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KLUWER ACADEMIC PUBLISHERS
BOSTON / NEW YORK / DORDRECHT / LONDON

Library of Congress Cataloging-in-Publication Data

Tumor Immunology and Cancer Vaccines/edited by Samir Khleif
p. ; cm. – (Cancer treatment and research; 123)
Includes indes.
ISBN 1-4020-8119-7 e-Book ISBN: 1-4020-8120-0
1. Tumors—Immunological aspects. 2. Cancer vaccines.
I. Khleif, Samir. II. Series.
[DNLM: 1. Cancer Vaccines. 2. Neoplasms—immunology.
3. Antigens, Neoplasm. QZ266 T9252 2004]
QR188.6.T8615 2004
616.99′4079—dc22 2004054847

Printed in the United States of America.

springeronline.com

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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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