. Development of a closed, semi-automated system for the generation of large-scale monocyte-derived DCs has been optimized by some groups (65, 66).

Peripheral-Blood-Derived DCs (PBDCs)

DCs can be also generated as circulating precursors from the blood by density-based purification techniques, after a period of in vitro culture (1–2 days) without cytokines (67, 68). During this time, DC precursors undergo maturation, become larger and less dense, which allows their purification by density-gradient centrifugation. Gradient solution lacking potentially immunogenic protein such as BSA has been employed including Percoll, Nycodenz, and metrizamide. DCs isolated in this manner possess potent allostimulatory activity and the ability to prime naïve $CD4^+$ T helper cells (69) and $CD8^+$ cytotoxic T lymphocytes (CTLs) (70). The use of density-based isolation is, however, limited by the low frequency of DC precursors in blood, and leukapheresis must be performed to generate sufficient numbers (on average 5×10^6 from the PBMCs of a single leukapheresis procedure) of DCs for vaccinations of humans.

$CD34^+$-Derived DCs

Human DCs can also be generated in vitro from $CD34^+$ hematopoietic progenitor cells. $CD34^+$ cells may be derived from bone marrow (71), cord blood (72), or purified directly from peripheral blood or after mobilization with cytokines such as granulocyte colony-stimulating factor (G-CSF) or GM-CSF (73). The generation of DCs in this way involves a positive selection using paramagnetic beads and in vitro culture with cytokines over 2 to 3-weeks. Final DC yields can be increased by expanding the progenitor pool (10–30 fold) prior to terminal DC differentiation. Some protocols include an initial expansion period [usually with Flt-3L, SCF or both, in combination with other cytokines] to boost DC progenitor cell numbers, followed by a differentiation step (which usually includes GM-CSF plus TNF-α) after culture with different combinations of cytokines, including TNF-α, Flt-3L, c-Kit, CD40 ligand (CD40L), SCF, GM-CSF or TGF-β(74). Both c-Kit and Flt-3L are transmembrane proteins on stromal cells that bind to tyrosine-kinase receptors and sustain DC progenitors (75), whereas TNF-α and CD40L block the granulocyte-differentiation pathway and stimulate the final maturation of DCs (3). $CD34^+$-DCs appear to be more efficient in the activation of tumor-specific CTLs than those derived from $CD14^+$ progenitors (76). In contrast to Mo-DCs, DCs derived from $CD34^+$ cells consist of two phenotypically and functionally distinct populations (77). One subset is similar to the epidermal Langerhans cells, and the other termed "interstitial/dermal DCs" is similar to those derived from blood monocytes (77). Immune responses to these unique LC containing preparations await evaluation in humans.

In vivo Generated DCs

An alternative approach is to expand DCs in vivo. Methods of stimulating DC mobilization and trafficking in vivo, allowing the native immune environment to naturally mature DCs, may overcome the functional limitations imposed by ex vivo culture; for example, the culture conditions used to expand cells ex vivo may significantly

affect their function and antigen-processing capabilities, or that DCs generated in culture may not traffic to draining lymph nodes in great numbers, thus limiting the development of systemic immunity. Treatment with the hemopoietic growth factor, Flt-3L (78), may potentially bypass the need for ex vivo culture and manipulation of DCs or their precursors. Results so far from the continuing human clinical studies have demonstrated that treatment with Flt-3L is well tolerated and can increase numbers of circulating DCs more than 20-fold (78, 79).

Maturation of Human DCs

DCs have multiple roles and dynamically shift phenotypes relative to their environment. DC phenotype and function may be affected by the precursor cells from which the DCs are derived, as well as by the factors used to effect differentiation or maturation.

Immature DCs can be further induced to mature by co-culturing with inflammatory stimuli including TNF-α, IL-6, IL-1β, pathogen-related molecules such as LPS, bacterial DNA, T cell–derived signals and prostaglandins such as PGE_2 or, alternatively, with a so-called monocyte conditioned medium (MCM) for an additional 3 days (80). The maturation process is associated with several coordinated events such as (a) expression of CD83, as well as the p55 actin-bundling protein fascin, an important controller of cytoskeleton remodeling; (b) loss of antigen-uptake capacity; (c) upregulation of T cell adhesion and costimulatory molecules (CD40, CD58, CD80, and CD86); (d) change in morphology, (e) expression of different cytokines genes; and (f) expression of chemokine receptors that guide DC migration into lymphoid organs for priming of antigen-specific T cells. Morphological changes accompanying DC maturation include a loss of adhesive structures, cytoskeleton reorganization, and acquisition of high cellular motility (81).

The use of DCs as adjuvants is supported by numerous animal studies with primarily mature DCs (82, 83), which have shown that the injection of tumor antigen–loaded DCs reliably induces tumor-specific CTL responses, tumor resistance, and in some cases, regression of metastases. However, in the majority of pilot clinical trials reported so far for humans, immature DCs have been employed (Table 2). Sporadic tumor responses are reported and the induction of tumor-specific CTLs by DC vaccination have been observed. Moreover, several reports have demonstrated that immature DCs but not mature DCs can induce tolerance to Ags used for vaccination (84, 85). Therefore, mature DCs have been used in recent vaccination protocols, especially when peptides are used as a source of antigen (86, 87). In contrast to immature DCs, mature DCs are much more potent in inducing Th1 and CTL responses in vitro and are resistant to immunosuppressive effects of tumor-derived IL-10. They also become migratory and travel to the local lymph nodes, where they present antigens in association with MHC to specific T cells. Several studies have shown that DCs generated in vitro must be matured to migrate optimally and to stimulate T cells efficiently (3, 88–90). In a small pilot trial, Jonuleit et al. vaccinated advanced, stage IV melanoma patients simultaneously with immature DCs and mature DCs loaded with different melanoma-associated peptide antigens. Both DCs populations

Table 2. Clinical Trials Evaluating DC-Vased Tumor Vaccines

Tumor Type	DC Characteristics	Form Antigen	Route	Maturation	Ref
Melanoma	Mo-DCs	Cocktail of peptide (MART-1, gp100, MAGE-3, Ty) or TL	intranodal	No	(95)
	Mo-DCs	MART-1, gp100	i.v.	No	(126)
	$CD34^+$-derived DCs	MAGE-1 and MAGE-3 or MART-1 and gp100 and Ty	i.v.	Yes	(99)
	Mo-DCs	MAGE-A1, MAGE-A3	i.v, s.c	No	(127)
	PBDCs	MAGE-3 or MART-1 plus rhIL-12	s.c.	No	(128)
	Mo-DCs	MAGE-3	i.v., s.c.	Yes	(87)
	Mo-DCs	MAGE-3	i.v., s.c.	Yes	(94)
	Mo-DCs	MART-1, gp100, Ty	i.v.	No	(129)
	$CD34^+$-derived DCs	MART-1, gp100, Ty, MAGE-3	s.c.	Yes	(130)
	Mo-DCs	gp100, Ty	i.v.	No	(131)
	Mo-DCs	TL	i.d.	No	(132)
	Mo-DCs	Panel of MHC class I and II-restricted peptides	s.c.	Yes	(133)
	Mo-DCs	TL	i.d.	No	(101)
Prostate carcinoma	Mo-DCs	PSMA peptides	i.v.	No	(134-137)
	PBDCs	PAP/GM-CSF fusion protein	i.v., s.c.	No	(138)
	PBDCs	PAP/GM-CSF fusion protein	i.v.	No	(139)
	PBDCs	Xenoantigen (mouse PAP)	i.d., i.v., i.l.	No	(18, 67)
	Mo-DCs	PSA RNA	i.v., i.d.	No	(140)
Colorectal carcinoma	Mo-DCs	Total tumor RNA	i.v.	No	(141)
	PBDCs	Total tumor RNA	i.v., i.d.	No	(142)
	Flt-3L mobilized PBDCs	CEA peptide	i.v.	Yes	(79)
Gastrointestinal carcinoma	Mo-DCs	MAGE-3 peptide	i.v.	No	(143)
Breast/ovarian cancer	Mo-DCs	HER-2/neu or MUC-1	s.c.	Yes	(144)
Gynaecological malignancies	Mo-DCs	Autologous TL	s.c.	Yes	(145)
B-cell lymphoma	PBDCs	Idiotypic protein	i.v., s.c. boost	No	(68)
	PBDCs	Idiotypic protein	i.v.	Yes	(108)
Pediatric solid Tumors	Mo-DCs	TL	i.d.	No	(100)

(*continued*)

Table 2. (*continued*)

Tumor Type	DC Characteristics	Form Antigen	Route	Maturation	Ref
Renal cell Carcinoma	Mo-DCs	Tumor-cell DC fusion	s.c, s.c boost	Yes	(146)
	Mo-DCs	TL	i.v.	Yes	(147, 148)
	Mo-DCs	TL	intranonodal s.c.	Yes	(149)
	Mo-DCs	TL	s.c	Yes	(150)
Multiple Myeloma	PBDCs	Idiotypic protein	i.v., s.c. boost	No	(110)
	Mo-DCs	Idiotypic protein	i.v.	No	(109)
	$CD34^+$-derived DCs	Idiotypic protein	i.v., s.c. boost	No	(111)
Breast, Papillary, Pancreatic Cancer	Mo-DCs	MUC1 cDNA transfection	i.d.	No	(151)
CEA-expressing malignancies	Mo-DCs	CEA peptide CAP-1	i.v.	No	(152)
Malignant glioma	Mo-DCs	Tumor cell-DC fusion	i.d.	Yes	(106)
	Mo-DCs	Tumor specific MHC class–I peptides	i.d.	No	(120)

Abbreviations: Ty, tyrosinase; TL, autologous tumor lysates; i.d., intradermal, PAP, prostatic acid phosphate; i.l., intralymphatic; CAP-1, carcinoembryonic antigen peptide-1; PSMA, prostate specific membrane Ag.; PSA, prostate specific Ag; MUC1, mucin 1.

were injected in different lymph nodes of the same patient. They demonstrated that mature DCs were capable of promoting a greater level of CTL and T helper reactivity measured in the circulating PBMCs (91). Another reason some groups support adoptive transfer of mature DCs is that immature DCs may lose their efficiency for T-cell stimulation once removed from exogenously supplied cytokines. Several studies have also reported that immature Mo-DCs can reverse into a macrophage after cytokine withdrawal, whereas mature DCs cannot undergo this reversion. However, as mentioned earlier, mature DCs have a lower capacity for uptake of exogenous antigens, such as RNA, proteins, or dead tumor cells. Different maturation protocols may also produce DC populations that are functionally distinct in terms of, for example, their ability to migrate, to produce cytokines, to stimulate T cells, and to induce T-cell cytokine secretion (92). These differences have important implications for decisions on the most appropriate type of DCs (immature or mature) for use in clinical trials.

Antigen Loading of DCs

Several forms of DC-mediated immunotherapy are currently being investigated with great intensity in clinical trials, using a wide variety of different vaccination protocols,

for numerous tumor types including melanoma, prostate cancer, AML, breast cancer, gastric cancer, lung cancer, renal cell carcinoma, gastric cancer, and others. A summary of some published clinical trials is presented in Table 2.

DCs can be pulsed with synthetic peptides or proteins derived from known tumor-associated antigens (TAA) such as MAGE-1 and MAGE-3, New York Esophagus (NY-ESO)-1, MUC-1, Her-2/neu, tyrosinase, carcinoembryonic antigen (CEA), or Melan-A/MART-1. For example, antigens of the MAGE family have been employed to induce melanoma specific immunity, first by using immature DCs (93) and later by using terminally mature DCs (87, 94, 95). The use of defined antigens for tumor immunotherapy has the clear advantage of being able to control the amounts of antigen administered, and to monitor the emerging response. The immunogenicity of defined peptide epitopes may be substantially increased by modifying the peptide sequence at amino acid residues that are crucial for the interaction with the MHC class molecules or with the specific TCR (96, 97). However, using peptides for DC loading has several intrinsic disadvantages. This approach is currently limited to that tumor type for which TAAs are identified. Moreover, the application of antigenic peptides is limited to use in patients who express a defined specific HLA haplotype. Finally, the majority of known TAA peptides are presented in association with MHC class I molecules and are recognized by tumor-specific $CD8^+$ T cells, whereas small numbers of TAA epitopes are presented in association with MHC class II molecules and are recognized by $CD4^+$ T cell (98).

To overcome such limitations, another approach is the use of whole proteins or multiple peptides as the source of antigen. Due to the broad spectrum of potentially recognizable peptides that can originate from each protein, this strategy allows the induction of immune responses against different epitopes that could be potentially restricted to multiple HLA alleles. Furthermore, the antigen-processing and presenting machinery could direct responses to important and immunodominant epitopes including both MHC class-I and class-II-restricted peptide antigens. This strategy has been followed for melanoma, using antigens against MAGE-1 and -3, tyrosinase, Melan-A and gp100 together with $CD34^+$-derived DCs (99)

Other approaches utilizing whole tumor cells as a source of antigen have been developed. Loading of DCs with tumor lysates or extract, obtained after repeated freezing and thawing or sonication of whole tumor cells, is one of the more established methods and has been used in a wide variety of tumor types (16, 100, 101). The studies showed induction of tumor-specific T-cell responses, including cytolytic activity and the production of immunostimulatory cytokines. DCs can also be transfected with either RNA coding for a specific tumor antigen or whole tumor RNA (46, 102). The ease in generating large quantities of nucleic acids gives RNA-based vaccines an advantage over tumor lysates, especially if multiple restimulations are needed using a small tumor sample. Moreover, tumor-restricted RNA can be enriched before loading by subtractive hybridization with RNA from normal tissues. Tumor-specific immune responses are thereby augmented, and the likelihood of autoimmunity generated from self-antigens is reduced. One weakness of this strategy is the unstable, labile nature of RNA. Because DCs are very effective at presenting

peptides from apoptotic cells (103), dying tumor cells (apoptotic bodies or necrotic cells) have also been used as sources for tumor antigens to load DCs (104, 105). Another strategy designed to deliver all antigens from tumor cells directly into the cytosol of DCs is by the fusion of DCs with tumor cells. The fused DC-tumor cells obtained are thought to combine the whole antigenic spectrum of the tumor with the powerful antigen capabilities. Studies using DC-tumor cell fusions have demonstrated the generation of tumor-specific CTL in vitro and antitumor immunity in vivo (106, 107). The important conceptual outcome of all these loading strategies is that tumor antigens would be processed by both the endocytic and proteosomal DC pathways and would be capable of stimulating both $CD4^+$ and $CD8^+$ T cells. Because these approaches do not require the definition of TAA or MHC haplotype of the patients they may provide for a broader clinical application.

Based on studies in a murine lymphoma model showing that vaccination with idiotype (Id)-pulsed DCs could generate a strong T lymphocyte anti-Id response and induce a protective antitumor immunity (15), several groups initiated clinical trials of Id-pulsed DC vaccination for patients with low-grade non Hodgkin lymphoma (NHL) (68, 108) and multiple myeloma (109–111). Both diseases are slowly, but inevitably, progressive malignancies, and generally express a unique immunoglobulin Id as a potential TAA. Recently, Timmerman et al. (108) reported a long term follow-up of 35 patients with follicular NHL, treated using this approach. They described that Id-pulsed DC vaccination can induce T-cell and humoral anti-Id immune responses, as well as durable tumor regression

The ability to manipulate DC function by gene transfer represents an attractive alternative strategy to enable some DC-based therapies, and does not require prior knowledge of the MHC type or relevant T-cell peptide epitope. The target genes transferred fall into two categories TAA and immunomodulatory proteins such as cytokines and costimulatory molecules. Available vectors include retroviruses, adenoviruses, lentiviruses, adenoassociated virus, herpes simplex virus, cationic liposomes, naked DNA and DNA-coated gold beads. The biologic effects of the transduction on DCs vary with the viral vector systems and the experimental conditions used in the studies. A comparison of various gene transfer methods in human DCs showed that adenovirus vectors was the most efficient in transducing human DCs, with transduction efficiencies exceeding 95% at higher multiplicity of infection (112). Potentially benefits and limitations of genetically-modified DCs for use in immunotherapy have been reviewed recently (113, 114).

Evaluation of Vaccine-Induced Immune Responses

One important objective of clinical vaccine trials is to devise in vitro immunological assays that correlate with clinical outcome, for use as surrogate markers of vaccine efficacy, and to make the results from different clinical studies comparable. The most crucial end-point reflecting the efficacy of antigen-specific vaccines is the induction of responses by $CD8^+$ T cells. To date, IFN-γ ELISPOT and recombinant MHC class I multimers loaded with the respective peptide appear to be the most commonly used and sensitive detection assays to measure CTL responses following DC

vaccination against a selected peptide antigen. Furthermore, tetramer staining can be used for in situ detection of peptide-specific CTL in biopsies of lesions and lymph nodes of cancer patients (115). An alternative approach allows the selective isolation of antigen-specific CTL by fluorescence activated cell sorting (FACS) (116). However, trends towards the use of more complex immunogens, such as whole proteins, require the development of efficient and sensitive methods for monitoring more complex immunologic effects as well. In the context of a vaccination trial using full-length tyrosinase (Ty) to immunize patients with metastatic melanoma, a monitoring technique was developed in which autologous DCs infected with a recombinant adenovirus encoding the Ty protein were used to assess the Ty-specific reactivity of fresh peripheral blood lymphocytes. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was used to measure the production of cytokine mRNA by T cells following a brief incubation with Ty-expressing DCs. Two out of ten patients enrolled demonstrated Ty protein-specific reactivity that increased during and after the period of vaccination. While one of these patients also reacted to an HLA-A1-compatible Ty peptide, the second did not recognize any of the known Ty epitopes, highlighting the importance of this technique for monitoring the effects of complex vaccines (117).

Preliminary results of clinical trials with DC immunization using ex-vivo-generated DCs appear contradictory, however. Fong et al. (79) examined in a phase I clinical trial, the ability of Flt-3L to increase the number of DCs in cancer patients as well as the ability of these expanded DCs to be harvested and used to immunize patients against CEA. They demonstrated that immunization with Flt-3L-expanded DCs loaded with an altered peptide ligand derived from CEA could lead to CEA-specific immunity and clinical responses. However, in a recent published phase II clinical trial, Rini et al. (118) reported that Flt-3L, although capable of inducing expansion of circulating myeloid and plasmacytoid DCs in patients with metastatic renal cell carcinoma, lacked significant clinical activity at the doses and schedules examined. Further studies will be necessary to ascertain the value, if any, of Flt-3L to generate clinically effective anti-tumor immunity.

There have been few clinical trials to date of DC-based immunotherapy for malignant brain tumors involving a total of 20 patients (Table 3). All have been Phase I clinical trials for the treatment of gliomas, utilizing multiple i.d. vaccinations comprised of 5-100 $\times$ 10^6 poorly characterized or immature bone-marrow derived, DCs. These DCs have been loaded with tumor antigen prepared in a variety of ways including tumor lysate (100), acid-eluted peptides (119, 120), gamma irradiated tumor cells (121), and by fusion to glioma cells (106). Likewise, *in vitro* and *in vivo* immune monitoring was highly variable. No objective clinical responses was observed, but increased lymphocytic infiltration of tumor has been reported (119, 120). Despite induction of lethal encephalitis in primates vaccinated with human glioma (122), treatment of humans has been well tolerated with minimal side effects, and autoimmune disease has not been reported to date. The utility of delayed-type hypersensitivity (DTH) and routine MRI imaging in assessing immune response and clinical efficacy has also been questioned. Preliminary reports of DC-based

Table 3. Clinical Trials of DC-Based Immunotherapy for Malignant Brain Tumors

Tumor Type	DC Characteristics	Form Antigen	Route	*In vitro* Response	*In vivo* Response	Ref
PNET	Immature (CD14-, CD80+, CD86+, CD83-, HLA-DR+)	autologous tumor + KLH	i.d.	increased T cell proliferation; ELISPOT	DTH for tumor and KLH in 1/2	(100)
AA, AO, GBM	MHC I+, II+, CD80+. CD86+, CD83-	irradiated and cultured autologous tumor	i.d.	increased IFNγ secretion	N.A.	(106)
GBM	high MHC I, II, CD80+, CD86+	allogeneic MHC-I Matched peptides	i.d.	increased T cell proliferation	increased CD3+ T cell proliferation	(119)
AA, GMB	CD14-, CD80+. CD86+, HLA-DR+	autologous tumor peptides	i.d.	Proliferation, cytotoxicity in 4/7	2/4 had increased CD45RO+ T cell infiltration	(120)
	N.S.	autologous tumor	N.S.	N.A.	N.A.	(121)

Abbreviations: PNET, Primitive neuroectodermal tumor; AA, anaplastic astrocytoma (ie WHO grade III astrocytoma); AO, Anaplastic oligodendroglioma; GBM, Glioblastoma multiforme (ie WHO grade IV astrocytoma); KLH, keyhole limpet hemocyanin; N.A., not applicable; N.S., not specified.

immunotherapy for newly diagnosed patients has been more promising, with several clinical responses observed and an extended median survival of over one year (123)

CONCLUSIONS

While DCs were difficult to isolate initially, these APCs can now be generated in large numbers in vitro and manipulated in multiple ways before administered back to a patient to induce anti-tumor immunity. Studies of dendritic cell biology in the laboratory and preclinical studies in the mouse have facilitated the implantation of clinical trials using DCs in the treatment of melanoma and other cancers. Importantly, there have been no reports of serious adverse events or significant autoimmune sequelae observed with DC vaccines apart from standard Grade I toxicities (124). Phase I clinical trials have established the feasibility of this approach against a number of human tumors, including renal cell carcinoma, melanoma, prostate carcinoma, cervical carcinoma, breast carcinoma, ovarian carcinoma, multiple myeloma, and intracranial tumors.

Animal studies and human cancer trials have shown that specific T-cell responses against tumors as well as tumor regression can be achieved with vaccines based on DCs. One challenge to DC-based tumor vaccines remains the difficulty of measuring that a clinically relevant immune response has been induced. Thus, one would wish to detect a clinically relevant frequency of tumor-specific cytotoxic T cells

and tumor-specific $CD4^+$ and $CD8^+$ T cells capable of producing inflammatory cytokines [reviewed in Ref. (82)].

The issues of optimal number of DCs as well as the frequency and route of administration (i.d., intranodal, s.c. or i.v.) remains uncertain, although compelling preclinical studies suggest that DCs should be administered either intradermally or intranodally, at regular intervals in the case of metastatic disease. In most published clinical trials, 4 to 70 million DCs have been administered at 2-week to 4-week intervals. DCs injected s.c. or i.d. could migrate to draining lymph nodes with varying efficiencies, although a significant number of cells remained at the injection site, whereas the i.v. route resulted in the dispersal of DCs to lung, liver, spleen, and bone marrow, but not to the peripheral lymph nodes (125). Unpolarized T-cell and antibody responses have been demonstrated with i.v. administration, whereas Th1 responses have been seen predominantly after i.d. and intralymphatic injections. Additional preclinical studies will be necessary to define the optimum DC population for use in clinical trials, with respect to the source and stage of maturation of DCs, and the stimuli used to generate them.

Despite unresolved issues, efforts continue to evolve DC-based antitumor vaccines. DC vaccination is currently being employed in an ever-increasing number of trials. Careful optimization of the most promising strategies, thoughtful selection of patient populations, and appropriate clinical trial design will be critical to the achievement of a reproducible determination of clinical benefit or failure of DC-based vaccines for cancer.

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9. UNDEFINED-ANTIGEN VACCINES

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INTRODUCTION

The immune surveillance hypothesis stated that tumors arose frequently, but were recognized and then eliminated by the host immune system because they expressed tumor-associated antigens (TAA) [1]. This theory was questioned because immune-deficient hosts did not exhibit an increased incidence of non-virally induced tumors. However, recent experiments in genetically manipulated mice, which have been made deficient in both the innate and adaptive immune systems, revealed that the immune system does indeed alter the incidence of both carcinogen-induced and spontaneous malignancies (immunoediting). Thus, the immune surveillance hypothesis has been resuscitated and has provided renewed impetus for cancer immunotherapy [2].

TAA recognized by T cells consist of two distinct types: shared antigens and individual tumor-specific antigens (TSA). Most of the well-characterized TAA are shared tumor antigens. These include tissue differentiation antigens, such as melanoma-associated antigens (MAA), e.g. MART-1 and gp100; cancer-testis antigens, e.g. MAGE and NY-ESO-1; and over-expressed normal antigens, e.g. Her2/neu, telomerase and Muc-1. Shared tumor antigens are usually weak "self" antigens, which are poorly immunogenic because the high-avidity T cells that recognize strong MHC-binding epitopes derived from these shared antigens have been deleted. Very few TSA have been described thus far; and except for idiotypic determinants on malignant lymphocytes, TSA result mainly from mutations that occur in individual tumor cells

within a specific patient. The lack of central tolerance to these antigens may make them more effective tumor rejection antigens. Another class of antigenic peptides comes from alternative open reading frames of mRNA that encode longer proteins, often using non-AUG as the initiation codon (e.g. CUG), or translation of introns and regions spanning the intron-exon junctions. These "cryptic" peptides could add a large number of TSA to the repertoire; however, it is not known whether cryptic translation also occurs in normal cells or if it is unique to transformed or virally infected cells [3].

A rationale for using undefined antigen vaccines to treat patients with cancer is that for the most common types of malignancy, e.g. carcinomas of epithelial origin, tumor antigens have not been well-characterized. Furthermore, in contrast to defined antigen vaccines, vaccines using intact tumor cells, or preparations derived from whole tumor cells, contain not only shared tumor antigens, but also many TSA, to which the host may not have developed tolerance.

The new immunoediting hypothesis stated that tumors arising in immune competent hosts after selection and sculpting processes would be less immunogenic than tumors that developed in immune-deficient hosts. A major challenge for the rational design of whole tumor cell vaccines will be to increase the immunogenicity of tumor cells so that the critical threshold for priming of the existing T-cell repertoire that can recognize and destroy tumor cells can be reached and to modulate host regulatory mechanisms that prevent recognition of many self antigens.

CROSS-PRIMING OF T CELLS. A major goal of vaccination in cancer patients is the induction of potent antitumor T-cell immune responses. Unless tumor cells metastasize to secondary lymphoid tissues, naïve T cells rarely have an opportunity to interact with and be activated by solid tumor cells, even if the tumor cells express MHC/peptide complexes on their surface. The activation of tumor antigen-specific T cells relies primarily on the cross-priming pathway, which involves antigen processing and presentation of tumor cell antigens by the host professional antigen presenting cells (APC), particularly dendritic cells (DC). After they take up tumor-derived antigens DC migrate into the T-cell zones of tumor-draining lymph nodes. Although most studies indicate that the major pathways for T-cell priming by tumor cells were mediated by host APC, equally strong evidence from Zinkernagel's group [4] indicated that localized solid tumors were largely ignored and cross-priming to antigens from solid tumors was minimal. Priming of CTL occurred only when tumor cells metastasized to or were injected directly into secondary lymphoid organs. Thus, the efficiency and physiological role of cross-priming has been challenged. The analysis of complex biological systems is greatly influenced by the model used and experimental design employed. Therefore, the generalization of any theory is difficult, and the truth probably lies between the two extremes. In fact, recent studies demonstrated that cross-priming of antigens derived from tumor cells required high levels of antigen expression, while antigens with a low level of expression were ignored by T cells [5] . In some situations, direct and cross-priming were found to be redundant, and the absence of either pathway led to T-cell priming by the other pathway [6]. The relative contribution of each pathway varied depending on many factors, such

as level of antigen expression, and localization of tumor cells. The contribution of cross-priming to T-cell mediated immune surveillance in cancer patients may not be possible to ascertain; however, clinical trials of vaccination with whole tumor cells that are designed to increase the efficacy of cross-priming may provide clues to whether the cross-priming pathway can be co-opted to produce therapeutic activity.

This review will focus on strategies that use undefined or ill-defined antigen preparations derived from whole tumor cells as the cancer vaccines. They include the following: whole tumor cells (both autologous and allogeneic); gene-modified cells (genes encoding cytokines, chemokines, and costimulatory molecules); cell-derived materials (lysates, apoptotic bodies, exosomes, heat shock proteins); and tumor-APC fusion cells. The basic concepts, preclinical development, and the preliminary data from current clinical trials will be described.

WHOLE CELL VACCINES

Early versions of whole cell vaccines usually consisted of killed tumor cells or tumor cell lysates mixed with bacterial adjuvants such as *Bacillus Calmette Guerin* (BCG) and *Corynebacterium parvum*. Although the mechanisms of the bacterial adjuvants are still not understood very well, the innate immune responses to various bacterial components play a critical role in bridging the innate immune response and adaptive immunity to tumor antigens. In contrast to the crude bacterial adjuvants used for the generation of early cancer vaccines, more recent generations of whole cell tumor vaccines have comprised genetically modified tumor cells using defined immune-modulating genes, including cytokines, chemokines and costimulatory molecules.

WHOLE CELL VACCINES WITH ADJUVANT. Bacterial adjuvants were used to boost anti-tumor immunity as far back as the 1890's [7, 8]. Studies using transplantable tumors demonstrated that the immunogenicity of whole tumor cell vaccines was enhanced by mixing tumor cells with bacterial adjuvants, such as BCG [9], *Corynebacterium parvum* [10] and the streptococcal preparation OK-432 [11]. Cancer vaccines were produced from autologous or allogeneic tumor cells. For an excellent historical perspective the reader is referred to a review by Oettgen and Old [7]. But for a few exceptions discussed below, the clinical benefits of these tumor vaccines were minimal. These studies were done before the "modern age" of immunology and the mechanisms for the immuno-potentiation effects of bacterial adjuvants remained a mystery until the recent discovery of innate immunity receptors for bacterial-derived molecules: the Toll-like receptor (TLR) family of proteins. Bacterial cell wall materials (LPS, lipoproteins and peptides), unmethylated genomic DNA (CpG motif), and flagellin bind to different TLR and cause maturation of dendritic cells and facilitate antigen presentation and T-cell activation. The involvement of other innate immune cells, such as macrophages and NK cells is less defined; however, their role in antitumor immunity should not be ignored. Building a bridge between innate and adaptive immunity may well be the mechanism to improve the efficacy of whole cell vaccines, a strategy that will be tested in the near future.

A few groups have continued to study this approach to vaccination. Morton and his colleagues at the John Wayne Cancer Institute have employed a vaccine

comprising three allogeneic melanoma cell lines with BCG as adjuvant [12] and Berd et al. used autologous melanoma cells that had been chemically modified with the hapten DNP mixed with BCG [13]. Single arm phase II studies of both vaccines indicated that vaccination improved the survival of patients with stage III disease and metastatic disease when compared to historical controls. Furthermore, clinical outcome appeared to be improved in patients who developed an immune response to the vaccine compared to patients that failed to respond. Both vaccines are being developed by private industry. Canvaxin® is now being compared to BCG in a randomized study of adjuvant therapy for patients with melanoma at high risk for recurrence (state III and IV disease after complete resection) [12]. These approaches have also been tested in other diseases. For example, based on the finding that TAA can even be shared among tumors of different histology, Canvaxin® was tested in patients with colon cancer. DNP-modified autologous tumor cells have been used in ovarian cancer. Two phase III adjuvant trials of an autologous tumor cell vaccine with BCG have been performed in patients with stage II and III colon cancer. One study, with 412 patients failed to detect a benefit for vaccinated patients [14] while another study with 254 patients reported an improvement in recurrence-free survival for stage II patients [15], but failed to show benefit for patients with stage III colon cancer. None of the vaccines described above has resulted in an improvement in survival and therefore, they have not become part of standard therapy in the US or Europe.

Gene-Modified Tumor Vaccines Cytokines and Chemokines

PRECLINICAL STUDIES. The cloning of cytokine genes and development of gene transfer technology ushered in a new era of cancer vaccines [16]. Dranoff et al. compared the efficacy of vaccination with B16 melanoma cells following gene transfer with a large array of cytokine genes using the MFG Moloney murine leukemia retrovirus as a vector. Tumor cells elicited the best protective immunity against tumor challenge if mice were vaccinated with irradiated gene-modified tumor cells that produced GM-CSF compared to cells that produced IL-1, 2, 3, 4, 5, 6, 7, 10, 12, 18, TNF-α, IFN-γ, SCF, G-CSF, M-CSF, Flt3-ligand, eotaxin, RANTES, MIP-1α, MIP-1β, or lymphotactin. The priming of antitumor CTL by GM-CSF gene-modified tumor vaccines was mediated primarily by host professional APCs [17]. Although similar comparisons have not been performed for the ever-growing number of newly discovered chemokines, SLC appeared to exhibit potent anti-tumor activity when expressed by tumor cells [18, 19]. The immune modulating activities of cytokines and chemokines are different, and a powerful synergism of the combination has been demonstrated between lymphotactin and GM-CSF or IL-2 [20].

Since autologous tumor cells contain both shared TAA and TSA, theoretically they would be preferable to allogeneic tumor cells, which contain only shared TAA, as vaccines. However, the wide application of autologous tumor cell vaccines has been hampered by the difficulty in obtaining adequate autologous tumor and the problems associated with the standardization of individual gene modified vaccines. Thus,

allogeneic tumor cell vaccines have been pursued more actively [21–23]. Although there may be a difference in efficacy between syngeneic and allogeneic whole tumor vaccines, cross protective immune responses have generally been observed [24]. A major concern was that the development of strong allogeneic responses might override the tumor-specific responses. However, the experimental evidence strongly indicated otherwise; the allo reactions enhanced the antitumor immune responses [25, 26].

CLINICAL TRIALS. The results of clinical trials for tumor cell vaccines transduced with IL-2, IL-4, IL-7, IFN-γ, IL-12 and GM-CSF have been reported [27]. Gene transfer to tumor cells has been accomplished with retroviruses, adenoviruses, lipofection or with DNA-coated gold particles. To date, melanoma has been studied most extensively, but cytokine-modified vaccines have also been used to treat patients with neuroblastoma, lung cancer, prostate cancer, ovarian cancer, kidney cancer, sarcoma, brain tumors and hematologic malignancies.

The most actively studied vaccines have been GM-CSF-transfected tumor cells. Phase I clinical trials of irradiated GM-CSF-transduced autologous tumor cells sponsored by Cell Genesys have been completed in patients with kidney cancer [28], prostate cancer [29] , melanoma [30] and non-small-cell lung cancer [31]. To circumvent the need for transfection of autologous tumor, a GM-CSF-producing bystander K562 cell line has been developed and used in combination with autologous NSCLC cells. Allogeneic GM-CSF-transfected cell lines have been used in the adjuvant setting for patients with pancreatic cancer [32] and to treat metastatic prostate cancer. Vaccination with GM-CSF-modified tumor cells has proven to be feasible and safe. Transduction of autologous tumors cells continues to be a challenge, but was successful in the majority of cases. The major side effect following vaccination was local tenderness at the injection site. Biopsies of vaccine sites, tumor nodules and DTH sites revealed dense dendritic cell, macrophage, granulocyte and lymphocytic infiltrates. Most patients exhibited DTH reactions to tumor cells after vaccination and individual patients developed tumor-specific CTL activity and produced high titers of antibody. None of the studies were designed to assess the clinical benefit of vaccination; however, tumor regression was observed in patients with melanoma, renal cell cancer and lung cancer. There was also a suggestion that pancreatic cancer patients treated at the highest dose of the allogeneic cell vaccine had an improvement in disease-free survival [32].

Costimulatory Molecules

In the two-signal hypothesis for T-cell activation, T cells are optimally activated when their T-cell receptor is engaged by a peptide/MHC complex and CD28 is engaged by a costimulatory molecule, CD80 or CD86 on the APC [33]. In contrast to professional APCs, most cells from solid tumors do not express costimulatory molecules. Therefore, gene-modified tumor cell vaccines were produced to make tumor cells better APCs by introducing genes for costimulatory molecules [34]. CD80 or CD86 transduction can convert tumor cells into professional APC's in vitro, although the precise role of such engineered APCs in the initiation of

antitumor T-cell responses in vivo is not clear. Since naïve T cells circulate primarily in lymphoid organs, and initial priming usually takes place within the secondary lymphoid tissues, direct activation of naïve T cells by tumor cells could occur only when tumor cell metastasize to lymphoid tissues [35, 36]. Two recent studies demonstrated a redundant role for both direct-and cross-priming [6, 37]. Cross-priming by host APCs was more efficient than direct priming by tumor cells even when tumor cells were engineered to express costimulatory molecules [37]. The characterizations of four additional CD28-related molecules and their ligands has not only increased the complexity of regulation of T-cell activation, but has also provided additional tools to augment antitumor immune responses [38]. Furthermore, members of the tumor necrosis receptor family (TNFR) family, which not only costimulate T cells, but also exhibit additional immunomodulatory effects on innate immunity present other opportunities to evaluate costimulatory molecules in tumor immunology. [39].

CLINICAL TRIALS. CD80 gene-modified tumor cells have been used to treat patients with metastatic renal cell cancer [40] and breast cancer [41]. In the former study, autologous tumor cells infected with an adenoviral vector that contained the CD80 gene were used; the latter trial employed an HLA-A2-matched allogeneic breast cancer cell line lipofected with a CD80-encoding DNA plasmid. Two of the 15 patients with renal cell cancer experienced a partial remission; however, the contribution of CD80 to vaccine efficacy was impossible to discern because treatment also included low-dose interleukin-2, which has activity in this disease [40]. Dols et al. administered their allogeneic breast cancer vaccine with BCG or GM-CSF as an adjuvant to 30 women with heavily pretreated metastatic breast cancer [41]. At the highest tumor cell dose significant tumor-specific T-cell responses were detected, but no objective tumor regressions were observed.

FUTURE DIRECTIONS. A new class of immune stimulatory receptors (NKG2D) expressed by NK cells, T cells and macrophages has the potential to modulate immune responses, including antitumor immunity [42]. Human NKG2D binds to MHC class I-related molecules (MICA, MICB), and CMV UL16-binding proteins (ULBP1,2,3), while mouse NKG2D binds to members of the retinoic acid early inducible 1 (Rae-1) family, minor histocompatibility antigen H60 and murine ULBP-like transcript 1 (MULT1) [43]. Expression of MICA and MICB was normally restricted to gastrointestinal epithelium, but can be induced by cellular stress and is upregulated after infections, and on tumor cells of epithelial origin, such as lung, breast, kidney, prostate, and colon tumors. Members of the Rae-1 family (Rae-1α, β, γ, δ, ε) are expressed during early embryogenesis, but absent in normal adult tissues. Like MICA and MICB, expression of Rae-1 is found on several mouse tumor cell lines [44]. MIC and Rae-1 play an important role in tumor immune surveillance. In humans, soluble MICA and MICB found in melanoma patients downregulate surface NKG2D expression on tumor-infiltrating T cells, which dampen their antitumor activity [45]. In mice, Rae-1 expression regulates the antitumor activity of $\gamma\delta$ T cells against cutaneous tumors [46]. Most importantly, mice vaccinated with tumor cells that express high levels of H60 or Rae-1 not only prevented tumor growth, but also induced CD8 T-cell dependent antitumor immunity [47, 48]. In another

study, however, no protection was observed despite complete rejection of primary challenge with Rae-1-transfected tumor cells [49]. While the efficacy of vaccination with B7-expressing tumor cells was dependent on inherent tumor immunogenicity, this was not the case for NKG2D costimulation. B16 melanoma cells that expressed B7 grew as rapidly as the parental line, but H60 or Rae-1 expressing B16 tumor cells were promptly rejected. Although the mechanisms by which NKG2D ligands induced antitumor CD8 T-cell responses remains to be determined, these ligands definitely add a new and potentially more powerful approach to the development of genetically modified tumor cell vaccines.

Cell-Derived Materials

LYSATES. Tumor cell-derived materials, including lysates (freeze-thaw, sonication and oncolysate [lysis by lytic viruses]), shed antigens, apoptotic bodies, exosomes, and enriched or purified heat shock proteins have been pursued as alternatives to whole cell vaccines. These materials should contain most of the antigens from tumor cells; however, there have been reports that lysates were less effective than inactivated whole tumor cells at generating antitumor immune responses [50].

Lysates from autologous tumor cells and allogeneic cell lines, with and without adjuvant, have been tested in clinical trials. The prototype lysate vaccine that is furthest along in development is Melacine®, a mixture of mechanical lysates from two allogeneic cell lines coadministered with the adjuvant DETOX [51]. The lysate contains the following melanoma antigens: gp100, the gangliosides GD2 and GD3, MART-1, MAGE-1, −2, −3, tyrosinase, TRP-1 and HMW-MAA. The adjuvant comprises bacterial cell wall skeleton and monophosphoryl lipid A. Melacine® has been under investigation since the early 1980's. It has modest antitumor activity, which may be enhanced by coadministration of interferon-alpha, in patients with metastatic melanoma [52]. This activity in patients with stage IV disease led the Southwest Oncology Group (SWOG) to perform a phase III observation controlled trial in patients with intermediate-thickness melanoma, 1.5-4.0 mm thick or Clark's level IV [53]. Six hundred eighty-nine patients were accrued; after a median follow-up of 5.6 years there has been no improvement in disease-free or overall survival among patients randomized to receive vaccine. However, among the 553 patients who had HLA typing performed, HLA-A2 and/or HLA-C3 positive patients who were vaccinated experienced a significant improvement in 5-year relapse-free survival (77% vs 63%, $p = .004$) [51]. These results suggest that HLA expression by the host may influence the efficacy of vaccine treatment. This may be due to direct effects on peptide presentation to T cells or indirectly by virtue of linkage to other polymorphic genes responsible for the actual vaccine benefit [51]. Further evaluation of Melacine® is planned in a much larger group of targeted patients who express HLA-A2 and/or HLA-C3.

Oncolysates prepared by infecting tumor cells with lytic viruses, such as Newcastle disease and vaccinia viruses, have been employed as cancer vaccines [54]. Oncolysates may elicit inflammatory responses against viral components that could augment antitumor immunity. Phase II studies in small numbers of patients with malignant

melanoma suggested an improvement in clinical outcome for patients vaccinated with oncolysates compared to historical controls. These preliminary results led to the performance of two randomized phase III clinical trials. Wallack et al. [55] compared vaccination with a vaccinia melanoma oncolysate prepared from four allogeneic cell lines to vaccinia injection alone in 217 patients with melanoma at high risk of recurrence following surgery. Hersey et al. [56] compared vaccination with a vaccinia oncolysate prepared from a single cell line to no immunotherapy in a similar group of 700 high-risk patients. Despite the promising results of the single arm studies, neither randomized trial demonstrated a statistically significant improvement in relapse-free or overall survival. Viral oncolysates have also been employed in the adjuvant setting for patients with completely resected colorectal cancer. Autologous colon cancer cells infected with Newcastle disease virus were used to treat 48 patients with completely resected colon cancer, [57]. The authors reported a significant improvement in 2-year survival compared to 661 historical controls. This soft clinical finding is similar to what has been observed in patients with melanoma and has not been confirmed in a controlled trial.

Another strategy employed by Bystryn and his colleagues at NYU, has been to construct a melanoma vaccine from antigens shed by tumor cells [58]. The rationale for this approach is that a polyvalent, partially purified vaccine that contains antigens expressed on the surface of tumor cells is likely to be biologically relevant. This approach is safe and results in antibody production and CD8 T-cell responses to a variety of MAA. In single arm studies this group observed improvements in recurrence-free and overall survival of vaccine-treated stage III patients compared to historical controls. This led to a double-blind, randomized, placebo-controlled trial in which despite the small number of patients (N = 38) there was a significant improvement in median time to disease progression for patients in the vaccine arm [58]. Confirmation of these results is needed before this treatment can be recommended in clinical practice.

Heat Shock Proteins (HSPs)

In an attempt to isolate immunogenic proteins from tumor cell homogenates (lysates) that can elicit protective antitumor immune responses, Srivastava surprisingly found that the key proteins were HSPs, ubiquitous proteins abundantly expressed in both normal and malignant cells (reviewed in [59]). HSPs purified from one tumor cell line were able to elicit antitumor responses that were specific for that tumor cell line only and HSPs from normal tissues failed to induce immune responses to any tumor cells. This enigma was resolved when HSPs were shown to be molecular chaperones that bound a large collection of peptides [60]. These peptides were proposed as the precursors to MHC-binding peptides [61]; however, currently the definitive proof is lacking and the precise process by which these peptides are transferred to MHC molecules in vivo is still not clear [62]. In addition, HSPs were to be able to activate host APCs via specific receptors, such CD91, LOX-1, and Toll receptors, and facilitate the cross-presentation of tumor antigens by host APCs [63–65]. Thus, HSPs have the unique ability to bridge innate and adaptive immunity, and can elicit

specific and potent antitumor responses in both prophylactic and therapeutic settings in multiple experimental tumor models [66].

Hundreds of patients with a variety of tumor types have been treated in phase I and phase II studies of autologous tumor-derived HSP-peptide complexes [67]. The requirement for autologous tumor limits the number of patients that can be treated, but vaccine preparation at a central laboratory facilitated the participation of multiple sites. Treatment was well tolerated and two complete responses were observed among 28 patients with advanced melanoma treated with HSP gp96-peptide complexes (Oncophage®; Antigenics, Inc.) [68]. The same group treated 29 consecutive patients who underwent potentially curative resection of colorectal liver metastases with autologous tumor-derived HSP gp96 [69]. A significant minority of patients in both studies produced a tumor-specific T-cell response following vaccination. There was a suggestion that production of a tumor-specific T-cell response correlated with clinical response in the melanoma study and disease-free and overall survival in the patients with colorectal cancer. Tumor-derived HSP-peptide complexes are currently being tested in two phase III trials—as adjuvant therapy compared to observation following nephrectomy in high-risk patients with renal cell cancer and as initial therapy compared to standard therapy (chemotherapy or immunotherapy) for patients with metastatic melanoma.

Exosomes

Exosomes are small membrane vesicles secreted by many different cell types as a consequence of fusion of multivesicular late endosomes/lysosomes with the plasma membrane [70]. APCs, such as B lymphocytes and dendritic cells, secrete MHC class-I- and class-II-carrying exosomes that stimulate T-cell proliferation in vitro [71]. Tumor-derived exosomes contain a rich source of antigens and HSP that can be transferred to DCs for cross-priming of T cells [72]. In addition, dendritic-cell-derived exosomes, when used as a cell-free vaccine, can eradicate established murine tumors [73].

Dendritic Cells

Dendritic cells initiate the T-cell response to many different antigens, including tumor antigens [74]. Because of their unique ability to activate naïve T cells, DC have been evaluated by many investigators. In mice, three major subsets have been identified; $CD11b^+$ *myeloid DC*, *lymphoid-like DC* that co-express CD8, and the IFN-α-producing *plasmacytoid DC* [75]. Similar subsets have been identified in humans. Studies of myeloid DC have been possible because large numbers of DC can be generated from GM-CSF-cultured $CD34^+$ hematopoietic progenitor cells in humans [76] and bone marrow-derived cells in mice [77]. Large numbers of plasmacytoid DC can also be produced from bone marrow cells following culture with Flt3 ligand [78]. Because both myeloid and lymphoid precursors can give rise to $CD8^+$ lymphoid-like DC, CD8 expression is not a reliable marker for lymphoid DC, but serves as a maturation marker [79]. Although mouse plasmacytoid DC can acquire CD8 expression after microbial stimulation, plasmacytoid DC are not the

precursor for $CD8^+$ DC found in lymphoid tissues in mice [80]. $CD8^+$ DC appear to be the primary APC for in vivo cross-presentation of cell-associated antigens (e.g. tumor cells, antigen-pulsed cells, cells infected with viruses and intracellular bacteria), soluble antigens, immune complexes and HSP [81]. Cross-presentation of antigens in the context of infection results in extensive T-cell activation and expansion, while cross-presentation in a steady state, without infection, results in premature T-cell activation and deletion [82]. Cross-presentation of antigens from growing or apoptotic tumor cells results in either tolerance, limited T-cell activation without tolerance, or priming [83–86]. The vastly different outcomes might be explained by the difference in tumor immunogenicity and level of antigen expression in the tumor models used for these studies. Cross-presentation of antigens by endogenous DCs has been demonstrated to be important for optimal T-cell expansion even when mature DCs were used as the vaccine [87]. Although the concept of cross-priming of T cells by host APC is well-accepted, its physiological significance and whether it can be manipulated for therapeutic intent in cancer immunotherapy are still hotly debated [4, 36]. Both GM-CSF-driven, monocyte-derived myeloid DCs and Flt3L-driven, CD34+ hemopoietic progenitor cell-derived lymphoid DCs have been used for clinical trials [88]. However, the optimal subset of DCs, and the best technique for the in vitro generation of DC to induce optimal antitumor immune responses in vivo has not been determined.

The number of clinical trials employing DCs has increased steadily in recent years. DC-based tumor vaccines have as many variations as tumor cell-based vaccines, including genetic modification of DC with cytokines, chemokines, cDNA encoding defined tumor antigens, mRNA, cRNA and genomic DNA from tumor cells, DC pulsed with recombinant tumor antigens, tumor lysates, apoptotic/necrotic cells and DC tumor fusion cells. There is also an extensive literature of peptide-pulsed DC. Only studies that employed DC in combination with undefined antigen preparations will be discussed further.

DC-Based Vaccines

Even when combined with bacterial adjuvants, direct immunization with tumor lysates was generally an inefficient method for T-cell priming. This may relate to rapid diffusion or insufficient uptake of tumor antigens by host APC. However, immunization with tumor lysate-pulsed DC greatly improved the efficacy of tumor lysates, underlining the importance of antigen presentation for efficient T-cell priming [89–91]. Presentation of exogenous proteins by APCs requires antigen acquisition, degradation, loading into MHC class I and II molecules and transport to the plasma membrane. There are at least three major routes by which DCs acquire and process exogenous antigens: receptor-mediated endocytosis, phagocytosis and macropinocytosis [92]. Accordingly, various approaches have been used to load tumor-derived antigens, including opsonized, apoptotic or necrotic whole tumor cells, exosomes, tumor lysates or HSPs enriched from tumor lysates. Soluble antigens, such as tumor lysates, are generally acquired via macropinocytosis, however,

receptor-mediated endocytosis may also be important for the acquisition of antigens included in the lysate, such as HSPs via CD91 and LOX-1 [64, 93], glycoproteins via C-type lectin receptors and lipoprotein via scavenger receptors [92].

Immature DC are more efficient than mature DC at antigen uptake; mature DC have reduced expression of antigen receptors and have down-modulated both macropinocytosis and phagocytosis. Stimulation of DC with TLR ligands leads to maturation with enhanced antigen processing and peptide-loading onto MHC molecules and transport of peptide/MHC complexes to the plasma membrane. A recent study suggests that the optimal conditions for MHC I and MHC II restricted antigen presentation are very different [94]. Antigen-loading before DC maturation is required for optimal CD4 T-cell activation, consistent with the notion that activated DC have a decreased ability to take-up exogenous antigens. Surprisingly, cross-presentation of antigen to CD8 T cells requires DC stimulation before antigen loading. Furthermore, only a subset of TLR ligands, those associated with viral infection (TLR3 and TLR9), were able to activate the MHC I restricted cross-presentation pathway [94].

CLINICAL STUDIES. There have been many trials of tumor lysate-pulsed DC in patients with advanced cancer [91, 95–100]. These trials include phase I studies in pediatric [91] or adult cancer patients [96], and other studies that combined components of phase I and II studies by employing different doses of DCs with various schedules in patients with a single disease entity [90, 95, 97, 98, 100]. Each study included fewer than 20 patients. Immature DCs produced by culturing peripheral blood mononuclear cells in GM-CSF and IL-4 were employed most commonly. They were cultured for 24 hours with a tumor lysate prepared by multiple freeze/thaw cycles before treatment. In other studies, TNFα [99] or a cocktail of TNFα, IL-6 and IL-1β were used to induce DC maturation [100]. Most investigators included KLH in the lysate, both as a reporter antigen and for its ability to provide help. Immunization was performed by a variety of routes e.g. intranodal, intradermal, subcutaneous or intravenous, and by a variety of schedules. Some investigators employed multiple routes of administration. None of the published studies have been powered to address the issue of efficacy; however, feasibility and safety have been demonstrated. Not all patients will have adequate tumor accessible for resection, but for those that do there was a high likelihood of generating suitable tumor lysate-pulsed DC for treatment. Except for mild fever with chills and arthralgia, and local injection site reactions, treatment was well tolerated. Occasional patients produced detectable serum autoantibodies, but none have been reported to be of clinical significance. The lack of side effects indicative of autoimmune reactions after immunization with a preparation so rich in self antigens may reflect the tight regulatory controls in patients, which could in turn explain the small number of responses that have been seen in each of these trials.

A summary of the immune findings reflects the difficulty in monitoring immune responses to undefined antigens. KLH responses can be demonstrated in the majority, but not in all of the patients to whom it was administered. DTH, ELISPOT and intracellular cytokine staining were performed to determine whether patients had

developed an immune response to autologous tumor antigens or peptides known to be expressed by their respective tumors. Immune responses were seen in some patients, but no correlation with clinical activity was detected. Complete responses were observed in patients with renal cell cancer [97], melanoma [90], fibrosarcoma [91] and cutaneous T-cell lymphoma [100].

Gene-Modified DC Vaccines

Gene-modified DCs can be used to increase T-cell activation. The genes that have been studied include GM-CSF [101], CD40L [102] and TNF [103], and T-cell growth factors, such as IL-7 [104], and IL-12 [105]. Gene modification of DC with the T-cell chemotactic chemokines, SLC [106] and lymphotactin [107], has also been shown to increase the efficacy of DCs pulsed with tumor-lysate or loaded with peptide. Interestingly, SLC gene-modified DCs were able to activate tumor-specific T cells without tumor involvement of draining lymph nodes, suggesting that the lymphoid neogenesis activity of SLC could induce a lymphoid-like structure in a vaccine site that enabled in situ T-cell priming [108].

DCs Transfected with Tumor Cell mRNA

Antigens from tumor cells can also be delivered into DC by transfection with mRNA extracted from tumor cells [109]. One great advantage of RNA-based vaccines is that RNA can be amplified after reverse transcription, providing an essentially endless supply for vaccination [110]. Tumor mRNA can even be prepared from single cells obtained by laser capture microdissection [111]. Autologous DCs transfected with mRNA for specific proteins (PSA and CEA) or total mRNA from tumor cells can stimulate T-cell responses, including CTL against specific antigens or against a broad array of unidentified tumor antigens [110]. Clinical trials using immature DCs transfected with PSA [112] and CEA-specific mRNA [113] have been performed, as well as a phase I study using total RNA in 15 patients with metastatic renal cell cancer [114]. This approach, which has been employed primarily by investigators at Duke University Medical Center is feasible and well tolerated. Tumor- or antigen-specific T cells have been induced in a majority of patients but there has been little evidence of antitumor activity in patients with established tumors.

DC/Tumor Fusion Hybrid Cells

Another approach to deliver tumor-derived proteins and mRNA into DC is to fuse tumor cells and DCs in a hybrid cell [115]. Hybrids can be created by chemical (PEG), electrofusion and biochemical (viral fusion proteins) technologies. PEG-mediated fusion is rather inefficient compared to electrofusion [116] or fusogenic membrane glycoprotein (FMG) mediated fusion [117]. Multiple vaccinations have been necessary to induce a strong antitumor immune response with fusion cells generated by PEG, whereas one immunization with hybrid cells generated by optimized electrofusion or FMG-mediated fusion was sufficient to induce a strong antitumor T-cell immune response that mediated rejection of established tumor in preclinical models [116, 117]. The difference in vaccine efficacy may be related to the fusion

efficiency of the different methods. This underscores the importance of optimization of fusion methods. Unfortunately, like most other vaccine strategies which lack standardization, it is not possible at this time to gauge the relative superiority of the different methods. Hybrid cells have been tested in clinical trials in patients with melanoma [118], renal cell cancer 119] and malignant glioma [102]. When reported, fusion efficiency was generally <20% using the PEG-based approach. Occasional tumor regressions were reported in patients with melanoma and glioma.

Summary

Our knowledge of the immune system and how it interacts with tumor cells continues to grow. With each advance in basic science comes a new opportunity to develop an effective treatment strategy. Many such opportunities have arisen in the past few decades and this chapter has attempted to describe how these new advances have been combined with a variety of undefined cellular antigen preparations in an attempt to develop effective cancer vaccines. None of the strategies described in this chapter have been sufficiently effective to become part of standard therapy. However, the approaches tested have generally been well-tolerated by patients with advanced cancer and the evidence of immunologic activity and examples of impressive clinical activity in a wide variety of malignancies, suggests that these strategies can be the building blocks upon which new advances are added and effective treatments developed.

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10. CANCER VACCINES IN COMBINATION WITH MULTIMODALITY THERAPY

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There has been a long-standing interest in manipulating the immune system for cancer treatment. Our evolving understanding of the fundamental principles of tumor immunology has accelerated the clinical development of cancer vaccines. A number of small cancer vaccine trials documented the induction of tumor-specific T cells in some patients, with some promising evidence of clinical response. Despite these encouraging early results, larger clinical trials testing cancer vaccines as a single intervention in patients with advanced cancers have been generally disappointing. Furthermore, while few trials have incorporated cancer vaccines directly into or in sequence with combined modality therapy, current data suggest that standard chemotherapy regimens can significantly curtail the vaccine-mediated induction of antitumor immunity. Here we review the development of granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting cell-based cancer vaccines in the context of these observations. Integrating these vaccines into standard cancer treatments should minimize the deleterious impact of tumor burden, and the negative influences of both pre-existing tumor-specific immune tolerance and the combined modality treatment regimens themselves. Moreover, carefully elucidating the pharmacodynamic interactions between traditional cancer treatment modalities and cancer vaccines in clinically relevant, preclinical models can identify novel combinatorial strategies with the potential for augmenting vaccine-activated, tumor-specific immunity. The scientific rationale underlying the development of future clinical trials should be based on both preclinical and clinical data in order to maximize the information gained,

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and to facilitate the development of the most effective vaccination regimens for incorporation into clinical practice.

INTRODUCTION

A significant focus of therapeutic cancer research over the last twenty years has been to develop the optimal combination of the traditional cancer treatment modalities, including surgery, radiation therapy, and drug-based therapies. Although optimal combined modality treatment can cure a significant percentage of leukemias and lymphomas, it is generally less effective against the solid tumors. The efficacy of combined modality therapy in solid malignancies is generally regarded to be primarily limited by a therapeutic drug resistance inherent to the tumor cell, and to a lesser extent by the collateral damage to normal tissues conferred by the imprecise specificity of radiation therapy and cytotoxic chemotherapy. This has led to increasing enthusiasm for the development of highly targeted treatment approaches that either disrupt the regulatory pathways that promote transformation and metastasis, or specifically deliver a therapeutic hit by targeting the drug to a structural phenotype specific to the cancer cell. Immunotherapy represents a unique biologically targeted treatment approach potentially capable of circumventing mechanisms of intrinsic therapeutic resistance by marshalling the patient's own immune system to reject the tumor. Intensive research both in applied biotechnology and molecular immunology has converged to facilitate the development of cancer vaccines as the fourth major cancer treatment modality, and a number of initial Phase II and III trials have suggested the promise of active immunization for cancer treatment (Table 1) (1–8). When combined with the three historical treatment strategies, tumor vaccines offer the added value of exquisite tumor specificity, minimal systemic toxicity, and a persistent and durable antitumor effect by virtue of immunologic memory. Despite these advantages, the efficacy of tumor vaccines is also potentially limited by the magnitude of the tumor burden, vigorous pre-established mechanisms of tumor-specific immune tolerance, and the potential antigenic plasticity of the tumor cells themselves. Here we focus on the development of GM-CSF-secreting cellular cancer vaccines, and review approaches for integrating these vaccines with the traditional treatment modalities of surgery, radiation, and drug-based therapies in additive or synergistic treatment regimens that maximize the efficacy and minimize the limitations of each individual therapeutic approach.

GENETICALLY MODIFIED GM-CSF-SECRETING TUMOR CELL VACCINES

Genetically modified granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting tumor vaccines represent an innovative cell-based cancer vaccine platform capable of activating immunity specific for solid tumors historically considered to be inherently nonimmunogenic. An early systematic analysis compared the ability of a variety of cytokines (including IL1, IL2, IL4, IL5, IL6, GM-CSF, IFNγ, and TNFα) delivered to poorly immunogenic B16F10 melanoma cells by retroviral transduction to effect tumor rejection (9). GM-CSF clearly stood out as the most potent inducer of an antitumor immune response capable of mediating tumor rejection in animals with small burdens of pre-established tumors (9). GM-CSF

Table 1. Summary of Recent Phase II and Phase III Trials of Cell-Based Cancer Vaccines

Vaccine	Patient Population	Phase	N	Intervention	Results
Hapten-modified autologous melanoma tumor cell Vaccine (ref 1)	Stage III/IV melanoma after regional lymphadenectomy	II	77	Vaccine	Improved 5 year survival in vaccinated patients > 50 years ($p = 0.011$), and who developed DTH > 5 mm ($p = 0.031$)
Allogeneic melanoma tumor cell vaccine (ref 2)	Metastatic melanoma after complete resection	II	77	Vaccine + BCG	Improved survival with the development of DTH and αTA90 IgM ($p < 0.0001$ for OS)
Sialyl-Tn-KLH + DETOX-B adjuvant (ref 3)	Metastatic breast cancer	II	23	Cy + Vaccine vs. Vaccine	Increased sialyl-Tn immune responses with Cy pretreatment
Autologous colon cancer tumor vaccine + BCG (ECOG 5283) (ref 4)	Stage II/III colon carcinoma after resection	III	412	Vaccine vs. Observation	No arm differences ($p = 0.73$ for OS); improved survival if vaccine site reaction > 1 cm ($p = 0.003$ for OS)
Allogeneic melanoma tumor cell vaccine (ref 5)	Metastatic melanoma after complete resection relapsed on vaccine therapy (trial in ref 16)	III	194	Vaccine vs. Vaccine + BCG	Improved survival in relapsed patients reinduced with more frequent vaccinations and more BCG ($p = 0.0178$)
Conjugated ganglioside (GM2—KLH) + QS-21 adjuvant (ref 6)	Stage IIB/III melanoma after resection	III	880	Vaccine vs. High dose IFNα2B	IFNα2B superior to vaccine ($p = 0.009$ for OS)
Allogeneic melanoma tumor cell vaccine (ref 7, 8)	Stage IB/IIA melanoma after resection	III	689	Vaccine vs. Observation	No arm differences; Improved survival in HLA-A2 and HLA-A3 vaccinees ($p = 0.004$)

Abbreviations: DTH, delayed type hypersensitivity; BCG, Bacille Calmette-Guerin; KLH, keyhole limpet hemocyanin; IFNα2B, interferon-α; OS, overall survival; RFS, relapse-free survival.

secretion at the vaccine inoculation site recruits and activates dendritic cells to take up and process antigen delivered by the tumor vaccine cells, crosspriming an effective antigen-specific immune response dependent on $CD4^+$ T helper type 1 and 2 cells as well as $CD8^+$ cytotoxic T cells (10). Dose-finding studies have suggested that increasing the number of tumor cells delivered, and thus the quantity of tumor antigen, augments the induction of antitumor immunity (11). The concentration of GM-CSF in relation to tumor cells is also important, with a minimum of 35 ng of GM-CSF/10^6 cells/24 hours for 4 to 5 days after inoculation *in vivo* required for the effective induction of antitumor immunity (11). The paracrine secretion of GM-CSF induces cellular infiltrates at the inoculation site that consist of predominantly macrophages, dendritic cells, and eosinophils up to three days after vaccination, evolving to mature lymphocytes and eosinophils at day seven (12, 13). These latter three observations thus establish the baseline parameters for designing and evaluating clinical trials testing GM-CSF secreting cancer vaccines alone and combined with other cancer treatment modalities.

Table 2. A summary of GM-CSF-secreting cancer vaccine trials reported to date. The majority of trials tested an autologous tumor cell vaccine platform. The 96 patient prostate cancer vaccine trial conducted by Simons et al and the 14 patient pancreatic cancer vaccine trial conducted by Jaffee et al both tested allogeneic tumor cell vaccine platforms. The induction of antitumor immunity was typically measured by the induction of delayed type hypersensitivity (DTH) to autologous tumor cells. The induction of DTH has not been reported for the 96 patient study of Simons et al, or the study of Neumanitis et al. The interim report of Neumanitis et al included only the first 12 patients of a planned 80 patient study. Reported clinical antitumor responses have been 25% or less in all of the reported trials. NR = not reported; *Interim analysis reported one partial response and one complete response.

Disease	Vaccine Platform	Number of Treated Patients	Intervention	Antitumor Immunity	Evidence of Clinical Antitumor Response	Reference
metastatic renal cell carcinoma	autologous	7	vaccine	yes	yes	12
metastatic melanoma	autologous	29	vaccine	yes	yes	14
metastatic prostate carcinoma	autologous	8	vaccine	yes	NR	15
metastatic prostate cancer	allogeneic	96	vaccine	NR	*	16
early and advanced NSCLC	autologous	12	vaccine	NR	yes	17
surgically debulked Stage II/III pancreatic carcinoma	allogeneic	14	vaccine in sequence with adjuvant chemoradiation	yes	yes	13

Promising results in preclinical studies prompted the clinical testing of both autologous and allogeneic GM-CSF-secreting tumor vaccines as a single therapeutic intervention in patients with metastatic renal cell carcinoma, metastatic melanoma, metastatic prostate carcinoma, and early or advanced non-small cell carcinoma of the lung (Table 2) (12–17). These initial trials established the safety of the vaccination strategy, and provided clinical validation of the relevance of antigen dose, level of GM-CSF secretion, and the cellular infiltrates at the vaccination site. The most recent clinical trials of GM-CSF-secreting cancer vaccines have tested treatment regimens that integrate these tumor vaccines into standard treatment modalities. The first reported clinical trial of GM-CSF-secreting tumor vaccines integrated with combined modality therapy for the treatment of pancreatic cancer was conducted by Jaffee and colleagues (13). They conducted a dose escalation trial testing a mixture of two allogeneic pancreatic carcinoma cell lines secreting GM-CSF at levels of 220 ng/10^6 cells/24 hours in patients with Stage II or Stage III pancreatic cancer after pancreaticoduodenectomy. Patients were initially vaccinated immediately after surgery and just prior to adjuvant chemoradiation. Those who remained disease-free after six months of aggressive adjuvant chemoradiation were eligible to receive an additional three monthly vaccinations. This study identified 5×10^8 vaccine cells (the highest dose tested) as a safe and bioactive vaccine cell dose for testing in future trials. Four of five patients who received this dose developed serum levels of GM-CSF that recapitulated the pharmacokinetics observed in preclinical models. No patient treated at lower vaccine dose levels developed detectable levels of serum GM-CSF. Clinically

significant delayed type hypersensitivity (DTH) responses ($\geq$1.0 cm) to dissociated autologous pancreatic tumor cells developed after the first vaccination in 1 of 3 and 2 of 4 patients who received 1×10^8 and 5×10^8 vaccine cells respectively. These 3 patients remained free of disease for over 2 years, suggesting a potential survival benefit. A Phase II trial of similar design to evaluate the efficacy of the integrated treatment regimen is now in active accrual. In the aggregate, these trials have demonstrated the safety of GM-CSF-secreting vaccines, with side effects limited primarily to local erythema and induration at the inoculation sites. While they have provided encouraging preliminary evidence of bioactivity, they have also more clearly defined the challenges facing the effective clinical development of cancer vaccines. These include the hurdle posed by established tumor burdens, the barrier of pre-existing tumor-specific immune tolerance, the potential plasticity of the antigenic profile displayed by the tumor as it continues to evolve, and the impact of standard cancer therapy on vaccine-activated immunity. As discussed below, integrating tumor vaccines appropriately with traditional treatment modalities can potentially surmount each of these obstacles to the efficacy of tumor immunotherapy, thereby maximizing the antitumor immune response.

CHALLENGES IN CANCER VACCINE DEVELOPMENT

The magnitude of the tumor burden is the first significant influence on the development of antitumor immunity (18). When tumors are small, they grow without accessing peripheral lymphoid tissues and are essentially ignored by the immune system. The tumor thus "sneaks through" immune surveillance at its earliest stages (18). Once it has reached sufficient size, the tumor invades local lymphoid tissue. This initiates an interaction with the immune system relatively late in tumor development. At this stage immune-mediated tumor rejection is determined by the relative balance between the growth kinetics and physical burden of the tumor cells compared to the intensity and diversity of the induced effector T cell response (19, 20). Superimposed on this imbalance, tumor cells employ several strategies to evade the developing immune response. Tumor cells can elaborate inhibitory cytokines (interleukin 10, transforming growth factor-β(TGF-β), and prostaglandin E_2) that inhibit the function of tumor infiltrating T lymphocytes (TIL) (21). They can also express surface fasL (CD95L), thus inducing the apoptosis of TIL engaged by the tumor cells (21).

Tumor-specific immune tolerance represents the second major barrier to the effectiveness of cancer vaccines (reviewed in 22). Vaccination for the prevention of infectious diseases sets the stage for a vigorous antigen-specific immune response capable of rejecting an exogenous infectious challenge. In contrast, tumor cells arise endogenously. Thus, with the exception of *de novo* genetic mutations, most tumor antigens are recognized as self. This typically results in either the central thymic deletion of those components of the T cell repertoire with the highest affinity for tumor-specific antigens, or their peripheral deletion by activation-induced cell death (AICD) in the setting of widely disseminated tumor (22). However, the process of thymic selection is imperfect, and allows the emigration of T cells that recognize self

antigen with low avidity or by virtue of specificity for a cryptic antigenic epitope not commonly seen by the immune system (23). This latent population of T cells may be activated under the appropriate conditions to exert potent antigen-specific immunity (24). Additionally, tumor cells do not evolve to present antigen effectively, and are typically detected by the immune system in the absence of an associated inflammatory response. These two factors lead to the presentation of tumor antigens in the absence of the costimulatory signals critical for immune activation, thereby rendering potentially responsive T cells unresponsive or anergic (22). Further, the compartmentalization of tumor antigens away from the immune system either by expression restricted to embryologic development or immunologically privileged sites, or by extremely low levels of antigen expression, can result in the peaceful coexistence of tumor and tumor-specific T cells that simply fail to see their target (22). If T cells are activated, a phenotypically skewed cytokine/chemokine receptor profile can render them functionally impotent by virtue of cytokine deviation and aberrant trafficking (22). Finally, novel subsets of dendritic cells and regulatory T cells have recently been characterized (22). These can downregulate the antigen-specific immune response, resulting in immune tolerance rather than immune activation. Thoroughly dissecting the impact of these diverse mechanisms of immune tolerance on the tumor-specific T cell repertoire available for therapeutic manipulation should facilitate the development of innovative combinatorial vaccination strategies for overcoming them.

The third challenge for effective tumor immunotherapy is the diversity and plasticity of the antigen expression profile of tumors themselves. Tumors can down-regulate the expression of tumor antigens targeted by an antigen-specific vaccine or therapeutic antibody, resulting in the outgrowth of antigen loss variants resistant to the therapeutic intervention (25, 26). The use of highly defined antigen-specific cancer vaccines is further limited by the fact that only a few tumor antigens are likely to represent true tumor rejection targets. Tumor rejection antigens are defined as antigens preferentially associated with a cancer cell that elicit an effective immune response capable of causing clinically meaningful tumor regression (27). The distinction between tumor antigens capable of eliciting an immune response and those that elicit an immune response that translates into a clinical response are well illustrated by studies of the natural and vaccine-induced immune responses in melanoma patients (28–30). Many patients have significant numbers of functional cytotoxic effector T cells specific for the melanoma antigens MART-1/Melan-A or gp100, but their disease continues to progress. Moreover, the numbers of antigen-specific T cells in these patients can be augmented with targeted vaccines, but this does not influence the immunodynamics of the antitumor response in a clinically meaningful way (28, 29, 31–33). Adding further complexity to the host-tumor interaction, tumors can also down-regulate multiple components of the antigen-processing machinery, including MHC Class I and Class II molecules, different proteosome subunits, and the TAP transporter (21). Importantly, these altered antigen-processing phenotypes have been found to correlate with poor clinical outcome (34).

These fundamental principles of tumor immunology can be combined with the lessons of traditional drug development to guide both the preclinical and clinical development of cancer vaccines. It is clear from the principles discussed above and the results of clinical cancer vaccine trials to date that cancer vaccines as a single treatment modality are not likely to have the potency required to overcome immune tolerance and surmount the tumor burden present in patients with established disease. Moreover, traditional drug development typically calls for early clinical testing in heavily pretreated patients with extensive disease. Both a greater number of prior chemotherapy regimens and close proximity to a prior chemotherapy treatment was recently demonstrated to limit the induction of carcinoembryonic antigen (CEA)-specific T cell prescursors in patients with advanced colorectal carcinoma treated with the canary pox vaccine ALVAC-CEA (35). Additionally, the serial ELISPOT analysis of post-vaccination, antigen-specific $CD8^+$ T cell responses of patients treated on the Phase I trial testing the integration of a GM-CSF-secreting pancreatic cancer vaccine with adjuvant chemoradiation also demonstrated the detrimental effect of close proximity to a prior chemotherapy treatment on vaccine-induced immune responses (Thomas, AM and Jaffee, EM, unpublished data). The mismatch between tumor growth kinetics and the intensity of the vaccine-induced antitumor response achievable with current vaccination regimens is a strong argument for testing vaccine therapy in patients with minimal or undetectable disease after standard therapy (19, 20). However, our own experience and the results of the ALVAC-CEA trial suggest that the potential negative impact of some standard treatment modalities on the potency of cancer vaccines must also be considered. The scientifically based sequencing of tumor vaccines with surgery, radiation therapy, chemotherapy, and biologically targeted therapy is thus a critical aspect of clinical cancer vaccine development that should be determined in relevant preclinical models when possible.

THE IMMUNOMODULATORY EFFECTS OF CHEMOTHERAPY

In cancer treatment, chemotherapy agents have been historically used for their direct cytotoxic activity on tumor cells. However, novel approaches to the use of chemotherapy have revealed that some drugs also have dose- and sequence-specific antiangiogenic or immunomodulatory effects (36–38). The frequent administration of Cyclophosphamide, Paclitaxel, Doxorubicin, or Vinblastine at very low doses (so-called metronomic administration) preferentially targets the tumor vasculature compared to tumor cells (37). Additionally, many chemotherapeutic agents (including Cyclophosphamide, the taxanes (Paclitaxel and Docetaxel), Doxorubicin, Melphalan, Gemcitabine and 5′-aza-2′-Deoxycytidine) can either potentiate or antagonize an antigen-specific immune response depending on the drug dose and timing in relation to an antigen exposure (Table 3). Here we review the immunomodulatory activities of these drugs in the framework of their impact on the antigen-specific immune response, existing mechanisms of tumor-specific immune tolerance, the tumor microenvironment, and tumor antigen expression profile of the tumor itself.

Table 3. The immunomodulatory effects of some chemotherapeutic and biologic agents

	Tumor Cell	Innate Immune Effectors	APC	$CD4^+$T Cell	$CD8^+$T Cell	B Cell
Cyclophosphamide				+	+	+
Doxorubicin	+	+	+		+	
Paclitaxel	+	+	+	+		
Melphalan	+			+	+	
Gemcitabine	+			−	−	−
5-aza-2′-Deoxycytidine	+					
Trastuzumab	+	+	+		+	
Rituximab	+	+	+			−

Chemotherapy and the Adaptive Immune Response

At standard doses, many chemotherapeutic drugs clearly suppress cellular immune responses; these include Cyclophosphamide, Paclitaxel, Gemcitabine, and Doxorubicin. Cyclophosphamide in particular has been widely used for its immunosuppressive effects in the treatment of autoimmune disease. Importantly, the timing of administration in relation to drug exposure is a critical determinant of the ensuing immune response (39). The humoral response to an antigenic challenge is markedly enhanced if Cyclophosphamide is given one to three days prior to antigen exposure (39). Similarly, treatment of animals with Doxorubicin three to five days prior to antigen exposure enhances the induction of adaptive cell-mediated immunity, likely by modulating cells of the monocyte/macrophage lineage (39). In contrast, administering Cyclophosphamide at the time of antigen exposure abrogates the primary antigen-specific humoral immune response in guinea pigs exposed to ovalbumin (40). Re-challenge of these same animals three months later again failed to induce an antigen-specific antibody response, although the animals did develop significant antibody titers to an unrelated antigen (41). This observation suggests that the simultaneous administration of Cyclophosphamide with antigen induces antigen-specific immune tolerance. Cyclophosphamide has a similar impact on the induction of contact hypersensitivity, a manifestation of cellular immunity.

In addition to modulating the induction of immunity to new antigens, Cyclophosphamide can also break both natural and acquired immune tolerance. For example, animals challenged with either syngeneic or autologous testicular cells develop significant delayed type hypersensitivity (DTH) reactions consistent with adaptive cellular immunity only if the challenge is preceded by Cyclophosphamide administration (42). Similarly, the treatment of mice with palpable MOPC-315 plasmacytomas initiates immune-mediated tumor rejection in 92% of animals, with cured mice retaining the ability to reject a subsequent MOPC-315 tumor challenge (43). The effect of Cyclophosphamide was abrogated by prior immunosuppression (44), and markedly diminished in mice with nonpalpable tumor burdens (a tumor rejection rate of 10%) (43), arguing for the importance of pre-existing antitumor immunity in

tumor rejection. Cyclophosphamide, Paclitaxel, and Melphalan can also promote a therapeutic balance between T helper type 1 and T helper type 2 lymphocytes (45, 46). As discussed below, we have found that both Cyclophosphamide and Paclitaxel treatment one day prior to an antigen-specific vaccination can reverse immunologic skew, favoring the development of antigen-specific T helper type 1 antitumor immunity capable of orchestrating the delay of tumor outgrowth in a tolerogenic murine model of breast cancer (46). Cyclophosphamide has also been reported to upregulate type I interferons, an observation that has been associated with the development of T helper type 1 immunity and ultimately correlated with an augmented production of $CD44^{high}$ memory T lymphocytes in the treated mice (47). Cyclophosphamide is also thought to abrogate regulatory T cell activity, although most of this data was generated prior to the resurgence of interest in suppressor T cell populations as they are currently defined (39).

The impact of Gemcitabine on adaptive immune responses is perhaps the least studied. Importantly, it has been reported to abrogate the humoral antigen-specific immune response. The administration of five doses of gemcitabine (120 μg/g every three days) to hemagglutinin (HA) T cell receptor transgenic mice completely abrogates HA-specific IgG responses in the context of a minimal to moderate augmentation of HA-specific T cell proliferation (48). We have found that Gemcitabine potently inhibits vaccine-induced T cell immunity (Jaffee EM, unpublished data). Gemcitabine may thus be a chemotherapeutic agent to avoid in combinatorial cancer vaccination regimens.

In summary, multiple chemotherapeutic agents significantly impact the adaptive immune response. Whether the influence is positive or negative depends largely on the agent under consideration, the dose given, and the timing of administration of the drug in relation to antigen exposure. These observations taken together argue for the careful pharmacodynamic analysis of cancer vaccines and chemotherapeutic agents in clinically relevant preclinical models rather than the simple addition of a vaccine to a treatment regimen considered to be the standard of care.

Chemotherapy and the Tumor Microenvironment

Chemotherapy can also modulate the tumor microenvironment, either discouraging or promoting the development of an effective antitumor immune response. The preferential antiangiogenic effect of metronomic chemotherapy could physically disrupt access to the tumor by the immune system, thus abrogating the efficacy of existing tumor-specific immunity. Furthermore, when integrated directly with active, specific immunotherapy, the metronomic scheduling is likely to inhibit the induction of antitumor immunity due to sequencing effects. Alternatively, chemotherapy can augment tumor-specific immune responses by inhibiting the secretion of immunosuppressive cytokines by the tumor, or by upregulating tumor cell expression of costimulatory molecules for effective antigen presentation. For example, Bleomycin has been shown to inhibit the production of tumor-derived TGF-β, thus partially abrogating the negative effect of the tumor cells themselves in the developing immune response (49). Melphalan can induce the secretion of tumor necrosis

factor-α (TNF-α) by tumor cells, thereby facilitating the development of a cytotoxic $CD8^+$ T cell response (50). The mechanism of TNF-α upregulation was more recently characterized, and was found to be dependent on the early production of interferon-β(51). This is reminiscent of the influence of Cyclophosphamide on the type I interferons discussed above. It is potentially important given the promotion of the T helper type I cytokine response by the type I interferons that is known to be critical for an effective antitumor response. Melphalan also upregulates the expression of both B7-1 and B7-2 in MOPC-315 tumor cells and host cells; a similar effect was demonstrated in P815 plasmacytoma cells (52, 53). Importantly, both Mitomycin C and γ-irradiation can also upregulate B7-1 (53). This suggests that some chemotherapeutic agents and/or radiation therapy could potentiate the induction of antitumor immunity by upregulating critical costimulatory molecules, thereby rendering the tumor cells themselves more effective antigen presenting cells.

Other cytotoxic drugs (Doxorubicin, 5-Fluorouracil, Gemcitabine, and Paclitaxel) and γ-irradiation can modify the tumor microenvironment by inducing tumor cell apoptosis (54, 55), potentially enhancing antigen presentation. Importantly, several groups have successfully combined apoptosis-inducing chemotherapy or γ-irradiation with intratumoral dendritic cell administration to induce effective antitumor immunity (56–58). Paclitaxel, which is known to upregulate proapoptotic molecules (59, 60) and phosphorylate bcl-2 (61), is of particular interest. Clinically, the degree of induction of apoptosis and mitotic arrest after one cycle of neoadjuvant Paclitaxel treatment was demonstrated to predict therapeutic response in women with locally advanced breast cancer (62). Further, the first dose apoptotic response correlated with the development of TIL in 67% of patients with clinical complete responses and pathologic residual disease (62). In contrast, only 25% of patients with a clinical partial response developed TIL (62). In addition to its potent apoptotic effects, Paclitaxel also has a variety of immunomodulatory activities (63). Paclitaxel is known to efficiently mobilize peripheral blood stem cells, likely through inducing GM-CSF secretion by macrophages and B cells (63). Thus, Paclitaxel may increase antigen presenting cells and cytotoxic T cells for participation in the induction of antitumor immunity, while simultaneously creating a potent source of tumor antigen in the form of apoptotic bodies produced due to the cytotoxic effect of the drug. Importantly, vigorous apoptosis itself can induce dendritic cell maturation (64). This process could be further facilitated by the lipopolysaccharide-mimetic effect of Paclitaxel, which results in the secretion of the proinflammatory factors interleukin 1β (IL1β), GM-CSF, TNFα, nitric oxide (NO), and interleukin 12 (IL12) (63).

The demethylating agents represent a novel class of chemotherapeutic drugs that exert an antitumor effect by promoting cellular differentiation (65). The drugs function by facilitating the re-expression of genes under active transcriptional repression as the result of methylation of important transcriptional regulatory regions. Importantly, restoring the expression of MHC Class I and cancer testis antigens by pretreating tumor cells with the demethylating agent 5′-aza-2′-Deoxycytidine *in vitro* can restore melanoma- and renal cell carcinoma-specific CTL activity (66, 67). These

results suggest that treatment of tumors *in vivo* with demethylating agents could circumvent the development of MHC Class I and antigen loss variants that sometimes underlie the failure of antigen-specific immunotherapy.

GM-CSF-SECRETING CANCER VACCINES AND MULTIMODALITY THERAPY

Given the cytoreductive and immunomodulatory potential of many anticancer drugs, the integration of these agents and GM-CSF-secreting vaccines for the treatment of locally advanced or metastatic cancer holds great appeal. Chemotherapeutic agents are commonly used for their cytotoxic effects, and are clearly immunosuppressive at standard doses. However, some can also either augment or reduce antigen-specific immune responses, depending on the drug dose and timing of administration in relation to the antigen exposure (38). Furthermore, emerging data suggests that therapeutic monoclonal antibodies could synergize with active vaccination by recruiting innate immune effectors (68). Two areas that clearly warrant further investigation are thus the integration of GM-CSF-secreting vaccines with traditional chemotherapy, and the integration of these vaccines with therapeutic, monoclonal antibody-based therapy.

Chemotherapy as a Vaccine Adjuvant for GM-CSF-secreting Vaccines

The sequencing of chemotherapy and GM-CSF-secreting tumor vaccines can be considered in the context of chemotherapy as a vaccine adjuvant, or in the context of the altered host environment created by autologous or allogeneic bone marrow transplantation and other types of lymphoablative therapy. The use of low to standard dose chemotherapy as a vaccine adjuvant has been examined in two preclinical models (46, 69). In the first, a variety of chemotherapeutic agents was tested in sequence with a GM-CSF-secreting CT26 colon carcinoma whole cell vaccine in BALB/c mice (69). In this nontolerogenic system, Cyclophosphamide (50–250 mg/kg) given one or two weeks prior to vaccination failed to increase the induction of CT26-specific $CD8^+$ cytotoxic T cells, whereas the drug given at the time of or subsequent to vaccination abrogated vaccine activity. Conversely, Doxorubicin (2–6 mg/kg) given one week prior to vaccine prevented immune induction, whereas similar doses given at the time of or subsequent to vaccination augmented CT26-specific $CD8^+$ T cell immunity. Vaccination followed by Doxorubicin treatment protected mice from a lethal CT26 tumor challenge, and cured 40% of mice with established tumor burdens. Doxorubicin alone cured 10% of such mice, whereas Cyclophosphamide plus vaccination or vaccination alone cured between 30–35% of tumor-bearing mice. Other chemotherapeutic agents tested in this system, including Vincristine, Vinblastine, Etoposide, Methotrexate, 5-Fluorouracil, Cytarabine, Cisplatinum, and Dexamethasone clearly reduced vaccine efficacy.

Because the CT26 model system is not characterized by antigen-specific immune tolerance, we extended these studies to the *neu* transgenic mouse (46). These mice are one of the most clinically relevant preclinical models for evaluating combinatorial immunotherapy regimens. Due to MMTV-driven expression of the protooncogene *neu*, the mice spontaneously develop *neu*-expressing breast cancers histologically

similar to those of patients (70), and have a pre-established *neu*-specific immune tolerance (71). The impact of *neu*-specific immune tolerance on vaccine-activated immunity is profound. Whereas parental FVB/N mice vigorously reject *neu*-expressing tumors in response to *neu*-targeted, GM-CSF-secreting vaccination, *neu* transgenic mice have at best a tepid antitumor response to the vaccine (71). In fact, tumor outgrowth rates are similar in *neu* mice who receive a mock vaccine and those who receive a *neu*-targeted, GM-CSF-secreting vaccine. Importantly, giving the vaccine in sequence with some chemotherapeutic drugs can partially overcome tolerance, and enhance vaccine efficacy in *neu* mice (46). Both parental FVB/N mice and tolerized *neu* transgenic mice demonstrate a more robust tumor rejection response to a *neu*-targeted, GM-CSF-secreting cellular vaccine preceded by either Cyclophosphamide (100 mg/kg) or Paclitaxel (20 mg/kg) one day prior to vaccination compared to vaccine alone. Similar to observations in the CT26 system, sequencing the vaccine with Doxorubicin (5–8 mg/kg) seven days later also resulted in an enhanced antitumor response, but only in the *neu* mice. Underscoring the importance of drug dose, the positive interaction between drug and vaccine diminished with nadiring T cell counts. Recapitulating previous observations, reversing the order of Cyclophosphamide, Paclitaxel, or Doxorubicin inhibited vaccine activity. Cisplatinum was not observed to have a positive interaction with the vaccine in *neu* transgenic mice. The activity of the drugs as a vaccine adjuvant was suggested by their ability to enhance the rejection of a subsequent tumor challenge, and confirmed by the documentation of augmented *neu*-specific T helper type 1 cellular responses by ELISPOT with combinatorial therapy compared with vaccine alone. A combination chemoimmunotherapy regimen including specifically timed Cyclophosphamide, vaccine, and Doxorubicin demonstrated the greatest potency, curing pre-existing tumor burdens in up to 30% of tolerized mice. A Phase I clinical trial testing timed sequential therapy with Cyclophosphamide, Doxorubicin, and a GM-CSF-secreting allogeneic cell-based breast cancer vaccine will explore the safety and bioactivity of this approach in patients with metastatic breast cancer. Additional trials testing GM-CSF-secreting vaccines preceded by Cyclophosphamide in patients with metastatic pancreatic cancer or metastatic non-small cell lung cancer are also planned.

GM-CSF-Secreting Vaccines and the Lymphocyte-Depleted Host

Lymphopenia-induced homeostatic T cell proliferation is a recently described mechanism for restoring the memory T cell compartment (72, 73). Manipulating the T cell repertoire with cancer vaccines during immune reconstitution after lymphoablative treatments might skew the immune system toward a desired antitumor specificity (74). Consistent with this concept, the selective induction and expansion of functional melanoma-specific T cells was documented in Rag-1-deficient, lymphopenic, tumor-bearing mice in response to a GM-CSF-secreting melanoma vaccine (75). The presence of this altered T cell repertoire correlated with significant tumor regression. Highlighting the potential clinical relevance of this phenomenon, several preclinical studies have demonstrated that vaccine-induced antitumor immunity

can be enhanced by vaccinating tumor-bearing mice with GM-CSF-secreting tumor vaccines during early engraftment after syngeneic or allogeneic T cell-depleted bone marrow transplantation (76, 77). Furthermore, altering the host microenvironment prior to the transfer of syngeneic tumor-specific T cells with sublethal irradiation in mice can result in an effective antitumor immune response as measured by CTL activity, IFN-γ secretion, and the persistence of memory T cells (78). The most recent study reported the efficacy of combined modality chemoimmunotherapy in the chemotherapy–resistant 4T1 model of metastatic breast cancer (79). Tumor-bearing animals underwent surgical resection, then treatment with a nonmyeloablative allogeneic stem cell transplantation where the conditioning regimen included irradiation and Cyclophosphamide. Subsequent treatment of animals with post-transplant donor lymphocyte infusions (DLI) and a tumor vaccine comprised of autologous tumor cells admixed with GM-CSF-secreting bystander cells resulted in potent systemic antitumor immunity capable of curing mice of metastatic mammary tumors. Similar results have been observed with the adoptive transfer of melanoma-specific TIL to patients with metastatic melanoma pretreated with a nonmyeloablative chemotherapy regimen consisting of Cyclophosphamide and Fludarabine (80). While the phenomenon of homeostatic proliferation has not yet been rigorously demonstrated in patients, this study suggests that it could be clinically relevant. Since many standard cancer therapies result in lymphopenia, carefully delineating the influence of radiation therapy and/or chemotherapy on the kinetics, persistence, and functional quality of antigen-specific immune reconstitution will be required for the effective application of cancer vaccines to the lymphopenic setting. Clinical trials evaluating the bioactivity of GM-CSF-secreting vaccines administered in the context of autologous or allogeneic bone marrow transplantation in multiple myeloma and acute myelogenous leukemia are currently underway.

GM-CSF-Secreting Vaccines and Therapeutic Monoclonal Antibodies

Combining GM-CSF-secreting vaccines with monoclonal antibody therapy is another promising area for research. Trastuzumab and Rituximab are two monoclonal antibodies that play clear roles in the management of breast cancers (81–83) and B cell lymphomas (84), respectively; a number of additional therapeutic monoclonal antibodies are currently in active clinical development (85). There is emerging data to suggest that humoral immunity may play an important role in antitumor immunity that has not been previously appreciated (86–91). For example, we demonstrated that the passive transfer of HER-2/*neu*-specific antibody and HER-2/*neu*-specific CTL results in a more robust antitumor effect than the passive transfer of either alone in the tolerogenic *neu* mouse model of breast cancer (88). Furthermore, we have also demonstrated that the combination of HER-2/*neu*-specific monoclonal antibodies and a GM-CSF-secreting HER-2/*neu*-targeted vaccine is more effective than either vaccine or monoclonal antibody alone in *neu* transgenic mice (92). Consistent with these observations, others have shown that humoral and cellular immunity can synergize to mediate the rejection of established lymphomas through a process dependent on $CD8^+$ T cells and $CD11b^+$, Fc-γ receptor-expressing macrophages (86, 87).

A number of mechanisms for synergism between humoral and cellular immune mediators have been suggested. Trastuzumab can exert an antitumor effect by inhibiting growth-promoting signaling pathways (93), and could render the tumor cell more sensitive to apoptosis (94). Like Rituximab, it can also recruit innate immune effectors by functioning as a nidus for the initiation of antibody-dependent cellular cytotoxicity (ADCC) (68). Furthermore, Trastuzumab has been recently demonstrated to enhance the lytic activity of MHC Class I restricted, HER-2/*neu*-specific CTL against HER-2-overexpressing breast and ovarian tumor cells (95). It was proposed that degradation of internalized HER-2/*neu* protein could increase the amount of HER-2/*neu* peptide epitopes available for loading onto MHC Class I molecules, thereby augmenting antigen presentation. Although not directly proven, support for this mechanism is provided by observations that Trastuzumab promotes the ubiquitination and degradation of HER-2/*neu* (96). Geldanamycin, an ansamycin antibiotic that also induces the ubiquitination and degradation of HER-2/*neu,* was demonstrated to augment the presentation of HER-2/*neu*-specific peptide epitopes by ovarian carcinoma cells, thereby enhancing the activity of antigen-specific CTL against treated HER-2/*neu*-overexpressing ovarian carcinoma targets (97). While recruiting innate immune effectors and augmenting antigen presentation are at least two potential mechanisms for the positive interplay between therapeutic monoclonal antibodies and cancer vaccines, potential also exists for antagonism. Rituximab can inhibit the induction of primary and secondary humoral immunity (98, 99), and can also abrogate the manifestation of antibody-mediated diseases (100) by eliminating both normal and malignant B cells responsible for antibody production. Thus, the integration of cancer vaccines with Rituximab might not be optimal when humoral immunity is thought to play an important role in tumor rejection. Regardless, these observations together demonstrate that integrating cancer vaccines with therapeutic monoclonal antibodies is an area that clearly warrants further research.

CONCLUSIONS AND FUTURE DIRECTIONS

Improvements in our understanding of tumor immunology have facilitated significant progress in the development of cancer vaccines. Early clinical trials have generated evidence for the safety of tumor vaccines, and have provided a suggestion of clinically significant bioactivity. They have also highlighted the challenges of cancer vaccine development. These include developing strategies for overcoming immune tolerance, and approaches for identifying the most active tumor rejection antigens for cancer vaccine formulation. Furthermore, these early studies highlight the importance of identifying important pharmacodynamic interactions between standard cancer treatment modalities and tumor vaccines. Surgical debulking is one approach for minimizing the impact of tumor burden, and patients with minimal residual disease are likely to be the most ideal candidates for vaccine therapy. The impact of chemotherapy on vaccine activity is a developing area of clinical research, with regard to both its positive and negative impact on the development of antigen-specific immunity. The impact of ionizing radiation on the immune response to cancer vaccines is an underdeveloped area that also warrants further investigation.

Finally, the advent of biologically targeted therapies such as the monoclonal antibodies Trastuzumab and Rituximab offer new opportunities for combining cancer vaccines with novel drugs in combinatorial treatment strategies with the potential for significant synergism. It is clear that the careful preclinical and clinical investigation of these issues will guide the most effective clinical testing of cancer vaccines, and facilitate their ultimate incorporation into standard clinical practice.

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III. VACCINE-ENHANCING STRATEGIES

11. CYTOKINE THERAPY FOR CANCER: ANTIGEN PRESENTATION

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What is the role of the immune system in cancer? The link between the two has long been apparent, as is illustrated by the high incidence of certain cancers such as Kaposi's sarcoma or lymphoma in individuals with the Acquired Immunodeficiency Syndrome (AIDS). It has been hypothesized that deficits in immune surveillance must therefore permit tumor cell growth under such circumstances. This chapter discusses the role of cytokines in the immune recognition of tumor cells.

INTRODUCTION

The host response to infection or malignant transformation is composed of the concerted actions of the innate and adaptive arms of the immune system. The cellular components of innate immunity are characterized by natural killer (NK) and phagocytic cells that rapidly and non-specifically attack foreign agents. Innate effectors subsequently provide the "danger" signals to activate cells of the adaptive immune system. The adaptive immune response includes B and T lymphocytes and displays antigen-specificity and immunologic memory. Central to the communication between innate and adaptive immunity are specialized "antigen presenting" cells (APCs) called dendritic cells (DCs). Immature DCs capture foreign antigens (or tumor antigens) in the periphery and migrate to lymphoid organs, where they mature and activate components of the adaptive immune system, e.g. antigen-specific $CD4^+$ helper T cells, $CD8^+$ cytotoxic T cells, and antibody-producing B cells.

The chief aim of cancer immunotherapy is to enhance the immune response against tumor targets. This can be accomplished via stimulation with tumor antigen (vaccination), provision of "tumor-killing" cells (adoptive cell transfer), and/or administration of growth factors called cytokines that regulate immune cells (1). Cytokine approaches for cancer therapy have three potential mechanisms of action. They can 1) directly induce cell death programs in tumor cells, 2) increase the number or activity of immune effector cells, or 3) increase the recognition of tumor cells by the immune system (2).

The underlying hypothesis behind cytokine therapy is that cytokines can help overcome deficiencies in the host immune response against cancer. Cancer cells escape immune surveillance through two key mechanisms. First, a state of immunodeficiency, either inherent or induced, can impair adequate anti-tumor immunity. An example of this would be a patient with AIDS and a profound T cell deficiency or a patient receiving chronic immune suppressive therapy to prevent allograft rejection by T cells (3). Second, the tumor or tumor microenvironment can establish a state of immune tolerance to tumor antigen and/or prevent proper tumor recognition and immune cell stimulation (4).

How can cytokines be utilized to correct a deficiency in tumor immunity? Systemic or local provision of specific cytokines may improve tumor antigen recognition and/or subsequent stimulation of anti-tumor immunity. For example, DCs are highly efficient APCs that can promote the antigen-specific adaptive immune response, including anti-tumor immunity. This chapter summarizes recent progress in cancer immunotherapy utilizing cytokines to enhance antigen presentation.

ANTIGEN PRESENTATION

A central step in the recognition of tumor cells by the adaptive immune system occurs through tumor antigen presentation. This process selectively activates tumor antigen-specific T cells or antibody-producing B cells. DCs often are called "professional" APCs due to their high efficiency in capturing, processing, and presenting antigens to T cells, thereby stimulating them and thus triggering the adaptive immune response.

DCs first were noted by Paul Langerhans in 1868, when he identified "Langerhans cells" with long, thin branches in sections of human epidermis (5). In 1973, Ralph Steinman identified "accessory cells" that facilitated the induction of a specific immune response from lymphocytes in mice and named these "dendritic" cells for their tree-like processes (6). Thirty years later, investigators have further characterized DC development and the role of DCs in the immune response, with important implications for tumor immunology.

Dendritic Cells: Phenotype

DCs are derived from bone marrow progenitor cells. Fully mature DCs are highly capable of antigen presentation. Mature DCs cells have numerous membrane extensions or processes (dendrites) that facilitate physical interaction with the environment and other lymphocytes (Figure 1). The surface phenotype of mature DCs includes

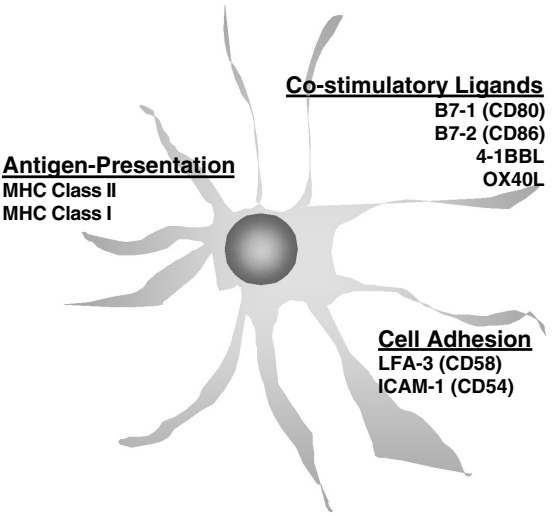

Figure 1. Mature dendritic cell. As "professional" APCs, mature DCs are characterized by the expression of MHC Class I/Class II molecules and CD1 for antigenic peptide and lipid presentation, cell adhesion molecules (ICAM, LFA) for homing to lymphoid tissues and initiating direct contact with lymphocytes, and ligands to provide co-stimulation (B7 ligands, 4-IBB, OX40L) for T cells.

high expression of MHC Class I and II molecules, co-stimulatory molecules (CD80-B7.1, CD86-B7.2), and cell adhesion molecules (ICAM-1, LFA-3) (7). In addition, DCs possess the intracellular machinery for processing proteins for antigen presentation, e.g. endosomes, lysosomes. DCs are negative for lineage markers CD3 (T cell), CD56 (NK cell), or CD19 (B cell) (7).

In addition to "danger" signals from infectious agents, immunoregulatory cytokines can induce the expansion and maturation of DCs in mice and humans. Initial studies demonstrated that cytokines are capable of differentiating mouse and human DC populations from bone marrow or peripheral blood precursors *in vitro*. These DCs are potent stimulators of T cells *in vitro* and are capable of promoting anti-tumor immunity *in vivo* (8–10). It is now known that cytokines such as Flt-3 ligand (FL) induce the expansion and differentiation of DC precursors in the periphery. Additional cytokines such as interleukin-4 (IL-4) and Granulocyte-Monocyte Colony Stimulating Factor (GM-CSF) differentiate DC precursors into immature DCs that are highly capable of antigen uptake. These cytokines have some functional redundancy in the development of DCs, as evidenced by genetically targeted mice deficient for GM-CSF or GM-CSF receptor α-chain that display normal hematopoiesis (11). Conversely, mice lacking FL have a reduced number of DCs (12). Lastly, cytokines including interferons (IFN) and tumor necrosis factor-αg (TNF-α) induce maturation of DC for maximal antigen presentation in local lymphoid tissues (Figure 2).

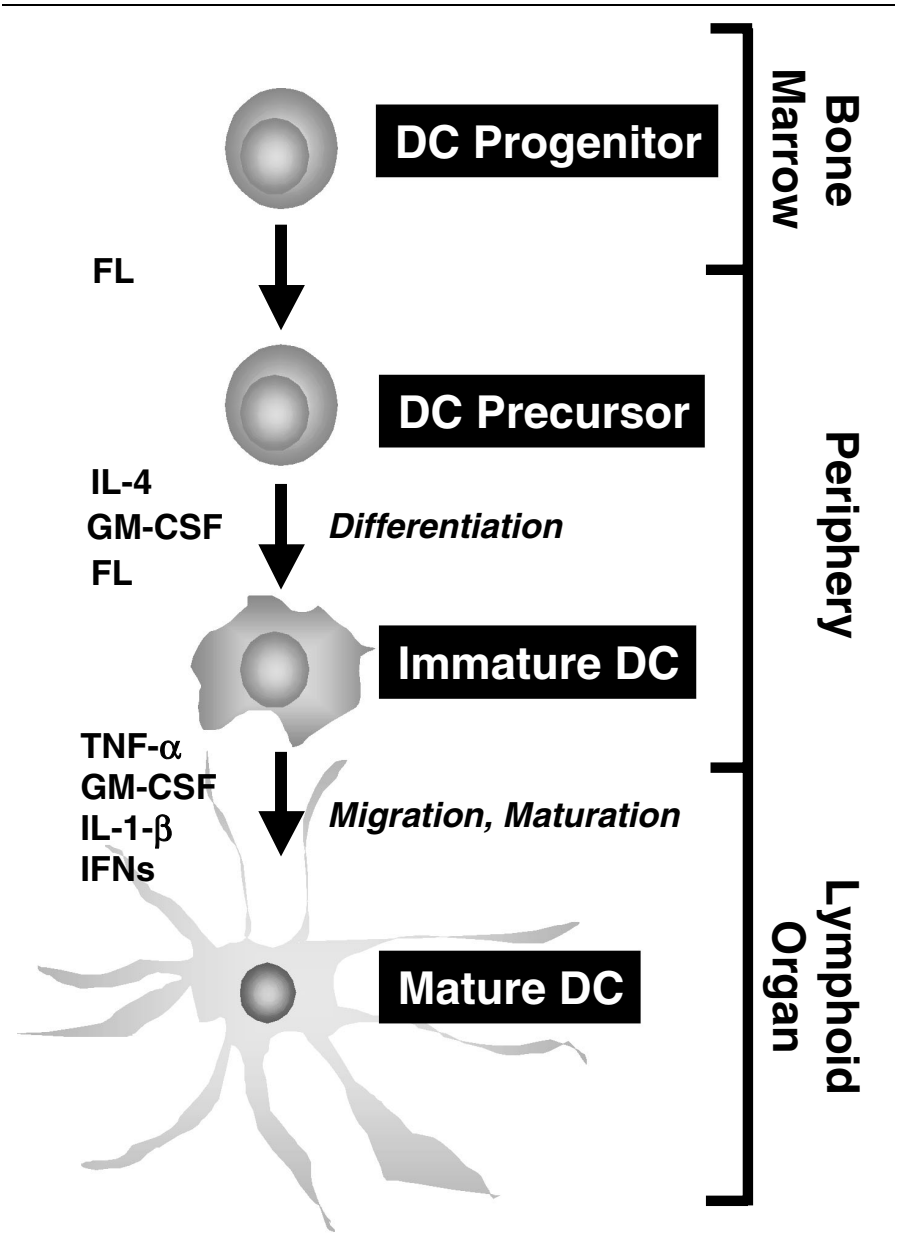

Figure 2. Dendritic cell differentiation and maturation. DC progenitors arise from the bone marrow, and circulate as either DC precursors or immature DCs in the periphery. Cytokines affect the differentiation, migration, and maturation of DCs. Immature DCs are specialized for the uptake of antigen. Additional cytokine stimuli or infectious agents induce maturation and migration of DCs to lymphoid tissues where mature DCs express genes important for antigen presentation and promote activation of the adaptive immune response.

Dendritic Cells: Therapy

Based on their efficient antigen presentation and ability to promote T cell activation, the potential application of DCs for cancer immunotherapy quickly was recognized. In 1996, several investigators demonstrated that DCs could be used to stimulate peptide-specific immunity *in vivo* (8, 10). Bone marrow-derived DCs were loaded with ova peptide and delivered to mice that subsequently were challenged with the EL4 murine thymoma cell line expressing the ova peptide (8, 9). These experiments demonstrated for the first time that peptide-loaded DC could be used to induce antigen-specific and protective immunity against tumors *in vivo*. Depletion of $CD8^+$ T cells abrogated this protective effect, while depletion of $CD4^+$ T cells had no effect, illustrating the role of DCs in tumor antigen presentation and the subsequent generation of tumor-specific $CD8^+$ cytotoxic T lymphocytes. In the same year, Paglia et al. successfully demonstrated that bone marrow-derived DCs pulsed with soluble protein (β-galactosidase) elicited protective immunity against β-gal expressing fibroblasts (10). Similarly, protection correlated with the expansion of $CD8^+$ T cells. This early work initiated a strong interest for developing a therapeutic strategy using *in vitro* expanded DCs that are "pulsed" or loaded with either tumor antigen peptides, tumor cell lysates, or transfected with tumor antigen genes (13). Strategies using *in vitro* generated DCs for anti-tumor immunotherapy are discussed with greater detail in another chapter of this text (J.J.Mule). Here, we will discuss the application of cytokines to enhance antigen presentation through the *in vivo* differentiation of DCs.

Dendritic Cells: Differentiation and Maturation

While vaccines utilizing *in vitro* generated DCs have demonstrated some success, there are three key advantages to cytokine therapies that generate APCs *in vivo*. First, cytokine-based therapies are "universal" since they do not require the preparation of autologous cell-based therapies such as *ex vivo* generated DCs. Second, cytokine therapies do not have the time constraints and contamination risks associated with the preparation of autologous cellular therapies. Lastly, cytokine therapies may elicit a broader immune response by allowing antigen processing and peptide selection to occur *in vivo*.

The following cytokines have been shown to or have the potential to enhance antigen presentation *in vivo*.

Colony Stimulating Factors (GM-CSF, G-CSF)

GM-CSF first was identified in the late 1970s from mouse lung-conditioned medium and was capable of stimulating granulocyte, macrophage and mixed lineage colonies from bone marrow (14). Signaling occurs through the GM-CSF receptor α chain and common βc, shared with IL-3 and IL-5 receptors, with subsequent activation of tyrosine kinases, mainly Janus family kinases (15). Early work with GM-CSF revealed its ability to induce proliferation of progenitor cells from bone marrow and leukemia cell lines (16, 17). In the late 1980s, several investigators demonstrated the

role GM-CSF in the differentiation of human erythroid, granulocyte, and monocyte/macrophage lineages from bone marrow and peripheral blood (18). Witmer-Pack et al. (1987) demonstrated that GM-CSF could prolong the survival of mouse Langerhans cells *in vitro* (19). Migliaccio et al. (1988) used GM-CSF and G-CSF to differentiate macrophage lineages *in vitro* from human bone marrow and peripheral blood (18). Differentiation of macrophages is significant as these cells had the potential to become effective APCs, and further, could be differentiated into DCs. Markowicz and Engleman (1990) reported that GM-CSF could prolong the survival of peripheral blood DCs and induce differentiation of DCs from peripheral blood monocytes (18). In contrast, Granulocyte Colony Stimulating Factor (G-CSF) alone does not appear to have a significant role in the differentiation of DC precursors *in vitro*, but may have some utility when combined with GM-CSF or other cytokines *in vivo* (19, 20).

Following Markowicz and Englemann's report on GM-CSF and human peripheral blood DC survival and differentiation in 1990, several other groups demonstrated that GM-CSF, alone or in combination with other cytokines, could differentiate DCs from DC precursors or progenitors *in vitro*. In 1992, Inaba et al. reported that GM-CSF could differentiate and cause proliferation of DCs from cultured mouse peripheral blood (20, 21). Caux et al. induced the differentiation of DCs from human $CD34^+$ bone marrow stem cells with GM-CSF and TNF-α(22). Santiago-Schwarz et al. similarly induced DC differentiation from human umbilical cord blood $CD34^+$ stem cells with GM-CSF and TNF-α (23). In particular, the development of multiple methods for generating large numbers of DCs *in vitro* was a critical step in the study of DC biology since previous studies had been limited by the small percentage (less than 0.5%) of DCs available from peripheral blood (22–24).

GM-CSF Provides Protective Immunity in Murine Models

Dranoff et al. sought to compare several cytokine gene therapies, including GM-CSF, in a mouse model of melanoma (25). In this model, mice were challenged with the B16 melanoma cell line and subsequently succumbed to fatal tumor burden within 15–40 days. Prior immunization with irradiated melanoma cells resulted in minimal improvement in survival and immunity. As a next step, mice were immunized with irradiated melanoma cell vaccines that had been retrovirally transduced with one of the following murine cytokine genes: IL-2, IL-4, IL-5, IL-6, GM-CSF, IFN-γ, and TNF-α. Immunization with GM-CSF-transduced tumors provided superior protection compared to all other cytokines tested (25). Further, the protective immunity established with GM-CSF-transduced tumor vaccination was significantly abrogated by prior depletion of either $CD4^+$ or $CD8^+$ T cells. The authors suggested that the immunostimulatory properties of GM-CSF lie in its ability to promote DC differentiation and antigen presentation, especially since the B16 tumors lacked expression of MHC Class II and thus likely were not capable of priming the $CD4^+$ T cell response themselves. They later reported that GM-CSF-transduced tumor vaccines expanded $CD11c^+CD80^+$ $CD86^+$ DCs *in vivo*, with a 40-fold increase in splenic DCs (26). This study was the first to make a broad comparison of cytokines as

Table 1. Cytokines and antigen presentation

Cytokine	Effects
GM-CSF	Proliferation and differentiation of myeloid, erythroid, granulocytic cells Increases antigen presentation through the expansion of DCs and DC precursors alone or in combination with other cytokines including IL-4 and TNF-α
FL	Proliferation of marrow progenitors Increases antigen presentation *in vivo* through the expansions of DC in mice and cancer patients
IL-4	Upregulates MHC I and MHC II in APCs Combination with GM-CSF promotes antigen presentation through differentiation of DCs from human peripheral blood and $CD34^+$ stem cells
IFN-α/β	Anti-viral activity May directly induce apoptosis in mouse and human tumors Activates macrophages Increases antigen presentation through upregulation of MHC Class I expression on both tumor cells and APCs Promotes DC maturation in combination with GM-CSF
IFN-γ	Produced by activated T cells and NK cells Anti-viral and anti-proliferative activity Major activator of macrophages Increases MHC Class I and II antigen processing, presentation, and expression in macrophages
TNF-α	Produced by leukocytes Enhances antigen presentation by monocytes Promotes antigen presentation through maturation of DCs in combination with GM-CSF

Abbreviations: GM-CSF, granulocyte-monocyte colony stimulating factor; DC, dendritic cell; TNF, tumor necrosis factor; G-CSF, granulocyte colony stimulating factor; FL, Flt3-ligand; IFN, interferon

cancer vaccine adjuvants using mouse models and placed GM-CSF at the forefront of cytokine therapies for cancer (25, 26). Could GM-CSF gene therapy or systemic administration of GM-CSF promote tumor immunity in patients with cancer?

Clinical Application of GM-CSF: Gene Therapy

Based on promising results from animal models using cytokine-transduced cancer cells and tumor antigen-loaded DCs as vaccines, Sanda et al. (1994) sought to retrovirally transduce human GM-CSF genes into prostate cancer cells from 10 patients (27). They showed that these cells secreted human GM-CSF in a gene-dose dependent fashion. This early work demonstrated the feasibility of cytokine gene therapy for producing autologous cancer cell vaccines. They hypothesized that co-expression of GM-CSF with the cancer cell vaccine would induce local differentiation of DCs capable of tumor antigen presentation. Subsequently, several Phase I clinical trials initiated treatment with preparations of GM-CSF–transduced autologous metastatic renal cell carcinoma, melanoma, and prostate cancer (28–30). Each trial proved to be safe with minimal toxicity to patients with late stage or metastatic cancer. These first studies demonstrated an immunological response to the vaccines as measured by delayed-type hypersensitivity reactions and antibody titers against the autologous

Table 2. GM-CSF and cancer immunotherapy

GM-CSF	Clinical Application
Recombinant cytokine	Differentiation of DCs and DC precursors *in vitro* from peripheral blood monocytes or bone marrow/cord blood stem cells Alone and in combination with other cytokines (IL-4, IFN-α, TNF-α, or FL) for *in vivo* expansion of mature DCs
Gene therapy	Autologous or allogeneic-tumor cell vaccines transduced with GM-CSF to promote local differentiation of DCs and increased antigen presentation Poxvirus delivery of GM-CSF as an adjuvant to tumor antigen or gene

tumors (31, 32). Local differentiation and infiltration of DCs in vaccination sites should likely have been considered as another end point of these vaccine approaches. Overall, these Phase I trials demonstrated safety and immunologic responses, suggesting approaches may be most beneficial in either in early stage cancers or in stages of minimal residual disease (33).

Clinical Application of GM-CSF: Recombinant Cytokine

Systemic administration of GM-CSF is well tolerated by patients and now is indicated for neutrophil recovery following chemotherapy and mobilization of peripheral blood progenitors (34, 35). Further analysis revealed that GM-CSF not only mobilizes peripheral blood progenitors, but also results in an increase in peripheral blood monocytes and DC precursors in normal healthy patients. Although recombinant GM-CSF can differentiate DCs from human bone marrow/cord blood stem cells and peripheral blood *in vitro*, GM-CSF is less effective as a DC differentiation agent when administered alone in humans. Combination treatment with GM-CSF and IL-4 efficiently expanded peripheral blood DCs ($HLA\text{-}DR^+$, $CD11c^+$, $CD83^+$) in patients with advanced cancer (36), while IL-4 alone does not differentiate DCs. Further, administration of GM-CSF in combination with G-CSF or FL to patients with cancer can significantly increase the number of peripheral blood DCs (37, 38). Direct comparisons of GM-CSF and cytokine combinations have not yet been studied in humans. To date, GM-CSF is the most commonly used vaccine adjuvant in cancer vaccines and currently is being used in clinical trials in the form of a systemically administered recombinant cytokine or via gene therapy with GM-CSF-transfected tumor cells or subcutaneous delivery of poxviruses expressing GM-CSF (30, 39–42).

Tumor Necrosis Factor-α (TNF-α)

TNF-α was the main component of the earliest cancer immunotherapy approach attempted in the 1890s by the surgeon William B. Coley (43, 44). Coley used bacterial extracts with tumor "necrosing" activity in patients with advanced cancer. Today, we know that TNF-α is mainly produced by macrophages and lymphocytes in

response to various infectious agents or cytokine stimuli. There are two receptors for TNF-α, TNF-R1 and TNF-R2 that are expressed ubiquitously. The main actions of TNF-α involve the direct induction of cytotoxicity and gene expression (45).

TNF-α and Antigen Presentation

In addition to the direct effects of TNF-α on tumor cells, TNF-α also regulates antigen presentation. In 1990, Zembala et al. reported that TNF-α increased the ability of human monocytes to present soluble protein antigen to autologous T cells *in vitro* (46). A possible mechanism for this enhanced antigen presentation was the upregulation of HLA-DR molecules after TNF-α treatment of monocytes. In 1992, two reports demonstrated that TNF-α helped to regulate the maturation of DCs from human stem cells, only in combination with GM-CSF (22, 23). The combination of GM-CSF and TNF-α increased the yield of $CD1a^+$ DCs by 10 to 20-fold from $CD34^+$ hematopoietic stem cells from umbilical cord blood. Culture with TNF-α alone had no effects on the differentiation of stem cells. TNF-α now is used with GM-CSF to mature DCs derived *in vitro* from human peripheral blood or stem cells (47, 48). Application of *in vitro* derived-DCs using TNF-α is described further in another chapter in this volume (J.J. Mule).

Thus, the use of TNF-α for tumor immunotherapy could be a two-pronged attack, first by directly inducing cytotoxicity in tumors and second by potentiating antigen presentation and subsequently promoting anti-tumor immunity.

Clinical Application of TNF-α

Systemic use of recombinant TNF-α for advanced cancers demonstrated little clinical benefit and caused dose-limiting toxicities that included hypotension, hepatotoxicity, malaise, fatigue, and thrombocytopenia (49). These toxicities are not surprising given the ubiquitous expression of TNF receptors. On a positive note, a different route of administration, "isolated limb perfusion," resulted in a clinical benefit with reduced toxicity for metastatic melanoma and sarcoma (50). Due to its toxicity, selection of TNF-α for *in vivo* cancer treatment most likely should be reserved for localized treatment and direct tumor cytotoxicity. Similarly, for cancer vaccines, TNF-α may have clinical utility if delivered locally at lower doses. One Phase I trial demonstrated that daily subcutanenous GM-CSF combined with continuous infusion of TNF-α could increase the number of Langerhans cells in the epidermis of cancer patients (51). However, given the higher efficacy of combined GM-CSF and IL-4 treatments to increase the number of DCs *in vivo*, recombinant TNF-α likely appears better suited for cancer vaccine strategies employing DCs generated *in vitro,* in combination with GM-CSF.

Flt3 Ligand

FL is a colony-stimulating factor that can induce proliferation, self-renewal, and differentiation of hematopoietic stem cells (52, 53). FL binds its receptor, Flt3, which is a receptor tyrosine kinase belonging to the family that includes c-KIT and PDGFR. Flt3 was cloned from pro-B cell lines and also is expressed in monocytic/myeloid

Table 3. History of Flt3-ligand

Year	Studies
1993	Murine FL cloned (52)
1994	Human FL homologue (53)
1996	FL therapy expands DCs in mice (56)
1997	FL therapy induces $CD8^+$ T cell-dependent tumor regression in mice (58)
2000	FL therapy increased number (48 and 44-fold) of $CD11c^+$ DCs in peripheral blood of healthy human subjects (59, 60)
2000	FL $^{-/-}$ mice have reduced splenic DCs (3 to 12-fold)
2002	FL therapy increased number of immature DCs in patients with metastatic renal cell carcinoma, with no effect on disease (61)
2002	FL adjuvant for HER2-neu vaccination promotes peptide-specific interferon-secreting T cells (62)

lineage and hematopoietic stem ($CD34^+$) cells from bone marrow or fetal liver. This pattern of expression led to the hypothesis that FL had an important role in early hematopoiesis. FL has been demonstrated to have an important regulatory role in DC biology in both mice and humans. (12, 54–56). This early work identified a rationale for the *in vivo* application of FL to promote DC expansion, antigen presentation, and subsequent adaptive (antigen-specific) immunity.

FL also plays an important role in regulating innate immunity in mice. In 1998, Shaw et al. showed that systemic administration of FL expands the absolute number of NK cells in mice and increases their cytotoxic activity (57). Thereafter, in 2000, McKenna et al. reported a marked deficiency of leukocytes, including NK cells, in the FL "knockout" mouse (12). These "knockout" mice had reduced numbers of B lymphoid and myeloid progenitors in the bone marrow, and a marked decrease in splenic DCs (3 to 12-fold) and NK cells (5-fold).

FL Expands DCs and Enhances Tumor Immunity in vivo

In 1996, Maraskovsky et al. administered FL to wild type mice and subsequently observed a significant expansion of DCs (56). After only nine days of daily intraperitoneal FL, they observed expansions of $CD11c^+$ MHC Class II^+ DCs in the spleen (17-fold), lymph nodes (4-fold), and peripheral blood (6-fold) compared to controls. In peptide-pulsing experiments and alloantigen-stimulations, these DCs were functionally mature.

In 1997, Lynch et al. hypothesized that FL-mediated expansion of DCs *in vivo* could provide protective anti-tumor immunity (58). They challenged mice with murine fibrosarcomas and administered daily FL for nine days. FL-treated mice displayed marked tumor regression and retarded tumor growth. Protection provided by FL was dose-dependent and could be abrogated by *in vivo* depletion of $CD8^+$ T cells but not $CD4^+$ T cells. Further, tissues surrounding tumors in FL-treated mice displayed greater DC infiltrates than untreated mice. Immunity was transferable through adoptively transferred splenocytes from FL-treated mice that successfully had rejected tumors.

Clinical Application of FL for Cancer Immunotherapy

Given the ability of FL to promote generation of DC precursors *in vivo*, FL was evaluated as a potential adjuvant for cancer vaccine strategies. In 2000, Maraskovsky et al. and Pulendran et al. reported the ability of FL, alone or in combination with G-CSF, to expand DCs in healthy human volunteers (59, 60). Pulendran et al. reported that administration of FL alone after ten days led to a 48-fold increase in $CD11c^+$ DCs ($HLA\text{-}DR^+$ $CD86^+$) and a 13-fold increase in $CD11c^-$ DCs ($HLA\text{-}DR^-CD86^-$) in the peripheral blood (59). Further, they reported that the $CD11c^+$ DCs had allostimulatory capacity when cultured with allogenenic $CD4^+$T cells *in vitro*, while this capacity was absent in $CD11c^-$ DCs. Maraskovsky's report confirmed that FL alone led to a 44-fold expansion of $CD11c^+$ DCs ($HLAD\text{-}DR^+CD86^+$), and that this population could be further differentiated *in vitro* with IL-4 and GM-CSF. $CD11c^+$ DCs expanded *in vivo* resembled immature DCs but were not as efficient at antigen uptake (60).

Recombinant FL was subsequently applied in patients with late stage cancer. FL was shown to be effective in mobilizing DC precursors in patients with melanoma or renal cancer (61). Again, FL expanded DCs (19-fold) with a partially differentiated DC phenotype, $CD11c^+$ $CD86^+$ $HLA\text{-}DR^+$, but $CD80^-$ $CD83^-$. More recently, FL has been combined with tumor antigen peptides in cancer vaccine trials. Disis et al. studied patients with HER-2/neu overexpressing malignancies and vaccinated them with HER-2/neu peptides, in combination with either recombinant FL or FL + GM-CSF (62). While this approach failed to promote proliferation of HER-2/neu-specific T cells, the immunization did increase the number of interferon-producing HER-2/neu-specific T cells. Infiltration and differentiation of APCs at the vaccine sites were not reported. This study demonstrated that systemic administration of FL as a vaccine adjuvant results in the mobilization of DCs capable of presenting tumor antigen and promoting T cell immunity against co-administered antigen (interferon-producing T cells).

Together, these pre-clinical and clinical data show that FL alone effectively expands DC precursors (44 to 48-fold) but that these DCs were not as effective at antigen presentation as the more mature DCs generated with GM-CSF. The most productive use of FL in cancer vaccines is in combination with other cytokines for either *in vivo* or *ex vivo* differentiation of DCs. Recombinant FL is safe, but its clinical use remains to be optimized and its production for continued clinical study in the U.S. needs to be renewed.

Interferons

Interferons (IFN) were first described in 1957 as antiviral cytokines by Issacs and Lindenmann when they demonstrated that IFN secretion could be induced by viral infection of chick embryo cells (63). Type I IFNs are highly heterogeneous with over 14 proteins produced in humans that include the major subtypes IFN-α, secreted by leukocytes, and IFN-β, secreted by fibroblasts (64). Type I IFNs signal through a Type I IFN-α or IFN-β receptor (65). IFN-γ is the only Type II interferon known and is secreted by activated NK cells and $CD8^+$ cytotoxic T lymphocytes. IFN-γ

signals through a single IFN-γ receptor expressed on nearly all cells, including tumor cells (65).

The first biological activity described for IFNs was their anti-viral function in virus-infected cells (63). IFNs induce three pathways that regulate virus-infected cells: two pathways inhibit protein synthesis and a third inhibits viral transcription (66). IFNs also demonstrate anti-proliferative and cytotoxic effects on cells, which led to their early use in cancer therapy. In addition to these anti-viral and anti-tumor activities, IFNs have an important role in linking innate and adaptive immunity. Early in the immune response, innate immune effector cells (NK cells) produce abundant IFN-γ, the strongest cytokine activator of macrophages. Activated macrophages respond to IFN-γ by producing cytokines (e.g. IL-12, IL-15, TNF-α) that further induce NK cell activity. These cytokines build a positive feedback loop, wherein macrophage secreted cytokines stimulate NK cells, and NK-derived IFN-γ further activate macrophages (67). Cytotoxic T cells also secrete IFN-γ as an effector molecule.

Type II IFNs are also thought to have direct cytotoxic and anti-proliferative effects on tumors (68). Neutralization of this effector cytokine in mice limited tumor surveillance in mice chemically induced with 3-methylcholanthrene (69, 70). When the IFN-γ-insensitive tumors from these mice were transferred to syngeneic mice, the tumors demonstrated lower immunogencity than IFN-γ-sensitive tumors. This process of the immune system shaping a tumor's phenotype has been termed "cancer immunoediting" (68).

IFNs and Antigen Presentation

IFNs induce the upregulation of MHC molecules in both immune cells and tumor cells. Type I IFNs induce expression of MHC Class I molecules, while IFN-γ upregulates both MHC Class I and II molecules. In addition, IFN signaling initiates the expression of genes important for antigen processing, including genes for the proteasome enzyme complex. The proteasome is essential for the proteolytic degradation of protein products into antigenic peptides for presentation by MHC molecules (65). Thus, IFNs can enhance antigen processing and presentation in not only tumor cells but also host APCs, including B cells, monocytes, and DCs.

In fact, recent reports demonstrated that Type I IFNs facilitate the differentiation of DCs from either $CD34^+$ bone marrow stem cells or peripheral blood monocytes (24, 71). Specifically, IFN-α can accelerate the maturation of DCs from immature DCs (71). Santini et al., reported that IFN-α, in combination with GM-CSF, could be used to quickly generate mature DCs from human peripheral blood monocytes *in* vitro and that these DCs were superior to those generated using IL-4 and GM-CSF, with respect to stronger stimulation in mixed leukocyte reactions and greater induction of human Ig from B cells (72).

Clinical Application of IFNs for Cancer Immunotherapy

Type I IFNs have had extensive clinical use for infections, multiple sclerosis, and several cancers (73–75). The many effects of IFNs likely are important in the mechanism

of action for each of these diseases. For example, IFN-α has been used to treat Hepatitis B or C infections, taking advantage of the anti-viral and immunological properties of IFNs (73). Further, Type I IFNs treatment of various tumor cell lines in vitro has shown cytotoxic and anti-proliferative effects. The effects of IFNs on antigen presentation have been studied and are most apparent in the treatment of melanoma and renal cell carcinoma (75). These studies, however, have focused on reactive cytotoxic and helper T lymphocytes as immunological endpoints, not activity of APCs.

One of the more successful applications of IFNs was in the early 1990s, when treatment with IFN-α was shown to induce remissions in 60–80% patients with early phase chronic myeloid leukemia (CML) (76). More recently, CML-specific cytotoxic T lymphocytes and antibody producing B cells have been implicated in those patients with CML who responded to IFN-α therapy (77–79). The effects of IFN-α on antigen presentation in these patients was first suggested when Wang et al. reported that DCs derived from bone marrow of CML patients were less effective in allo-stimulation than normal bone marrow-derived DCs (78). Molldrem et al. identified peptides from Proteinase 3 that were highly expressed in myeloid leukemias, and recognized by cytotoxic T cells that in turn could lyse the leukemia cells (80). Using MHC Class I tetramers and a Proteinase 3 peptide called PR1, they demonstrated that patients responsive to IFN-α2b therapy displayed PR1-specific T cells, while non-responders lacked these T cells (79). Lastly, Paquette et al. reported that IFN-α and GM-CSF could differentiate DCs *in vitro* from the peripheral blood or bone marrow of CML patients, better than IL-4 and GM-CSF. Furthermore, they demonstrated that CML patients responding to IFN-α had an increase in bone marrow DCs. They hypothesized that IFN-α could facilitate antigen presentation directly from leukemic cells through upregulation of MHC molecules and other DC antigens (81). Additional evidence in breast and colon cancers suggests that IFN-α treatment may also directly increase expression of select antigens on tumor cells (82).

In contrast, cancer patients treated with Type II IFNs as single agents have had poor responses as single agents for cancer treatment. Type II IFNs have been largely studied as effector molecules of the immune response that act in a positive feedback loop to strengthen both innate and adaptive effectors. Although IFN-g strongly increases MHC gene expression in APCs, the clinical trials completed thus far for solid and hematologic malignancies have shown little promise (2). Despite these results, IFN-γ has shown some efficacy for the treatment of chronic myelogenous leukemia (83).

These studies suggest that in addition to direct cytotoxic effects on tumor cells, IFNs, especially IFN-α may mediate clinical responses through the upregulation of MHC molecules on tumor cells, increased tumor cell antigen expression, and increased differentiation and maturation of DCs.

CHALLENGES FOR CYTOKINE THERAPY

Cytokines that can increase the number of APCs and enhance tumor antigen presentation on tumor cells or on host APCs, may improve T cell recognition and responses to such antigens. While a direct role for cytokines in minimal residual disease is

possible, a role in cancer vaccine strategies seems more promising. While preclinical and clinical data using GM-CSF, IL-4, FL, IFNs, and TNF-α have shown potential, however, optimal use of these cytokines may depend upon the specific cancer and tumor antigens. Successful clinical application of cytokine strategies will most likely require more preclinical and clinical studies. Mechanisms to make these cytokines available for such work should be rapidly developed by the National Cancer Institute in collaboration with industry.

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12. TINKERING WITH NATURE: THE TALE OF OPTIMIZING PEPTIDE BASED CANCER VACCINES

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INTRODUCTION

Methodological progress at the end of last century in the identification of tumor associated antigens specifically recognized by cytolytic T lymphocytes (CTL) made possible the characterization of numerous peptides (p) presented by class I Major Histocompatibility Complexes (MHC). These p-MHC complexes are the ligands for clonotypically distributed T cell receptors (TCR). The latter are heterodimers of α and β chains bearing structural homology with immunoglobulins. As such, they are composed of constant and variable segments. Three hypervariable regions can be identified, of which the Complementary Determining Region 3 (CDR3) is both the most variable and involved in the interactions with the peptide amino acid residues in the p-MHC complex (1, 2).

The tumor associated antigenic peptides identified thus far are derived from a large variety of cellular polypeptides. These may include regular proteins from different cellular compartments, isoforms encoded by alternatively spliced genes, the products from alternative open reading frames, mutated genes or frameshifts and may even result from the transcription of the antisense strand of DNA (3–5). In one case, the antigenic peptide was shown to be generated by a protein splicing mechanism, thus far unknown in the eukaryotic world (6). Thus, the variety of cell biological mechanisms uncovered so far as giving rise to antigenic peptides reveal the highly opportunistic nature of tumor recognition by CD8 T lymphocytes. This is the result

of two concurrent mechanisms leading to antigen recognition by T lymphocytes. On one hand, the process of antigen recognition involves a sophisticated molecular apparatus able to discriminate as few as 1–10 p-MHC complexes on the surface of the antigen presenting cell that normally display up to 10^4–10^5 p-MHC complexes (7). Thus, the system has evolved to attain an exquisite sensitivity and is endowed with powerful discrimination and amplification properties. On the other hand, the repertoire of αβ T cells is shaped by positive and negative selection processes during thymic development. As a result the majority, up to 95% of immature thymocytes that successfully rearrange TCRs, are eliminated from the mature repertoire (8, 9). The coupling of these two thymic selection forces ensures that the TCRs expressed by T cells exported for immunesurveillance in the periphery possess sufficient affinity for interaction with self MHC molecules but are depleted of potentially dangerous TCRs with high affinity for self p-MHC complexes. As a result self tolerance is firmly established. However, this does not mean absence of autoreactive T cells. In fact, these cells exist in the peripheral T cell repertoire but possess a low to intermediate avidity for a high number of self antigens (10–14). Thus, despite the considerable diversity of tumor associated peptides, many of those identified thus far are derived from conventional polypeptides that are expressed by both normal and tumor cells.

Practical issues in specific therapy of cancer favour the use of those antigens that are expressed in the maximum number of patients with a given type of tumor. In this regard, most of the antigenic peptides from mutated gene products are poor candidates for widely applicable vaccines because their expression is limited to individual tumors. Consequently, the best candidates for vaccine development in terms of cancer population coverage are those derived from self antigens. Thus, an expected limitation of these generic cancer vaccines is the existence of self tolerance. Such constraints have been clearly demonstrated in studies conducted in experimental mouse models. For instance, large differences in TCR avidity for a p53-derived class I restricted T cell epitope can be measured when comparing the TCR repertoires of wild type mice and p53-genetically deficient counterparts (15). Similar findings were reported in a transgenic mouse model system for CTL recognizing a dominant viral antigen (16). Another elegant illustration of this phenomenon comes from the comparative analysis of the HLA-A2 restricted CD8 T cell repertoire for a tyrosinase-derived melanoma associated antigen in strains of mice expressing or not the *tyrosinase* gene product (17).

Attempts to vaccinate patients with such peptides result in the selection of a specific CTL response. Moreover, appropriate immunization may elicit a tumor protective response mediated by the low avidity CD8 T cell repertoire in a mouse model (18). Interestingly, it has been shown in well defined animal models that similar or even more efficient CTL responses can be obtained by immunization with peptides modified at key residues (16, 19–23). These studies clearly demonstrate that this class of peptide analogues have the ability to mobilize the intermediate/low avidity T cell repertoire and induce protective anti-tumor CTL responses. Here we review the sequence and structural basis of p-MHC complexes formation and show how antigenic peptide modifications can improve the peptide's immunogenicity.

PEPTIDE BINDING TO MHC

Sequence Analysis

To fulfil their immunological functions, MHC Class I molecules have evolved to bind with sufficient affinity a large number of peptides with widely divergent amino-acid sequences. Sequence analysis of the peptide population obtained by elution of immunoaffinity purified MHC class I molecules (24) revealed the presence of allele-specific binding motifs. For instance, *HLA-A*0201* encoded molecules selectively bind peptides with L, M or I at the second position of the peptide, or P2, and V or L at PΩ, i.e. the residue occupying the carboxyl terminal position of the peptide. In contrast, P2 needs to be a Y or F and PΩ an I or an L in peptides binding to the mouse K^d molecule as well as in those binding to the human HLA-A24 molecule (24–26). Nearly identical results were obtained by a completely functional approach using substituted peptides as competitors of antigenic peptides to inhibit lysis of chromium labelled targets by H2 K^d restricted CTL clones (27). These results suggested that there are distinct amino acid residues in the peptide that are directly involved in MHC binding, while the remaining peptide residues are relatively unconstrained at the sequence level.

Since this pioneering work, large amounts of data have been collected on the nature of peptide sequences restricted to different MHC allelic products. For instance, the MHCPEP database (28) has been regularly updated and contains information on peptide binding for several MHC together with experimentally determined affinity constants for MHC. There are currently over 2500 peptide sequences known to bind HLA-A2, allowing statistics to be made, though care should be taken since the peptide population is subject to certain bias (28). Table 1 shows the occurrence of each of the twenty naturally occurring amino acids at specific peptide positions for the panel of peptides known to be naturally associated with HLA-A2 in somatic cells. The first number is computed for the entire database, and the second one for high affinity peptides only (246 sequences). The main anchor residues described above are clearly still predominant and the Table provides information on which alternate residues are allowed.

Due to the strong selectivity at positions P2 and PΩ, these two residues are referred to as *main* or *primary* anchor residues and those occupying the less selective positions P1, P3 and PΩ-3, PΩ-2 to as *secondary* anchor residues (29). An additional regular feature of MHC class I binding peptides is their defined length of 9–10 residues. Indeed, despite the identification of occasional T cell epitopes whose optimal length is clearly at variance with this rule (e.g. (30, 31)), the large majority of known T cell epitopes as well as sequenced MHC class I-associated peptides are nona or decapeptides. Thus, relatively simple sequence motifs can be defined for sets of peptides binding to well defined class I MHC molecules, both from murine and human origin. These motifs can be defined by three components. The first, the primary anchor residues. The second, the secondary anchor residues and the third component is a defined peptide length.

The sequence motifs described here find their explanation in the specific p-MHC architecture in three dimensions, detailed in the next chapter. An immediate

Table 1. Anchor residues specificity for the different pockets of HLA-A2.

Amino acid	P1	P2	P3	P(Ω-3)	P(Ω-2)	P(Ω)
A	13/6[a]	4/3	10/5	9/3	13/10	5/4
C	0/1	0/0	1/0	2/0	1/1	0/0
D	0/0	0/0	6/2	1/1	2/0	0/0
E	2/0	0/0	2/1	1/0	2/0	0/0
F	10/13	0/0	5/10	9/13	13/25	0/0
G	11/23	1/0	7/8	5/7	4/7	1/1
H	1/0	0/0	2/1	1/0	3/2	0/0
I	6/5	13/15	4/4	7/6	5/3	10/6
K	9/8	0/1	2/3	2/1	1/2	0/0
L	7/11	60/64	17/25	9/7	11/8	28/30
M	2/1	7/6	1/2	1/1	0/0	1/1
N	1/1	0/0	5/3	1/1	2/2	0/0
P	1/0	0/0	7/9	8/7	8/14	0/0
Q	2/1	0/0	1/0	2/3	2/1	0/0
R	3/4	0/0	1/0	1/0	1/0	0/0
S	6/5	0/0	5/5	5/7	3/3	0/0
T	2/2	4/3	2/1	5/4	3/2	3/2
V	4/3	3/1	5/4	15/24	9/7	42/52
W	1/2	0/0	4/4	0/0	2/2	0/0
Y	5/6	0/0	3/4	2/4	2/1	0/0

[a] The first digit represents the percentage of the particular amino acid residue in all the peptides known to bind to HLA-A2, the second one represents the same quantity for the set of high affinity binding peptides only.

application of these insights has been the design of computer algorithms to identify candidate peptides in proteins of known sequence that would bind a given MHC class I molecule. Two such computer programs have gained wide recognition: the bioinformatics and molecular analysis section (BIMAS) algorithm (http://bimas.dcrt.nih.gov/molbio/hla_bind, (32, 33)) and the one based on the SYFPEITHI database (http://syfpeithi.bmi-heidelberg.com, (34)). Another algorithm with the same purpose has been reported recently (35). These algorithms have been thoroughly reviewed recently (36). Numerous studies have been performed leading to the identification of potential T cell epitopes in proteins of interest for immunotherapy of infectious or autoimmune diseases and cancer. Such an approach has been dubbed "reverse immunology" to emphasize the fact that, in contrast to the initial methods leading to CTL-defined antigen identification, the starting "reagent" is the bioinformatic tool and the end-product the isolation of the peptide-specific T cell (37).

Structural Analysis

A major step in the understanding of peptide binding to MHC molecules was the elucidation, by X-ray crystallography, of the three dimensional structure of the p-MHC complex. It allowed to derive general rules on the strategy of peptide binding by MHC, see for example (38–40). So far, the structures of around 60 different p-MHC complexes have been solved by X-ray crystallography with

Non-Natural Peptide Antigen Analogues Resistant to Biodegradation

The efficient use of antigenic peptides as therapeutic agents may be limited by the high sensitivity of peptides to degradation by peptidases present in biological fluids (82, 83). Initial *in vitro* studies showed that peptide degradation by proteases in serum could decrease the presentation of exogenous antigenic peptide by MHC on the surface of presenting cells (84, 85). In addition, the degradation of the antigenic peptide *in vitro* was correlated with a diminution of their persistence *in vivo* (86). Further studies indicated that local persistence of the antigenic peptide could be associated with the induction of an optimal immune response (87). Together, these factors could limit the immunogenicity of the antigenic peptide as tumor vaccine. Thus, rendering antigenic peptides resistant to degradation by peptidases could have important implications in the design of efficient peptide based vaccines for immunotherapeutic treatment of cancer.

To this end, an effective approach consists in introducing structural modifications to the antigenic peptide. This has been showed for both MHC class-I (88–90) or class-II (91) restricted antigenic peptides. The structural changes in the antigenic peptide involve either a variety of chemical modifications of the peptide bond (92–95) or substitutions with non-natural amino acids (a.a.) (88, 89, 96). A drawback, however, was the simultaneous appearance of dramatic negative effects on the MHC binding properties of the antigen and/or on the recognition by antigen specific T lymphocytes (88, 89). Such detrimental effects must be minimised in order to use such peptidase resistant pseudopeptides as efficient therapeutic compounds. To preserve the antigenicity and immunogenicity of the antigenic peptides, a more rational approach was taken to introduce minimal modifications of the peptide structure. In this approach, the knowledge of the degradation mechanism of the antigenic peptide guides the choice of structural modifications targeted to the appropriate position in the peptide's structure, the one(s) susceptible to proteolytic attack (88).

The detailed mechanism of tumor antigenic peptide degradation was initially determined by the analysis using an on-line HPLC mass spectrometry (HPLC/ESI-MS) method of the degradation fragments generated after incubation of the antigenic peptide in human serum for various periods of time (97). In line with the findings of this study, the analysis of the degradation of the MelanA/MART-1 related peptide $MelanA_{26–35}$ A27L indicates the involvement of aminopeptidases and di-peptidyl-carboxypeptidases (Figure 6) (94). Interestingly, the degradation of the antigenic peptides from their amino- and the carboxy-terminal ends was found to be sequential and no endopeptidase activity was involved. The kinetics of amino- and carboxy-terminal degradation can be different from one peptide to another leading to slightly different degradation profiles. The analysis of the degradation of Melan-A/MART-1 nona- and decapeptide related peptides suggest that the nature of the amino-terminal residues has a direct or indirect effect on both amino- and carboxy-peptidase enzymatic activities (94).

The degradation model of the peptide $MelanA_{26–35}$ A27L predicts that the peptidase sensitive bonds are the first (Glu^1-Leu^2) and the eighth (Leu^8-Thr^9). Within

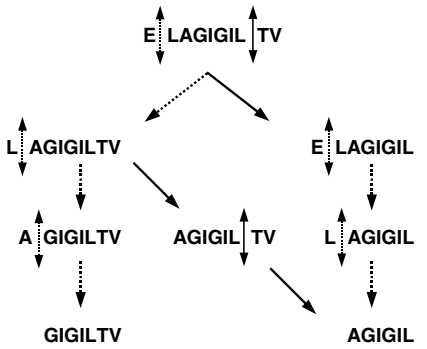

Figure 6. Degradation model of Melan-A_{26-35} A27L antigenic peptide.
The degradation model of the antigen Melan-A_{26-35} A27L (ELAGIGILTV) has been established from qualitative and quantitative data obtained from HPLC-mass spectrometry analysis. The peptide is cleaved by aminopeptidases (dotted arrows) and dipeptidyl-carboxypeptidases (full arrows). Reprinted with permission from (94). Copyright 2001. *The American Association of Immunologists, Inc.*

the MelanA_{26-35} A27L sequence, the peptidase sensitive peptide bonds were targeted for the introduction of a variety of structural modifications. Protection of the N-terminal and C-terminal ends of the peptide was explored by acetylation and amidation, respectively. Peptides with backbone modifications such as reduced or retro-inverso peptide bonds were also synthesised. Finally, substitutions of peptide residues by non-natural amino acids such as the D series amino acids, β-amino acids, cyclic amino acids, N-hdroxylated amino acids or methylated amino acids (NMe-amino acids or αMe amino acids) were also investigated (94).

Although the introduction of one structural modification in only one of the sensitive peptide bonds does not significantly improve the half-life of the peptide in human serum (94), most of the structural modifications were efficient to locally protect the peptide against peptidase. The short half-life of the mono-protected analogues was related to the degradation of the non-protected end of the peptide. We have shown that only the analogues carrying both amino- and carboxy-terminal modifications of the peptide were fully protected against degradation and display a remarkable stability in the serum with a half-life superior to 24 hours, compared to 2 minutes for the natural peptide (94).

Not all the structural modifications were efficient to locally protect the peptide against peptidase activities. We have found that the amidation of the carboxy terminal end of the peptide were not fully protective against the degradation. The doubly protected analogues with amidated C-terminal ends display a half-life between 11 to 20 hours. This result confirms previous observations showing that the Angiotensin Converting Enzyme (ACE), the most abundant dipeptidylcarboxy peptidase in the serum, was able to cleave peptide with amidated carboxy-terminal ends (98, 99).

Structural modifications of antigenic peptide could have a negative impact on its immunological properties (88, 89, 96). The first important biological property

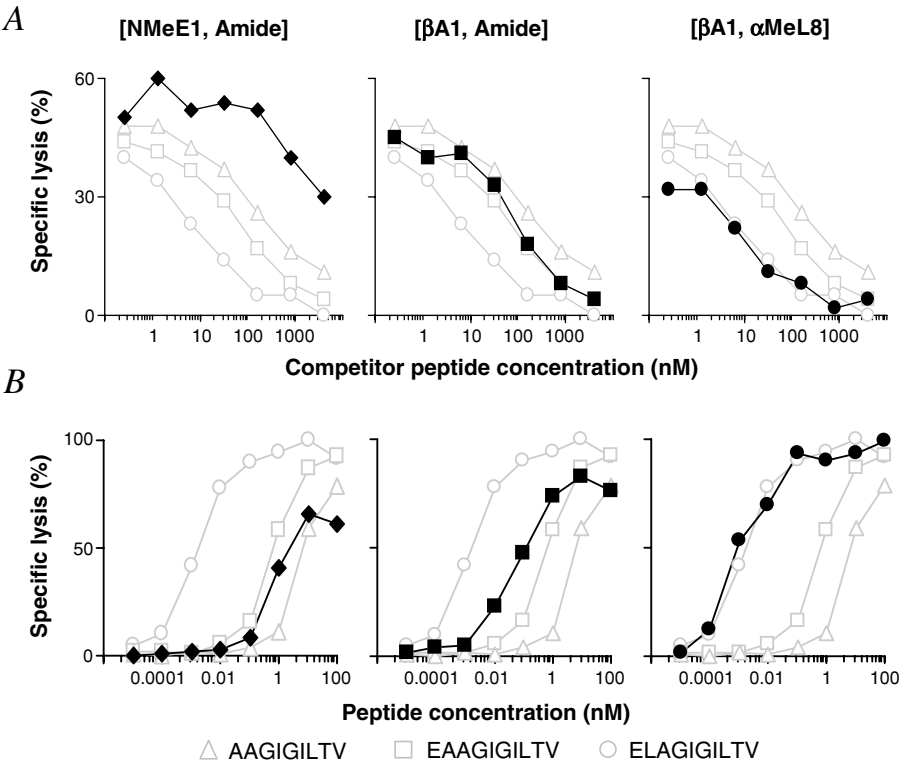

Figure 7. HLA-A*0201 binding affinity and Melan-A antigen specific CTL recognition of the Melan-A_{26-35} A27L non-natural analogues. **A**, HLA-A*0201 binding affinity of the Melan-A_{26-35} A27L non-natural analogues were determined using a competition assay based on the inhibition of the recognition of the tyrosinase$_{368-376}$ peptide by a specific T cell clone 156/34. **B**, Melan-A antigen specific CTL recognition of the Melan-A_{26-35} A27L non-natural analogues was determined using a chromium release assay. In each graph, the data corresponding to the Melan-A_{26-35} A27L protease resistant analogues, indicated in the upper part of each panel, are in black (filled symbols). The HLA-A*0201 binding (A) and antigenic (B) properties of the Melan-A related peptides are in grey (open symbols).

affected by structural modifications of the antigenic peptide is its binding to MHC molecules. We have shown that most of the structural modifications of MelanA_{26-35} A27L have a negative effect on the MHC binding properties of the antigen (94) (Figure 7A). Not surprisingly, this could occur even when the MHC non-anchor residues are modified (88, 94). In case of the amino- and carboxy-modified analogues, the negative effects of the modifications are always additive. Structural modifications could modify the peptide ability to fit properly the MHC peptide-binding groove. For example, methylated or cyclic residues could increase the steric hindrance. Other studies indicate that the peptide backbone flexibility could be reduced by the introduction of retro-inverso or reduced peptide bonds (100, 101). Molecular modeling of the non-natural analogues in the MHC binding groove indicates that

the hydrogen bond network between MHC residues and the peptide backbone or the peptide ends could be disturbed (88, 100). In rare cases, structural modifications of the peptide could have a positive impact on MHC binding. Indeed, non-natural antigenic peptides including structural modifications such as β-amino acid (96) or N-hydroxylation (102) have been shown to display a better affinity for the MHC compared to the unmodified peptide. In our study, the introduction of β-amino acid or N-hydroxylation within the first peptide bond of the MelanA$_{26-35}$ A27L peptide did not significantly change binding to HLA-A*0201 (94). We have also shown that other structural modifications such as α-, or N-methylation of appropriate residues in the peptide sequence have minimal effects on the MHC binding properties of the non-natural analogues. We have identified MelanA$_{26-35}$ A27L non-natural analogues bearing both amino- and carboxy-terminal structural modifications with MHC binding properties very similar to the unmodified peptide. In addition to the MHC binding affinity, the peptide-MHC complex stability could also be considered to determine if the non-natural analogues could be efficiently presented by the MHC (96).

The second aspect of the antigenicity that could be impaired by structural modification of antigenic peptides is the efficiency of recognition by specific T cells. After normalizing the efficiency of T cell recognition to the change in binding to MHC ((94), Figure 7B), non-natural analogues could be recognised in very different ways by the T cell receptor. Previous studies, including ours, reported that non-natural antigens with reduced peptide bond, although showing similar or improved binding to MHC, were poorly recognised by antigen specific T cell clones (94, 95, 101). Other studies have shown that recognition of the non-natural analogues could be clone specific depending on the region of the peptide recognised by each clone (88, 95), illustrating how the peptide backbone and structural modifications of non-anchor residues could modify the shape adopted by the peptide in the MHC binding groove and ultimately affect the recognition by the specific T cell receptor.

Fortunately, in some cases, structurally modified antigenic peptides can be recognised efficiently by antigen specific T cells. In our studies, we have identified amino- and carboxy-modified analogues of MelanA$_{26-35}$ A27L that were efficiently recognised by a Melan-A specific T cell line ((94), Figure 7B). We have shown that the non-natural peptides bearing α-methylation, N-hydroxylation or β-amino acid were recognised by the Melan-A specific T cells within a concentration range similar to that of the non-modified peptide in cytolytic assay (94). Thus a stepwise approach to design the non-natural analogues of MelanA$_{26-35}$ A27L allows the identification of fully protected peptides against peptidases with a binding to the MHC and a recognition by the Melan-A specific T cells similar to the non-modified peptide (Figure 7).

However, even when they are efficiently recognised by antigen specific T cells, the non-natural analogues can yet trigger T cell effector functions different from those triggered by the native antigenic peptide (100, 103). Thus, structural modification of the antigenic peptide could have profound effects on the functional properties of

the antigen, and the effector functions of the antigen specific T cells recognising the non-natural analogue must be carefully checked.

An essential requirement for their use as therapeutic agents is that the non-natural peptide analogues must be able to induce an efficient antigen specific immunity after vaccination mediating recognition and elimination of tumor cells expressing the antigen. The immunogenicity of the non-natural analogues resistant to peptidase degradation was initially tested *in vitro*. Using the ELISpot method, a previous study described the cross-reactivity of T cells induced by Melan-A_{27-35} non-natural analogues with the non-modified peptide (89). In our study, we used HLA-A*0201/MelanA_{26-35} A27L tetramers to quantify the number of Melan-A specific cells induced after *in vitro* stimulation of PBMC from healthy donors with the structurally modified analogues. We showed that amino- and carboxy-modified analogues, efficiently presented by the MHC and recognised by the antigen specific T cells, were able to induce the expansion of Melan-A specific cells from PBMC of healthy individuals (Figure 8). Three MelanA_{26-35} A27L protease resistant analogues induced a higher number of Melan-A specific cells than the non-modified peptide, indicating that protection against proteolysis could significantly enhance the *in vitro* immunogenicity of the antigenic peptide.

More importantly, the Melan-A specific $CD8^+$ T cells induced by stimulation with some of the protease resistant Melan-A peptide analogues were also able to recognise not only the unmodified MelanA_{26-35} A27L peptide, but also Melan-A_{26-35} or MelanA_{27-35} native peptides (Figure 8). Finally, the Melan-A specific T cells induced by the non-natural Melan-A analogues showed robust cytolytic activity against Melan-A expressing melanoma tumor cell lines, indicating their ability to recognise the Melan-A antigen naturally processed and expressed by tumor cells (94). The immunogenicity of structurally modified antigenic peptides has been also shown *in vivo.* Indeed, immunization of mice with antigenic pseudopeptides with reduced bonds induced a native antigen-specific immune response and conferred a better resistance against lethal challenge with tumor cells expressing the target antigen (104).

CONCLUSION

From the above considerations, one can draw the following strategy for rational peptide modifications; the two main anchor positions (P2 and PΩ) should be considered first as they might be less prone to modify the overall peptide conformation. These positions should be replaced by the well known main anchors for the MHC molecule of interest. Alternatively, non-natural aminoacid modifications can also be used. Theoretical prediction techniques can be used to select modifications that do not alter the conformation and that do not affect TCR affinity using free energy calculations. The secondary anchor positions are much more delicate to use as they are less deeply buried than the main anchors and can interact with TCR directly or through a conformational change of the peptide. Here theoretical structure based approaches are expected to play an important role. Again natural or non-natural

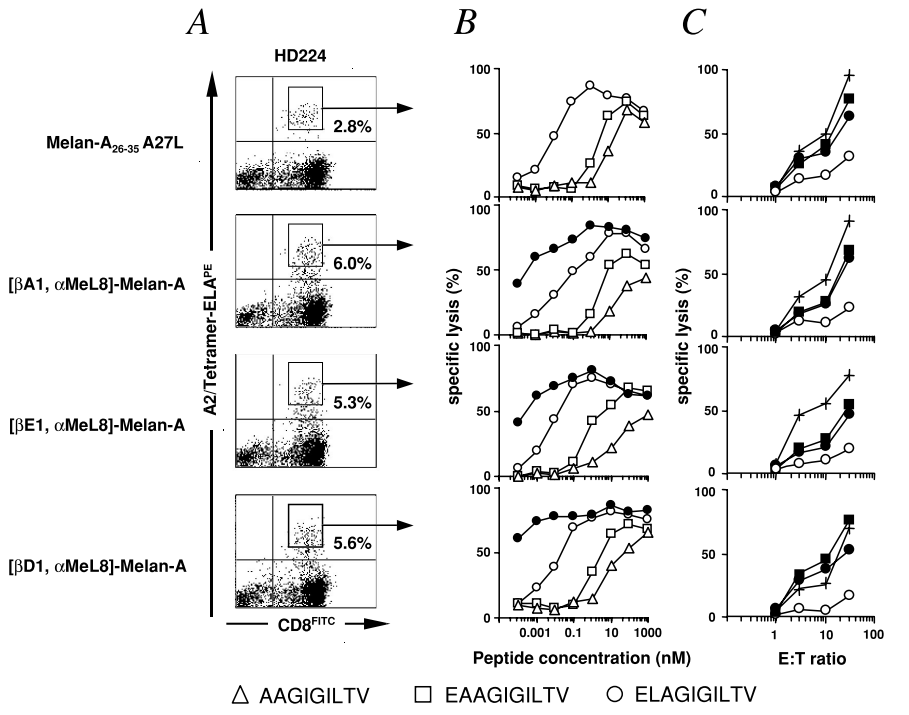

Figure 8. Antigenic specificity of and tumor recognition by CTL induced with the non-natural Melan-A analogues. After *in vitro* stimulation of PBMC from a healthy donor (HD224) with the Melan-A peptides, the tetramer$^+$ CD8$^+$ lymphocyte populations were sorted on a FACSvantage cell sorter. These populations were then expanded to sufficient cell numbers and their cytolytic activity was measured. **A**) Dot plot representation of the flow cytometry analysis of PBMC cultures after *in vitro* stimulation with Melan-A_{26-35}A27L or with the doubly modified analogues. The gate defined for cell sorting of the tetramer$^+$ CD8$^+$ populations is represented. **B**) Melan-A peptide recognition by the sorted tetramer$^+$ CD8$^+$ lympphocytes from the corresponding gate shown in panels A (Melan-A_{27-35}, open triangles; Melan-A_{26-35}, open squares; Melan-A_{26-35}A27L, open circles; Melan-A doubly modified analogue, full circles). **C**) Melanoma tumor cell recognition by the sorted tetramer$^+$CD8$^+$ lymphocytes from the corresponding gate shown in panels A. Cytolytic assay was performed with ^{51}Cr-labeled tumor cells (Me 275 and Me 290 : HLA-A*0201$^+$, Melan-A$^+$; NA8-MEL : HLA-A*0201$^+$, Melan-A$^-$) with increasing effector to target cell (E:T) ratios. (Me 275, full squares; Me 290, full circles; NA8-MEL, open circles, NA8-MEL sensitized with the Melan-A peptide, cross symbols). Reprinted with permission from (94).Copyright 2001. *The American Association of Immunologists, Inc.*

substitutions can be used. Finally, backbone or side chain modifications can be used to improve peptide's protease resistance.

In the past 7 years, intensive efforts were deployed to overcome the limitation of antigenic peptide degradation in biological fluids in order to design efficient therapeutic tumor vaccines. Structurally modified antigenic peptide analogues fully resistant to exopeptidase degradation with an improved *in vitro* immunogenicity were successfully designed. Such compounds could be very attractive candidates to

improve the efficacy of peptide based tumor vaccines. Nevertheless, further studies are required to extend and validate this approach to an increased number of tumor derived antigenic peptides. Finally, to consider the use of non-natural antigenic peptides as therapeutic agents, the safety and the *in vivo* immunogenicity of these compounds should be determined more precisely in clinical studies. It can be anticipated that such stable peptides should be carefully confined in such way that they selectively reach mature professional antigen presenting cells. The risks of inducing tolerance when delivery of immunogenic peptides allows prolonged systemic persistence has been demonstrated in an animal model (105).

In summary, any peptide modification should be followed by both MHC binding assays and analysis of the CTL fine specificity of recognition for a large panel of clones. Some modifications might abrogate recognition by some of the TCR but not by others as the affected part of the epitope might be more or less important for each individual TCR. Tinkering with nature offers many opportunities but it should be carefully monitored and the many variants of natural peptides should undergo a rigorous, experimentally driven selection process.

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13. TUMOR IMMUNOLOGY AND CANCER VACCINES

Adoptive Cellular Immunotherapy of Cancer: A three-signal paradigm for translating recent developments into improved treatment strategies

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ADOPTIVE CELLULAR IMMUNOTHERAPY

In 1909 Paul Ehrlich proposed the idea that immunological defenses provide a normal host with some resistance against malignant cells. Subsequently, Medawar documented that the transfer of immune cells could mediate graft rejection (1, 2). This seminal finding provided the basis for the work of Mitchinson (3, 4), and Brncic, Hoecker and Gasic (5) who in the early 1950's documented that the adoptive transfer of lymphoid cells from immunized mice could confer immunity to tumor challenges. In the following Decade investigators began to immunize pigs and rabbits with patient's tumors to obtain immune cells. Subsequently, peripheral blood, lymph node and spleen cells from these animals were adoptively transferred to the peritoneum or pleural cavity of patients with cancer (6) (7). At the same time, Nadler and Moore immunized pairs of melanoma patients with each other's tumors. Following subcutaneous transplant of malignant cells, patients exchanged white blood cells with reports of clinical improvement in approximately one third of the patients (8). These studies provided the base on which other investigators, taking advantage of developments in the field of cellular immunology, helped evolve the application of adoptive cellular immunotherapy to where it is today. This chapter will review these developments, provide an overview of the preclinical animal models, their translation into clinical trials and the exciting new strategies that have evolved from

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an in-depth analyses of basic T cell immunobiology. The focus of this chapter will be on T cells and the "three-signal" paradigm (9) for improving efficacy of adoptive immunotherapy. This focus emanates from the appreciation that T cells have the unique capacity to recognize unique or shared determinants on tumor cells, mediate destructive anti-tumor effects, replicate to mobilize additional effector T cells and differentiate into memory cells that can provide long-term anti-tumor immunity.

In 1954, Billingham, Brent and Medawar first used the term "adoptive immunity" to describe "an immune state transferred from one animal to another by immunologically activated cells" (10). Today, adoptive T cell immunotherapy of cancer can be defined as the transfer, to the tumor-bearing host, of T cells with anti-tumor properties that can mediate therapeutic effects through direct and/or indirect mechanisms. The earliest studies used fresh isolated immune cells, obtained by repeated immunization of naïve animals with irradiated, killed or sub-tumorigenic doses of tumor, or by ligation or excision of a growing tumor. Subsequent developments allowed for the isolation of tumor-specific T cells in a more clinically applicable setting. A key finding of most studies is that adoptive transfer of tumor-specific T cells routinely eliminates micrometastatic disease and provides long-term anti-tumor immunity to treated animals. While results of a recent clinical trial appear to be promising (11), the majority of clinical studies to date only occasionally report objective responses. Why are objective clinical responses so infrequent and why do the patients which respond, in contrast to their animal counterparts, often relapse? In our opinion it is essential to address a number of fundamental questions before it will be possible to consistently develop effective adoptive cellular immunotherapy for cancer. These questions include: Which T cells mediate tumor regression and what are their properties? Are the same cells responsible for mediating the initial tumor regression and maintaining long-term immunologic memory and preventing recurrence? Are there cells that can suppress or regulate the generation of anti-tumor T cells to be used for adoptive transfer? Can these cells suppress the immediate anti-tumor activity or the development of effective immunological memory following adoptive transfer? How many T cells are required to mediate tumor regression? What can be done to generate more and/or better T cells for adoptive immunotherapy? Following adoptive transfer the majority of transferred T cells die, is it possible to manipulate the environment to expand and maintain the transferred T cells? What is required to promote trafficking of transferred T cells to tumor sites? We will review studies in animal models that address some of these questions, however the importance of these findings ultimately lies in whether their translation into clinical trials improves outcomes.

T CELL-MEDIATED TUMOR REGRESSION

The vast majority of preclinical adoptive immunotherapy studies identified CD8+ T cells as the primary mediators of tumor regression (12–17) and implicated cytolytic activity as the central effector mechanism (18–22). Tumor regression could usually be achieved by the adoptive transfer of cytolytic $CD8^+$T cell clones or bulk cultures, in the absence of CD4 help, if IL-2 was administered to the recipient animal. Subsequent

studies demonstrated that significant therapeutic activity could be obtained by the adoptive transfer of tumor-specific T cells that lacked cytolytic activity at the time of transfer (23–25). However, a subsequent study documented that non-cytolytic $CD8^+$ T cells could acquire cytolytic activity following interaction with tumor in vivo (26). This highlights a major limitation of studies that attempted to identify the mechanism of effector T cell-mediated tumor destruction in vivo indirectly by characterizing the in vitro phenotype of transferred T cells.

Are Cytotoxic T Cells Responsible for Tumor Regression?

Lymphocyte-mediated cytotoxicity is traditionally defined as the ability of effector T cells to lyse ^{51}Cr-labelled target cells in a 4- to 6-hour in vitro assay. This cytolytic event is mediated by either the perforin-dependent granule exocytosis pathway and/or the Fas ligand (FasL)/ Fas pathway (27). The development of mice with mutations in the genes encoding either perforin or FasL allowed the first direct examination of whether these pathways were essential for T cell-mediated tumor regression. In both instances, adoptive transfer of tumor-specific T cells generated from perforin or FasL deficient mice were as capable of mediating regression of pulmonary metastases and cure of animals with systemic tumor as wild type (wt) T cells (28). Since the tumor used in these studies was resistant to killing via the Fas/FasL pathway, the experiments with PKO effector T cells argued against a role for the cytotolytic pathway in T cell-mediated tumor regression. However, cured PKO mice were less resistant to a subsequent s.c. tumor challenge (29). Consistent with this observation, tumor-specific PKO effector T cells mediated regression of pulmonary (30) or intracaranial tumor and pulmonary metastases, but were ineffective against the same tumor at a subcutaneous site (31). In this model, regression of subcutaneous MCA-205 sarcomas required the adoptive transfer of effector T cells that expressed perforin. Other studies suggested that perforin was required for regression of EL4 tumor located in the peritoneum (31). Thus it appears that the mechanisms T cells utilize to mediate tumor regression vary depending on the location of the tumor and are also likely be dependent on the tumor type.

CD4+ T Cells can also Mediate Tumor Regression

The early 1980s saw the accumulation of substantial evidence arguing that $CD4^+$ T cells could also be effective mediators of tumor regression. Fernandez-Cruz and colleagues were the first to show that adoptive transfer of noncytolytic $CD4^+$ T cells could mediate regression of tumors in rats (32). Subsequent studies by Greenberg and colleagues, confirmed that adoptive transfer of nonlytic $CD4^+$ T cells in combination with cyclophosphamide could mediate regression of the FBL3 leukemia in mice (33). However, since this tumor was strongly immunogenic, induced by virus and the CD4 response directed against viral genes was therapeutic, there were questions about how translatable this observation was to tumor immunology (34). Interest in $CD4^+$ effector cells waned in the mid 1980s as cells with a CD8 phenotype

and/or cytolytic function were reported to mediate regression of newly-induced "weakly" immunogenic tumors (35) (36) (37). While CD4 T cells played a critical role in priming cultured CD8 effector T cells; tumor regression was mediated by the adoptive transfer of cultured CD8 T cells (38). These same studies showed that both CD4 and CD8 T cells were required when non-cultured "fresh" immune spleen cells were used for adoptive transfer, but since the clinically applicable cell was one that could be expanded by in vitro culture, most investigators focused on the CD8 T cells. Interest in CD4 T cells was further reduced by evidence that their in vivo depletion did not affect IL-2-induced T cell-mediated regression of large 10-day pulmonary metastases (39).

Recently, Hu and colleagues, used mice that were congenitally deficient in MHC class II-restricted CD4 T cells (MHCII KO) to reevaluate the role for endogenous CD4 T cell help in tumor regression mediated by adoptive transfer of CD8 effector T cells. Unlike previous studies where animals were transiently depleted of CD4 T cells by mAb, the MHCII KO animals could not recover endogenous help. Similarly to previous reports, adoptive transfer of CD8+ T cells was highly effective at eliminating pulmonary metastases at the time animals were sacrificed, ten days following adoptive transfer. However, when survival studies were performed, CD8+ T cells that were effective at curing established tumor in wt mice failed to cure any tumor-bearing MHCII KO mice (40). Thus, in this model, where adoptive transfer of CD8 T cells, but not CD4 T cells, is effective at mediating tumor regression in wt mice, endogenous CD4 T cells are playing a critical role. These results, summarized in Figure 1, suggest that CD4 T cells promote the maintenance and/or development of memory CD8 T cells with therapeutic activity (40) and provides a rationale for why the transfer of both CD4 and CD8 T resulted in the dramatic objective responses seen in a recent clinical trial (11).

One reason for the failure of CD4 T cells to mediate tumor regression in the preceding studies may have been that a greater number of tumor-specific cells were required to mediate the regression of pulmonary metastases than were transferred. However, the detection of tumor-specific class II-restricted CD4 responses is problematic and is less advanced than that for CD8 responses. With the recent identification of class II-restricted tumor-associated antigens, improved culture techniques for expanding and monitoring CD4 T cells and a new appreciation for the role of CD4 T cells in developing and maintaining immunity, there has been a renewed interest in the antitumor effector function of CD4 T cells (reviewed in (41)). Exploiting magnetic bead methods, Kagamu and Shu isolated $CD62L^{low}$ TDLN cells and, after culture, obtained CD4 effector T cells that were highly polarized to a tumor-specific type 1 cytokine profile, secreting greater than 100 fold more IFN-γ than IL-4, and cured mice of intracranial tumor in adoptive transfer studies (42). A recent report from Mattes and colleagues identified CD4 T cells as mediating the regression of pulmonary and visceral metastases of a CTL-resistant tumor (43). Together these reports document that the CD4 T cells are capable antitumor effectors that need to be understood and harnessed to improve immunotherapeutic approaches to treat cancer.

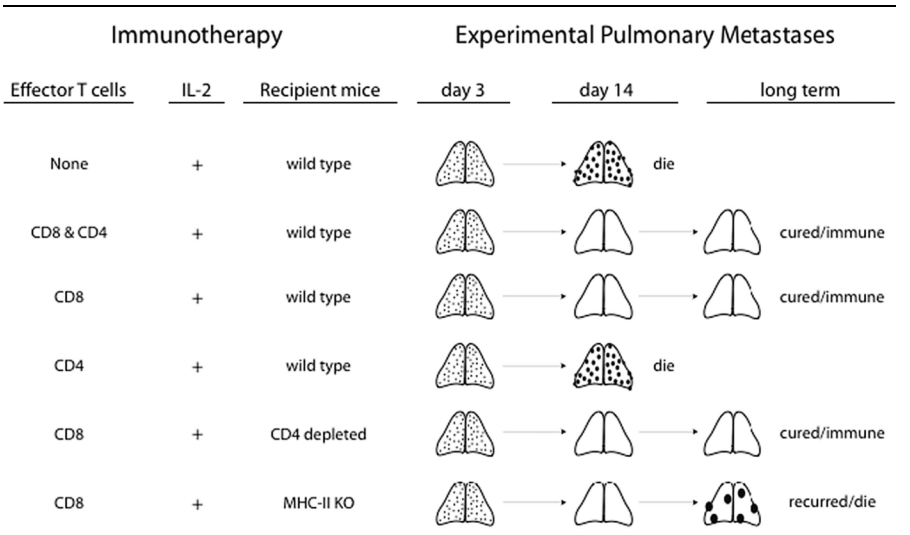

Figure 1. Tumor-vaccine draining lymph node T cells were activated with anti-CD3 for 2 days and then expanded for 3 days in CM supplemented with 60 IU/ml IL-2 to generate 'effector' T cells. Effector T cells generated from intact mice contained both $CD8^+$ and $CD4^+$ Effector T cells. $CD8^+$ effector T cells were generated from mice depleted of $CD4^+$ T cells by treatment with anti-CD4 mAb. $CD4^+$ T cells were generated from mice depleted of $CD8^+$ T cells by treatment with anti-CD8 mAb. Effector T cells were adoptively transferred into mice with 3-day established pulmonary metastases that were otherwise untreated (wild type), treated with anti-CD4 mAb (CD4 depleted), or genetically deficient of classical $CD4^+$ MHC class II-restricted T cells (MHC II KO). Mice were sacrificed at 10 to 14 days following adoptive transfer and the number of pulmonary metastases enumerated or followed for survival.

Type 1 and Type 2 Cytokine Responses—the Ying and Yang

What mechanisms are non-cytolytic CD8 and CD4 T cells using to mediate tumor regression? An observation of adoptive transfer studies using effector T cells deficient in either perforin or FasL was that the effector T cells released "inflammatory" type 1 cytokines (IFN-γ or TNF-α) in response to in vitro stimulation with specific tumor. This is likely an important observation as the immune response is not limited to inflammatory cytokines. In the early 1970s it was suggested that an immune response was a composite of two antagonistic T-cell populations (44, 45). Today it is generally accepted that these two T-cell populations, composed of both $CD4^+$ T helper (Th) cells and $CD8^+$ T cytotoxic (Tc) cells, can be segregated based on their cytokine release patterns (46–54). A type 1 T cell selectively secretes, IFN-γ, TNF-α, TNF-β/LT and/or IL-2, whereas type 2 T cells secrete IL-4, IL-5, IL-6, IL-10, and/or IL-13. This simplistic view of immune regulation has become more complex with the description of Th3 cells, that immunosuppress through the secretion of TGF-β (55), and the observation that some IL-4 is required to obtain type 1 T cell responses (56).

The differentiation of T cell subsets along a type 1 or type 2 path depends on the type of antigen, the route and dose of entry of the antigen, the nature of costimulatory signals provided, as well as the genetic background of the host (57–60). In addition

the most clearly defined factor determining T cell differentiation is the cytokine environment present at the initiation of the immune response. IL-4 is the dominant cytokine influencing type 2 polarization (61, 62), signaling through the IL-4 receptor and activating Stat-6 (63–65) and the transcription factors, c-Maf and GATA-3 which commit the T cell to type 2 differentiation (66) (67). In contrast, IL-12 and IFN-γ are the cytokines that drive type 1 polarization (68, 69). IL-12 directly influences type 1 polarization by activating Stat-4 in naïve T cells (70). More controversial are the effects of IFN-γ, which likely augment type 1 polarization indirectly by upregulating expression of IL-12 by macrophages (71). However, since type 1 responses are not completely abrogated in IL-12 p40 deficient mice (72) and IL-12 poorly induces T-bet, a transcription factor associated with type 1 differentiation, it has been suggested that IFN-γ may also directly influence type 1 polarization. Supporting this reasoning is the observation that T-bet expression is dependent on Stat-1, which is efficiently induced by IFN-γ (73). Other cytokines, such as IL-18 and IL-1α were thought to act as polarizing signals, but both molecules appear to amplify responses that are already polarized (74). The antagonistic nature of these T cell populations is evident in that the cytokines that drive polarization of their respective phenotypes inhibit polarization toward the opposing phenotype. The most dominant effect is exerted by IL-4, which induces type 2 polarization and inhibits type 1 polarization (75, 76). If IL-4 reaches a certain threshold at the beginning of an immune response then a type 2 cytokine profile will dominate that response. One way IL-4 exerts its effect is by down-regulating expression of IL-12Rβ2, thereby blocking the effects of IL-12 on differentiating T cells (77). IL-4 also inhibits type 1 polarization indirectly by downregulating IL-12 production of dendritic cells (78, 79).

Cytokine Profile is Relevant to Disease

The relevance of type 1 or type 2 polarized T cell responses to disease states was first appreciated in infectious disease models (reviewed in (53)). An example is the protection against the parasite, *Leishmania major* (*L. major*), that is associated with a type 1 polarized immune response and this protection is lost in IL-12Rβ2 deficient mice (80) (81). Mouse strains that are susceptible to *L. major* infection mount predominantly a type 2 immune response. Converting the type 2 immune response toward a type 1 response, in these susceptible mice, provides protection from an infection with *L. major* (82). The process of promoting a non-therapeutic immune response, as seen in the mouse strains that are susceptible to *L. major*, has been termed 'immune deviation' (83). However, it must be noted that all immune pathologies cannot be explained by immune deviation. For example, the cell-mediated disease experimental autoimmune encephalomyelitis (EAE) is believed to be mediated by Th1 immune responses that can be ameliorated by Th2 responses. Yet susceptible strains of mice that have the IFN-γ gene disrupted are still susceptible to EAE whereas resistant strains of mice become susceptible to EAE when this gene is disrupted (84). Whether other type 1 cytokines, such as TNF-α, might be responsible for the pathology observed in EAE is unclear and only underscores the complexity of the immune system. Nonetheless, appreciation of immune deviation has changed

the way immunologists look at many disease states. But is this paradigm relevant to cancer?

A Type 1 Cytokine Response is Essential for Tumor Regression

Various studies have implicated the type 1/ type 2 paradigm in the regulation of the host's immune response to cancer however the significance of these two T-cell populations has been controversial (85–91). While several reports had made correlations between type 1 responses being therapeutic (87, 88), Hu and colleagues were the first to show that a poorly immunogenic tumor that failed to prime therapeutic T cells for adoptive immunotherapy, had not ignored the tumor, but had initiated a tumor-specific type 2 response that was non therapeutic (86). Vaccination with the same tumor that was lipofected with an allogeneic MHC class I gene, primed T cells that exhibited a tumor-specific type 1 cytokine profile and were therapeutic in adoptive transfer studies. These results suggested that the failure of adoptive immunotherapy, in this model, was not the result of T cells failing to recognize the tumor; rather, it was because the host mounted an ineffective (type 2) immune response. Winter and colleagues examined whether other poorly immunogenic tumors (tumors where vaccination fails to provide protection from a subsequent tumor challenge) stimulated a tumor-specific type 2 cytokine response in the vaccinated host. Examining a panel of tumors, ranging from strongly to poorly immunogenic, they showed that immunogenic tumors primed immune responses that were highly polarized towards a type 1 cytokine response, while a type 2 cytokine response was dominant for poorly immunogenic tumors (85). Further, To and colleagues, working in the weakly immunogenic MCA-205 tumor model, showed that tumor-specific CD4 T cells polarized to a type 1 response were significantly more therapeutic than CD4+ T cells polarized to a type 2 cytokine profile (89). In contrast to these findings other groups have suggested that type 2 polarized T cells can mediate tumor regression (92–94). However, a careful study using adoptively transferred type 1 or type 2 polarized OT-1 transgenic T cells demonstrated that the type 2 T cells were markedly less therapeutic than T cells from type 1 polarized immune responses (95). Additionally, the therapeutic type 2 response was non-therapeutic if the T cells were adoptively transferred into IFN-γ knockout mice (96). Thus therapeutic type 2 polarized T cells were dependent on the type 1 cytokine, IFN-γ, derived from the recipient, underscoring the importance of a type 1 immune response. Chamoto et al., used the same tumor model system, but transferred non-transgenic T cells and reported that only the adoptive transfer of Th1 cells, but not Th2 cells, induced tumor-specific cytotoxic Tc1 cells in the recipient that led to eradication of the tumor mass *in vivo* (94). In another study the adoptive transfer of Th2 cells led to tumor eradication; however by inducing necrosis with an infiltration of inflammatory cells into the tumor mass (97). It is unclear from the design of this last study whether type 1 cytokines played a major role in mediating tumor destruction. Recently, Mattes and colleagues reported that type 2 CD4 T cells mediated, through an eosinophil-dependent process, the regression of pulmonary and visceral metastases of a CTL-resistant tumor (43). Thus, while the majority of studies support the importance of

a type 1 response, there are some examples of type 2 responses mediating tumor regression.

IFN-γ is Critical for in situ Priming but not Effector Function or Protective Memory

In the absence of IFN-γ vaccination fails to provide protective antitumor immunity (98, 99). In spite of the lack of protection, effector T cells generated from TVDLN of GKO mice exhibited tumor-specific cytotoxicity and adoptive transfer of GKO effector T cells mediated complete regression of pulmonary metastases in both wt and GKO mice (99). Further, mice cured of experimental pulmonary metastases were resistant to a secondary tumor challenge documenting that IFN-γ is neither required to maintain a protective memory response or to mediate regression of subcutaneous tumor. These results are generally consistent with an earlier study by Peng et.al. using the MCA-205 sarcoma model (31). However, this report found that effector T cells from GKO mice were less effective than wt effector T cells, requiring transfer of greater numbers of effector T cells or combination with radiation of the tumor-bearing host. In contrast to these two reports, studies from Prevost-Blondel et. al., using effector T cells from GP33 TCR-tg wt or GKO mice did not see regression of pulmonary metastases when IFN-γ was absent from their system (100). However, TCR-Tg effector T cells from GKO mice were able to mediate efficient regression of pulmonary metastases in wt hosts, excluding the possibility that these GKO effector T cells were not efficiently "primed". The authors suggest that given the low level of class I on their B16 the lack of therapeutic efficacy may be related to a dependence on IFN-γ to upregulate expression of class I on the tumor in vivo. In the studies of Winter et. al., it is interesting that the effector T cells from GKO mice did not develop a tumor-specific T2 cytokine profile. Subsequent studies showed that effector T cells generated from GKO mice secrete TNF-α in response to stimulation with specific tumor, providing a possible alternative mechanism for these T cells to mediate tumor regression.

Perforin, IFN-γ and TNF—A Triad of Effector Molecules

While the aforementioned studies using gene knock-out or mutant mice deficient in a single effector molecule examined the requirement for that molecule in T cell-mediated tumor regression, they failed to rule-out the likelihood that the immune system of the deficient host would utilize other elements of the immune system to compensate for absence of that molecule. To evaluate whether perforin and IFN-γ were able to compensate for each other when either was absent, T cells from mice deficient in both molecules were evaluated for their ability to mediate tumor regression in the poorly immunogenic B16BL6-D5 melanoma model. While slightly less effective than T cells from either PKO or GKO mice, effector T cells from perforin-IFN-γ double ko (PKO/GKO) mice could eliminate pulmonary metastases and cure mice of three day established tumor (101). Analysis of the effector T cells revealed they maintained a type 1 cytokine profile, secreting TNF–α in response to stimulation with specific tumor. Poehlein and colleagues went on to show that the therapeutic efficacy of these T cells could be blocked by treatment with soluble TNF

receptor. These results suggest that a triad of effector molecules exist, any of which may be able to mediate tumor regression. However, strategies that can generate all three responses will likely be the most effective. Consistent with this hypothesis are earlier reports that the administration of type 1 cytokines (IFN-γ, IFN-α, and or TNF-α) together with the adoptive transfer of a cytolytic tumor-specific T cell clone further augmented therapeutic efficacy (102, 103). Since the additional cytokines were not expected to support T cell growth better than IL-2, which was administered to all animals, the authors suggested that the IFN-γ, IFN-α or TNF-α worked to augment the susceptibility of the tumor to killing by cytolytic T cells, or that the cytokines mediated antitumor effects independent of CTL.

A Type 1 Immune Response Correlates with Therapeutic Response in Humans

In 1994, Kawakami and colleagues showed a strong correlation with the adoptive transfer of T cells exhibiting tumor-specific IFN-γ secretion and objective clinical response (104). While a highly selected group of patients, this report supports the link between a type 1 cytokine response and tumor regression. Subsequently, Lowes and colleagues, identified an increase in TNF-β (LT-α) mRNA in melanoma lesions undergoing spontaneous regression and absent in progressing nodules (105). Since TNF-β is a type 1 cytokine it provides additional support for this paradigm.

However, in tumors that are not undergoing regression, elevated levels of type 2 cytokines have been observed (106). Another study of melanoma and renal cell carcinoma patients reported that T cells from the peripheral blood of patients with no current evidence of disease displayed Th1 responses to the tumor-associated MAGE-6 peptide (107). Additionally, this report identified that the majority of patients with active disease had a MAGE-6 peptide-specific type 2 immune response, characterized by secretion of IL-5. Other studies have observed tumor-specific type 2 cytokine responses in patients that have progressed following immunotherapy. Meijer and colleagues detected a mixed tumor-specific type 1 and type 2 cytokine response in the peripheral blood of some patients following adoptive immunotherapy (108). Mixed type 1 and type 2 responses have also been reported in patients receiving a breast cancer vaccine (109). While to our knowledge there is no direct evidence that a tumor-specific type 2 response can suppress the therapeutic activity of tumor-specific type 1 effector T cells, it is expected that an established tumor-specific type 2 memory response would interfere with endogenous epitope spreading and the propagation of newly primed tumor-specific type 1 T cells (110–112).

A Three-Signal Paradigm for Effective Generation of Therapeutic T Cells

Overall the above noted data strongly argue that a type 1 cytokine response is critical for T cell-mediated tumor regression in most models. Further, evidence for immune deviation in additional animal tumor models (85, 86, 88) as well as evidence from clinical studies correlating a type I response with therapeutic effects (104, 113), spontaneous regression (105) or absence of disease (107) provides additional support for the hypothesis that the development of a tumor-specific type 1 response is critical for effective immunotherapy. These early findings led, in 1999, to the proposition of a three-signal paradigm for the development of effective T cells for adoptive

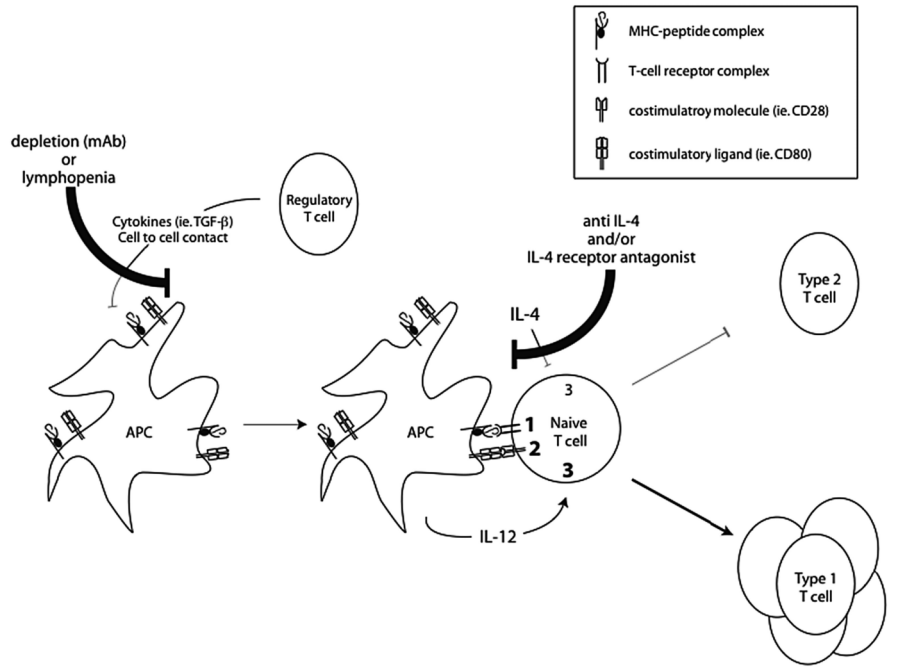

Figure 2. Generation of therapeutic effector T cells requires three signals. This includes the well established first "two signals"; including T-cell receptor interaction with MHC-peptide complexes (**signal 1**) and the interaction of costimulatory molecules with their ligands (**signal 2**). The third signal (**signal 3**) polarizes the responding T cell along a type 1 (Th1/Tc1) phenotype. This third signal promotes generation of a type 1 response by blocking type 2 polarizing signals (ie. IL-4 receptor antagonists) and providing type 1 polarizing signals (IL-12).

immunotherapy (9). The first signal is that of specific antigen, presented by MHC to the TCR. The second is a costimulatory signal(s) and the third are those that polarize the developing immune response to a type 1 cytokine profile and/or block the development of a type 2 cytokine response. This three-signal paradigm provides a basic strategy to induce, augment and maintain the generation of T cells with therapeutic activity. Figure 2 provides a schematic of how this paradigm might be used to optimize the development of a tumor-specific type 1 immune response. This includes the elimination of factors and/or cells that interfere with these three signals. Combining these strategies with homeostasis-driven expansion and regulatory T cell depletion (discussed below) provide a basis for a new generation of adoptive cellular immunotherapy trials.

MANIPULATING THE DEVELOPING ANTI-TUMOR IMMUNE RESPONSE

Since the transfer of T cells with a type 1 polarized tumor-specific cytokine response exhibited enhanced therapeutic efficacy a possible strategy for adoptive

immunotherapy is to examine whether type 2 polarized responses could be "repolarized" toward a type 1 phenotype. T cells from lymph nodes draining a poorly immunogenic tumor that routinely develop a non-therapeutic tumor-specific type 2 cytokine response could be "repolarized" towards a tumor-specific type 1 cytokine response by *in vitro* culture with anti-IL-4 mAb and exogenous IL-12. Coincident with expression of the type 1 cytokine response these T cells exhibited therapeutic efficacy in adoptive transfer studies (85). The mechanism of this effect is unclear. Did the altered cytokine milieu actually change the cytokine profile of the tumor-specific type 2 cells that were not fully differentiated (114, 115) or did the IL-12 enhance expansion of a small population of tumor-specific type 1 T cells from the mixed population of tumor-specific type 1 and type 2 T cells residing in tumor-vaccine draining lymph nodes (116)? While a better understanding of the mechanisms operational in these short-term animal experiments will provide important information, applying these strategies to patients with advanced disease will likely be different and require additional research with clinical specimens to optimize the conditions for obtaining a tumor-specific type 1 immune response. However, a recent report from Knutson and colleagues shows that CD4 T cells from vaccinated cancer patients maintained antigen-specific type 1 CD4 T cells better when cultured with IL-12 and IL-2 than standard culture conditions (117).

In addition to manipulating the cytokine environment, infectious disease models have provided additional insight into strategies that can influence T cell polarization. For example, strain-specific differences within dendritic cells influence the ability of inbred mice to mount polarized immune responses that protect against *L. major* (118). Adding to the complexity, various groups have segregated dendritic cells based on their ability to induce type 1 or type 2 T cell responses (119–121). The role of costimulation by antigen-presenting cells in skewing immune responses is another potential target for augmenting the generation of tumor-specific T cells for adoptive cellular immunotherapy. CD80 (B7-1) and CD86 (B7-2) have been among the most studied ligands of T cell activation, are critical for priming tumor-specific T cells in vivo and may also affect T cell polarization (122) (123) (60) through a series of interactions; including the strength of signal through the T-cell receptor and differential upregulation of costimulatory molecules (57). Additionally, constimulation may broaden the repertoire of tumor antigens recognized by the responding T cells, allowing them to respond to subdominant epitopes (124). This approach, together with polyclonal stimulation with anti-CD3 in vitro, has shown efficacy for improving expansion of effector T cells for adoptive immunotherapy (125,126).

While not a polarizing signal, strategies that block the inhibitory signal provided to CD28 through interaction with CTLA4 have already been shown to substantially augment immune responses in preclinical models and clinical trials (127) (128) (129) (130). CD137 (4-1BB) is a costimulatory molecule expressed on activated T cells that appears to augment type 1 polarization. *In vitro* activation of tumor-vaccine draining lymph node cells with antibodies to CD137, CD3, and CD28 increased tumor-specific IFN-γ secretion more than activation with anti-CD3 and anti-CD28.

In adoptive transfer studies T cells activated with all three antibodies were more effective mediators of tumor regression than T cells activated with anti-CD3 and anti-CD28 (131, 132). Another co-stimulatory molecule is CD134 (OX40). This member of the TNF receptor family is expressed on recently activated CD4+ T cells. Ligation of CD134 augments proliferation and cytokine secretion and has been shown to augment the immune response to weakly immunogenic tumors (133–136). While some controversy exists, most data suggests that CD134 stimulation augments both type 1 and 2 immune responses (137–140). Thus, providing co-stimulation via CD134 will likely have the greatest therapeutic effect when it is administered with a type 1 polarizing signal or in adoptive transfer studies where tumor-specific CD4 T cells are already polarized towards a type 1 cytokine profile.

Costimulation Following Adoptive Immunotherapy

Another way to augment the effectiveness of adoptive immunotherapy is by supplying costimulatory signals to the T cells following adoptive transfer. While a number of investigators have transfected tumors with CD80 for use as vaccines (122), Bai and colleagues showed that administering an anti-CD28 mAb augmented the therapeutic efficacy of tumor-specific (Tg-TCR) T cells (141). In conjunction with adoptive immunotherapy, the administration of antibodies against 4-1BB or OX40 also increased therapeutic efficacy of the transferred T cells (142)(143).

An alternative to providing costimulatory signals is to block inhibitory signals provided by host cells or the tumor. Blocking the inhibitory effect of CTLA-4 is one approach, but as yet we know of no studies that have combined this strategy to promote survival or activity of antitumor effector T cells in adoptive immunotherapy studies. However, given its activity in combination with vaccines, these studies will certainly be performed soon. B7-H1 is a B7 family molecule that provides an inhibitory signal to T cells and is expressed by some tumors (144). Recently, Strome and colleagues showed that by blocking B7-H1 in vivo at the time of adoptive immunotherapy it was possible to increase therapeutic efficacy against a B7-H1 expressing tumor (145).

SUPPRESSION AND REGULATORY T CELLS

In addition to the inhibitory signals noted above, the immunogenic potential of tumor antigens is dependent on a variety of host immune mechanisms that avoid autoimmunity by ensuring tolerance to self antigens. There is accumulating evidence that immunological tolerance is maintained by T cell-mediated suppression of self-reactive T cells. T cell-mediated regulation is not a new concept as lymphocyte populations that can suppress antigen-specific immune responses were described more than three decades ago (146, 147). The suppressive lymphocyte population was characterized mainly by function since molecular markers defining this population were unavailable at the time. Subsequent studies demonstrated that $CD4^+$ T cells were responsible for the T cell-mediated suppression (148–150). A suppressive lymphocyte population was further characterized as the $CD4^+$ T cell subpopulation expressing the IL-2Rα chain (CD25) that if depleted prior to T cell transfer into athymic

nude mice resulted in autoimmune disease (151). Other groups demonstrated that elimination of $CD25^+$ T cells resulted in the development of autoimmune diseases such as colitis and diabetes (152–156). This $CD25^+CD4^+$ T cell population, which represents 5–10% of peripheral $CD4^+$ T cells in mice, is nonproliferative to antigenic stimulation *in vitro* and potently suppresses the proliferation of other $CD4^+$ or $CD8^+$ T cells (157–159). Recently, other markers associated with $CD25^+CD4^+$ regulatory T cells have been described, including GITR (160, 161), CTLA-4 (162), Foxp3 (163, 164), CD103 (165), $CD45RB^{low}$ (166, 167) and Lag-3 (CD223) (168). Human $CD25^+CD4^+$ regulatory T cells isolated from peripheral blood express $CD45RO^+$, CD62L, and CTLA-4 and can suppress the proliferation of $CD25^-CD4^+$ T cells (169). It appears that $CD25^+CD4^+$ regulatory T cells require antigen-induced activation to become suppressive, but their suppressive function is antigen independent (170, 171). They mediate suppression via secretion of immunosuppressive cytokines, TGF-β and IL-10 (172–174), but some mechanisms appear to require direct cell-cell contact (175–177).

Early on it was postulated that the elimination of "suppressor" T cells could enhance effective antitumor immunity in tumor-bearing hosts (178). Studies from North and Colleagues showed that CD4+ T cells from tumor-bearing mice could inhibit therapeutic adoptive cellular immunotherapy (148). These results, under appreciated for the past 15 years, correspond closely to what we now appreciate as regulatory T cells. Recent studies have shown that depletion of $CD25^+$ cells augmented both $CD4^+$ and $CD8^+$ T cell responses against tumor (179–183). Additionally, *In vivo* depletion of $CD25^+CD4^+$ regulatory T cells using the anti-CD25 mAb, PC61, promoted regression of several leukemias, sarcomas, and a myeloma (184). Reactivity to the tumor-associated antigen, tyrosinase-related protein-2 (TRP-2), was enhanced in $CD25^+CD4^+$ depleted mice challenged with B16 melanoma and injected with an antibody that blocks CTLA-4 (181). Furthermore, depletion of $CD25^+CD4^+$ regulatory T cells in tumor-vaccinated mice augmented the sensitization of tumor-vaccine draining lymph node cells, which upon adoptive transfer demonstrated augmented antitumor therapy. In support of an important role for IL-2 in the developing anti-tumor immune response, enhanced activity of tumor-vaccine draining lymph nodes did not occur if depletion of $CD25^+CD4^+$ regulatory T cells was performed after tumor vaccination (183).

The accumulation of data obtained from tumor models has led to a search for $CD25^+CD4^+$ regulatory T cells in humans. Increased percentages of $CD25^+CD4^+$ T cells expressing TGF-β were present in non-small cell lung cancer (NSCLC) tumor-infiltrating lymphocytes and ovarian cancer tumor-associated lymphocytes compared to PBMCs from normal patients (185). $CD25^+CD4^+$ T cells isolated from NSCLC could inhibit proliferation of autologous peripheral blood T cells (186). Similarly, $CD25^+CD4^+$ T cells from pancreas or breast cancer involved lymph nodes suppressed the proliferation and secretion of IFN-γ of activated $CD8^+$ cells or $CD4^+CD25^-$ cells (187). Clinical trials designed to deplete $CD25^+CD4^+$ regulatory T cells or block their function will be important steps to determine whether these cells, which play such an important role in preclinical models, are obstacles

to effective immunotherapy in humans. One treatment that is already available to delete CD25+ cells is the IL-2-diptheria toxin fusion protein (ONTAK), which could provide a novel strategy for depleting $CD25^{+}CD4^{+}$ regulatory T cell population in patients (188).

WHEN IT COMES TO TUMOR-SPECIFIC T CELLS "MORE IS BETTER"!

In 1955, Mitchison was the first to demonstrate that the adoptive transfer of greater numbers of tumor-specific T cells increased therapeutic efficacy (189). This paradigm is now well established in all adoptive transfer models we know of (102, 190, 191). While obtaining increased numbers of tumor-specific T cells in animal models is often a trivial problem, simply requiring additional mice, obtaining increased numbers of tumor-specific T cells in patients with cancer represents a therapy-limiting obstacle to potentially effective treatment. What limits the expansion of T cells responding to vaccination or following adoptive transfer into a tumor-bearing host? In normal non-lymphopenic hosts homeostatic mechanisms work to maintain a constant level of lymphocytes and the expansion of tumor-reactive T cells is limited. However, in lymphopenic hosts, T cells expand to fill the void. Several hypotheses have been proposed to explain homeostasis-driven T cell proliferation. One of the earliest to be suggested was the "space" hypothesis. Here, the creation of space allows responding tumor-specific T cells to expand, unencumbered by their neighbors and/or inhibitory signals those neighbors may provide (192–195). A second is the "suppressor cell" hypothesis, which emphasizes the selective elimination of "suppressor" or $CD4^{+}CD25^{+}$ regulatory T cells by the lymphopenic insult resulting in increased expansion of tumor-reactive T cells (196–203). This research has provided solid information on the requirements for T cell expansion in lymphopenic hosts and provides insight into how this information may be exploited to buttress antitumor immunity.

What Controls Homeostatic Proliferation?

The expansion of CD4+ and CD8+ T cells in lymphopenic hosts is dependent primarily on TCR - peptide interactions (204) (205), with clonal competition for these interactions being a limiting factor for ultimate expansion (206). However, these interactions are not the sole mechanism for expansion, as an IL-7-mediated component can also drive proliferation independent of TCR signaling (207) and IL-12, which does not play an essential role in T cell expansion, can augment the response (208). Further, IL-2, costimulation via CD28 or interactions with 4-1BB/4-1BBL and CD40/CD40L are not required for homeostasis-driven proliferation (209, 210). During expansion naïve T cells express a "pseudo" memory phenotype and never revert back to a naïve phenotype (211). Importantly, T cells undergoing homeostasis-driven expansion are hypersensitive to antigen stimulation with a lowered threshold for activation and expression of effector functions (209, 212, 213).

Augmented Priming of T Cells in Vaccinated Lymphopenic Hosts

Mackall and colleagues were the first to appreciate how exposure to antigen during immune reconstitution of a lymphopenic host could be exploited to skew the

T cell response to vaccination (204). Subsequently Borello and colleagues showed how vaccination, with a GM-CSF transduced B-cell lymphoma, A20, in the post bone marrow transplant (BMT) setting could augment therapeutic efficacy more effectively than in a normal host (214). They also showed how this strategy led to increased expansion of tumor-reactive T cells by reconstitution with trace levels of transgenic TCR T cells reactive with the model antigen expressed by the tumor. Asavaroengchai and colleagues used tumor lysate-pulsed dendritic cells to vaccinate mice post BMT against the MT-901 mammary cancer (215). This provided effective protection in challenge studies and reduced outgrowth of pulmonary metastases in animals with established tumor prior to BMT and vaccination. Hu and colleagues transfused normal spleen cells into either irradiated or congenitally lymphopenic Rag1-/- mice and vaccinated with a GM-CSF secreting tumor vaccine. Tumor vaccine-draining lymph nodes (TVDLN) harvested from reconstituted lymphopenic mice contained an increased frequency of tumor specific CD4+ and CD8+ T cells and were significantly more effective in adoptive transfer studies (216, 217). This demonstrated that neither BMT nor the cytokine storm accompanying whole body irradiation were required to obtain the beneficial anti-tumor effects provided by vaccinating a lymphopenic host. Dummer and colleagues reported how lymphopenic animals, either sublethally irradiated or Rag1 -/- mice, reconstituted with normal lymph node cells were more resistant to tumor challenge then either control or lymphopenic animals (218). This response, in non-vaccinated animals, correlated with an increase in tumor-specific cytotoxicity and IFN-γ release. Together, these reports suggest, that by initiating vaccine strategies during periods of lymphopenia-driven T cell expansion it is possible to skew the T cell repertoire towards tumor antigens, resulting in a dramatic expansion of tumor-specific T cells. Since adoptive immunotherapy has been limited to tumors where it is possible to generate substantial numbers of tumor-specific T cells, this maneuver could be used to obtain tumor-specific T cells in malignancies where it has previously been impossible to do so.

Adoptive Immunotherapy in Lymphopenic Hosts Augments Therapeutic Activity

Homeostasis-driven proliferation can also have a tremendous impact when exploited at the efferent stage of immunotherapy. The adoptive transfer of antigen-specific lymphocytes into a lymphopenic host, has been shown to augment the effector activity of the transferred cells. This was first shown by Harris and colleagues, who in 1954 reconstituted irradiated rabbits with immune lymph node cells and documented substantial increases in antibody production (219). Over the intervening decades the observation that immunosuppression prior to adoptive transfer improves therapeutic efficacy has remained consistent (220) (221) (222) (36). While the creation of space, elimination of suppressor cells or a direct anti-tumor effect were frequently used to explain the advantage conferred by immunosuppression, in some models it is likely that all three mechanisms played a role in the augmented antitumor effect. Based on the preponderance of preclinical data supporting its use, many clinical studies used immunomodulatory doses of cyclophosphamide prior to infusion of transferred cells even though there was little clinical evidence to support its use. The recent success of stem cell transplant strategies in renal cell cancer (223, 224) prompted Rosenberg

and colleagues to reevaluate the degree of immunosuppression employed prior to adoptive transfer. Their success in adoptive cellular immunotherapy in melanoma patients receiving a mix of tumor-specific T cell clones and bulk CD4+ and CD8+ TIL following a non-myeloablative conditioning regimen (discussed latter—(11)) has created a great deal of enthusiasm in the field of adoptive immunotherapy (225). The unparalleled expansion and persistence of tumor-specific T cells, greater than 70% of circulating CD8+ T cells in some patients, will undoubtably lead to additional trials of this approach in melanoma and other malignancies.

While trials exploiting homeostasis-driven proliferation at the afferent limb (priming) or the efferent limb (transfer of effector cells) are underway or have recently been reported. Given the strong preclinical and recent clinical data it is only a matter of time before studies will be instituted that exploit the advantages of homeostasis-driven proliferation at both the afferent and efferent stages of the immune response. In the following sections we will review clinical trials of adoptive cellular immunotherapy, in some cases discussing preclinical studies that were relevant to the clinical trial. Table I presents an overview of adoptive cellular immunotherapy strategies that made their way to clinical trials for patients with cancer.

ADOPTIVE CELLULAR THERAPY USING NON-SPECIFIC CELLS

Macrophages

Macrophages play an important role as antigen presenting cells and as effector cells in humoral and cellular immunity; however, their role in mediating anti-tumor effects is not well established. The infiltration of macrophages and monocytes into many different tumors, e.g. colon carcinoma (226) (227), mammary carcinoma (228) and melanoma (229), has been shown, although the degree of infiltration can vary greatly even in tumors of the same histology (230). Preclinical adoptive transfer experiments with in vitro activated macrophages revealed their potential to inhibit pulmonary metastases (231) and to induce regression of transplanted human melanoma cells in SCID mice (232). Although activated macrophages were effective at inhibiting the formation of metastases, they were generally ineffective at mediating regression of established tumors (233). Additionally, strategies that activated macrophages in vivo, using lymphokines or synthetic analogs of muramyl dipeptide, had limited success mediating tumor regression (233) (234). Further, macrophages can release growth factors that promote angiogenesis (235) and support tumor growth (236) complicating their application to treat cancer.

However, these preclinical studies were sufficiently encouraging that clinical trials employing adoptive transfer of macrophages were performed. To increase the number of macrophages in the peripheral blood prior to leukapheresis, patients were pretreated for 7 days with GM-CSF. Harvested macrophages were stimulated in vitro with IFN-γ and LPS and adoptively transferred (237). Macrophages accumulated at sites of tumor metastases (238), but no objective responses were observed in the treatment of 22 colorectal cancer patients (239) (240) (241), 10 melanoma patients (242) and 11 non small cell lung cancer patients (NSCLC) (243). A recent study

Table 1. Adoptive Cellular Immunotherapy of Cancer in Humans

Donor	Vaccine	Cell source	Cultured	Transferred	Ref
Fresh cells					
Xenogeneic					
Pigs	autol tumor	PBMC, LN?	no	peritoneum	(7)
Rabbits	autol tumor	spleen?	no	pleural cavity	(6)
Allogeneic					
Non-related	autol tumor	leukocytes	no	i.v.	(8)
Cultured cells					
Syngeneic					
Autologous	none	leukocytes	yes/autol tu	i.p.	(8)
Monocytes					
	GM-CSF*	PBMC	Yes /IFN-γ LPS	i.v.	(239–243).
	none	PBMC	Yes /IFN-γ	i.vesicularly	(244)
NK/LAK cells					
	None	PBMC	yes/TCGF	i.v.	(262)
	none	PBMC	Yes /IL-2	i.v.	(269–272)
	none	PBMC	Yes /IL-2	i.p.	(273, 274)
	none	adh PBMC	Yes /IL-2	i.v.	(278).
T cells					
	none	PBMC	Yes /PHA	i.t.	(251)
	none	PBMC	Yes /PHA	i.v.	(252)
	none	TIL	Yes /tumor/IL-2	i.v., intra arterial	(290–295)
	IFN-α	TIL	Yes /tumor/IL-2	i.v.,	(296).
	none	CD8 TIL	Yes /tumor/IL-2	i.v.	(297)
	none	PBMC	Yes /anti-CD3	i.v., + IL-2	(280)
	tumor	LN	Yes /tumor/IL-2	i.v.	(305).
	tumor	LN	Yes /anti-CD3/IL-2	i.v.	(309–311)
	tumor	PBL	Yes /anti-CD3/IL-2	i.v.	(312).
	none	CD4 + PBL	Yes /anti-CD3/IL-2	i.v., + IL-2	(283)
	autol tumor	LN	Yes /Bryostatin+Ca+IL-2	i.v.	(342)
	autol tumor	LN	Yes /SAg/IL-2	i.v.	(314, 317–319)
	gene-mod tu	LN	Yes /anti-CD3/IL-2	i.v.	(108, 322)
	peptide	PBMC/TIL	cloned	i.v.	(331, 332)
	none	PBMC	Bulk IVS	i.v.	(333)
	gp100	PBMC	cloned	i.v. N-Myelo#	(334).
	Mixed %	TIL	cloned + Bulk	i.v. N-Myelo#	(11).

*GM-CSF administered to increase recovery of Monocytes from PBMC.
#Non Myeloablative chemotherapy administered prior to T cell infusion.
%Some patients previously treated on other vaccine trials.

repeatedly administered, intravesicularly, IFN-γ activated macrophages to 17 patients with superficial bladder cancer following transurethral tumor resection. 15 patients received 6 or more infusions of 0.9 to 2.5×10^8 activated macrophages. Urinary IL-8 and GM-CSF were markedly increased and the recurrence rate in the 12 months following initiation of therapy was significantly less than the year preceding the first adoptive transfer (244) (245). Future studies will be needed to confirm these promising findings. Another possible strategy to improve the efficacy of this approach would be to combine adoptive transfer of macrophages with bi-specific antibodies

that exploit FcγR or FcαR expression of macrophages to target these effector cells to antigens expressed on cancers (246) (247) (248).

Phytohemagglutinin-Activated Killer Cells (PAK)

Phytohemaglutinin (PHA) is a plant lectin that is mitogenic for T cells. PHA-activated peripheral blood lymphocytes mediate cytolytic activity against fresh autologous and allogeneic tumor targets, but not normal cells, through lectin-dependent cellular cytotoxicity (249, 250). While the adoptive transfer of PHA-activated killer cells (PAK) directly into the tumor (251) or intravascularly (252) had little therapeutic success, the later was an important step as it proved the feasibility of expanding large numbers autologous lymphocytes and adoptively transferring them back to cancer patients.

Lymphokine—Activated Killer Cells (LAK)

The discovery that it was possible to generate lymphokine-activated killer (LAK) cells, that could lyse fresh "non-cultured" tumor cells, simply by culturing lymphocytes for three days with a high concentration of IL-2 created a good deal of excitement in the 1980's (253) (254) (255). The earliest studies used lectin-stimulated lymphocytes to generate T cell growth factor (TCGF) that was used as the source of IL-2. The availability of recombinant IL-2 rapidly expanded the number of investigators studying LAK cells (256). Initially controversy existed over whether LAK activity was derived from T cells or NK cells. The earliest studies using TCGF characterized LAK cells as being T cells while later studies identified them as being NK cells (254) (255) (257) (258) (259) (260). Subsequently it was shown that the variations in LAK precursor phenotype were due to whether TCGF or recombinant IL-2 were used to generate LAK; TCGF induced both T cells and NK cells to generate LAK activity while IL-2 worked predominantly on NK cells (261).

The first clinical trial of "LAK" cells adoptively transferred TCGF cultured PBMC, that exhibited lysis of autologous tumor, into three patients. There were no clinical responses, but relatively few cells were given and patients did not receive systemic IL-2 (262). The failure of this approach would subsequently be predicted from animal models which showed that therapeutic efficacy of LAK cells was optimal when maximal numbers of cells were transferred and the maximal tolerated dose of exogenous IL-2 was administered (263) (264) (265). Translation of these observations to the clinic required that patients first be treated with IL-2 alone. The first clinical studies administered Jurkat cell-derived, or recombinant IL-2 alone at different doses, routes and schedules to 39 cancer patients. No therapeutic effect was observed in these trials but the toxicity of IL-2, particularly the increased vascular permeability was identified (266–268). The knowledge that IL-2 could be administered safely paved the way for trials that combined adoptive cellular therapy with IL-2 support. A prospective randomized trial of high dose IL-2 alone or in conjunction with LAK cells in patients with melanoma, renal cancer, colorectal cancer, non Hodgkin lymphoma and other tumors revealed no significant difference between the two groups with 10 CR and 4 PR among 85 patients treated with IL-2 and LAK versus 4 CR

and 12 PR among 79 patients treated with IL-2 alone (269). These findings were similar to those of the cytokine working group (270–272).

One explanation for the failure of adoptively transferred LAK cells to mediate tumor regression is the possibility that they are not able to traffic in large numbers to the tumor site. To overcome this roadblock, local administration of LAK cells was studied in patients with peritoneal disease. One study treated patients with ovarian cancer and reported 1 PR for 10 patients treated (273). A second study reported 2 PR for 10 ovarian cancer patients and 5 PR for 12 colon cancer patients treated by intra-peritoneal administration of LAK cells and IL-2 (274). Both studies identified ascites, abdominal pain and intraperitoneal fibrosis as limitations to this approach (273, 274).

Another explanation for the limited success of adoptive immunotherapy is that patients treated on these trials generally have advanced disease that is more difficult to treat. Since preclinical models predict that adoptive immunotherapy would be more effective against minimal residual disease some have sought to combine adoptive immunotherapy in the adjuvant setting. Kimura and colleagues performed a prospective, controlled study that randomized stage IIIA NSCLC patients, following curative resection of locally advanced primary lung cancer, to either no adjuvant therapy, chemotherapy alone or chemotherapy and adoptive immunotherapy with LAK cells and IL-2. Patients receiving chemo-immunotherapy had a significantly better 5-year survival rates (53.4%, n = 25) versus chemotherapy alone (33.4%, n = 26), or no additional adjuvant therapy (15.3%, n = 13) (275). These encouraging results may be due to applying immunotherapy in the adjuvant setting and/or to the combination of adoptive immunotherapy under conditions that support homeostasis-driven proliferation of adoptively transferred cells. Future studies will address both components.

Enriching for the subset of LAK cells that mediate anti-tumor effects is another approach to increase the efficacy of adoptive immunotherapy. IL-2-activated NK cells selected by their adherence to plastic surfaces (adherent NK cells, ANK) were shown to have increased anti-tumor activity in vitro and in animal models (276, 277). However, initial clinical trials with adoptively transferred ANK have had little success (278).

Anti-CD3 Activated PBL

In 1989 Anderson and colleagues reported that anti-CD3 activated spleen cells exhibited cytolytic activity against NK-resistant tumor targets and mediated regression of 5 day established pulmonary metastases (279). An advantage of this approach compared to LAK cells was a substantially increased yield of activated killer cells. A clinical trial of this approach adoptively transferred PBMC that had been activated by an overnight in vitro culture with anti-CD3. Subsequently, all patients received systemic administration of IL-2 to support in vivo expansion of the transferred T cells. In this trial all patients received low dose cyclophosphamide for the purpose of reducing "suppressor" cell activity. There was one PR in 24 treated patients (280). Additional preclinical studies using anti-CD3 stimulation of naïve spleen cells demonstrated that

the dominant mediator of therapeutic activity was a CD4+ T cell. In this model, transfer of anti-CD3 activated CD4 T cells was more effective than the transfer of anti-CD3-activated CD8 or bulk unseparated spleen cells (281). Based on this and other in vitro human studies supporting this concept (282), Curti and colleagues performed a second clinical trial using CD4 T cells obtained by negative selection of leukapheresis product (283). Isolated CD4 T cells were activated with anti-CD3 and expanded in IL-2 (90 IU/ml) for 4 days prior to being adoptively transferred with all patients receiving systemic IL-2. An interesting component of this trial was that PBMC were obtained from patients following cyclophosphamide treatment at a time when their WBC count was dropping towards its nadir or as the WBC was recovering from its nadir. Recovery of CD4 T cells was greatest when leukapheresis was performed when the WBC was dropping following cyclophosphamide administration. Most patients were treated with CD4 T cells obtained in this way. There was 1 CR and 2 PRs for 31 treated patients, but 8 of 17 patients with the greatest CD4 expansion exhibited some anti-tumor effects.

ADOPTIVE CELLULAR THERAPY USING TUMOR-SPECIFIC T CELLS

Tumor-Infiltrating Lymphocytes

Reports of tumor-specific T cells in the peripheral blood of melanoma patients encouraged the idea that the immune system might be exploited to treat melanoma (284) (285) (286) (287) (288). However, the inability to regularly isolate these tumor-specific T cells or obtain them in large numbers was one limitation to their application in adoptive transfer studies (262). This changed when it was discovered that by culturing freshly isolated tumor preparations with high doses of IL-2, it was possible to routinely generate cultures of Tumor-infiltrating lymphocytes (TIL). In preclinical studies the TIL exhibited tumor "specificity" and were 50 to 100 times more effective at reducing established pulmonary metastases than LAK cells (36) (16). Consistent with preclinical reports, TIL generated from some melanoma patients could lyse autologous tumor targets specifically (289). The initial clinical trial of TIL administered with IL-2 and cyclophosphamide (25 mg/kg), showed 11 partial responses in the 20 patients treated (290). A subsequent study of 86 melanoma patients reported an objective response rate of 34% with 5 CR and 24 PR. In this study approximately 2/3rds of the patients were pretreated with cyclophosphamide (25 mg/kg) 36 hours prior to adoptive transfer. However the overall response rate was not different: 31% for TIL + IL-2 and 35% for cyclophosphamide, TIL + IL-2 (291).

A general disadvantage faced by immunotherapists, is that most patients treated on adoptive immunotherapy trials have advanced and bulky disease that is difficult to treat. Recently, there have been two reports using adoptive immunotherapy with TIL as adjuvant therapy for resected stage III-IV melanoma, i.e. patients with minimal residual disease. In one trial, lymph node metastases from stage III patients, rendered disease free by surgery, were randomly assigned to receive either TIL plus interleukin-2 (IL-2) for 2 months, or IL-2 only. Eighty-eight patients entered the trial with 44 in each group. While there was no difference in overall survival for the

2 groups, subset analysis suggested a significantly increased survival for that group of patients with only a single invaded lymph node that received TIL and IL-2 (292). Labarriere and colleagues further analyzed the in vitro properties of TIL generated from 40 patients where autologous melanoma cell lines were available and showed that patients receiving TIL specific for autologous tumor, measured by intracellular staining for IFN-γ, had a longer relapse free interval than patients receiving TIL that were not specific (113). The second trial treated 25 stage III and IV patients with TIL and IL-2. Eight of 22 stage IIIC (>3 lymph nodes involved) patients that received between 0.27-to 85 $\times$ 10^{10} TIL were disease free at a median follow-up of 5 years (293).

The initial success in treating melanoma patients encouraged investigators to consider other malignancies. Kradin and colleagues, generated TIL and treated 7 patients with adenocarcinoma of the lung, but saw no responses where there was greater than 50% reduction at all sites (294). Bukowski and colleagues treated 18 renal cell carcinoma patients with TIL alone or supported with escalating doses of IL-2 and saw no objective clinical responses and were unable to generate TIL from 7 patients (295). While TIL from most patients exhibited non-specific cytotoxicity, TIL from one patient exhibited tumor-specific cytolytic function. A subsequent trial from Belldegrun and colleagues pretreated patients with IFN-α prior to radical nephrectomy for TIL generation. TIL were then adoptively transferred and patients received IL-2 and IFN-α (296). TIL were generated in 11 of 11 patients attempted and there were 2 CR and one surgical CR. Subsequently, a large multi-center trial of CD8 TIL for renal cancer enrolled 178 patients, randomizing 160 to either TIL + IL-2 (n = 81) or IL-2 alone (n = 79). Of the 81 patients enrolled to TIL + IL-2 only 72 were eligible for TIL therapy and sufficient numbers of T cells could be grown on only 39 patients (297). Intent-to-treat analysis demonstrated objective response rates of 9.9% (8/81) and 11.4% (9/79), which was not different for the two groups. Notable differences between this trial and the 86 patients treated by Rosenberg and colleagues were the requirement to transport tumor specimens to a central facility, the isolation of only CD8+ TIL for expansion and infusion, the relatively low number of T cells infused in some patients and the selection of patients with renal cancer.

Why didn't more renal cancer patients respond? In concert with some clinical trials, investigators looked for surrogate markers of therapeutic effector T cells. The first report of a correlation was provided by Aebersold et al., who showed that tumor-specific cytolytic activity of TIL was associated with clinical responses in melanoma patients (298). Another study of selected melanoma patients observed a significant correlation between the adoptive transfer of gp100-specific, IFN-γ secreting T cells and tumor regression (104). However, of the renal cancer studies listed above, only one reported functional analysis of TIL cultures and only a single patient was noted to express autologous tumor-specific cytolytic activity. More extensive characterization of TIL in vitro function might have provided additional insights about why the response rate is so low. However, immunological monitoring in renal cancer patients has been somewhat limited by the absence of defined renal cancer-specific/associated antigens. Recently, determinants have been identified that

appear to represent common renal cancer tumor antigens; brightening the prospects for immunological monitoring in this disease (299) (300). However, since the generation of renal cancer-specific T cells from TIL is a rare event (295, 301, 302), better ways of generating renal cancer-specific T cells are needed before increased response rates are likely to be seen for this disease.

Tumor Vaccine-Draining Lymph Node T Cells—in vitro Sensitization

While tumor-specific T cells can be grown from TIL of most melanoma patients, it is not possible to reliably generate autologous tumor-specific T cells for adoptive transfer from most other tumors. One potential problem with TIL is that the T cells present at the tumor site may be suppressed by association with tumor factors and/or T reg cells and be ineffective at mediating tumor regression. Simultaneous with the identification of TIL, Shu and colleagues, showed that T cells from the lymph nodes draining a progressively growing tumor were a good source of anti-tumor T cells. These tumor-draining lymph node (TDLN) T cells, following in vitro sensitization (IVS) with tumor cells and IL-2, exhibited tumor-specific cytolytic activity in vitro and were highly therapeutic in adoptive transfer studies (37, 303, 304). It was speculated that this approach, utilizing in vitro activated tumor vaccine-draining lymph node T cells (TVDLN) or vaccine primed lymph nodes (VPL) might circumvent some of the obstacles associated with TIL. The clinical application of IVS-TVDLN cells was studied in 17 melanoma patients and 3 renal cell cancer patients. In these studies irradiated autologous tumor cells were combined with Bacillus Calmette Guérin (BCG) and used as the vaccine. TVDLN were harvested 10 to 14 days later, stimulated with cryopreserved autologous tumor and expanded in media containing 600 IU/ml of IL-2 and subsequently transferred to the patient in combination with IL-2. One patient receiving adoptively transferred T cells developed a partial response compared to none in the IL-2 control cohort (305). An interesting observation from this study was that T cell transfer conferred DTH reactivity to autologous tumor while vaccination and IL-2 treatment alone did not. A serious limitation of this strategy was the requirement for large numbers of tumor cells to perform IVS.

Tumor Vaccine-Draining Lymph Node T Cells—Anti-CD3 Activation

Frustrated by the limitation of the prior trial, Shu and colleagues investigated alternatives to using tumor to drive in-vitro expansion of T cells with therapeutic efficacy. They found that stimulating TVDLN cells with anti-CD3 appeared to mimic antigen-specific stimulation, supporting the maturation of T cells that were specific for the tumor used in the vaccine. In animal models anti-CD3-activated TVDLN cells mediated regression of tumors in the brain, skin and lung (24, 306) (307, 308). In a clinical trial 11 melanoma and 12 renal cell cancer patients were vaccinated with autologous tumor cells and BCG. The TVDLN were activated with anti-CD3 and IL-2 and adoptively transferred in combination with IL-2. A 33% response rate was achieved in RCC patients (2 PR and 2 CR), while only one PR was observed in melanoma patients (309). A subsequent Phase II trial of 39 stage IV renal cell cancer patients reported 4 CRs and 5 PRs for an overall response rate of 27% (310). An

interesting observation of this report was the correlation of clinical response with the transfer of T cells that exhibited a high IFN-γ: IL-10 ratio for tumor-specific cytokine release. Chang and colleagues applied this same approach to 6 patients with advanced advanced head and neck cancers. Patients were vaccinated with autologous tumor and BCG, TVDLN were harvested, expanded with anti-CD3 and IL-2, and infused into patients who received 15 doses of IL-2 (311). Analysis of infused TIL for 4 of 5 patients identified tumor-specific secretion of IFN-γ and GM-CSF but no IL-4 or IL-10 (3 of 3 patients reported). No objective clinical responses were observed. A similar approach vaccinated 21 NSCLC patients with autologous tumor cells and GM-CSF. After 2 vaccinations lymphocytes were activated with anti-CD3, expanded in IL-2 and patients received T cells numbering between 0.5-to 6.1 $\times$ 10^{10}, with 18 patients receiving more than 1.6 $\times$ 10^{10} T cells (312). Median survival of all 21 patients was 18.6 months, with a 1-year survival of 51.6%.

Tumor Vaccine-Draining Lymph Node T Cells—Superantigen Activation

The staphylococcal enterotoxins also known as microbial superantigens are small proteins that can cross link distinct Vβ subunits of the TCR with MHC Class II molecules and lead to selective expansion of T cells, which express the appropriate Vβ TCR. Preclinical studies demonstrated that staphylococcal enterotoxin C or staphylococcal enterotoxin B two potent microbial superantigens, exhibited to specific IFN-γ release and were therapeutic in the treatment of murine pulmonary (25) and intracranial metastases (313). In humans, staphylococcal enterotoxin A (SEA) is the most potent mitogen, and activates greater than 80% of human T cells (314). There have been four clinical trials performed by Shu and colleagues where SEA-activated TVDLN were expanded in low dose IL-2 in vitro and adoptively transferred to patients with advanced disease. A major difference between these four studies and most other adoptive cellular immunotherapy trials is that they did not provide systemic IL-2 to support in vivo survival of transferred T cells. This strategy was undertaken based upon this group's preclinical data showing that adoptive immunotherapy with activated TVDLN was more effective against intracaranial and subcutaneous tumor when systemic IL-2 was not provided (315) (42) (308) (316).

In the first clinical trial, ten patients with malignant glioma were vaccinated with irradiated autologous tumor cells and received GM-CSF. The TVDLN cells were stimulated ex vivo with SEA, expanded in IL-2 and then expanded a second time with anti-CD3 and IL-2 in order to generate high numbers of T cells. Patients were administered 10 mg/kg cyclophosphamide and 24 to 48 hrs later had their T cells infused. Three patients experienced a PR lasting 6, 7 and >13 months (317). A subsequent trial was performed with newly diagnosed gliomas. Patients were vaccinated with autologous tumor and GM-CSF. In this study harvested TVDLN cells were activated once with SEA, expanded in low dose IL-2 for 6–8 days and adoptively transferred to patients pretreated with cyclophosphamide (314). Four patients experienced a PR lasting 11, 14, 17 and >29 months. A third clinical trial repeated this strategy in patients with metastatic renal cell carcinoma. Patients were vaccinated with autologous tumor mixed with GM-CSF and TVDLN were activated

by culture with SEA, expanded in IL-2 and adoptively transferred. All patients pretreated with cyclophosphamide and no systemic IL-2 was administered. There was 1 PR reported for 20 patients treated (318). The fourth trial was undertaken in patients with unresectable squamous cell carcinoma of the head and neck (SCCHN). Patients were treated as noted above and sufficient T cells were obtained to treat 15 of 17 patients enrolled. There were no objective clinical responses in this trial (319). Overall this approach had the greatest success in Glioma where there were 7 PRs for 22 patients treated.

Tumor Vaccine-Draining Lymph Node T Cells—Gene Modified Vaccines

Although murine models show that vaccination with poorly immunogenic tumors fails to sensitize therapeutic T cells, genetic modification of these ineffective vaccines can convert them to effective inducers of effective T cells for adoptive immunotherapy. Modification with an allogeneic MHC class I gene or a construct encoding GM-CSF are two approaches that are effective in preclinical models (320, 321). The first to enter clinical trials was modification of alloantigen-modified autologous tumor. Patients received unmodified autologous tumor cells and BCG in one extremity and HLA-B7-lipofected autologous tumor in an alternate extremity. Ten to 14 days later TVDLN draining both vaccine sites were isolated and expanded independently so that possible differences in vaccine effectiveness could be evaluated by in vitro assays. For adoptive cellular therapy anti-CD3 activated TVDLN draining both vaccine sites were combined and administered together with systemic IL-2. This report failed to observe any responses in 9 melanoma and 11 renal cancer patients treated. In contrast to preclinical studies that saw lipofected vaccines shifting the tumor-specific T cell response towards a type 1 cytokine profile (86), here the HLA-B7-modified vaccine promoted a tumor-specific IL-5 response that was higher than that observed for the TVDLN draining autologous tumor and BCG (108). Given this and other findings of tumor-antigen specific IL-5 in patients with progressive disease, future investigations will need to explore whether tumor-specific IL-5 responses might interfere with therapeutic efficacy or be a marker of other type 2 cytokines that have that ability.

Chang and colleagues used a similar trial design to study the effectiveness of a GM-CSF-transduced autologous tumor vaccine to prime tumor-specific T cells for adoptive immunotherapy. Five melanoma patients were vaccinated with autologous tumor transduced with a vector encoding GM-CSF in one extremity and unmodified tumor cells alone at a different site (322). TVDLN were harvested 7 days later, activated and expanded separately so that immunological comparisons could be made and the two populations of T cells were combined for adoptive transfer. Four patients received T cells and IL-2 with one patient undergoing a CR. Tumor-specific immunological monitoring was possible on 2 patients, but did not include the patient with the CR. While there were no consistent differences between tumor-specific T cells primed by either vaccine, the yield of TVDLN cells was consistently greater in the LN-draining the GM-CSF-modified vaccine. While this report of Chang and colleagues is preliminary, the application of GM-CSF secreting autologous tumor

vaccines is an approach that is already showing promising results in NSCLC (323). The combination of a GM-CSF transduced-tumor vaccine with other strategies (eg. Non-myeloablative conditioning and/or CD25+ T cell depletion) and adoptive cellular immunotherapy will certainly be tested in clinical trials in the near future.

Selected Tumor-Specific T Cells

An underlying tenant of adoptive immunotherapy is that if you could select and transfer only tumor-specific T cells with therapeutic activity you could improve the response rate of treated patients. Thus strategies that identify the tumor-specific T cells maybe crucial to the success of adoptive immunotherapy. Recently, Kagamu and colleagues developed an approach to select the subset of T cells that have been specifically sensitized to tumor during vaccination (315). T cells that expressed a low level of L-Selectin (L-selectin^{Lo}), a well-established marker for recently activated and memory T cells (324) (325) (326) (327), were isolated using magnetic bead technology, expanded in vitro and studied for their ability to mediate regression of the weakly immunogenic MCA-205 (315). Consistent with its ability to mark recently activated "responding" T cells, the L-selectin^{Lo} TVDLN T cells contained all of the therapeutic activity. Others have repeated this observation using therapeutic vaccine strategies in different tumor models with similar success (86, 328).

But is this strategy translatable to cancer patients? Currently efforts are underway to characterize the in vitro anti-tumor properties of L-selectin^{Lo} TVDLN T cells from cancer patients (343). Combining this approach with the polarizing signals suggested above (three-signal paradigm) this approach might improve not only the therapeutic efficacy but also help to reduce the high costs of expanding large numbers of cells for AIT.

Tumor-Specific T Cell Clones and Lines

In review of the proceeding clinical trials, one limiting factor has been the requirement to generate large numbers of tumor-specific T cells. For many cancers, there is still a paucity of evidence that this feat can be routinely accomplished. However, for melanoma and EBV associated malignancies, while technically challenging, it is a therapy, which can be attained by experienced laboratories. Proof of the feasibility and efficacy of this approach was provided by Walter and colleagues, who reconstituted cellular immunity against cytomegalovirus (CMV) in patients following bone marrow transplant (329). While the development of anti-virals limited the application of this technology for CMV, it paved the way for its application in cancer. Stimulated with tumor cells or APC and T cells are cloned or cultured in bulk to generate CTL lines. Rooney and colleagues have used adoptive immunotherapy with EBV-specific CTL lines to either prevent or treat EBV-induced lymphoma in allogeneic transplant recipients. In their prevention study they successfully generated CTL lines for 69 of 70 patients attempted. None of 39 evaluable patients developed EBV lymphoma, contrasting with an incidence of 11.5% in a control population from the same institution (330). This same group also reported CTL transfer was

effective at treating lymphoma in two patients who received CTL transfer following onset of disease.

Unfortunately, the efficacy of Tumor-specific $CD8^+$ T cell clones has been disappointing. In 2001 Dudley reported on 13 patients treated with CD8+ T cell clones reactive with gp100 epitope (331). The first 12 patients received T cell clones alone with no evidence of clinical response. Eleven of these patients and one previously untreated patient went on to infusions of T cell clones and either subcutaneous or intravenous IL-2. There was one minor response in a patient receiving T cells and intravenous IL-2. Subsequently, Yee and colleagues transferred T cell clones generated by in vitro culture with autologous dendritic cells pulsed with an HLA-A2-restricted peptide for either MART1/MelanA or gp100 (332). Clones were selected for their ability to lyse antigen positive tumor targets in ^{51}Cr-release assays. They reported 2 minor responses but no PR or CR in 10 patients treated with 4 cycles of CTL infused at two-week intervals. An interesting component of their study was an internally controlled comparison of how T cell survival was affected by IL-2 administration. The median T cell survival following the initial infusion of CTL, when no IL-2 was administered, was 6.68 days. This compared to a median T cell survival of 16.92 days.

Mitchell and colleagues obtained T cells by leukapheresis and used tyrosinase peptide-pulsed Drosophila cells transduced with HLA-A2.1, CD80, and CD54 to prime/expand T cells in IVS (333). T cells were adoptively transferred and patients immunized with peptide. One PR was seen in 10 patients treated with adoptive transfer of 10^8 cells.

Tumor-Specific T Cell in Non-Myelo Ablated Patients

Dudley and colleagues reported on a similar trial that transferred Tumor-specific $CD8^+$ T cell clones into patients who first received a non-myeloablative regimen of cyclophosphamide and fludarabine (334). The first 6 patients received no systemic IL-2 treatment following T cell infusion. The next three received 15 doses of 72,000 IU/Kg and the remaining six received 720,000 IU/Kg every 8 hours till tolerance (mean 11 doses). There were no clinical responses for the 15 patients enrolled. One reason for the disappointing result of these studies could be the use of T cells directed against a single antigenic epitope. A recent report using a human MART-1-specific T cell clone for the adoptive immunotherapy of human melanoma in SCID mice showed that this treatment leads to immunoselection of MART-1 antigen-loss variants and treatment failure (335).

Rosenberg and colleagues performed a subsequent trial where patients received the same non-myeloablative chemotherapy regimen and high dose IL-2 but instead of receiving Tumor-specific $CD8^+$ T cells alone, received a mixture of $CD4^+$ and $CD8^+$ T cells. In striking contrast to previous trials where $CD8^+$ T cell clones or lines rapidly disappeared from circulation, some patients on this trial had T cells expand and persist at frequencies as high as 75% of $CD8^+$ T cells for 120–140 days (11). The most striking finding of this report was that 6 of 13 patients had

objective clinical responses. These observations raised several important questions. First, why did a high percentage of patients respond? Is it solely a result of transferring $CD4^+$ T cells that may contain some HLA-DR-restricted tumor-specific cells? One supposition is that it is likely a combination of creating "space" in the lymphopenic host and deleting $CD4^+$ T reg cells that promotes the expansion and persistence of transferred T cells. A second supposition is that the expansion and persistence of the transferred T cells relies on the presence of some tumor-specific $CD4^+$ T cells. There is some evidence from preclinical models that $CD4^+$ T cells play a valuable role in maintaining anti-tumor immunity long-term (336) (40).

Approaches to Expand Tumor-Specific T Cells

The encouraging results seen in trials combining CD4 and CD8 T cells with non-myelo ablation will lead to additional trials of this approach. As noted above, a three-signal paradigm has been proposed to generate T cells with therapeutic efficacy. However, methods are still needed for the large-scale expansion of antigen-specific T cells. Artificial APC is one approach that is being explored. Maus and colleagues developed used a cell line that expressed ligands for the TCR, CD28 and 4-1BB and have used it to expand functional CD8 T cells (337). Others have used beads coated with either anti-CD3 and anti-CD28 (338). This approach can rapidly expand T cells and has already seen application in a clinical trial (339). A similar approach generated a bead with a soluble class I molecule and anti-CD28. An advantage to this approach is that it can be loaded with specific peptides (340). Multiple expansions with this approach triggered expansion of T cells that retained antigen-specific function. A similar approach provided TCR signaling to CD 4 T cells with good results (341).

Conclusions

From our perspective a great deal has changed in the past few years. We now appreciate that tumor-specific T cells have at least a triad of properties (perforin, IFN-γ, and TNF) that they can utilize to mediate tumor regression. We also have a basic understanding of in vitro methods to polarize primed T cells towards a "therapeutic" type 1 cytokine profile (IFN-γ and TNF). Additionally, combining vaccination at a time when host T cells are undergoing homeostasis-driven proliferation has been shown to dramatically increase the frequency of tumor-specific T cells generated by the host. The discovery of CD25+CD4+ regulatory T cells at tumor sites and the success of combining adoptive transfer of CD4 and CD8+ TIL with a non myeloablative conditioning regimen that includes fludarabine, a drug that that preferentially decimates CD4+ T cells, are likely to be related. The availability of antibodies or ligands that block negative signals (CTLA4) or provide costimulatory signals (4-1BB, OX40) will be extended or initiated soon. The next several years should prove particularly informative as trials incorporating combinations of strategies make their way to the clinic.

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IV. CLINICAL TRIALS DESIGN

14. CLINICAL TRIAL DESIGNS FOR THERAPEUTIC CANCER VACCINES

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1. INTRODUCTION

Many aspects of the usual paradigm for the clinical development of cytotoxic anticancer drugs are not appropriate for therapeutic cancer vaccines (1). In this chapter we explore some of these differences and recommend designs and strategies more suited for the development of effective therapeutic vaccines.

2. DOSE-ESCALATION STUDIES

2.1. Safety Studies

Phase I studies usually involve dose escalation in cohorts of three patients, starting with a very low dose, in an effort to identify a maximally tolerated dose. Phase I studies are generally conducted in patients with advanced metastatic disease who have failed all other available treatments. Tumor vaccines are often based on DNA constructs, viral vectors and cytokines that have been determined as safe in previous clinical trials. Peptide vaccines generally seem inherently safe as long as the cytokine adjuvants are used in combinations and doses previously demonstrated to be safe. Consequently, no phase I safety study should be required for most therapeutic cancer vaccines.

On the other hand, a novel virus or plasmid used as a recombinant vaccine vector for the first time should be evaluated for safety. A dose escalation design may be

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appropriate but patients whose immune systems have not been compromised by extensive chemotherapy are the most relevant subjects. If such vectors are proven to be nontoxic at substantial doses, then subsequent dose-escalation safety trials using the same vectors but with different recombinant inserts may not be required.

2.2. Immunogenicity Studies

Feasibility issues limit the maximum doses of certain vaccines that can be produced for administration to patients. In many cases, the dose selected will be based on pre-clinical findings or on practical considerations.

For cancer vaccines, it is not always the case that more is better. In studies of peptide vaccines based on non-mutated melanoma antigens, in vitro analysis did not reveal any correlation between peptide dose and the generation of specific T cell reactivity from the peripheral blood lymphocytes of vaccinated patients (2, 3). Thus, for subsequent trials using similar peptides, an intermediate fixed dose of 1 mg was chosen for vaccination, bypassing repetitive phase I studies.

Dose ranging to find the *minimal active dose* may be feasible but the 3–6 patients per dose level used in conventional toxicity trials may not be adequate. Those small sample sizes are only sufficient to exclude high toxicity rates. Suppose that an assay is used in a binary manner to define immunogenic response. Table 1 shows the probability of no immunogenic responses in n patients as a function of the true immunogenic response probability. If one wants a dose at which the immunogenic response probability is at least 30% say, then if you observe no immunogenic responses in 7 patients it would be appropriate to escalate to the next dose level.

Korn et al. (4) defined a sequential procedure for finding a biologically active dose, although not necessarily the minimal active dose. During an initial accelerated phase one patient per dose level is treated until a biological response is seen. After the first response is seen, cohorts of 3–6 patients are treated per dose level. With 0–1 biological responses among the 3 patients at a dose level, escalate to the next level for the next cohort of patients. With 2 or 3 responses out of the 3 patients, expand the cohort to a total of 6 patients. With 5 or 6 biological responses out of the 6 patients, declare that dose level to be the biologically active level and terminate the trial. With fewer than 5 biological responses out of the 6 patients, a new cohort of 3 patients is accrued at the next higher dose level, etc. Korn et al. (4) describe some of the statistical properties of this sequential design.

Table 1. Finding the minimum active dose

Probability of immunologic response	Number of patients treated at dose	Probability of no immunologic responses
0.20	11	0.09
0.25	9	0.08
0.30	7	0.08
0.40	5	0.08
0.50	4	0.06

Trying to determine whether there is a dose-response relationship involves comparing immunological responses for different dose levels. Such trials, if designed properly, require larger sample sizes. Consider, for example, planning a study of two dose levels to test whether there is a relationship between dose and immunologic response. If the immunologic response probabilities at the two dose levels are 50% and 90%, then 20 patients treated at each dose level are required for a one-sided statistical significance level of 0.10 and a statistical power of 0.90 (5). Larger sample sizes are required to detect smaller differences. Using more than two dose levels allows one to treat somewhat fewer patients at each dose level, but the total number of patients required to detect a dose-response relationship will actually be much larger than if only two dose levels are tested. This is because the two most extreme dose groups are the most informative for detecting a dose-response relationship.

Trying to characterize the shape of the dose-activity relationship or finding an optimum biologic dose is an even more ambitious objective that is rarely practical in a phase I tumor vaccine study.

3. PHASE II STUDIES

The general objectives of the phase II vaccine trial are similar to those of the phase II cytotoxic trial. The primary objective is to determine whether the regimen has biologic activity that is likely to translate into patient benefit. The second objective is to optimize the regimen.

With cytotoxics, the generally accepted endpoint for phase II trials is objective tumor response; that is, tumor shrinkage by at least 50%. Tumor shrinkage is not a direct measure of patient benefit, although it sometimes is predictive of benefit. The most commonly accepted direct measures of patient benefit are survival, disease free survival and symptomatic relief. Therapeutic effect on these endpoints cannot be reliably established outside of a phase III trial with an appropriate control group not receiving the experimental therapy. Investigators sometimes like to infer that a regimen prolongs survival because the responders live longer than the non-responders, but this analysis has long been known to be invalid (6,7).

Tumor shrinkage is generally used as the endpoint for phase II trials of cytotoxics for two reasons. First, because response represents biological activity that can be attributed to the therapy (i.e. tumors rarely shrink spontaneously by 50%). Secondly, if the degree, duration and abundance of responses are sufficient, then it is plausible to hope that tumor response may translate into patient benefit. There are many cytotoxic regimens which were active in phase II trials but which subsequently had no identifiable effect on survival in phase III trials. Torri et al. (8) performed a meta-analysis of randomized trials to quantify the relationship between improvement in response rate and improvement in survival outcome for advanced ovarian cancer studies. They found that a very substantial improvement in response rate was necessary to have any identifiable effect on survival.

For phase II tumor vaccine studies, clinical endpoints and/or immunological endpoints are commonly used. Clinical endpoints include tumor shrinkage, reduction in tumor marker levels or delay in time to tumor progression.

Table 2. Optimal two-stage designs (8)

Target response rate (p_1)	First stage sample size (N_1)	Maximum sample size (N)	Number of responses required for activity (A)	Probability of early termination
20%	12	37	4	.54
25%	9	24	3	.63
30%	7	21	3	.70
35%	6	12	2	.74

3.1. Single-Arm Trials Using Clinical or Immunologic Response Rate

If tumor shrinkage is the endpoint, then phase II designs used for cytotoxics can be employed (9, 10, 11). Simon's "optimal two-stage" designs are widely used for phase II cytotoxic trials to test whether a regimen has a response rate above a background level p_0 (10). Frequently, $p_0 = 0.05$ is used. With clinical response, this assumes that no more than 5% of the patients will have apparent responses caused by variability in response assessment or spontaneous remissions. The 2-stage design incorporates an early termination point, which allows the investigator to discontinue patient accrual if a desired endpoint has not been achieved in the first stage of the trial.

At the conclusion of the clinical trial, the regimen will be declared active or inactive. Table 2 shows several designs with 10% false positive rate, 10% false negative rate and $p_0 = 0.05$. The false positive rate (α) is the probability of declaring the regimen active when the true response probability is p_0. The false negative rate (β) is the probability of declaring the regimen inactive when its true response probability is the target response rate p_1, the level of activity that we wish to be able to detect. In the first stage, N_1 evaluable patients are entered and treated. If no responses are observed, then the trial is terminated and the regimen is declared inactive. Otherwise accrual continues to a total of N evaluable patients. At that point accrual is complete. If the total number of responses is at least A, then the regimen is declared active. The last column of the table indicates the probability of early termination after the first stage when the true response probability is p_0. For example, if $p_0 = 5\%$ and the target response rate is 25%, then 9 patients are treated in the first stage of the trial. If no responses are observed, the trial is terminated. Otherwise, accrual is continued to a total of 24 patients. If at least 3 responses are seen in the 24 patients, the regimen is declared active. The probability of declaring a regimen active when it's true response rate is 5% or less is 10%. The probability of missing the activity of a regimen with a true response rate of 25% is 10%. With a regimen having a response rate of 5%, the probability of stopping after only 9 patients is 63%. This design with $p_1 = 25\%$ and $p_0 = 5\%$ seems reasonable for many initial vaccine trials using tumor regression as endpoint.

An optimum two stage design can also be used with a binary immunologic response endpoint. In such a case, however, the values of p_0 and p_1 will generally be much higher than for a tumor regression endpoint. Optimum two-stage designs for any values of p_0, p_1, α, and β are easily generated using computer program OTSD

(optimum two-stage design) available at http://lib.stat.cmu.edu/designs. The required number of patients depends strongly on the difference p_1-p_0.

A variety of alternative single-arm designs for evaluating binary endpoints have been published. For example, Garnsey-Ensign developed three stage designs (11), and Thall and Simon (12) developed continuous monitoring Bayesian designs. The essential characteristics of this class of designs is that the endpoint is binary and that the objective is to evaluate the response rate of the regimen on it's own, not in comparison to the response rate for some other regimen. If the objective is comparative, then even for a single arm trial, the design and method of analysis should take into account the variability in the estimate of response rate for the external control regimen. Methods such as that of Makuch and Simon (13), Dixon and Simon (14) and Thall and Simon (12, 15) attempt to take that variability into account.

For therapeutic vaccines, the current situation for most diseases (other than melanoma) is that few if any partial or complete tumor responses have been observed with any regimen but that varying degrees of immunogenicity have been obtained. It is usually very difficult to compare the degree of immunogenicity obtained with different regimens by different investigators because of differences in assays, variation in procedures and reagents, and differences in patient selection. It is even difficult to compare the degree of immunogenicity obtained by the same investigator in different studies with different regimens because of assay variability. There is also generally some uncertainty in what measures of immunogenicity are most appropriate. There are currently no measures that can be considered true surrogates for clinical response.

It takes fewer patients to determine whether a regimen causes any clinical responses than it does to compare it to another regimen with regard to immunologic response rate. The optimum two-stage design recommended above for evaluating clinical response rate has a first stage of only 9 patients. Consequently, a reasonable phase II development strategy is to design phase II trials using the optimal two-stage design for distinguishing a 5% clinical response rate from a 25% clinical response rate with error rates of 10%. If after accruing the 9 patients in the first stage, no clinical responses are seen, then the trial is terminated. If one or more clinical responses are seen, accrual should continue unless the level of immunologic response is so inadequate that the investigator would like to make some modifications to the regimen. In cases where accrual is terminated after 9 patients because of lack of clinical responses, the immunological activity of the regimen for the 9 patients accrued will provide information for modifications of the vaccine regimen.

3.2. Multiple Arm Screening Trials Using Immunological Response Rate

One of the complexities of therapeutic vaccine development is the many options available for attempting to enhance immunological recognition of a specified tumor antigen. In addition to the vector or mode of presentation of the antigen to the immune system, there are alternative adjuvants, preparative regimens, routes and schedules of administration. Because of the difficulty and time required for clinical trials, it is best to optimize vaccines to the extent possible using animal models. Nevertheless, there may be several vaccine candidates available for clinical trial. One

approach would be to perform a two-stage 9–24 patient clinical trial on all candidate regimens, stopping at 9 patients unless partial remissions are seen. An alternative strategy is to perform a multi-arm phase II trial to optimize the regimen with regard to immunogenicity before focusing on clinical endpoints. The reason for using a multi-arm randomized phase II design is to ensure comparability of patients on the different regimens and to control for assay variability.

3.2.1 *Factorial Screening Designs*

Two types of randomized phase II trials are potentially relevant for optimizing a vaccine regimen. One method is the use of a phase II factorial design. Suppose that there are m binary factors that represent dimensions in which a basic vaccine may be modified. For example, one factor might be route of administration and another might be whether a specified adjuvant is administered. Since there are m binary factors, there are 2^m possible combinations of levels of the factors. For example, let the levels of each factor be denoted 0 or 1. Then with two factors the possible combinations of factors are (0,0), (0,1), (1,0), and (1,1). The study is conducted by randomizing N patients into the 2^m treatment groups. If the factors affect immunogenicity independently, then in comparing the two levels of one factor, one can ignore the other factors. Actually, a stratified or model based analysis is more powerful than simple pooling, but the point is that the two levels of each factor are compared based on the assumption that the difference does not depend on the levels of the other factors. Consequently, comparing level 0 of a factor to level 1 of that factor involves comparing average immunogenicity for the N/2 patients with the factor at level 0 to average immunogenicity for the N/2 patients with the factor at level 1. The trial is sized, i.e. N is selected, for m independent two-arm comparisons involving N/2 patients per arm, not for one 2^m arm comparison. If there are 3 factors ($m = 3$), then there are $2^3 = 8$ arms to the trial. If $N = 32$, then 4 patients are randomly assigned to each of the arms, but the comparisons of the levels of each factor involve comparing average immunogenicity for two groups of 16 patients. The value of N is selected based on the manner in which immunogenicity is measured (e.g. continuous scale or binary), assay and biological variability among patients, and size of difference to be detected. Suppose, for example, that immunogenicity is measured on a continuous scale, and let x denote the change in immunogenicity from baseline for a patient after treatment. Assume that x is approximately normally distributed and let σ denote the standard deviation for x for different patients receiving the same vaccine regimen. Let δ denote the size of the difference in mean value of x we wish to be able to detect in comparing vaccine groups, and let α and β denote the type 1 and type 2 error rates for the comparison. Then N/2 patients are required in each of the two groups with:

$$N = 4\left(\frac{z_{\alpha/2} + z_{\beta}}{\delta/\sigma}\right)^2 \tag{1}$$

where $z_{\alpha/2}$ is the $100(\alpha/2)$'th percentile of the standard normal distribution and z_β is the 100β'th percentile. For 5% type 1 error and 80% power, we have $z_{\alpha/2} = 1.96$

and $z_\beta = 0.84$. To detect a difference in means that represents one standard deviation of inter-patient variability in immunogenicity requires $N = 32$ patients randomized. This gives 16 patients in each level of each binary factor. With 3 binary factors, there are 8 treatment groups. Hence randomly assigning 4 patients per treatment group will satisfy this requirement. The required sample size is very dependent on the δ/σ ratio. Reducing σ by improving the assay reproducibility will increase this ratio for a fixed δ. The quantity σ reflects both biological variability and assay variability, and so using a more homogeneous group of patients may also serve to reduce σ.

3.2.2. *Randomized Selection Design*

An alternative approach to optimizing a vaccine regimen is to conduct a randomized phase II trial of the variants and to select the regimen that has the best average immunogenicity in the trial. This type of approach has been described by Simon et al. (16), Strauss and Simon (17) and Yao et al. (18). The analysis does not result in any conclusions of which factors are important to immunogenicity or which regimens are significantly better than which other regimens, but merely a selection of a regimen which is most promising for further investigation. There are two approaches to establishing sample size per treatment group for such selection designs. One approach, described in Simon et al. (16) is to require that the sample size per treatment be large enough to assure with high probability that if one treatment is superior to all other treatments by a specified amount δ, then it will have the largest sample mean and will therefore be selected. With normally distributed measures of immunogenicity, the probability of correct selection depends on the ratio of δ/σ and on the number of treatment arms. If there is one best arm and the rest are inferior by δ, then the probability of correct selection decreases as the number of arms increases. Table 3 shows the sample size required to have a probability of correct selection of 0.90 as a function of δ/σ and the number of treatment arms. For δ/σ values of 0.75 or greater, the design requires fewer than 15 patients per arm for up to 8 randomized arms.

Another approach to establishing sample size for randomized selection designs is based on the assumption that the true mean immunogenicity for a regimen can be regarded as a random draw from some hypothetical super-distribution of activity levels (17, 18). By studying more regimens in a randomized trial, one has a greater chance of including a very active regimen. If the number of patients available for

Table 3. Number of patients per arm for randomized selection design

Number of treatment arms	Patients per arm $\delta/\sigma = 0.5$	$\delta/\sigma = 0.75$	$\delta/\sigma = 1.0$
2	13	6	4
3	21	9	6
4	24	11	6
5	27	13	7
6	30	14	8
7	31	14	8
8	35	15	9

Table 4. Expected immunogenicity of selected regimen for randomized selection designs that utilize 50 patients assumes $\mu = 0$ and $\nu = 1$

Number of regimens	Patients per regimen	Expected immunogenicity of selected regimen		
		$\sigma = 2$	$\sigma = 1$	$\sigma = 0.75$
2	25	0.52	0.55	0.56
5	10	0.99	1.10	1.13
10	5	1.15	1.40	1.44
25	2	1.14	1.60	1.73

the trial is fixed, there is a trade-off between the number of arms in the trial and the number of patients per arm. If we assume that the super-distribution is normal with mean μ and standard deviation ν, then we can compute the expected mean immunogenicity level for the regimen that has the best sample mean in the randomized K arm selection trial. Table 4 shows the expected mean immunogenicity levels as a function of the number of randomized arms and the sample size per arm when the total number of patients is fixed at 50. Four cases are shown: 2 arms of 25 patients, 5 arms of 10 patients, 10 arms of 5 patients, and 25 arms of 2 patients. The mean μ and standard deviation ν of the super-distribution are set at 0 and 1 respectively, and the table shows results for different values of the standard deviation σ of immunogenicity measurement for patients receiving the same vaccine regimen. It can be seen from the table, that in most cases the best treatment is identified by studying 25 treatment arms, each with only 2 patients. This is not really practical and reflects the unrealistic assumption that an unlimited number of regimens are available and that the activities of these regimens are independent and can be regarded as draws from a normal distribution. Nevertheless, the model does highlite the principle that there is an opportunity cost to studying few regimens thoroughly. The approach provides some justification for screening many regimens with a smaller sample size than is used for non-selection based trials.

3.3. Controlled Phase II Trials with Time to Tumor Recurrence or Progression Endpoint

Therapeutic vaccines may be more effective in patients with lower tumor burdens, and may slow progression rather than cause regression of bulk tumor. Patients without clinical evidence of disease may have more intact immune systems and be more appropriate candidates for tumor vaccines than patients with more advanced measurable metastatic disease.

Evaluating the effect on a regimen on time to progression of sub-clinical disease is very problematic in a single arm phase II trial. It is easy to devise a definition of disease stabilization, i.e. lack of recurrence or progression for a specified period of time, but the validity of the definition depends on the existence of data that establish that such stabilization does not occur in the absence of treatment. This is difficult to establish reliably because of the usual difficulties of identifying comparable non-randomized controls and because of special difficulties involved with measuring time to disease progression in a consistent manner for different cohorts of patients. Consequently,

use of disease stabilization or time to progression as an endpoint in single arm trials should only be considered when data from a specific set of contemporaneous controls from the same institution are available. In such a case, rather than attempting to define disease stabilization as a dichotomous endpoint (e.g. present or absent based on some threshold), it is preferable to compare the time to progression for the patients in the phase II trial to the distribution of time to progression of a specific set of control patients not receiving the vaccine regimen. Dixon and Simon (14) provide formulas for computing the number of patients required in the single arm trial.

Phase III trials are generally randomized comparisons of a new regimen compared to a standard treatment using an endpoint of established medical importance to the patient such as survival or quality of life. Phase III trials are usually planned using a 5% type one error parameter (α) because the results of phase III trials are viewed as definitive and are used as a basis for marketing approval and practice guidelines. In the development of cancer vaccines, there is a role for what might be called a "phase 2.5" trial (1). Such a clinical trial would also be randomized, but may use an endpoint measuring biological anti-tumor activity even though the endpoint might not be established as a valid surrogate for survival or quality of life. The phase 2.5 trial might also be based on an elevated statistical significance level since the objective of the trial would not be for marketing approval or for establishing general practice guidelines.

To detect a large effect of a treatment in delaying tumor progression in a rapidly progressive disease such as pancreatic cancer or melanoma with visceral metastases does not require many patients in a randomized trial. With exponentially distributed times to progression, a 40% reduction in the hazard of progression corresponds to a 67% increase in median time to progression. In order to have 80% power ($\beta = 0.20$) for detecting this size of effect using an $\alpha = 0.10$, only about 87 patients are required (assuming accrual rate of about 3 patients per month, median time to progression of 12 months for control group and follow-up time of 24 months after end of accrual) (19). Hence, with 44 patients randomized to vaccine and the same number randomized to control, one can conduct a randomized "phase 2.5" trial for evaluating whether the vaccine reduces the hazard of progression by 40%. This design would be a "phase 2.5" design because of the unconventional use of a one-sided $\alpha = 0.10$ significance level and because time to progression might not be established as representing clear patient benefit. The phase 2.5 design is similar to the phase III design in the respect that it contains a control group for evaluating the experimental regimen and the intent is comparative.

Statistical power for detecting a specified reduction of the hazard of an event is determined by the number of events, not the number of patients. The number of events required to have power 1-β for detecting a treatment effect of size δ with a one-sided statistical significance level of α is approximately:

$$E = 2\left(\frac{z_\alpha + z_\beta}{\ln(\delta)}\right)^2 \tag{2}$$

where δ is the ratio of median time to events if the distributions are exponential (19). In the calculation of the previous paragraph, $\delta = 1.67$, $\alpha = 0.10$, $\beta = 0.20$ and

consequently E = 35 events. The number of patients needed to obtain 35 total events depends on the accrual rate, accrual period and follow-up period. With a slowly progressive disease, it may take many patients to be entered in order to observe a specified number of events unless the follow-up time following the close of accrual is very long. If the disease is rapidly progressive and all patients are followed until progression, then only 35 patients need to be randomized to observe 35 events.

Two different vaccine regimens can be evaluated in a randomized controlled phase II trial with time to progression endpoint by utilizing a three arm design. One arm would be the control group that does not receive either vaccine. For separate evaluation of each vaccine group without adjustment of the significance level for the fact that two vaccines are being evaluated, the number of events and patients required increases by one third compared to the two-arm trial. For example, if 44 patients per arm are required for the two-arm trial above, then 44 patients per arm are required for the three arm trial.

Trials using time to progression endpoints can be terminated early if interim results are not promising. One simple strategy is to perform an interim analysis when half of the planned total number of events have been observed. Accrual can be terminated if the number of events in the treatment group is greater than the number of events in the control group at that time. This interim analysis does not effect the type 1 error rate and causes negligible loss in statistical power (20). More sophisticated and efficient interim analysis plans for early termination when results are not promising are also possible (21). For a trial with multiple vaccine arms and one control arm, the interim monitoring can be used to evaluate each vaccine arm and stop accrual to those for which results are not promising.

Randomized phase 2.5 trials may be structured so that all patients first receive tumor reduction with other modalities prior to randomization.

4. PHASE III TRIALS

Phase III trials are generally randomized comparisons of a new regimen compared to a standard treatment using an endpoint of established medical importance to the patient such as survival or quality of life. Phase III trials of therapeutic cancer vaccines do not differ in important respects from phase III trials of conventional treatments; a randomized trial is required in both cases with a medically relevant endpoint and an appropriate control group.

5. SUMMARY

Therapeutic cancer vaccines have characteristics that require a new paradigm for phase I and phase II clinical development. Effective development plans may take advantage of some of the following observations:

Dose ranging safety trials are not appropriate for many cancer vaccines.

Dose ranging trials to establish an optimal biologic dose are often not practical. We have presented an efficient design of Korn et al. (4) to identify an immunogenic dose.

Vaccine efficacy can be efficiently evaluated with tumor response as endpoint utilizing a two stage design with only 9 patients in the first stage. If no partial or complete responses are observed in the initial 9 patients, accrual to the trial is terminated.

Optimization of vaccine delivery by comparing results of single arm phase II studies using immunological response as endpoint is problematic because of assay variation and potential non-comparability of patients in different studies.

Randomized screening studies can be used to efficiently optimize vaccine immunogenicity. Efficiency in use of patients depends on having assay variation and inter-patient variability small relative to the difference in immunogenicity to be detected.

Phase II studies using time to progression as endpoint are most interpretable if they employ randomized designs with a no-vaccine control group. Such designs may use an inflated type 1 error rate, and need not be prohibitively large if patients with rapidly progressive disease are studied. Interim monitoring plans may effectively limit the size of the trials by terminating accrual early when results are not consistent with the targeted improvement.

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15. CLINICAL TRIAL DESIGN AND REGULATORY ISSUES FOR THERAPEUTIC CANCER VACCINES

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INTRODUCTION

Tumor immunology began, over 100 years ago, with the observation that inflammation induced by infectious agents or their products could induce tumor regression (1). While numerous attempts to produce either active or passive immunity to tumors have been based on models of successful vaccines for infectious agents, graft rejection of foreign antigens, or induction of breaks in self-tolerance, the potential of the immune system to prevent and control tumor growth has yet to be fully exploited. Stimulated by the identification of human tumor rejection antigens, an emerging understanding of human immunobiology, and advances in biotechnology, the last decade has seen a marked increase in clinical trials of therapeutic cancer vaccines (2–11). Methods to identify effector T-cells and their epitopes (4, 6, 7, 12–17), to augment immune responses with cytokines (18–24) and costimulatory molecules (25–28), to manipulate regulatory T-cells, and to use dendritic cells to present tumor antigens (29–33), have entered the clinical arena. As illustrated in Figure 1, these advances have led to a steady increase in the number of Investigational New Drug Applications (INDs) filed by the Division of Cancer Treatment and Diagnosis (DCTD), National Cancer Institute (NCI), starting in 1990.

Cancer vaccines speak to the hope of biologic control of cancer with minimal toxicity. The earliest attempts to use vaccines consisted of using the patient's own killed or lysed tumor cells or tumor cell lines as a vaccine, or to create anti-sera for passive immunity (34–39). More recently, cancer vaccines often utilize well-defined,

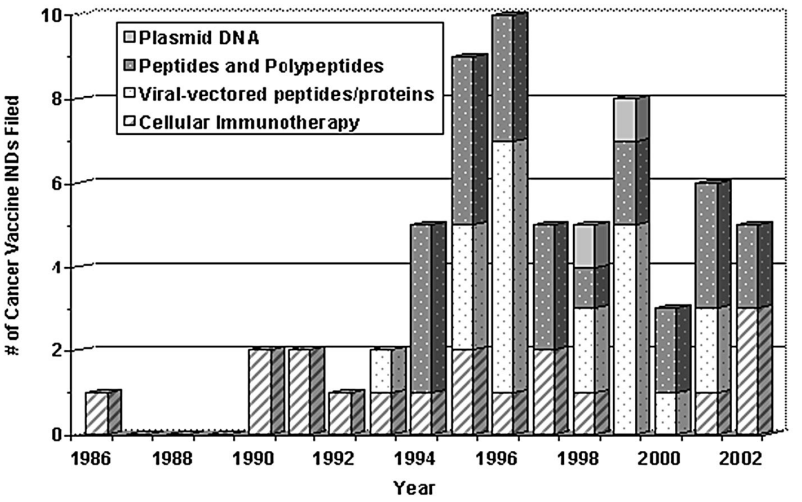

Figure 1. The number of Investigational New Drug Applications (INDs) for vaccines filed by the DCTD, NCI for each of the years from 1986–2002. The number of vaccines is further delineated by type of vaccine (i.e. cellular therapy, peptides and polypeptides, viral vectored peptides/proteins and plasmid DNA.)

purified tumor-specific (40–46) and tumor-associated antigens (47–51), in various forms including synthetic peptides and larger proteins (41, 47, 52–54), peptide-pulsed dendritic cells (29, 55–57), plasmids (58), and virus vectors (59–69). In this chapter we provide guidance on the regulatory and clinical issues relevant to cancer vaccine trials. It should be noted that the scientific evaluation of non-cytotoxic agents is an ongoing process, with the Food and Drug Administration (FDA) review of investigational products evolving as clinical experience with these products is attained. Information provided in this chapter should be used as a framework or guide in considering product issues and the design of clinical trials. The FDA should always be consulted prior to the filing of an IND. One method of discussing product and clinical issues for a specific product with the FDA is the Pre-IND meeting. Reference to a FDA guidance on Pre-IND meetings is provided at the end of this chapter.

REGULATORY REVIEW ISSUES

In order to conduct a clinical trial with an experimental biologic agent, an IND application must be submitted to the Center for Biologics Evaluation and Research (CBER) of the FDA as well as to a local or central Institutional Review Board (IRB). Gene therapy products are also subject to further review by the National Institutes of Health (NIH) (see below).

In June 2003, CBER was reorganized and several offices were transferred to the Center for Drug Evaluation and Research (CDER). Therapeutic vaccines remain under the auspices of CBER. In particular, gene- and cell-based cancer vaccines are

the regulatory responsibility of the Office of Cellular, Tissue and Gene Therapies in CBER. For the categorical distribution of biologics amongst CBER and CDER, see the Federal Register: June 26, 2003 (vol. 68, No. 123) or the web site http://www.fda.gov/cber/transfer/transfer.htm. General guidance on IND submission can be found at http://www.fda.gov/cber/ind/ind.htm.

Cancer vaccines range over a broad spectrum of biological products and combinations of products. These products include plasmid DNA, RNA, tumor cells, tumor cell lysates, peptides, proteins (including immunoglobulin idiotypes), recombinant viruses, peptide-pulsed dendritic cells, as well as passive immunization agents such as antibodies and adoptive cellular therapy utilizing antigen-specific T-cells. Many are combined with adjuvants such as incomplete Freund's adjuvant (water-in-oil emulsions), saponins, monophosphoryl lipid A, and aluminum salts (alum) as well as cytokines and immune stimulating agents.

Within each of the above categories many different products are being evaluated in preclinical models or are in clinical trials already. Retrovirus, vaccinia, canarypox, fowlpox, adenovirus, adeno-associated virus, herpes and lentivirus are currently being used as expression vectors to carry cytokines, costimulatory molecules, differentiation antigens and tumor-associated antigens in an attempt to generate a therapeutic immune response. For example, the DCTD, NCI has evaluated 28 different poxvirus, two adenovirus, and three plasmid vectors expressing recombinant vaccine products alone or in combination. Tumor-cell vaccines gene-modified to contain immunostimulatory molecules, dendritic cells, dendritic/tumor cell fusions, and even bacteria can be enlisted to present tumor antigens. NCI has sponsored seven INDs for whole-cell vaccines. Peptide vaccines that represent basic tumor antigenic epitopes are well defined, relatively inexpensive, and easy to manufacture and administer. The ability to create agonist peptides by changing critical amino acids that bind to MHC or T-cell receptor molecules, adds to the potential efficacy and complexity of peptide vaccines. Passive immunization or adoptive immunotherapy clinical trials sponsored by the NCI have included the use of tumor-infiltrating lymphocytes (in some cases retrovirally-transduced with a marker gene or cytokine), autologous peripheral blood lymphocytes peptide-sensitized *ex vivo*, cloned T-cells, and expanded activated T-cells. Because cancer vaccines cover such a wide spectrum of products, they are subject to a wide variety of product-specific FDA regulatory guidelines and guidances in addition to the general FDA guidelines for all biologic products.

Oftentimes, multiple products are used together or in sequence in order to boost an immune response. For example, a vaccinia-vectored vaccine may be used as a priming immunization, followed by boosts with a fowlpox-vectored vaccine containing a gene encoding the same antigen, given along with GM-CSF (70). To facilitate a multi-agent trial such as this, the original IND filing should include all of the products. A single IND might also be used to compare a number of similar candidate vaccines for the purpose of selecting the most promising one for further development. One example would be an IND that includes multiple peptides representing CTL or CD4+ epitopes of a particular differentiation antigen in the early

phase of drug development (phase 1/2). By late phase 2/3, information relevant to the chosen product should be made into a new IND to support further development and eventual licensure. The DCTD's NCI's experience with the early development of vaccines and regulatory agencies is described below. *Please note that the NCI's experience may not be universally applicable as each vaccine brings unique issues that should be considered on a product specific basis.*

Gene-based Vaccine Products

Cancer vaccines that contain recombinant DNA (rDNA) such as DNA plasmids and viral-vectored vaccines are a special case, which may be subject to NIH review. Cancer vaccines are included in this category for review by the Office of Biotechnology Activities (OBA) in the NIH Guidelines for Research Involving rDNA Molecules (Section V: Appendix M-VI-A). A footnote to Appendix M states that, *"Human studies in which induction or enhancement of an immune response to a vector-encoded microbial immunogen is the major goal, such an immune response has been demonstrated in model systems, and the persistence of the vector-encoded immunogen is not expected to persist are exempt from Appendix M-1"*. Since this footnote identifies agents that are not subject to OBA submission, this is meant to imply that cancer vaccines containing *human* genes are subject to the NIH Guidelines and therefore require submission to OBA for review. This applies to studies that utilize any federal funding for recombinant DNA research and those for which the sponsor or institution conducting the study receives such funding. Protocols plus supporting documentation must be submitted to the Office of Biotechnology Activities (OBA), NIH per the NIH Guidelines for Research Involving Recombinant DNA Molecules, Section 1-C (see http://www4.od.nih.gov/oba/). These protocols must also be submitted to the Institutional Biosafety Committee (IBC), as well as to the Institutional Review Board (IRB). IRB, IBC and FDA review and approval are not required prior to OBA submission, but are necessary prior to the start of the clinical trial.

PRODUCT ISSUES

FDA guidance on the manufacture of biological products is based on ensuring the identity, potency, purity, stability, bioavailability and safety of the product prior to human use. In general, there should be an adherence to the current Good Manufacturing Practices (cGMP) (21 CFR 210–211), with full cGMP adherence by the time clinical studies reach phase 3. These general principles apply to all cancer vaccines. Below, we will discuss select issues most relevant to cancer vaccine development.

An important product issue that is frequently overlooked in early trials is the development of a potency assay. This assay should be an *in vivo* or *in vitro* measure of the biological function of the product. While such a potency assay is not mandatory for early clinical development, in our experience, an assay measuring the intended biologic activity is essential for interpreting clinical results, especially those based on individualized products such as cellular vaccines. Prior to phase 3 clinical trials or, for that matter, any clinical trial that is intended to support

product registration, a validated potency assay based on biological function must be utilized (FDA-NCI Workshop on Tumor Vaccines, December 10–11, 1998, http://www.fda.gov/cber/minutes/workshop-min.htm). The assay must be robust, sensitive, specific, quantitative, and reproducible (FDA communication).

As biological agents, many cancer vaccines are manufactured using animal-derived reagents including fermentation broth, serum, amino acids, transferrin, albumin, enzymes, and lipids. Since 1991, the FDA has issued several guidances and Letters to Manufacturers, regarding the use of ruminant-derived reagents and the FDA's concern about potential transmission of classic and variant Creutzfeldt-Jakob disease (see http://www.fda.gov/cber/bse/bse.htm). There is evidence that variant Creutzfeldt-Jakob disease may be associated with the causative agent of bovine spongiform encephalopathy (BSE). Therefore, the FDA has requested that, materials derived from ruminants that have resided in countries where BSE has been diagnosed, or where they are unable to assure that BSE does not exist, not be used in product manufacture. (Letter to Manufacturers of Biological Products- Recommendations Regarding BSE, April 2000). The United States Department of Agriculture (USDA) maintains a list of countries at risk for BSE (9CFR 94.18). Because this list continues to grow as more and more ruminants infected with BSE are identified, it may be preferable if no ruminant-derived (or even animal-derived) products are used in the manufacture of cancer vaccines, regardless of their country of origin. Regarding products manufactured prior to the issuance of FDA guidance on BSE, FDA advisory committees acknowledged that risks posed by the use of bovine materials are theoretical and negligible, but they also advocated that there should be public disclosure regarding these risks and that materials from countries on the FDA BSE list be replaced as soon as possible. (Special Joint Meeting of Transmissible Spongiform Encephalopathy and Vaccines and Related Biological Products Advisory Committees, July 27, 2000) In general, if any animal-derived reagents of unknown origin were used during product manufacture, the Informed Consent should contain this information as well, noting the unlikely but possible risk of BSE transmission. Practically speaking, the origin of all animal-derived raw materials should be listed in the IND/Master File submission.

Gene-based Vaccine Products

Individual categories of cancer vaccine agents have particular product issues worth specific mention. Gene-based vaccines less than 40 kilobase pairs in length must be entirely sequenced prior to phase 1 clinical trials. For vectors 40 kilobase pairs or greater in length, the insert plus flanking regions (e.g., 500–1000 base pairs upstream and downstream), as well as transcriptional control regions for the inserted transgene, any other portion of the vector genome manipulated during derivation of the vector, or any regions with known toxic effects should be sequenced prior to phase 1 clinical trials (communication from FDA). A comparison of the sequence with existing human sequences using the Basic Local Alignment Search Tool (BLAST) is also recommended in order to detect any potentially harmful sequences and homology with human proteins (http://www.ncbi. nlm.nih.gov/BLAST/).

Whole Cell and Tumor Lysate Products

Whole cell and tumor lysate vaccines are complex mixtures and as such present challenges with respect to lot-to-lot consistency. (FDA-NCI Workshop on Tumor Vaccines, December 10–11, 1998, http://www.fda.gov/cber/minutes/workshop-min.htm) Lot-to-lot reproducibility must be demonstrated with respect to identity, purity, and potency. To achieve consistency, one must have a defined isolation, culture and expansion procedure that yields a well-defined cellular product. Products must be characterized with respect to morphology, immunophenotype, and function. It is desirable to identify several immunologically relevant antigens on the tumor cells and demonstrate their consistency from lot-to-lot. The proportion of irrelevant contaminating cells may need to be quantified in order to assess purity. Where appropriate, contamination with live tumor cells must be quantified. If cells or lysate are cryopreserved, validated assay methods should be used to demonstrate that there has been no change in viability, phenotype, and function upon thawing. Criteria for standardization are still in development for most products.

Other Issues for Gene- and Cell-based Products

In March 2000, the Office of Therapeutics, CBER sent out letters to sponsors of all gene-based INDs and Master Files requesting specific manufacturing information in an attempt to determine if current standards for the manufacture and testing of products were being followed (Dear Gene Therapy IND or Master File Sponsor Letter, March 2000). In particular, they requested manufacturing data, a summary of quality control/quality assurance (QC/QA) procedures, and a description of the clinical oversight and monitoring programs. Requested manufacturing data included product characterization and testing (methods, specifications, results), disqualification of lots, stability program, and a listing of all products made in the facility. More details on CBER's requests regarding gene therapy products can be found at http://www.fda.gov/cber/ltr/gt030600.htm. These issues should be addressed in any gene transfer or cellular therapy IND and updated in subsequent Annual Reports. More recently, similar types of letters have been sent to sponsors of cell therapy INDs. Amongst other items the FDA requested a description of the QC/QA programs for cellular therapy products that included the qualification program for cells, critical reagents, and equipment; and product tracking/labeling, as well as personnel qualifications and procedures for auditing contractors.

Long-term follow-up of patients is required for all gene-based products. The FDA's Biological Response Modifiers Advisory Committee has recommended that long-term follow-up extend over 15 years and should focus on the collection of clinical information pertaining to *de novo* cancer, neurologic, autoimmune, and hematologic disorders. (Biological Response Modifiers Advisory Committee Meeting, October 24, 2001) Unexpected medical problems including information on hospitalizations and medications should be collected. For retrovirus-based products in particular, testing for replication-competent retrovirus should be conducted on patient samples. In some cases, the clonality of vector integration sites should also be assessed using patient samples.

PRECLINICAL SAFETY/EFFICACY ISSUES

Preclinical studies must support the safety and rationale for the proposed clinical dose and schedule. Studies should be conducted in the most relevant species and model available utilizing a dosing schedule close to or identical to the proposed clinical use. There are many biologic products that are species-specific and, therefore, may be best tested in a pharmacologically relevant species, not necessarily a traditional toxicology species (71). For some agents, such as human cytotoxic T-lymphocytes, there may be no relevant animal model. In such cases, extremely cautious phase I safety testing may be most appropriate. Pharmacokinetic studies should also be conducted where appropriate. Preclinical toxicology studies should be designed to determine a dose range and potential dose-related toxicities in order to monitor safety in human trials. Animal models of disease can be utilized to address efficacy and, at the same time, some safety issues (72). For cancer vaccines, immunological activity should be studied, either *in vitro* or *in vivo*, depending on feasibility. Murine models expressing human transgenes for MHC molecules or tumor antigens are available to examine MHC restriction and tolerance. Cancer vaccines are intended to stimulate the immune system so one must separate intended effects from aberrant, toxic effects (immunotoxicity).

If there is prior human experience that demonstrates the safety of a class of agents, then this may be sufficient to support entry of a new member of this class into clinical trials. For example, extensive clinical data demonstrating the safe use of a tumor antigen plus extensive experience with vaccinia and avipox vaccines may allow a new poxvirus vaccine product containing the gene for the same tumor antigen, to enter into phase 1 clinical trials without further *in vivo* toxicity testing. However, additional non-clinical safety studies in relevant animal models may be needed prior to large-scale clinical trials in, for example, less critically ill or a more heterogeneous patient population. The FDA should be consulted regarding this option. Toxicology studies are required for first generation vectors, those with new molecular entities and for agents when adverse events have been observed with a similar vector or transgene construct. Compliance with Good Laboratory Practice (GLP) regulations is generally expected for all non-clinical studies supporting safety. (ICH: Guidance on Preclinical Safety Evaluation of Biotechnology-derived Pharmaceuticals, July 1997).

The FDA will be reviewing the pharmacology/toxicology studies to assess the risk versus benefit of the product. The patient population, the severity of the disease, as well as the availability of alternative therapies will be considered during this process. Particular categories of cancer vaccines will have particular pharmacology/toxicology issues and these are discussed below.

Gene-based Products

Biodistribution studies to evaluate the tissue distribution, persistence, and integration potential of a vector must be conducted with gene transfer agents. Such non-clinical safety studies can identify potential target organs for toxicity (distribution of vector to non-target sites) in addition to determining the potential for germline transmission.

Information regarding the level and duration of gene expression *in vivo* should also be obtained whenever possible.

A biodistribution study would be expected pre-phase 1 for a first generation vector or a new molecular entity, a change in formulation, a change to systemic route of administration, or if the transgene has the potential to induce toxicity if expressed in non-target tissue. (June 14, 1999 Recombinant DNA Advisory Committee presentation by Anne Pilaro, CBER/FDA) A biodistribution study does not necessarily need to be performed prior to phase 1 studies if there is already extensive human experience with that class of vectors and the inserted product or expression cassette is not expected to influence the toxicity or the biodistribution of the product. A bridging study comparing the pharmacological activity and transfection efficiency of two related vector preparations, may supplant the need for toxicology testing as well (72).

Peptide Vaccine Products

Peptide vaccine products are often considered to be of low risk. Since peptides themselves do not usually have any biological activity except for their intended immunogenicity, there is often more concern about the toxicity of the adjuvant rather than the peptide. For novel adjuvants, preclinical studies should be conducted with the adjuvant, using single and repeat dosage with the route used in clinical studies, and usually in more than one species. If toxicology studies exist and there is previous human experience with a particular adjuvant alone and in combination with other antigens, then a complete toxicology study may not be needed, if the antigen-adjuvant combination does not pose a special risk.

INTRODUCTION TO CLINICAL TRIALS

As we begin the 21st century, there are still no therapeutic cancer vaccines licensed for use in the United States. This reflects the fact that no phase 3 clinical cancer vaccine trials have been consistently successful in demonstrating a clinical benefit. There are currently 15 or more phase 3 clinical trials in progress in a variety of cancer types and which utilize different therapeutic approaches. The wide variety of vaccine products and approaches also reflects the fact that there is no consensus regarding the best way to produce an effective anti-tumor response and no clear understanding of how best to develop cancer vaccines that are very likely to be effective.

The development of an effective therapeutic cancer vaccine presents a difficult problem. Due to the number of combinations of potential tumor-specific and tumor-associated antigens, adjuvants, and varieties of strategies for immunization that could be used for a therapeutic cancer vaccine, it is impossible to test them in a systematic manner using clinical end points while immunologic endpoints still require validation. In addition, current stratagems for designing and producing vaccines are often based as much on intellectual property owned by individual companies or investigators as on experimental data. In our experience, independent clinical investigators have initiated most therapeutic cancer vaccine development. Sponsoring companies, the majority of which are biotechnology companies, usually have a limited number

of products in development and are relatively new to therapeutic vaccine development. With a few exceptions, the pharmaceutical industry has limited experience in the development of therapeutic cancer vaccines. The few companies that specialize in vaccines develop prophylactic vaccines intended to protect against infection (73). Effective methods for choosing potential candidates based on pre-clinical data and for conducting phase 1 and phase 2 trials in an efficient manner are progressing along with our ability to manipulate the human immune system. Toward this end some standardization of study methods and immunologic evaluation is essential in order to compare and contrast results from different studies.

In one respect there is a currently approved vaccine for cancer. A vaccine already exists that prevents cancer by preventing infection by the cancer-causing virus, Hepatitis B (hepatoma). In addition, a vaccine for the human papilloma virus (cervical cancer) has had clinical success. Also, there is interest in a vaccine for the bacteria, H. pylori for the prevention of ulcers, which would also have the benefit of preventing gastric cancer. Licensing guidelines for a papilloma virus vaccine discussed at a November 2001 meeting of the Vaccine and Related Products Advisory Committee follow more generally the path of traditional vaccines with the most significant discussion centered on the use of virologic measures as surrogate end points for tumor prevention rather than reduction of the incidence of dysplasia as a clinical end point (http://www.fda.gov/ohrms/dockets/ac/01/transcripts/3805t1.doc). There is much less experience to guide the development of therapeutic vaccines. At least one vaccine, Melacine™ for prevention of recurrence of resected stage II melanoma, was approved for use in Canada but not approved by the U.S. FDA as clinical trials failed to meet primary end points. Further interest in the Melacine™ vaccine was stimulated by a retrospective subset analysis showing a 30% survival advantage for patients who are HLA A1 and/or C3 positive. Further clinical studies will need to be conducted to determine efficacy in this patient subset. The complex biologic, clinical, and regulatory questions raised by the development of this whole cell lysate vaccine were extensively discussed at an ODAC meeting (http://www.fda.gov/ohrms/dockets/ac/02/transcripts/ 3838t1.pdf). One comment deserves close attention by anyone engaged in therapeutic cancer vaccine development. Ultimately, it is prudent to get FDA input before putting in 7 to 10 years, tens of millions of dollars, and hundreds of patients in clinical trials to make sure it will satisfy not only the Agency but also what expert advisors feel is appropriate. Prior to study implementation, the FDA will provide a special protocol assessment upon request. This is a procedure whereby the FDA will review a proposed phase 3 study whose data will form the primary basis for an efficacy claim. They will assess whether it is adequate to meet the scientific and regulatory requirements identified by the sponsor (Guidance for Industry: Special Protocol Assessment, May 2002).

Regulatory guidelines perhaps have most impact at two stages of clinical trials. The first is during the pre-clinical development and the safety evaluation of the product in order to meet the requirements for filing an IND and conducting a phase 1 trial. The second is for the design and interpretation of phase 3 trials to meet

the statutory requirements for demonstration of safety and efficacy and providing labeling information required to license and define the use of the drug.

PHASE 1 CLINICAL TRIALS

Phase 1 trials are typically designed to test the safety of an agent and to determine a recommended phase 2 dose to evaluate efficacy. The typical dose escalation trial of cytotoxic drugs is premised on determining the maximum dose of any agent or combination that can be given (74). Phase 1 clinical trials with cancer vaccines on the other hand are designed to determine not only the safety of the vaccine but the optimal dose for eliciting the measured immune response which may involve a threshold as well as an upper limit. Since immunologic responses are generally not dose-dependent beyond a threshold response, demonstration of an immunologic response in a significant proportion of patients might be a reasonable end point for phase 1 trials. There are two significant ways that, in our experience, vaccine trials differ from more traditional trials of phase 1 cytotoxic drugs. First, the direct toxicity of vaccines and adjuvants is often minimal; the most frequent toxicities include local reaction, adenopathy, and constitutional symptoms such as fever, headache, and fatigue. Systemic toxicity when observed is most often associated with cytokines such as GM-CSF or IL-2, which are often given to enhance immunologic responses. Second, autoimmunity is a consideration for any vaccine that enhances immunologic responses. For example, IL-2 is frequently associated with autoimmune hypothyroid disease. Recently an agent thought to enhance immunologic responses, anti-CTLA-4 antibody, has been administered in conjunction with cancer vaccines, and has been reported to be associated with autoimmune reactions, sometimes severe.

The patient population selected for phase 1 trials has typically been patients with incurable or otherwise untreatable metastatic malignancy and this tradition has been maintained for vaccine trials. Since immune responses may be impaired in patients with advanced disease, it is possible that safety and efficacy questions may require studying patients with less advanced disease. The ability to administer high doses of vaccines and the understanding that the largest dose may not be the optimal dose requires an evaluation of the targeted immunologic response to determine a biologically active dose. Beyond safety, phase 1 (or early phase 2 trials) may be very useful to identify appropriate phase 2 end points, obtain and validate immunologic studies or surrogate end points, and evaluate factors in patient selection (including the presence of the target, the ability of the patient to mount an immune response, and the immunologic susceptibility of tumor). The average phase 1 trial may take between 6 to 24 months to complete depending on size, accrual, immunization schedules, and the time required between patient cohorts to allow dose escalation. In our studies, phase 1 trials have ranged from very simple dose escalation designs requiring no more than 12–18 patients to more elaborate phase 1/2 designs that gather significant information on immunologic efficacy and clinical activity of both single and combination agents. Clinical studies should have a study design, end points, defined patient population, and correlative studies that can be related to clearly identified objectives. The more information that is obtained regarding the biology

and potential anti-tumor mechanisms in early trials, the more likely meaningful information would be obtained to support later trials. The number of currently active phase 1 trials of therapeutic cancer vaccines is difficult to estimate but probably represents the introduction of 50–100 new products over the past two years.

PHASE 2 CLINICAL TRIALS

Phase 2 clinical trials are usually disease and stage specific since it is assumed that this would identify a reasonably uniform population and provide consistent results in larger trials. Since most phase 2 trials are not randomized, the selection of patients may be the critical factor in trying to establish efficacy based upon historic comparison and in the failure to replicate apparently successful trials in larger settings. The primary objective of phase 2 trials defines the range of responses at an optimal dose and schedule from phase 1 trials. For conventional cytotoxic drugs this is most conveniently accomplished using measurements of decreases in tumor size to define an objective response as a decrease in overall tumor burden. The Response Evaluation Criteria in Solid Tumors (RECIST) defines an objective response as a 30% or greater decrease in the sum of long diameters of measurable tumor (75). Other disease specific criteria are available for lymphoma, myeloma, and PSA responses in prostate cancer. Whether or not these objective criteria represent the most appropriate way to evaluate the clinical benefit of vaccines is a matter for continued analysis. The presence of partial responses may demonstrate activity but often not clinical benefit, while mixed responses, with some lesions resolving while others progress, perhaps reflects local determinants of the ongoing battle between tumor and T-cell. Occasionally pathologic complete response can be determined when biopsy shows absence of viable tumor but lesions are still measurable. While vaccines for infectious diseases are evaluated after a single course there is reason to think that therapeutic vaccines would require continued boosting or even retargeting of antigens as part of maintenance regimens for patients with responses or stable disease. Such long term protracted approaches remain to be evaluated. And, while complete responses are most frequently associated with the potential for long-term survival for an individual patient, the lack of objective responses may not capture ongoing anti-tumor activity that could be reflected by increases in time to progression and overall survival.

Since tumors may initially progress while patients are being vaccinated, trials should attempt to allow sufficient time for the patient to develop an immunologic response before concluding a vaccine is not effective. In addition, there are clear examples reported in which a tumor may respond after initial progression if enough time is permitted to complete a vaccine regimen and observe a response (76). An effective vaccination is most likely to be completed in the adjuvant setting. In patients with advanced disease, progression may limit the therapy. Modifications to allow continued treatment after limited progression need to be evaluated.

Designs for cancer vaccine trials that could be used for efficient evaluation of candidate vaccines have been reviewed. Randomized phase 2 designs have been suggested that could serve to identify promising agents and regimens, utilizing clinical progression endpoints or surrogate markers (77). The appropriate sizing of phase 2

trials may vary from small trials that demonstrate large treatment effects to larger trials intended to show smaller differences. Asking phase 3 efficacy questions that require randomization, survival endpoints, and appropriate controls of smaller phase 2 designed trials may be highly misleading. For example, a randomized design in patients with renal cell cancer using monoclonal anti-VEGF antibody, bevacizumab, while demonstrating only a 10% objective response rate succeeded in demonstrating a significant increase in time to progression in patients receiving the antibody. The critical factor that made this study successful was a carefully constructed, conducted, credible study design, but all depended on the strong effect of the agent, representing a hazard ratio of 2.5 compared to placebo. However, it is clear from this illustration that a less active agent would not have been likely to have a significant result (78).

PHASE 3 CLINICAL TRIALS

Phase 3 trials intended to support regulatory marketing approval require, substantial evidence of efficacy from adequate and well-controlled investigations (79). Studies must allow a valid comparison to a control group and adequate quantitative assessment of the drug's benefit, which is interpreted as prolongation of life, a better quality of life or an established surrogate. An application must also provide sufficient information to allow the product label to describe the effective and safe use of the agent in a defined population. The Federal Food Drug and Cosmetic Act requires that drugs be safe for intended use and an amendment in 1962 to that Act codifies the efficacy requirements.

In 1992 an addition, Subpart H (Accelerated Approval of New Drugs for Serious or Life Threatening Illnesses), to the new drug application regulations allowed accelerated approval on the basis of a surrogate end point such as response rate or time to progression, if it appears to provide benefit. The accelerated approval requires post-marketing studies to demonstrate the treatment is beneficial. Of 57 drugs approved between 1990 to 2002, about one third were approved based on survival and almost one half were approved based on response rate.

CONCLUSIONS

What is the appropriate end point for a therapeutic vaccine? Although we have begun to see objective tumor responses in some patients, response rates of 10 to 20% are not generally adequate to use as end points in phase 2 trials. In order to see reliable differences in survival in a phase 2 trial, a very striking treatment effect in a well-controlled setting is probably required. As a practical matter, to show benefit, most phase 3 studies of therapeutic vaccines have used overall survival in advanced disease or time to recurrence either in the adjuvant setting or following complete resection with a high risk of recurrence. It is important to understand some of the reasons phase 3 vaccine trials have consistently failed to meet expectations following promising phase 2 trials. Phase 2 trial results may be strongly influenced by patient selection and retrospective analysis of subgroups, which may contribute to inadequate phase 3 trials even when based on positive phase 2 data. However, even with well designed trials, rational vaccine development beyond the empiric evaluation of individual products will require a deeper understanding of human tumor immunobiology.

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SELECT SOURCES OF REGULATORY GUIDANCE

Title 21 of the Code of Federal Regulations

Informed Consent of Human Subjects (21 CFR 50, Subpart B).
Institutional Review Boards (21 CFR 56).
Good Laboratory Practices for Nonclinical Laboratory Studies (21 CFR 58).
Current Good Manufacturing Practice in Manufacturing, Processing, Packaging, or Holding of Drugs; General (21 CFR 210).
Current Good Manufacturing Practice for Finished Pharmaceuticals (21 CFR 211).
Investigational New Drug Application, Part 312 (21 CFR 312).
Adequate and Well-Controlled Clinical Trials (21 CFR 314.126).
Biological Products: General (21 CFR 600).
General Biological Products Standards (21 CFR 610).

FDA/ICH GUIDANCES, GUIDELINES AND POINTS TO CONSIDER

(http://www.fda.gov/cber/guidelines.htm)

Good Clinical Practice in FDA-Regulated Clinical Trials (http://www.fda. gov/oc/gcp/)
E6 Good Clinical Practice: Consolidated Guideline May 1997.
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16. IMMUNE MONITORING

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A fundamental aspect of developing effective cancer vaccines is the process of evaluating the immune response to such therapeutic interventions. Despite the availability of a number of immunologic assays for evaluating tumor antigen-specific immunity, no single test has emerged as an unequivocally superior metric for judging the efficacy of cancer vaccines. Nonetheless, there have been several technologic developments that will likely impact the search for the ideal biomarker for tumor-specific immunity. The available tools, major advancements, and current challenges in this quest for reliable immune monitoring techniques are the focus of this chapter.

1. INTRODUCTION

When patients are treated for minor ailments with highly effective agents, such as the treatment of a local bacterial infection with an appropriate oral antibiotic, the initial response to treatment typically can be evaluated on clinical grounds alone. For most cancer patients with life-threatening conditions, the scenario is quite different. In many cases, the only available systemic therapies may produce a modest to poor response, and months to years may be required to confirm any survival benefit. Although clinical evaluation may include a variety of laboratory tests and imaging studies, the ability of many such tests to demonstrate a true benefit to survival is poor. Because immunotherapy remains a largely unproven therapeutic modality for the treatment of cancer, similar issues arise in the setting of cancer vaccines.

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A second major obstacle in the evaluation of immune responses to vaccines is identifying the optimal patient population to be evaluated in clinical trials. Early phase studies are typically undertaken in patients with advanced disease, in part because the relative impact on patient survival and quality of life in case of an adverse event is expected to be small in a patient with a short life expectancy. If a vaccine is found to be safe, subsequent studies may also be performed in patients with minimal to no measurable disease following treatment, or in patients with earlier stage disease. Some investigators believe that tumor vaccines are not (and may never be) potent enough to eradicate extensive tumor burden, and that these patients may simply be too immunosuppressed to mount an adequate response. Patients with earlier stage disease generally have lower tumor burden and may be more immunocompetent than patients with advanced disease. They may have such long survival, however, that large numbers of patients would have to be followed over a number of years in order to prove a vaccine effective. Consequently, many investigators have focused on patients with advanced disease whose treatment has eradicated gross or "measurable" disease. In theory, these patients should be relatively immunocompetent despite a relatively short recurrence-free and overall survival. Despite these efforts, cancer vaccines to date have exhibited at best modest clinical impact regardless of the patient population examined.

Another barrier to progress in immune monitoring is an incomplete understanding of relevant immunoregulatory mechanisms. It is likely that identification of critical signaling events necessary and sufficient for stimulating antitumor immunity will lead to recognition of reliable biomarkers for effective antitumor vaccines. Because no existing cancer vaccines induce sufficiently robust antigen-specific T cell responses, there is no standard vaccine preparation available for evaluating immune monitoring assays. Instead, investigators are left with the task of trying to optimize immunologic assays based on T cell responses that in many cases may be difficult to detect above background.

Finally, despite the publication of thousands of articles on active specific immunotherapy for the treatment of cancer, few properly controlled, prospective, randomized trials have been reported. Numerous vaccine strategies nonetheless have been abandoned as ineffective. This has made interpretation of the literature in this field difficult and has promoted skepticism about the promise of tumor vaccines. The challenge of developing and systematically testing methods of immunologic monitoring to identify promising vaccine candidates is therefore one of the most important objectives in immunotherapy at this time. This article will describe how existing strategies have been used to address this problem and will discuss technologic advancements that may facilitate the development of reliable biomarkers for clinically effective antitumor immunity.

2. PRINCIPLES OF IMMUNOLOGIC MONITORING

Although it is likely that many elements of humoral and cellular immunity have some influence on the ability to generate antitumor immune responses, most investigators agree that T cells play a pivotal role. Therefore, T cell biology has been a primary focus of active specific immunotherapy for a number of years and will be the focus

Table 1. Proposed properties of an ideal immune monitoring assay

Property
Reliable indicator of true clinical efficacy/effect on survival
Reliable indicator of true tumor-specific immunologic activity
Superior sensitivity, specificity, reproducibility
Ability to perform test on minute quantity of biological material
Adaptability to automation and high throughput analysis
Time-efficient, cost-effective

of this chapter on immunologic monitoring. Previous chapters have described key immunologic events that appear to be critical for the induction of potent antitumor T cell responses. An open question is which of these processes most closely reflect the true clinical impact of tumor vaccines, and how such processes can be evaluated in a simple assay system.

Most assay systems designed to quantitatively measure phenotypic and/or functional properties of T cell populations contrast sharply with those used to evaluate humoral responses to prophylactic immunizations against infectious agents. In the latter case, regardless of the complex immunologic processes that ultimately lead to an antibody response, evidence has shown that a reliable biomarker for clinical efficacy of such immunizations is the titer of neutralizing antibodies present in the serum (1, 2). While it is possible that a reliable serum biomarker for antigen-specific T cell immunity exists, none has been identified thus far. Hence, most existing assays for the measurement of antitumor activity depend upon phenotypic and/or functional properties of T cells themselves. This has presented challenges along several fronts: (i) determining the most appropriate source of T cells, such as peripheral blood, the vaccination site, lymph nodes draining the vaccination site, or the tumor itself; (ii) optimizing the procurement, processing, storage and (if necessary) shipping of cells without compromising cell viability and function; (iii) identifying the phenotypic and/or functional properties of T cells that most accurately reflect antitumor immunity; (iv) establishing which subpopulations of T cells should be evaluated (e.g., CD4+ [helper], CD8+ [cytotoxic], CD25+ [regulatory] T cells); (v) selecting the appropriate timing for immunologic monitoring following vaccination; and (vi) selecting the specific methods for evaluating tumor-specific T cell properties that most likely reflect the true antitumor response. In view of these challenges, an ideal immunologic monitoring technique might have the following properties similar to those listed in Table 1. Unfortunately, at the present time no method of immune monitoring has all these characteristics.

3. TECHNIQUES OF IMMUNE MONITORING

A variety of methods have been used to evaluate the antitumor response to immunotherapy. These may be divided into clinical and immunologic assessments, the latter being the focus of this chapter. The standard endpoint according to which all methods of assessment ultimately must be compared is overall survival. As discussed

earlier, however, judging the efficacy of each individual candidate tumor vaccine on the basis of overall survival is extremely costly in terms of time and resources, and is therefore impractical. Evaluation of clinical response by measuring tumor shrinkage is common practice, and standard guidelines for this method of evaluation are available (3). Unfortunately, measurement of tumor shrinkage is a relatively unreliable technique for determining the response to immunotherapy, and it does not contribute to an understanding of underlying regulatory mechanisms.

The development of immunologic assays to evaluate tumor-specific T cell responses has been an area of intense investigation. Such immunologic tests comprise both in vivo and in vitro approaches. Both types of tests have been utilized extensively in the evaluation of cancer vaccines and are reviewed herein.

3.1. In Vivo Testing: Delayed Type Hypersensitivity

Delayed type hypersensitivity (DTH) testing is a classical method for measuring type IV (cellular) immune responsiveness. This technique involves administering an intradermal injection of an antigen preparation and recording the amount of erythema and induration produced after 48 to 72 hours. This response is believed to reflect antigen-specific activation of CD4+ T cells to release T-helper 1 cytokines such as interferon-gamma (IFN-γ) in the region of the injection site (4). These cytokines recruit monocytes and other inflammatory cells to the site and cause an increase in vascular permeability and extravasation. CD8+ T cells also appear to have the capacity to mediate such a response (5).

DTH testing requires little training, does not require sophisticated or costly equipment, and can be performed readily in the clinic or at the bedside. This technique of immune monitoring consequently has played a prominent role in the monitoring of immunotherapy trials. The procedure for applying DTH testing to immune monitoring is not standardized, however. Doses of peptide antigens, for example, may vary from the low microgram to milligram range and typically are administered in volumes of 0.1 to 1.0 ml (6–8). Preparations may contain one or more peptides, adjuvant(s), antigen-presenting cells and/or a variety of other agents designed to enhance immune reactivity. The procedure for measuring the response (erythema and induration) and the definition of a positive result also varies among studies, and there is a significant subjective component. Due to the lack of standardization of the injection procedure, measurements, and interpretation, meaningful comparison of DTH results among immunotherapy trials may can be difficult.

Another problem with DTH testing is that responses are not always antigen-specific. In one study, for example, erythema and induration were noted in response to injections with peptide-loaded dendritic cells (DC), but DC that were not loaded with any antigen produced a similar DTH response (9). In another study in which patients were injected with CEA peptide-loaded DC, some patients without apparent erythema or induration at the injection site were noted to have inflammatory infiltrates on histopathologic examination (10). The potential for nonspecific contributions to the DTH reaction may be particularly apparent when adjuvant agents are used. For example, GM-CSF, a cytokine included in some vaccine

preparations, itself may induce a DTH response (11). Responses to other antigens in the vaccine preparation may also occur, as may occur when fetal bovine serum is used to cultivate cells during vaccine preparation (12).

Although not all studies have demonstrated DTH reactivity to be a reliable marker of antitumor immunity, some have shown concordance with other assays and/or an association with clinical response. In a study of HER2/neu peptide vaccine, a DTH induration of diameter 10 mm or greater was associated with an antigen-specific proliferative response, while lesser degrees of induration were not (6). Hsueh and colleagues reported that tumor-specific DTH reactivity was associated with a significantly better overall survival in stage IV melanoma patients with no measurable disease who were treated with a polyvalent allogeneic cellular melanoma vaccine (13).

Because DTH testing is easily incorporated into the design of immunotherapy trials and appears to have some relationship to antigen-specific immunity, it will likely remain an integral part of the immune monitoring repertoire until a superior approach is available. However, in the interim, a system of standardizing the application of this test to immune monitoring could enhance its utility.

3.2. In Vitro Methods

3.2.1. *Sources of Specimens for Immune Monitoring*

The optimal source of specimens for use in immune monitoring assays is unknown. Most assays of antigen-specific T cell immunity are based on evaluation of T lymphocytes themselves. There are many potential sources of lymphocytes, such as the peripheral blood, tumor deposits, injection sites, or draining lymph nodes. Because the peripheral blood is the most convenient source of T cells, it has been the most widely utilized. Whether the peripheral blood T cell response to a vaccine correlates with the clinical response has been called into question, however. Lee and colleagues administered a gp100 peptide vaccine with or without IL-2 and found that the only subset of patients who showed evidence of a clinical response did *not* have detectable gp100-specific T cells in the peripheral blood (14). One explanation offered for this was that the tumor-specific T cells in patients with clinical responses migrated to tumor deposits or other site(s). Consistent with this hypothesis, some tumors are known to harbor tumor-specific T cells (15), but the presence of such tumor-infiltrating lymphocytes (TIL) does not necessarily correlate with clinical response (16).

Another potential source of T cells is the DTH (vaccine injection) site, but whether these antigen-specific T cells would have similar properties to TIL is unclear. Draining lymph nodes may also contain antigen-specific T cells, although such T cells have been found even in healthy patients without malignances (17). Nonetheless, this is a particularly attractive source of T cells in part because of the frequency with which regional lymph node dissections are performed for melanoma and other malignancies.

Recently, Slingluff's group reported a promising method for harvesting tumor-specific T cells from lymph nodes of melanoma patients using a procedure adapted

from the sentinel lymph node biopsy technique (18). The lymph node directly draining the peptide vaccine site (the sentinel immunized node, SIN) was harvested from each of five patients. While CTL activity could be demonstrated in T cells from the SIN in 5/5 patients, similar activity was identified in only 2/5 patients with T cells from the peripheral blood. Further work will be necessary to determine whether procedures such as this will replace peripheral blood as a source of T cells for monitoring the immune response to cancer vaccines.

An alternative strategy to examining T cells themselves is to identify a surrogate biomarker for T cell activity in tissues or body fluids. Most previous studies in which serum markers were used as indicators of immune responsiveness to cancer vaccines have focused on the measurement of antibodies or immune complexes (19–21). This strategy represents an attractive option because of the difficulty of procuring, processing and storing T cells in a manner such that antigen-specific T cell activity is properly retained. One example of this approach is the analysis of serum T cell cytokine levels. While the measurement of serum cytokine levels has not yet been demonstrated to be of value in immune monitoring, this strategy could assume a more dominant role with the advent of increasingly sophisticated proteomics instrumentation and methodologies.

Regardless of the source of T cells or other specimens, the procedures for collection, processing, and in some cases storage of these samples may be critical to an accurate readout of immune assays. For example, whether and how best to cryopreserve peripheral blood mononuclear cells (PBMC) could have a dramatic impact of T cell activity. In part because it is generally easier to preserve molecular rather than cellular integrity and activity, a progressive shift toward molecular approaches appears likely.

3.2.2. *Types of In Vitro Methods for Immune Monitoring*

Although there is no gold standard technique for immune monitoring, a number of assays are currently in use, and there has been no shortage of novel approaches or improvements in existing ones in the published literature. These techniques may be categorized according their common properties, as shown in Table 2. Although increasingly the divisions among these categories are becoming blurred and some strategies may incorporate a combination of these features, the conceptual framework is useful.

First, these methods may be distinguished according to whether they examine *cell biological* or *molecular* properties. For example, T cell proliferation and microcytotoxicity assays reflect biological processes that may occur in response to antigen-specific T cell activation, but do not indicate the specific molecules responsible for these processes. In contrast, one can measure specific cell surface molecules or cytokines that are believed to be involved in these processes without directly examining such T cell functions. A second category applies specifically to molecular techniques and is becoming increasingly useful in light of the rapid rate of technologic advancement in this field. This regards the class of molecules that are detected by molecular techniques: *DNA*, *RNA* or *proteins*.

Table 2. Properties of selected in vitro immune monitoring assays

Method	Approach: Cell biological (CB) or Molecular (M)	Molecular Analysis Detects: DNA, RNA or Protein	Assay Parameter(s) Examined: Primarily Phenotypic or Functional
T Cell Proliferation	CB	—	Functional
Microcytotoxicity	CB	—	Functional
Tetramer analysis	M	Protein	Phenotypic
Spectrotyping	M	DNA	Phenotypic
TCR subfamily analysis	M	RNA or Protein	Phenotypic
ELISA	M	Protein	Functional
ELISpot	M	Protein	Functional
CFC	M	Protein	Phenotypic and Functional
Proteomics	M	Protein	Phenotypic and/or Functional

Abbreviations: RT-PCR, reverse transcriptase polymerase chain reaction; TCR, T cell receptor; ELISA, enzyme-linked immunosorbent assay; ELISpot, enzyme-linked immunospot; CFC, cytokine flow cytometry.

Finally, in vitro immune monitoring assays can be classified according to whether they evaluate *phenotypic* or *functional* properties of T cells. For example, quantitating the frequency of a T cell population based on a cell surface marker is a phenotypic assay, while cytolytic assays are functional. Although cytokine based methods in one sense could be viewed as phenotypic assays, these experiments typically involve measuring cytokines produced in vitro over a certain time period in response to antigen-specific stimulation and are generally categorized as functional assays.

A number of modifications also may be made to virtually any of these assays, but do not themselves define distinct categories. For example, in order to address the relatively poor signal-to-noise ratio of microcytotoxicity assays, one or more rounds of in vitro T cell stimulation and expansion may be carried out prior to analysis. Such manipulations may be performed prior to virtually any immunologic assay, although one could argue that this should not be necessary for the detection of truly significant antigen-specific T cell responses. Other platforms, such as multiplex and high-throughput analyses, represent additional modifications that may be applied to a variety of immune assay systems.

3.3. Assays for Immune Monitoring

3.3.1. *Assays that Measure Cell Biological Process*

Traditional immune monitoring techniques monitor cellular processes that are associated with antigen-specific T cell stimulation. Among these are T cell proliferation and microcytotoxicity assays. The use of these techniques in conjunction with limiting dilution analysis (LDA) enable one to obtain an estimate of T lymphocyte precursor frequency.

3.3.1.1. T Cell Proliferation Assay

The T cell proliferation assay is generally believed to reflect the amount of CD4+ T lymphocyte proliferation in response to stimulation with a specific antigen. This method involves incubation of the patient's T lymphocytes (or PBMC) and antigen with or without added antigen-presenting cells (for example, irradiated autologous or HLA-matched dendritic cells). After approximately three to five days, cells are pulsed with tritiated thymidine, and DNA synthesis is quantitated by measuring the amount of tritiated thymidine incorporated into DNA using a gamma counter. Relative proliferation may be estimated using a stimulation index, which is defined as the ratio of the [radioactivity incorporated into T cells following stimulation *with antigen*] to the [radioactivity incorporated into the control T cells in the *absence of antigen*].

The evaluation of in vitro T cell proliferation as an index of tumor antigen-specific immunity induced by vaccine therapy has been a fundamental component of the immune monitoring assessment (22–24). A major advantage of this strategy is its relative simplicity. Furthermore, it is not very labor-intensive and requires only standard reagents and equipment that are found or easily obtained at most research facilities. Its disadvantages from a technical standpoint include the lengthy incubation period of several days and its requirement for radioactivity. Of particular concern, because the proliferation assay evaluates a process (DNA synthesis) that is not associated specifically with antigen-specific activation of T cells, results may be influenced by nonspecific stimulation. Not surprisingly, proliferation assay results have not correlated strongly with clinical activity of cancer vaccines (22, 25). Some of the drawbacks of the traditional proliferation assay may be circumvented with a recently described flow cytometry-based strategy for evaluating proliferation (26).

3.3.1.2. Microcytotoxicity

The ability of CD8+ cytotoxic T lymphocytes (CTL) to lyse target cells may be monitored directly with microcytotoxicity assays. In this technique, effector T cells or PBMC are mixed with target tumor cells at a variety of effector:target ratios, and target cell lysis is measured after a specified time interval. The traditional approach involves using target cells loaded with a radioactive tracer such as (51) Chromium prior to the assay and measuring the quantity of tracer released during a defined incubation period. Percent specific lysis may be calculated from the amount of tracer released using the following formula:

$$\%\ \text{Specific Lysis} = \frac{\text{Total Release} - \text{Background Release}}{\text{Maximum Release} - \text{Background Release}} \times 100\%,$$

where *Total Release* is the amount of radioactivity released into the medium in the presence of effector cells, *Maximum Release* is the amount released when target cells are completely lysed with detergent, and *Background Release* is the amount released in the absence of effector cells. Percent specific lysis is then plotted versus the effector:target ratio for each assay condition.

A distinct advantage of this approach is that the T lymphocyte mediated lysis of tumor cell targets in vitro is relevant to the desired effect of tumor vaccines in vivo. However, the tracers may not be taken up avidly by certain types of target cells, and the (51) chromium-based assay requires handling of radioactive material. Although a europium-based fluorometric cytolytic assay is available, this technique has not been applied as widely as the (51) chromium-based method, possibly due to problems with reproducibility. Due to the relatively poor signal:noise ratio of the microcytotoxicity assay, detection of tumor-specific CTL activity directly from PBMC or other sources of T cells may be difficult or impossible, and therefore one or more in vitro stimulations of T cells are often necessary prior to performing the assay. These in vitro manipulations appear to amplify, but may also distort the relative magnitude of tumor-specific CTL present in the patient. In addition, because autologous tumor cells may not be readily available for use as targets, alternative targets such as HLA-matched tumor cell lines or tumor antigen-loaded antigen-presenting cells, may be used instead. Consequently, the relevance of microcytotoxcity assays to the true tumor-specific CTL activity induced by tumor vaccines has been called into question.

Alternative methods for evaluating CTL cytotoxicity in vitro have been developed in order to circumvent some of the problems associated with the traditional (51) chromium release assay. For example, a flow cytometry-based technique in which target cells are stained with a green fluorescent membrane dye (DiO_{18}) and the nuclear dye propidium iodide (27). The percentage of target cells in each well that are lysed during an incubation period may be calculated because all cells are stained by the membrane dye, but only nonviable cells take up the propidium iodide. An alternative cytotoxicity assay is based on the flow cytometric detection of target cell Annexin I expression, which increases in response to attack by effector T cells (28, 29). These alternative methods do not involve radioactive materials and are not dependent upon the ability of target cells to take up tracer material as in the release assays. Whether the results of these alternative cytotoxicity assays will predict clinical response to tumor vaccines remains to be determined.

3.3.1.3. Limiting Dilution Analysis

The classical approach to determining cytotoxic T lymphocyte precursor frequency is through LDA (30). In this technique T cells are serially diluted into a large number of wells of a microtiter plate, and cells are stimulated with an antigen of interest. A Poisson distribution analysis is used to calculate the proportion of wells with a given T cell number that contained at least one antigen-specific precursor at the beginning of the stimulation. This allows an estimate of the antigen-specific T cell precursor frequency to be calculated. LDA is labor-intensive, cumbersome, and may be difficult to reproduce. Although this method is worthy of mention as a foundation for discussion of microcytotoxicity assays, its utility in the context of cancer vaccine trials is limited.

3.3.2. Assays that Detect Molecular Markers of Immunity

As knowledge regarding the molecular basis of immunity and tumor immunology has grown and molecular techniques have become more powerful, there has been an increasing focus on detection of molecular biomarkers for tumor-specific immunity. Because of an incomplete understanding of these processes at the molecular level, however, it is unclear which factors would most accurately reflect the state of the immune system and would serve as the best surrogate markers of immunity. Nonetheless, several strategies already are in use, and a number of exciting modifications and entirely novel approaches have recently been described or are currently in development.

3.3.2.1. Techniques Based on Molecular Determination of Phenotype

Phenotypic and functional immunologic analyses are complementary methodologies. It appears likely that these two approaches increasingly will be used in conjunction in immune monitoring protocols and ultimately will merge seamlessly into one analysis. Purely phenotypic analyses are useful in that detailed quantitative and qualitative information can be obtained regarding a particular T cell population of interest. However, functional activity of such cell populations can only be inferred.

3.3.2.1.1. T cell receptor v region analysis. Antigen-specific T cells may be quantitated by examining the frequency of cells that harbor specific T cell receptor (TCR) V-J region sequences. The basis of this method is that a significant T cell response to a vaccine should elicit an expansion of T cells that express specific J-alpha, J-beta, V-alpha and V-beta chains that correspond to recognition of the index antigen. Flow cytometry may be used to estimate the frequency of such a T cell response with antibodies that recognize specific subfamilies of the TCR alpha or beta chains (31). Alternatively, polymerase chain reaction (PCR) technology may be used to derive similar information (32, 33).

TCR V region analysis requires only a small amount of specimen and may be performed on T cells isolated directly from the peripheral blood without prior ex vivo expansion. Although it is somewhat cumbersome, an automated, rapid, fluorescence-based variation of the PCR technique has been developed for detecting complementarity-determining region 3 (CDR 3) length analysis of TCR gene families (34). This approach is able to distinguish among polyclonal, oligoclonal and monoclonal CDR3 distributions.

3.3.2.1.2. Mhc-peptide tetramer analysis. An alternative approach to quantitating antigen-specific T cells is to use a flow-based technique in which fluorophor-conjugated complexes bind specifically to a TCR of interest. Tetramers consist of tetrameric complexes of a particular type of major histocompatibility complex with a defined peptide that binds to that major histocompatibility (MHC) complex. Four such complexes are bound together to a fluorophor-conjugated avidin molecule through avidin-biotin linkages, allowing for an extremely stable tetrameric unit. Such tetrameric complexes have been shown to bind stably and specifically to

cognate antigen-specific T cells (35). An alternative reagent that may be used to detect antigen-specific T cells is a dimer of MHC-peptide complexes that is bound together through an antibody (36, 37). A tetramer may be prepared for virtually any MHC class I-restricted peptide, provided the sequence of the immunogenic peptide is known and a stable tetramer can be synthesized.

MHC-peptide tetramers have proven to be a useful tool for quantitating antigen-specific T cells in the context of immunotherapy trials (14, 38, 39). One group has used tetramer staining and flow cytometry to sort antigen-specific T cells isolated from the peripheral blood or lymph nodes (40). They found that these T cells produced cytokines in response to antigen-specific stimulation.

Although the availability of tetramers represents a significant technologic advancement in immune monitoring, these reagents have several limitations. First, tetramers can only be developed for peptides of a defined sequence for a specific antigen, and stable tetramers may be difficult to synthesize for certain peptides. Although tetramers are available for a variety of MHC class I-restricted peptides, tetramers for class II-restricted peptides have been introduced more recently (41, 42). Therefore, far fewer tetramers are available for MHC class II-restricted peptide antigens than for class I peptides. Furthermore, tetramers are HLA class-restricted, most available tetramers being restricted to HLA-A2.01. Consequently, any particular tetramer is only useful in a minority of patients. Finally, although functional activity has been documented in antigen-specific T cells detected with tetramers, the precise composition and function of tetramer-positive cells is not completely clear at this point. One approach to circumventing this problem is the combined use of tetramer staining with other types of flow cytometry based analyses, such as cytokine flow cytometry (43). There also appear to be phenotypically distinct subsets, such as memory versus effector T cells, that are not evident unless appropriate markers (e.g., CD45RA/CD45RO) are examined (31).

3.3.2.2. Techniques Based on Cytokine Production

Several immunological methods are available that evaluate antigen-specific T cell activation by measuring some functional property of T cells, such as the elaboration of cytokines. Although nonspecific stimulation of T cells, for example by mitogens, may induce the production of cytokines, antigen-specific cytokine production usually can be determined by cytokine-based methods if appropriate controls are incorporated. An advantage of these approaches is that the pattern of cytokines detected reflects whether the immune response has primarily a T helper 1 (Th1) or Th2 bias. In addition, cytokine-based methods can be used to quantitate T cell responses. Finally, most of these techniques are fairly sensitive and, in theory, could detect a specific antitumor T cell response at a frequency of one in 1,000 T cells or lower.

3.3.2.2.1. Cytokine elisa. The enzyme-linked immunosorbent assay (ELISA) is a widely used immunoassay for the detection of specific proteins both in the research and clinical laboratory setting. This assay is easily standardized, is reproducible across investigators and institutions, and kits are commercially available for the detection of a variety of cytokines. In this assay, antigen-specific stimulation of T cells or

PBMC is carried out in microtiter plates by the methods previously described. After a defined incubation period, usually about 24 to 48 hours, supernatants are tested for the cytokine(s) of interest using a sandwich immunoassay with a colorimetric or fluorescence based detection method. This typically involves coating nitrocellulose-bottom microtiter plates with a monoclonal antibody against the cytokine of interest, adding an aliquot of each T cell supernatant, adding an enzyme conjugated form of the anti-cytokine antibody, and incubating with a substrate that is converted to a soluble colored product by the conjugated enzyme. The reaction product can be measured by colorimetric or fluorometric detection, and the relative amount of cytokine released in each well is then determined using a linear regression plot created using a cytokine standard.

In the context of cancer vaccine trials, the cytokine most frequently examined using this method is IFN-γ, which reflects a Th1 pattern of cytokine production. A number of studies have reported significant antigen-specific cytokine release by T cells from patients treated with tumor vaccines. However, experiments typically have been performed using T cells previously treated with one or more rounds of in vitro stimulation. In addition, although the quantity of cytokine released may be related to the strength of the immune response, the precise frequency of antigen-specific T cells present in a sample cannot be determined by this method. Still more concerning is the relatively poor correlation of cytokine release data with clinical response.

3.3.2.2.2. Elispot. The ELISpot (enzyme-linked immunospot) method is based on similar principles to those of the ELISA and represents a modification of the latter technique. This assay is set up in a similar fashion as the ELISA, but the T cell stimulation is performed directly in the nitrocellulose-bottom microtiter plates pre-coated with antibody against a particular cytokine. As the cytokine of interest is released during the course of the incubation period, some of it binds to the nitrocellulose in the region of the T cell of origin in a pattern reflecting its concentration gradient. This results in a "footprint" of bound cytokine at that site. At the end of the assay, cells are washed away with a detergent. The plate is then developed as in the ELISA, but using a substrate that produces an *insoluble* colored product, which appears as a spot on the bottom of the plate. The number of spots per well is counted and is expressed as a frequency relative to the total number of input T cells or PBMC.

Unlike the ELISA, the ELISpot allows an estimation of the antigen-specific T cell frequency. Data analysis may be more cumbersome and subjective, however, due to the large number of spots that must be counted amidst a variable amount of background staining. Special automated plate readers are available that address both of these concerns. Kits available for performing ELISA and ELISpot assays are expensive, however, and sending ELISpot plates for professional reading or purchasing an ELISpot reader represent considerable added costs. Modifications of the ELISA may circumvent some of these problems. For example, one group has developed a multiplex, fluorescence-based immunoassay that requires about 100-fold less primary antibody than the ELISA and is conducive to high-throughput analysis (44). The recently developed Lysispot assay represents another derivative of the ELISpot (45).

This method enables one to quantitate cytokine-secreting T cells and cytotoxic T cells simultaneously. Interestingly, the investigators found that cytokine secretion and cytotoxicity can be independently regulated.

The ELISpot has been demonstrated to have excellent sensitivity (approximately 1 in 100,000) and specificity for antigen-specific T cell responses and to be reproducible across laboratories (20, 45, 46). A number of groups have used this technique as a method for immune monitoring in clinical trials (48–51). Consequently, the ELISpot increasingly is being touted as a standard component of the armamentarium of immune monitoring tools for cancer vaccine trials.

3.3.2.2.3. Flow cytometry based cytokine detection. Flow cytometry based methods also may be used to evaluate antigen-specific cytokine production by T cells following the administration of a vaccine. In cytokine flow cytometry (CFC), T cells or PBMC are stimulated in vitro with an antigen or cellular target of interest in the presence of stimulating anti-CD28 monoclonal antibody, which enhances costimulation. The secretion inhibitor brefeldin A is added so that cytokines elaborated during the assay build up within the cell of origin. At the end of the assay, the T cells are fixed, permeabilized, and stained with commercially available fluorophor-conjugated monoclonal antibodies against cell surface markers (e.g., CD8), an activation marker (i.e., CD69), and one or more intracellular cytokines (e.g., IFN-γ). Multi-parameter flow cytometry is used to analyze the frequency of T cells within the population of interest that stain positively for CD69 and the cytokine of interest. These "double-positive" T cells are presumed to represent functional antigen-specific T cells.

CFC has a number of advantages. It evaluates not only the relative amount of cytokine produced (i.e., relative fluorescence intensity), but also the frequency of the cytokine-producing cells. Assays are relatively easy to set up, and typical incubation periods range from four to six hours. It is also quite sensitive, allowing detection of T cells present at a frequency of one in 10,000, provided the background is adequately low. Furthermore, any T cell subpopulation of interest may be examined. In addition, with proper instrumentation, multiple cytokines may be analyzed simultaneously. The method may be even more powerful in combination with other flow-based methods, such as tetramer analysis, which enables one to precisely determine the concordance of cytokine production with the antigen-specific T cell population of interest as defined by its MHC-peptide specificity. Finally, CFC has also been adapted so that cytokine production by other immune cells of interest, such as DC, may also be examined (52). Therefore, the flow cytometric platform for cytokine production in principle allows for the acquisition of a large amount of quantitative and qualitative information of direct relevance to describing the T cell response to a tumor antigen of interest.

CFC unfortunately has several pitfalls. Although this technique is extremely sensitive, the background is variable and may be high enough that the limit of detection is determined more by signal:noise ratio than by the theoretical sensitivity. Like many assays of cellular immune function, it is highly dependent upon the quality of input cells and is critically dependent upon proper specimen acquisition and processing.

Not only is this method dependent upon access to costly multi-parameter flow cytometry instrumentation, but the types of instruments that allow analysis of several cytokines and cell surface markers simultaneously (i.e., with more than four or five channels) are extremely expensive and are not available at most centers. Finally, cells must be fixed prior to permeabilization, and therefore live cells cannot be analyzed. This eliminates the ability to perform functional and molecular analyses on specific cytokine-producing subpopulations of cells.

Improvements in the technology for flow-based detection of cytokine-producing cells are occurring at an alarming pace and will likely increase the value of this method in immune monitoring over the next several years. For example, one modification allows the detection of cytokine-producing cells without prior fixation (53). This technique utilizes an anti-CD45/anti- IFN-γ bispecific monoclonal antibody-antibody conjugate to capture elaborated IFN-γ on the surface of the T cell of origin. Application of this technology in conjunction with MHC-peptide tetramers enabled the investigators to identify live, cytokine-secreting antigen-specific T cells.

3.3.2.2.4. Detection of cytokine mrna. The detection of cytokine messenger RNA (MRNA) is yet another approach to evaluating cytokine production by antigen-specific T cells. Quantitative reverse transcriptase-PCR (RT-PCR) represents one method of measuring the abundance of a particular transcript in a sample and has been applied to the detection of cytokine mRNA (54–56). Kammula applied this technology to the evaluation of T cell responses in a melanoma peptide vaccine trial (57). In this study, cytokine mRNA levels of T cells within PBMC and within fine needle aspirates of tumor tissue were examined. The authors demonstrated that this method could be used successfully to examine antigen-specific T cell immunity in response to tumor vaccines.

3.2.2.3. Proteomics in Immune Monitoring

Proteomics is the study of the proteome, the protein complement of the genome. The goal of proteomics is to elucidate not only protein composition, but also protein state and function and the nature of protein-protein interactions. Proteomics is increasingly being applied to challenging areas of medical research. This trend is related to technologic advancements in mass spectrometry instrumentation and software, as well as the automation and miniaturization of analytical tools for protein analysis. The application of proteomics to clinical problems already has led to the development of a promising serum test that may be useful for the early detection of ovarian cancer (58).

Immunoproteomics, the application of proteomics to immunologic problems, offers novel strategies for target antigen identification and for monitoring the immunologic response to therapeutic interventions. Proteomics has not yet been applied as a tool for immune monitoring in cancer vaccine trials, but offers substantial promise in this regard due to a number of theoretical advantages.

The ability to analyze serum, PBMC or other specimens that have previously been cryopreserved could increase the feasibility of multi-center trials and enhance the reproducibility of immune assays. With existing technology, multiplex

immunohistochemical analysis of a wide array of cytokines and regulatory proteins simultaneously from a minute quantity of biological material is feasible. In addition, the availability of monoclonal antibodies against a variety of immune regulatory proteins phosphorylated on specific residues should enable investigators to obtain information about the regulatory mechanisms underlying tumor-specific immunity. In addition, protein profiling may allow evaluation of the immune response to a cancer vaccine from a drop of serum, and this method does not require knowledge of the identity of specific peptide species that best predict immune responsiveness. Despite the theoretical advantages of proteomics for immune monitoring, however, significant work will be necessary to establish whether this approach will have an eventual role in the context of cancer vaccine trials.

4. STATISTICAL CONSIDERATIONS IN IMMUNE MONITORING

Without question, as the reliability and standardization of immune monitoring tests improve, the importance of proper statistical data analysis will only grow. Like the methods themselves, however, there is a lack of standardization and consensus among investigators with regard to the optimal methods for data analysis. Progress toward standardized biostatistics for immune monitoring will be expedited if several fundamental problems can be addressed. These include variability in the readout of immunologic tests, lack of consensus regarding definitions used in the interpretation of tests, and failure to apply appropriate methods of statistical analysis.

4.1. Variability in Immunologic Assays

Three sources of variability of the immunologic assay results are encountered: variability within the same specimen, variability among specimens from the same patient drawn at different times, and variability among patients. Variability in the results of a single immunologic test performed on the same specimen results largely from error that is inherent in measurements associated with each step of the assay (e.g., slightly more or less reagent is pipetted into a well). Contributions from the instrument and operator both may be minimized through proper quality control, but cannot be eliminated. Of course, it is critical to avoid systematic contributions to this variability. For example, wells on the edge of a microtiter plate may loose more volume due to evaporation over time than wells further in the center of a plate, thereby affecting the concentration of reagents and introducing a systematic error into the results obtained from those wells. Most sources of systematic error can be mitigated through standardization and automation of as many steps in immunization and immune monitoring protocols as possible.

The measure of variability of a test on individual specimens is the coefficient of variation, which is defined as [standard deviation $\div$ mean] $\times$ 100%. Low coefficients of variation indicate that the reproducibility of the assay is good. The reproducibility or reliability of an assay can also be assessed by attempting to replicate the measurement on the same specimen at different times and places. In this case, variability is also introduced by differences in equipment used, differences in handling or storage of the specimen, and the analysts performing the assays. The agreement among laboratories

or analysts (inter-observer), or even within the same analyst (intra-observer), can be described by the kappa statistic, which is defined as [agreement beyond chance] ÷ [amount of agreement possible beyond chance].

Characterizing the variability in the results obtained from the same patient over time is essential for interpretation of results of studies involving immunologic manipulations such as vaccines. Presently, this type of variability cannot be controlled because the immune system reacts continuously to a variety of poorly defined factors (e.g., stress or invasion by micro-organisms). It is possible to vaccinate subjects with control foreign antigens, such as kehole limpet hemocyanin (KLH), at the same time the tumor vaccine is administered. This should allow an estimation of the temporal variability in immune responsiveness.

Finally, there is significant patient-to-patient variability. This arises, in part, from a variable degree of immune responsiveness or competence. Such differences among patients may be related to variations in tumor burden or overall medical condition. Specific characteristics of the tumors themselves also could influence responsiveness, such as the relative complement or abundance of the tumor antigens that are represented in the vaccine. In addition, some patients will have detectable baseline, pre-immunization antigen-specific T cells, while others may not. One strategy that can decrease patient-to-patient variability is the selection of similar patients for a trial, although this may not be feasible in studies restricted to uncommon malignancies or certain HLA types. Another approach to this heterogeneity among patients is to appropriately standardize immune responses during data analysis. For example, if a pre-immunization tumor antigen-specific T cell population is detectable, then one may examine the *increase* in antigen-specific T cell frequency. Standardization of responses to those against control antigens also should enable one to control for the variable overall state of immune competence among patients.

4.2. Definitions and Statistical Methods in Data Analysis

Perhaps the most important issue for the statistical analysis of results from immune assays remains the definition of what comprises a positive or negative result. Unlike many situations in medicine in which there is general agreement on how to define an entity (for example, a malignancy is defined by a number of microscopic features), there is no consensus on how to define an immune response. Because of this, there is no established gold standard against which other assays may be compared. Consequently, it has been difficult thus far to provide clear determinations of the sensitivity or specificity of a given assay. The recent workshop by the Society for Biological Therapy (59) intended to provide recommendations on the assays that might provide the most utility in monitoring clinical trials is an excellent first step in this regard. Nonetheless, the cut-offs chosen for positive and negative responses remain largely arbitrary. Our group has suggested that one way to empirically determine the appropriate cut-offs for positive and negative immune responses is to base them on responses to a potent immunogen, such as the cytomegalovirus (CMV) matrix protein, pp65. We performed ELISPOT, tetramer, and CFC assays on PBMC from

CMV-seropositive and seronegative donors. This enabled us to identify a cut-off point between the two groups at which the sensitivity, specificity and accuracy were acceptable.

A final concern in the evaluation of immunologic assays is the appropriate application of valid statistical tests. This issue arises particularly often with regard to multiple comparisons. Typically, immune assays are performed at several time points during a vaccine study, and several assays may be performed at each time point. This raises the possibility that a false positive will arise by chance. Clearly, it is important to use stringent methods of adjustment (such as Bonferroni corrections) to determine whether differences are truly statistically different.

5. SUMMARY AND CONCLUSIONS

A wide array of immunologic tests are available for immune monitoring in cancer vaccine trials, and the number of novel assays and technical modifications continues to burgeon. Because only a small fraction of all proposed vaccine trials tested in phase I-II trials, for practical reasons, will ultimately move forward to be tested in phase III trials, there must be a system of establishing the most promising immunization strategies. This evaluation of cancer vaccine will require standardization of the immune assays and statistical methods used in immunologic monitoring. Furthermore, the use of a systematic approach to evaluating and adopting novel technologies for immunologic assessment would likely lead to timely implementation of more reliable, practical and cost-effective methods of immune. It should be the goal and expectation that this rational approach to immune monitoring will allow the critical appraisal of the most promising vaccine candidates in the context of pivotal, multi-center trials.

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