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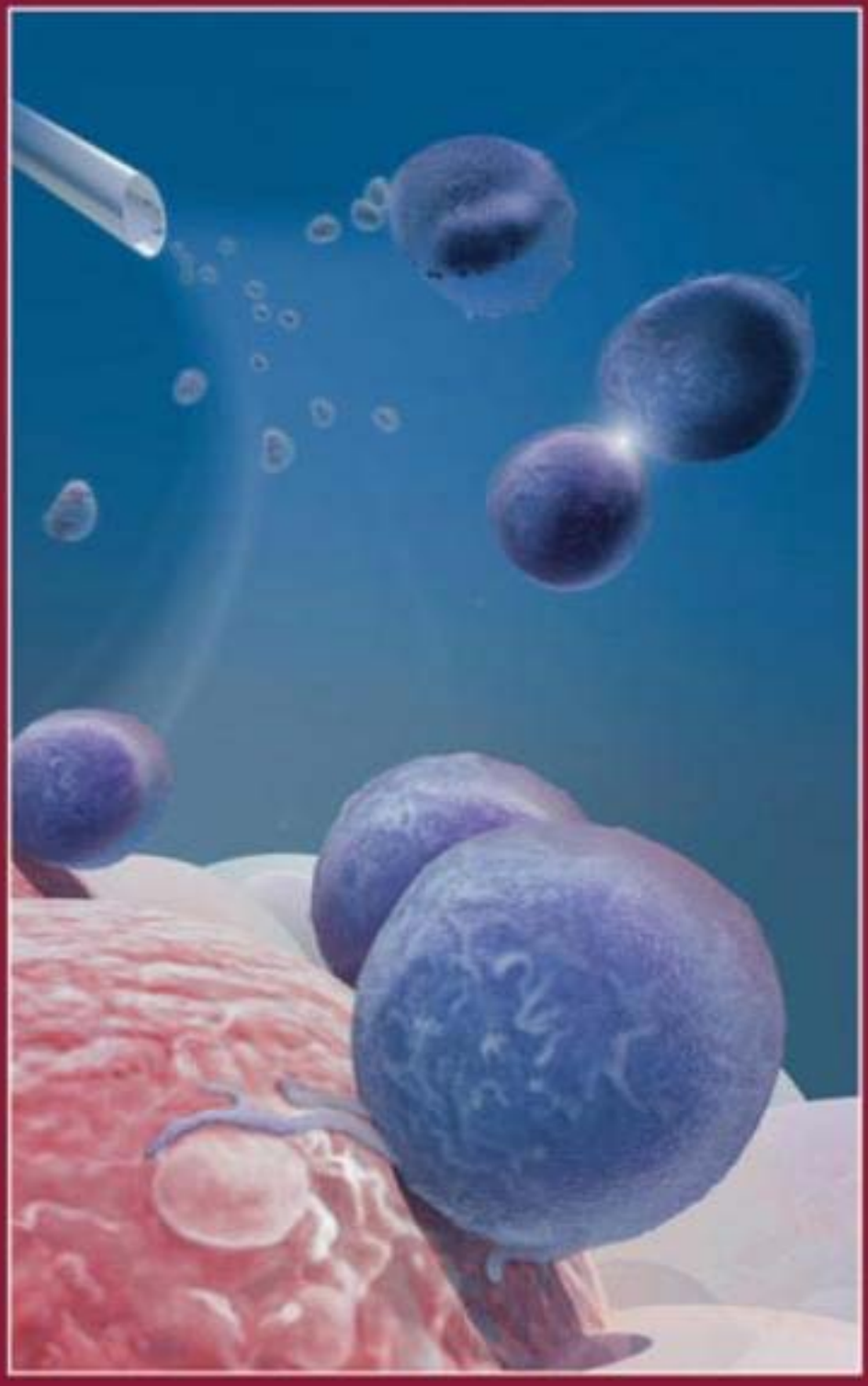
Cancer Vaccines and Tumor Immunity



Rimas J. Orentas • James W. Hodge • Bryon D. Johnson

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FOREWORD

CANCER VACCINES AND CANCER IMMUNOTHERAPY: NEW PARADIGMS

Jeffrey Schlom

The field of cancer vaccines is currently in an active state of both preclinical and clinical investigation. Hypothesis-driven preclinical studies in both in vivo rodent models and employing in vitro human systems are readily being translated into science-driven clinical trials. The basic concept in the use of vaccines for cancer therapy is to define strategies in which the immune system recognizes tumor-associated antigens that are not otherwise being recognized in the tumor-bearing host. There are numerous components of the immune system that vaccines have been shown to activate. These include cytolytic CD8 T cells, helper CD4 T cells, antibodies, natural killer (NK) cells, and other components of the innate immune system. Indeed, recent (as of 2007) studies are demonstrating a more intimate association than had been previously thought between the innate and the adaptive immune systems in cancer immunity. Equally important are the findings that a range of suppressor cells, such as regulatory T cells, immature macrophages, and other immunosuppressive entities and signals must be dealt with for the effective generation of an immune response and antitumor immunity. Both classical chemotherapeutic drugs, such as cyclophosphamide, and monoclonal antibodies directed against immune suppressive elements such as CTLA4 (cytotoxic T lymphocyte antigen 4), are examples of strategies that are actively being investigated.

A major aspect of this book is the elucidation of the wide breadth of vaccine vehicles and strategies that are being employed to enhance the activation of the host immune system to tumor-associated antigens. Anticancer vaccines that are being investigated include the use of recombinant viral vectors, recombinant bacterial vectors, peptides and polypeptides, carbohydrates, proteins, whole tumor cells, and

yeast, to deliver antigens to dendritic cells and other antigen-presenting cells. The area of immunostimulants to enhance vaccine efficacy is an equally important field of research. Strategies discussed in these chapters include the use of T cell costimulatory molecules in vectors, and biological adjuvants to activate the immune system, such as cytokines, CpG motifs, and bacillus Calmette–Guérin (BCG).

One issue frequently raised in the use of cancer vaccines is antigenic heterogeneity of tumors and tumor cell antigen escape variants. More recent clinical studies employing whole tumor cell vaccines and viral vector-based vaccines have provided clinical evidence of the phenomenon of "antigen cascade" or "epitope spreading" postvaccination. This well-studied phenomenon in preclinical models has been shown to be the result of cross-presentation of tumor antigens to antigen-presenting cells as a consequence of some tumor cell destruction postvaccination. Consequently, the host immune system is exposed to tumor antigens other than those in the vaccine.

The analysis of the clinical activity of therapeutic cancer vaccines, as is the case with other new targeted therapies, may well require a new paradigm. The classical paradigm for cancer therapeutics has been to first demonstrate reduction in the size of measurable tumor masses by the established RECIST criteria (*response evaluation criteria in solid tumors*, which looks for a greater than 30% reduction in tumor volume; further details available at `http://ctep.cancer.gov/guidelines/recist.html`). Often, however, these reductions in tumor size are concurrent with substantial toxicity to the patient, and in some cases with minimal or no demonstrated increase in patient survival. For example, a randomized clinical study comparing higher vs. lower doses of interleukin 2 (IL-2) showed more tumor regressions in patients with metastatic renal cell cancer employing the higher dose of IL-2, but no statistical increase in patient survival when compared with the lower dose. Similarly, while adoptive transfer of T cells has been shown to dramatically reduce tumor burden in subsets of melanoma patients, to date, no randomized trial has demonstrated an advantage in patient survival using this approach as compared to the use of IL-2 alone. Cancer vaccines, on the other hand, induce a dynamic process that may or may not result in the substantial reduction of tumor masses by RECIST criteria, but may result in tumor stasis, low levels of toxicity, and most importantly, increased patient survival. For example, this paradigm has recently been demonstrated in small-molecule-targeted therapies such as the use of sorafenib. In a randomized phase III trial involving 903 patients with metastatic renal cell carcinoma, treatment with sorafenib resulted in only 1/451 complete responses, and $<10\%$ of patients demonstrated partial responses by RECIST criteria. However, treatment with this targeted therapy demonstrated a statistically significant increase in patient survival leading to FDA approval.

Unlike the evaluation of classical chemotherapeutic agents, radiation, or adoptive transfer of immune cells, the administration of cancer vaccines generates a dynamic process in the host immune system. Furthermore, it may take multiple vaccine cycles, even in the face of progressive disease, for the full biologic effect of vaccines to take place. For example, in three different randomized phase II trials, employing three different types of prostate cancer vaccines, there were few demonstrations

of tumor shrinkage using RECIST criteria, but substantial evidence of increases in survival of prostate cancer patients in each of these trials. Thus, it would be misguided, and a classic example of "paradigm paralysis," to consider only the use of RECIST criteria for evaluation of any targeted therapy, especially cancer vaccines.

Preclinical and clinical studies have also provided evidence that cancer vaccines are most efficacious when used in patient populations of low tumor burden, relatively early in the disease process, and not following treatment with multiple cycles of different chemotherapies. The "failure" of some cancer vaccines in phase III trials may well be due in part to ill-designed clinical studies in inappropriate patient populations. Phase III trials with more sophisticated vaccines and more appropriate patient populations are necessary, and are ongoing and planned. More effective monitoring of immune cell levels and function, prior to as well as postvaccination, along with monitoring of immune suppressive cell subsets and entities, may also well lead to better-designed clinical trials and perhaps the ability to identify those patients or patient populations most suitable for a given vaccine regimen or strategy.

The next frontier for cancer vaccines will most likely be their use in combination therapy, that is, in combination with standard-of-care therapies such as chemotherapy, radiation therapy, hormonal therapy, or monoclonal antibody–based therapies, or in combination with new experimental targeted therapies. Cancer vaccines are well suited for combination therapies because of their demonstrated minimal toxicity in clinical trials. Preclinical studies, moreover, have recently shown that low-dose radiation of tumor cells as well as certain chemotherapeutic agents can alter the phenotype of tumor cells by upregulating molecules such as Fas, adhesion molecules, major histocompatibility complex (MHC), and tumor antigens, and thus render tumor cells more susceptible to T-cell–mediated attack. There is also emerging evidence that certain chemotherapeutic agents may inhibit suppressor cell populations. A demonstrated synergy or combinatorial efficacy of cancer vaccines with standard-of-care agents will undoubtedly lead to the use of vaccines earlier in the disease process and/or in patients with lower disease burdens.

It has also been demonstrated, as evidenced in the different chapters of this book, that different types of vaccines and vaccine strategies can activate different components of the immune system. Studies have now demonstrated the efficacy of diversified prime and boost vaccine strategies, using two different vaccine types. It is hoped that as the field matures, vaccines developed by two different commercial entities can eventually be combined or used sequentially to take advantage of this phenomenon. Moreover, as evidenced in this book, numerous tumor-associated antigens are now being targeted by the various vaccines. As the field matures, it is also anticipated that patients will be able to receive different vaccine types targeting multiple tumor-associated antigens.

The recent FDA approval of a human papilloma virus–based vaccine toward the prevention of cervical cancer is clearly an important landmark. It is hoped that as the field of therapeutic cancer vaccines matures, the vaccines and vaccine strategies

developed can also ultimately be applied to the destruction of preneoplastic lesions in high-risk patient populations.

Taken together, the field of cancer vaccines is now staged to exploit the use of (1) more sophisticated vaccines and vaccine strategies to greatly enhance both the level and intensity of host antitumor immune responses, (2) strategies to eliminate or reduce immunosuppressive entities, (3) combinatorial therapies with conventional as well as experimental agents, (4) better designed clinical trials in more appropriate patient populations, and (5) survival of patients as a primary clinical trial endpoint.

PART I

INTRODUCTION

1

CANCER VACCINES: PROGRESS AND PROMISE

Rimas J. Orentas, Bryon Johnson, and James Hodge

1.1 INTRODUCTION

The increasingly sophisticated understanding that we have of how tumors and the immune system coevolve in the host is leading to a steady stream of new ways to apply this knowledge through the design of cancer vaccines. The rapidity with which these vaccine approaches can in some cases be translated into a clinical trial is a testimony to the years of fundamental medical research that has given rise to the current fields of bone marrow transplantation, adoptive immunotherapy, antibody therapy, and experimental vaccine development. As the science of cancer vaccines has advanced, so has a panoply of regulatory and institutional hurdles that seems to threaten the use of these therapeutic advances, especially in the traditional setting of an academic medical center. In this introductory chapter we will outline the field of cancer vaccines as a whole by highlighting the contributions that follow in subsequent chapters. Our hope is that this entire volume will serve as a reminder as to how far the science of cancer vaccines has come, and also as an inspiration to keep pushing forward with experimental vaccine trials because of the pressing

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need that remains for cancer patients and their families: an effective and durable therapy.

Background

From the dawn of modern oncology, two fundamental observations were made about interactions between the immune system and cancer: (1) in some cases, if the tumor mass or the draining lymph nodes were heavily infiltrated with lymphocytes, the patient did better [1,2], and (2) it was recognized that certain immunodeficiencies were associated with the development of cancer [3]. This lead to two related issues: how tumors arise in the face of an otherwise healthy immune system, and whether tumor formation is a relatively rare or common occurrence. In light of these issues a paradigm arose in the midtwentieth century that held sway for considerable time, namely, that of tumor immunosurveillance. Tumor immunosurveillance theories proposed that tumor formation, or at least the cellular mutations that lead to cancer, occur with a regular frequency and that the immune system is able to recognize and for the most part eliminate aberrant cells [4].

Current Perspectives

Today the immune system and cancer are not thought to interact in this way, and the shaping of both the tumor phenotype and the immune response to it is recognized as a mutual evolutionary process [5]. Also, our ideas about tumor formation have changed. Rather than viewing cancer as the expression of a single mutational event or oncogene, tumor formation is now clearly recognized as a stepwise process that may take considerable time to develop into clinical disease [6]. Nevertheless, studies seeking to demonstrate immune surveillance gave us the tools to study how the immune system recognizes and eliminates cellular substrates (i.e., tumors). The term *cancer vaccine* is used because we are emphasizing the goal of immunotherapy, namely, to recognize and eliminate malignant cells. For effective vaccination, appropriate protein/peptide targets must be identified and immune regulatory T cell responses that have evolved with the tumor must be countered. How we study and implement these two components is a central theme of this book.

1.2 ADJUVANTS: ENHANCING INTERACTIONS BETWEEN INNATE AND ADAPTIVE IMMUNITY

Adjuvants are compounds known to stimulate the immune system through deposition effects and the inclusion of compounds now known to stimulate the innate immune system [7]. While heat-killed mycobacterium suspended in mineral oil has been the standard adjuvant in animal studies, the severity of delayed-type hypersensitivity (DTH) reactions to this formulation has prohibited its use in the clinic [8]. The administration of another well-characterized bacterial strain is

perhaps the best-known bacterium-based adjuvant system in humans. The attenuated mycobacterium known as *bacillus Calmette–Guérin* (BCG) was originally developed as a tuberculosis vaccine. In some instances, where activation of the innate immune system in situ is sufficient, adjuvant administration alone may be therapeutic. The anticancer effects of BCG instillation, specifically in bladder carcinoma, are clearly documented in Chapter 3. Although BCG has clear and powerful effects, it must be given in repeated or maintenance doses demonstrating that while adjuvant-like effects alone may be able to control cancer in situ, expansion of more specific cancer stem cell targeting or a longlived immune memory response has not occurred. There is still more we can learn from BCG therapy as part of its effect may by attributable to the uptake of BCG by specific integrins on the tumor cell surface, leading to antigen-specific immune responses as well. Thus, what was once considered primarily an adjuvant-like effect may convert tumor cells that preferentially bind and internalize BCG into targets of the adaptive immune response.

In the study of adjuvants, many have used lipopolysaccharide-like structures of the bacterial cell wall to stimulate immunity. However, the immunostimulatory effects of complex saccharides may be far broader than simple adjuvant effects. In Chapter 2 Wilson and Danishefsky present a compelling new argument that appropriately constructed glycoconjugates have the ability to evoke strong antitumor antibody responses on their own. And as with other vaccine responses, a strong antitumor glycoprotein IgG response correlates with a strong antitumor T cell response. Unlike oligopeptide vaccines, glycoconjugates present a number of challenges in their synthesis. Nevertheless, because of their heterogeneity and structural complexity, we are only beginning to scratch the surface in terms of discovering appropriate immune targets. Immune responses to glycoconjugate vaccines such as Globo-H-KLH in adjuvant (a hexasaccharide found expressed on breast cancer, conjugated to keyhole limpet hemocyanin (KLH) and administered in adjuvant QS21) are harbingers of renewed interest in targeting tumor-associated oligosaccharide antigens. Currently, both multimolecular and multivalent antigen strategies are being developed and evaluated in clinical trials.

The most direct means to initiate an adjuvant effect is to stimulate the innate immune system with known toll-like receptor ligands. The family of toll-like receptors (TLRs) appears to play a pivotal role in the generation of different classes of innate immune responses by differential detection of conserved pathogen-expressed molecules. Bacterial CpG DNA and synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG are potent inducers of the innate immune system, including dendritic cells (DCs), macrophages, and natural killer (NK) and NK T cells. In Chapter 4 Speiser and Krieg examine methods of enhancing vaccine-induced immune responses by activation of selected TLR. In particular, the targeting of TLR9 has emerged as a powerful tool in the generation of Th1 adaptive immunity, and has shown promise for enhancing the efficacy of cancer vaccination. The studies performed to date show great promise for the clinical application of TLR9 activation with CpG ODN for enhancing the clinical outcomes from cancer vaccination. The safety of TLR9 activation with CpG ODN appears good, and its

selective and strong biological effects hold promise for further development and application in larger numbers of patients.

1.3 ANTIGEN-SPECIFIC THERAPY: NOVEL PRESENTATION OF PEPTIDE AND PROTEIN ANTIGENS

Many of the advances made in cancer vaccine therapy have featured novel means to present cancer-associated peptide or protein antigens to the immune system with a goal to both break immune tolerance and expand antigen-specific effector cells. The most direct means is to directly use a recombinant peptide, protein, or DNA plasmid vector as a vaccine. The use of polyepitope vaccines, synthesized and injected directly as peptides, or as expressed by DNA plasmid or adenoviral vectors, is highlighted in Chapter 5. Rather than focusing on a single cancer-associated epitope, polyepitope vaccines allow for either multiple-peptide antigen targets, or multiple epitopes from the same antigen that are presented by different MHC alleles, to be expressed in a single engineered peptide product. Polyepitope vaccines for melanoma and Her2/*neu*$^+$ breast cancer are currently in phase I trials. In these trials, plasmid-based DNA vectors are used to express the polyepitope. Intriguing studies are also under way with plasmid DNA vectors in subjects who are papilloma virus–seropositive, with the intent to induce protective immunity. The use of DNA vaccines is further explored in the context of cervical cancer in Chapter 6. Transfection of antigen-presenting cells with a DNA vaccine can be used to engineer the intracellular routing of the protein antigen of interest and thus enhance the vaccine effect. For example, inclusion of a lysosomal targeting motif can preferentially target the class II MHC pathway and lead to better CD4 T cell responses. Peptide motifs that can target antigens to either class I MHC or proteasomal processing compartments have also been described. In this model of cancer vaccination, vaccine constructs can include, in addition to the tumor-specific antigen, proteins that promote interaction between antigen-presenting cells (APCs) and responding T cells, or genes that prevent apoptosis of transfected APCs and thereby allow for a better vaccine effect. As the science of defining what an ideal APC actually is advances, so will our ability to engineer these responses with plasmid DNA vectors.

The advantage of peptide or DNA constructs is that they can be directly synthesized in the laboratory, and therefore they are simpler to use than biological vectors (i.e., modified pathogens) for presenting cancer-associated antigens to the immune system. In Chapter 7 Wansley explores the evolution of recombinant poxviruses as cancer vaccines, and discusses potential new directions for combination therapies. These recombinant viruses have been made to elicit tumor-specific immune responses by modifying the virus to express a variety of tumor-associated antigens, including carcinoembryonic antigen (CEA), prostate-specific antigen (PSA), and MUC1. In order to augment the immune response to these tumor-associated antigens, a vaccinia vector expressing three costimulatory molecules—B7-1, ICAM-1, and LFA-3 (TRICOM)—has been developed, and has exhibited some efficacy in the clinic. More recent studies have examined combining TRICOM vectors

with various cytokines [such as interleukin 2 (IL-2) and granulocyte–macrophage colony-stimulating factor (GM-CSF)], and with standard-of-care radiation therapy. Both of these approaches have shown promise in humans, and are being tested further. Since poxviruses have been shown to be safe in humans both alone and combined with other standard-of-care therapies, the use of these vectors in combination with other anticancer therapies is very promising.

Biologic vectors, including whole bacteria, have also been used as a vaccine platform, and we highlight this field by focusing on the work of Paterson and colleagues in Chapter 8 with *Listeria monocytogenes*. *Listeria* is an intracellular pathogen, and as such, when used as a bacterial vaccine vector, it is relatively free of the effects of neutralizing antibody. Up to four repeated doses have been given in experimental animals with minimal antivector responses. A bacterial vector can be regarded as the ultimate adjuvant, as a myriad of innate immune-stimulating structures are present. *Listeria* is of special interest because once it is internalized by a professional APC such as a dendritic cell, it escapes the phagolysosome, enters the cytosol, and is able to load both class I and class II major histocompatibility complex (MHC) with engineered antigen. The safety profile of administering a live (albeit attenuated) strain of bacteria in cancer patients, especially those who may have a congenital immunodeficiency, is a concern. This concern can be countered with either clearance of the bacterial vector with antibiotic chemotherapy or the use of psoralen and UV-treated or irradiated *Listeria*. As with other DNA vectors, DNA expression vectors transfected into *Listeria* allows for generation of fusion proteins that route the antigenic protein to the appropriate intracellular compartment or appropriate degradation machinery, which appears to greatly enhance the immune response to those sequences. The use of bacterial vaccines as vaccine platforms is still at an early stage with respect to clinical translation, and phase I studies are just getting under way.

In addition to viral and bacterial vectors, exciting new work described by Munson and colleagues (see Chapter 9) focuses on recombinant nonpathogenic brewer's yeast, *Saccharomyces cerevisiae*, as novel vectors for cancer immunotherapy. This vector platform triggers both innate and adaptive immune responses, delivers polypeptide antigens that are effectively processed into a full complement of appropriate-sized peptides competent for presentation by MHC class I and class II pathways, and elicits potent T cell immune responses against tumor cells expressing target antigens. This chapter discusses methods for engineering yeast to express tumor antigens and the unique properties of recombinant yeast in the activation of innate and adaptive immune responses. The broad applicability of the yeast-based immunotherapy to elicit protective T cell immune responses has been demonstrated in preclinical studies with numerous foreign, mutated, and overexpressed antigens, and several recombinant yeast-based vaccines are currently being evaluated clinically.

1.4 CELL-BASED CANCER VACCINES

Cancer immunotherapy can also be approached through the use of tumor cells as a platform to initiate a therapeutic immune response. Tumor cells by themselves are

typically not immunogenic. However, as the science of determining how an effective APC initiates an immune response advance, these strategies are being used to alter tumor cells and render them as loci of immune stimulation. The most general method for cell-based immunotherapy is to use a single representative cancer cell as a universal vaccine for all patients with that same type of cancer. Some investigators consider this approach as suboptimal as it is allogeneic, where the MHC type of the vaccine and the patient do not match, and the immune system may be distracted from generating a tumor–antigen-specific to an allospecific response. Others argue that an allogeneic vaccine will be effective for just this reason, and that the alloimmune response will serve to amplify the cancer–antigen-specific response. This issue will be addressed in Chapter 10, where Copier and Dalgleish discuss the use of whole tumor cells as vaccines in this unique approach to cancer therapy, as tumor-specific antigens do not have to be known a priori for the vaccine to be effective. These vaccines rely on the idea that there are multiple tumor antigens within the cells themselves against which the immune response can be activated. Highlighted are clinical trials where allogeneic cell lines were used as vaccines for prostate cancer and melanoma, and showed extended survival as compared to the control arms. The modification of whole-cell vaccines to elicit a more robust immune response has been addressed in several ways, by the addition of BCG to the vaccine, or the modification of the cells to express costimulatory molecules or secrete cytokines. While autologous whole-cell vaccines are now being tested in combination with chemotherapy, future work is expected to address the efficacy of combination therapy with allogeneic whole-cell vaccines.

Research on the use of an allogeneic vaccine in the context of existing chemeotherapeutic treatment is highlighted in Chapter 11. Chemotherapy for cancer can enhance or modulate immune-based approaches. In mouse models of breast cancer where a GM-CSF-producing cell-based vaccine is combined with chemotherapy in *neu* transgenic mice, low doses of cytoxan (cyclophosphamide, CY), and paclitaxel (PTX) were found to augment vaccine activity if given prior to vaccination, but not if given after. In contrast, low doses of doxorubicin (DOX) were found to augment vaccine activity if given after vaccination, but inhibited vaccine activity when given prior to immunization. These effects are attributable to a combination of inhibiting T-regulatory (Treg) activity, altering immunologic skewing toward a Th2 response and activation of CD8 cells. Combinations of cell-based vaccines with antibody (HER2/*neu*-specific) therapy also augment antitumor immunity, and antibody therapy alone is also enhanced by CY. A human allogeneic, GM-CSF-secreting, breast tumor vaccine is now being evaluated clinically. It is composed of two cell lines, SKBR3 and T47D, both of which have been genetically modified to secrete human GM-CSF by plasmid DNA transfection. These have been tested both in sequence with standard breast cancer therapeutics and more recently in combination with tumor-specific antibody.

The idea of using a cell-based vaccine to induce antitumor immunity brings a renewed focus to the type of effector cells vaccines are able to expand, and to the Treg cell system that coevolves with the tumor in the host [9]. In Chapter 12

the latest findings on manipulating the Treg cell networks are presented. Growing evidence has demonstrated that cancers utilize active mechanisms to block host antitumor immunity. Significant evidence implicates $CD4^+CD25^+$ Treg cells (Tregs) as important mediators of active immune evasion in cancer. Four strategies to inhibit Treg numbers or activity are highlighted: removal by depletion, interference of trafficking, inhibition of differentiation, or blocking of Treg function. The FDA-approved fusion protein denileukin diftitox (Ontak) has received attention recently as a potential agent to deplete functional Treg. As Ontak theoretically depletes any T cell bearing IL-2 receptors (including effector T cells), its utility may be limited in some settings. Ontak has been shown to reduce Treg numbers in the blood of some patients with cancer. In Chapter 12 Rüter discusses strategies to block Treg activity and presents preliminary data in this regard.

The mechanisms by which bone marrow transplantation induces antitumor immunity are numerous, and yet to be fully defined. They include direct cytotoxic effects and antigen release initiated by the preparative regimen, graft-versus-tumor effects, and the expansion of antigen-specific antitumor effector cells. High-dose chemotherapy or radiotherapy for solid tumors followed by hematopoietic stem cell transplantation (HSCT) reduces tumor burden, but many patients still relapse with disease following this intensive treatment as a result of incomplete elimination of tumor cells, inadequate graft-versus-tumor effects, and delayed immune reconstitution after HSCT. In Chapter 13 Jing and Johnson summarize recent work in their lab, and the work of other investigators, utilizing experimental mouse models of human autologous HSCT to determine the optimal parameters for inducing effective vaccine-induced antitumor immunity early after HSCT. Their work highlights the effectiveness of using a cell-based vaccine as a means to induce antineuroblastoma immunity. The animal data indicate that immune status early after HSCT is crucial to the success of early posttransplant vaccination, and that transfer of immunocompetent lymphocytes may be required if autologous HSCT is to be effectively used as a platform for tumor vaccination.

In Chapter 14 Gress and Sportes discusses the clinical use of HSCT (both autologous and allogeneic) as a platform for tumor vaccination. As shown by their research group, high-dose therapy followed by hematopoietic stem cell rescue can provide a time window for vaccination against residual tumor cells before the patient relapses with disease. Similar to the preclinical data, the clinical data also suggest that the ability to effectively administer tumor vaccines early after HSCT may be dependent on the adoptive transfer of "naive" or preactivated lymphocytes collected prior to transplant. Furthermore, in pediatric patients or young adults, thymus-mediated T cell reconstitution at later timepoints posttransplantation provides rationale for prolonged tumor vaccine administration to prevent late-disease relapses. The use of T-cell-depleting nonmyeloablative chemotherapy as an alternative vaccine platform to HSCT is also discussed.

Cell-based vaccines, administered directly or in the context of HSCT are designed to introduce a locus of immune activation that can generate antitumor effector cells that can then circulate throughout the body to sites of distant disease. Conventional treatment such as surgical removal of tumor followed by radiation

and chemotherapy may prevent effective immune recognition of cancers due to the loss of a major source of antigens, and damage to preexisting cytotoxic T lymphocytes (CTL) by radiation and chemotherapy. In Chapter 15 Drs. Yu and Fu discuss an alternative strategy, the use of the primary tumor as the site of CTL priming prior to surgical resection. It has been demonstrated that the creation of lymphoid-like structures within a tumor can lead to the rapid recruitment of naive lymphocytes and expansion of $CD8^+$ T cells. Strategies utilized for the generation of these tertiary lymphoid structures include the use of lymphotoxin α and β, and another member of the TNF family, LIGHT (see Section 15.5, for a definition of this acronym). LIGHT expression within the tumor environment recruits naive T cells and generates tumor-specific CTLs that can survive and exit the microenvironment to patrol peripheral tissues and eradicate disseminated metastases. These strategies could prove a potent strategy for enhancing antitumor immunity and permitting a clinically desirable outcome for cancer patients.

The identification of immune costimulatory molecules was a key advance in the engineering of cancer vaccines. With a view toward the future application of more recently described immune costimulatory molecules, in Chapter 16 Dr. Leiping Chen's group describes new molecules that may be included in cell-based vaccines or as targets of immunomodulation in their own right. The molecules that are currently being brought to phase I clinical trials for therapeutic applicability are members of both the PD-1/B7-H1/B7-DC pathway and the CD137/CD137L pathway. Exciting advances can still be made in learning how both positive and negative signals mediated by immune costimulatory molecules orchestrate antitumor immune responses.

1.5 DEFINING EFFECTIVE CLINICAL RESPONSES

The development and use of cancer vaccines has a long history in experimental medicine. However, to gauge the effectiveness of a cancer vaccine, standard ways to evaluate the immune response must be formulated. Chapter 17, from Dr. Kaufman's laboratory, introduces the various immune assays that are currently being used to monitor responses in clinical cancer vaccine trials. In Chapter 18, the final contribution in this volume, Dr. Whiteside discusses in detail the laboratory services necessary to support a cancer vaccine trial, the assays and limitations of immune monitoring for the detection of tumor-specific T cells after cancer vaccine administration, and the advantages of having a central laboratory operated as a good laboratory practice (GLP) facility to produce the vaccine and perform the immune monitoring assays.

In this volume we have attempted to present some of the best current research on cancer vaccines. We did not include sections devoted to adoptive immunotherapy or dendritic cell strategies for vaccination, as these could serve as volumes in their own right. We also did not concentrate on the identification and testing of new cancer antigens, as this field is also vast and exploding with new targets, due in part to the revolution in gene expression profiling and completion of the

human genome project. Our aim was to present examples of how, once identified, antigens can be translated into effective loci of immune stimulation. Cancer vaccine development is still evolving as it changes from a small-scale academic enterprise into an applied science requiring a large clinical infrastructure. Our hope is that we have stimulated further interest in all of these fields, and that the mechanisms for cancer vaccine development presented in this volume will continue to develop. We could think of no better goal than to offer patients a whole new palette of therapeutic options on the basis of our ability to induce tumor immunity.

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PART II

ADJUVANT THERAPY: ENHANCING THE ENDOGENOUS IMMUNE RESPONSE

2

FULLY SYNTHETIC CARBOYHYDRATE-BASED ANTITUMOR VACCINES

Rebecca M. Wilson and Samuel J. Danishefsky

2.1 INTRODUCTION

The development of a clinically effective means by which to incite the immune system to recognize and eliminate tumor cells is a longstanding ambition in cancer research. Despite the potential therapeutic implications of such an advance, the practical difficulties facing the field of tumor immunology are substantial. The most obvious challenge is the identification of a mechanism by which to induce the immune system to selectively recognize tumor cells as "other." In this regard, our laboratory (and others) have taken particular note of the finding that malignantly transformed cells often exhibit aberrant levels and types of cell surface carbohydrates, which are typically displayed as either glycoproteins or glycosphingolipids. It is thus conceivable that, through exposure of the immune system to appropriately presented tumor associated carbohydrate antigens, it may be possible to set into motion an immune cascade that would culminate in the elimination of tumor cells presenting the targeted carbohydrates [1].

Cancer Vaccines and Tumor Immunity
Edited by Rimas Orentas, James W. Hodge, and Bryon D. Johnson

Encouragingly, studies have revealed that, although exposure to tumor-associated oligosaccharides alone is not sufficient to evoke an immune response, when tumor-associated carbohydrate antigens are presented to the immune system as glycoconjugates, appended to the appropriate immunogenic carrier molecules (such as the KLH carrier protein [2]), antibody formation may be observed. These antibodies are sensitive primarily to the carbohydrate sectors of the glycoconjugates, rather than to the immunogenic carriers. Importantly, the antibodies thus raised are capable of recognizing and binding to tumor cells that overexpress the carbohydrates in question.

Clearly, at the conceptual level, the prospect of a carbohydrate-based antitumor vaccine construct appears to be quite attractive. It bears note, however, that a number of practical issues would need to be addressed if one were to undertake to develop a truly useful vaccine of this type. First, there is a great deal of heterogeneity of cell surface carbohydrate expression, even within a particular cancer type [3]. In fact, the level and type of cell surface carbohydrates may vary over the lifetime of a single tumor cell. For this reason, it would seem unlikely that a simple monovalent vaccine construct (i.e., one based on a single tumor-associated carbohydrate antigen) could, in practice, be sufficiently powerful to serve as a broadly useful therapeutic device. From this perspective, it is more probable that a clinically successful vaccine candidate would incorporate multiple tumor-associated carbohydrate antigens.

A second complicating factor lies in the nature of the tumor-associated antigens themselves, and in their ability to induce powerful immune responses. In fact, many tumor antigens are self-antigens or autoantigens, and for this reason it may be difficult to induce the immune system to generate antibodies against these particular antigens. A further challenge, broadly applicable to all carbohydrate-based vaccines, lies in the generally accepted belief that these types of antigens invoke a humoral response, which is T-cell-independent. Thus, introduction of the vaccine may yield a shortlived B-cell-mediated IgM response with poor memory and without the critical support that is typically provided by T cells. It should be noted, however, that several studies have presented evidence of cases in which carbohydrate-based antigens have in fact been able to invoke T cell responses [4].

It is clear, however, that despite the undeniable challenges of such an undertaking, the potential therapeutic benefits of developing a clinically useful carbohydrate-based anticancer vaccine would be tremendous. The potential to harness the considerable resources of the human immune system in the treatment and even prevention of cancer serves as a powerful impetus for this research program. Since the mid-1980s, our laboratory, through key collaborative efforts, has been striving to address some of the obstacles outlined above through the rigorous design, synthesis, and evaluation of increasingly potent anticancer vaccine constructs [5].

2.2 CARBOHYDRATE SYNTHESIS

Although it is possible to obtain carbohydrates through isolation from natural sources, it is exceedingly difficult to isolate adequate quantities of structurally

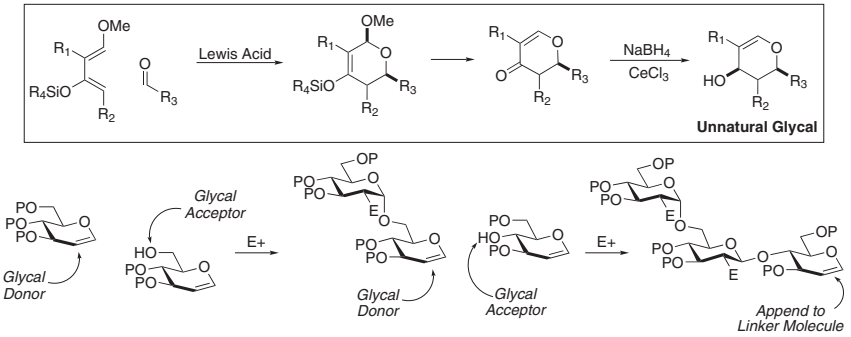
homogeneous material, as carbohydrates often exist in living systems as difficult-to-separate heterogeneic mixtures. For the purposes of rigorous and controlled evaluation of the immunologic properties of tumor-associated carbohydrate antigens, it would clearly be advantageous to have access to single, homogeneous glycoforms. Our laboratory has long been devoted to the development of novel and improved methods for the de novo synthesis of structurally homogeneous carbohydrates and glycopeptides.

The synthesis of oligosaccharides presents a unique set of challenges. Unlike the two other major forms of biological oligomers (oligopeptides and oligonucleotides), the assembly of oligosaccharides requires the generation of new stereogenic centers during the oligomerization step. In order to achieve reasonable levels of efficiency in the preparation of complex oligosaccharides, it is critical that these new stereocenters be created with very high levels of selectivity. Furthermore, protecting group issues are significantly more complex in the case of oligosaccharide assembly.

Our group's interest in oligosaccharide synthesis first arose in the context of the development of a novel cycloaddition reaction leading to the selective formation of dihydropyrones. In the context of this early disclosure, we began to consider whether the types of functionality obtained through this type of transformation could be applied to the larger challenge of carbohydrate synthesis. We took note of the fact that the glycal motif, accessible through our novel cycloaddition methodology, might serve as a convenient building block in the synthesis of oligosaccharides. Of particular interest was the fact that glycals, which contain only three hydroxyl groups instead of five, possess an olefinic functional component that may serve as a potential site of differentiation in the oligomerization process. Although we were not the first to recognize that glycals could serve as efficient *donors* (e.g., through the process of iodoglycosidation), our major contribution was in recognizing that these building blocks could potentially serve as valuable glycosyl *acceptors*. Thus, we postulated that, through an appropriately conceived series of protecting group manipulations, glycals might be used exclusively to iteratively fashion complex oligosaccharide domains. These synthetic oligosaccharides would, of course, terminate in glycal-type olefinic functionality, and the application of this strategy would thus require the development of efficient methods for their derivatization and appendage to appropriate linking motifs.

In the end, we were able to demonstrate the broad utility of this glycal assembly method for the synthesis of complex oligosaccharide domains. Additionally, a number of methods have been developed for achieving the requisite derivatization of the terminating glycal and its stereoselective appendage to linking functionalities. These synthetic concepts, outlined briefly in Scheme 2.1, have been reviewed extensively in other settings [6].

In short, since the mid-1980s, we have continued to develop techniques that allow us to efficiently and selectively synthesize increasingly complex carbohydrate sectors. These major advances in the synthetic chemistry of oligosaccharides, taken as a whole, have provided us with "freedom to operate" in designing and implementing the syntheses of a number of very complex carbohydrate-based vaccine



Scheme 2.1 Glycal assembly strategy toward carbohydrate synthesis.

constructs, some of which could conceivably serve as agents of real therapeutic value. In the following sections, we provide an overview of the evolution of our fully synthetic carbohydrate vaccines program, as well as a brief prospectus on future directions in this field.

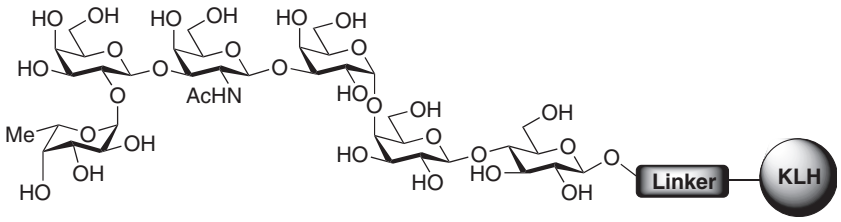
2.3 SYNTHETIC CARBOHYDRATE-BASED VACCINES

Unimolecular Monovalent Vaccines

Our first vaccine constructs were monovalent in nature, composed of a single antigen conjugated to an immunogenic carrier molecule, such as KLH. In order to achieve appreciable levels of immunogenicity in mouse or human settings, these vaccine conjugates must be coadministered with an immunoadjuvant, in our case, QS21 [7]. One of the original constructs to be synthesized in our laboratory was the Globo-H-KLH conjugate [8]. Globo-H, a hexasaccharide, was first isolated from the human breast cancer cell line (MCF-7) and later found to be overexpressed in a variety of other cancer cell lines, including colon, lung, ovarian, and prostate [9]. At the time, the synthesis of the Globo-H antigen was a significant undertaking, and provided an important platform for the evaluation of our glycal assembly protocol, outlined above (see Scheme 2.1).

The Globo-H-KLH vaccine performed well in immunologic investigations [10]. Preliminary studies conducted on mice revealed this vaccine construct to be capable of producing strong IgM and weaker IgG responses, as determined by ELISA screening. Importantly, the antibodies thus obtained were found to be specific to Globo-H, reacting only with Globo-H-positive (MCF-7) cell lines, as determined by flow cytometry analysis, and not with Globo-H-negative (B78.2 melanoma) cells. Furthermore, the antibodies raised were very effective at inducing complement-mediated cytotoxicity of MCF-7 cells.

On the basis of these encouraging preclinical results, the Globo-H-KLH conjugate was advanced to phase I clinical trials against both prostate and breast cancer.



Scheme 2.2 Globo-H-KLH.

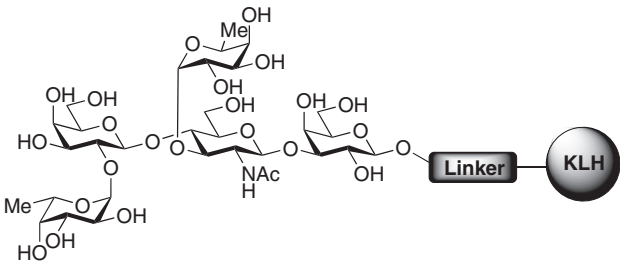
It should be briefly noted that, although the exclusive purview of a phase I clinical evaluation is the establishment of the safety profile and lowest optimally immunogenic dosage of a drug candidate, when conducting clinical trials with a vaccine candidate, an evaluation of the patient antibody titers following inoculation can provide some useful insight into the ability of the construct to induce specific antibody formation in human settings.

In this context, it was encouraging to find that, in the clinical trial directed toward prostate cancer, Globo-H-KLH demonstrated immunogenicity across a range of tumor burden levels, and its ability to induce antibody formation was apparently independent of the patient's cancer stage. Importantly, each patient evaluated produced IgM antibodies that were specific to Globo-H and reactive with Globo-H-positive cells. Furthermore, the titers obtained were capable of inducing complement-mediated lysis in cell lines overexpressing Globo-H [11].

One important biomarker of prostate cancer progression—prostate-specific antigen (PSA)—was monitored prior to, throughout the course of, and following vaccine treatment. In two of five patients evaluated for this marker, it was found that the slope of the log (PSA) concentration, measured as a function of time, decreased following the course of treatment with the Globo-H-KLH vaccine. We hasten to add that, because of the small number of patients and the checkered nature of the cohort population, these data certainly do not presage a pattern of expected behavior in a more managed clinical setting.

A breast cancer trial with the Globo-H-KLH construct yielded similarly encouraging results. In this trial, 16 of 27 patients exhibited significant (at least threefold) increase in IgM reactivity, as determined through fluorescence-activated cell sorter (FACS) analysis. Importantly, despite the fact that Globo-H is endogenously expressed on the surfaces of normal pancreatic cells, there did not appear to be any clinically significant toxic effects associated with exposure to Globo-H in either trial. Rather, any reaction to the treatment appeared to be due to the use of the immunoadjuvant [12].

As stated above, one must be particularly cautious in drawing conclusions from these preliminary findings obtained from a small pool of patients. Nonetheless, the results generated during the course of the phase I clinical trials for breast and prostate cancers certainly give encouragement for continuing and upgrading the program. Phase II/III clinical trials for breast cancer are planned to commence shortly.



Scheme 2.3 Lewis^y-KLH.

Lewis^y (Scheme 2.3) is a pentasaccharide that is overexpressed in a number of tumor cell lines, including ovarian, prostate, and breast cancers. One possible impediment to the employment of Lewis^y as an effective antigen is the fact that it is endogenously expressed in normal epithelial cells. We thus anticipated some potential difficulties in invoking a potent immune response to this particular antigen.

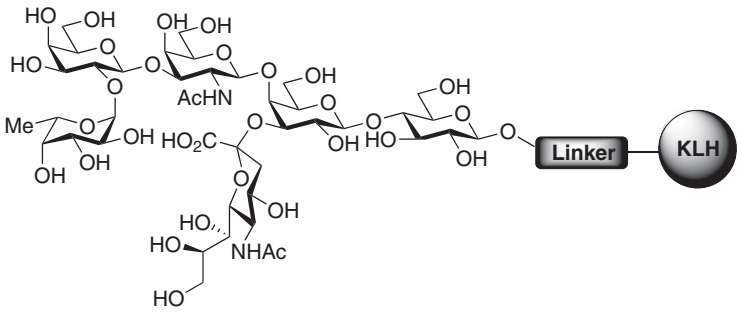
Nonetheless, on the basis of promising preclinical findings, Lewis^y-KLH was advanced to a phase I clinical trial against ovarian cancer [13]. The vaccine construct was universally well tolerated in patients, and antibody responses were observed in the majority of patients (16 of 24), with 8 individuals exhibiting high-titer IgM responses. As would be found with most carbohydrate vaccine constructs evaluated through this program, the induction of IgG antibodies was much less common. In the case of Lewis^y, only four patients exhibited clear IgG responses. Eight of the sera obtained following vaccination were clearly reactive with Lewis^y-positive OVCAR-3 (ovarian cancer) cells, as determined by FACS analysis. There was also a strong correlation between the FACS reactivity and the ability of the sera to effect complement-dependent lysis of OVCAR-3 cells.

In summary, although the Lewis^y antigen most likely lacks the potency required to be effective in a monovalent setting, it was envisioned that Lewis^y could serve as a valuable component of a potential multiantigenic vaccine.

More recently, we synthesized the fucosyl-GM1 antigen (Scheme 2.4), which is typically overexpressed on the surfaces of small cell lung cancer (SCLC) cells but which is not found on normal cell surfaces. The fucosyl-GM1-KLH conjugate was evaluated in a phase I setting against SCLC and found to be capable of inducing IgM antibodies at levels as low as 10–30 μg in the majority of patients inoculated. In six of eight patients expressing antibody, the IgM titers obtained were capable of eliminating fucosyl-GM1-expressing SCLC cells through complement-mediated lysis [14].

Clustered Vaccines

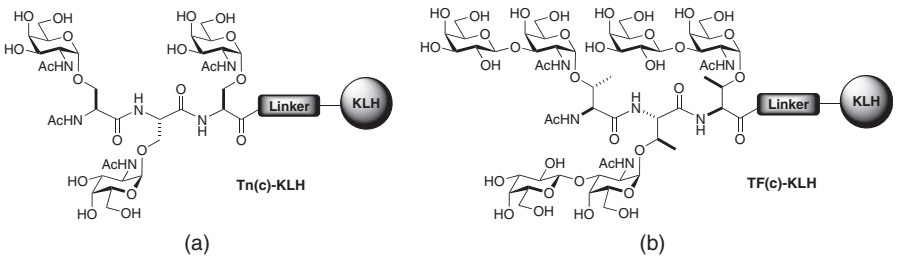
As our laboratory achieved a growing level of sophistication, in terms of both our synthetic capabilities and our understanding of the nature of tumor associated cell surface carbohydrate expression, we developed the ability to advance our program



Scheme 2.4 Fucosyl-GM1-KLH.

beyond the realm of simple monovalent antigen–KLH constructs, and to begin to evaluate increasingly complex vaccine candidates. In this context, we took note of the fact that, in the mucin family of glycoproteins, cell surface carbohydrate epitopes are often expressed on adjacent serine or threonine residues in clusters of two to five [15]. These clusters appear to be the preferred targets of monoclonal antibodies, and as such, we came to consider the possibility of achieving enhanced levels of immunogenicity by clustering our own synthetic carbohydrate antigens on a single-peptide backbone. This clustered glycopeptide would then be conjugated to KLH carrier protein in much the same way as the monovalent constructs. Through some important synthetic insights and advancements, the challenge of efficiently preparing carbohydrate clusters was solved at the level of chemical synthesis, and we were able to gain access to a number of trimeric clustered (c) vaccines, including Tn(c) [16], TF(c) [16a,17], STn(c) [18], 2,6-STF(c) [19], and Lewis^y(c) [20]. A number of these clustered constructs are being evaluated in preclinical and clinical settings.

At this stage, both the Tn(c)-KLH and TF(c)-KLH clusters (see Scheme 2.5) have separately been advanced to phase I clinical trials against prostate cancer [16c,17]. The results of each trial have provided some cause for optimism. First, high titers of IgM *and* IgG antibodies were observed in the majority of patients in both trials. Furthermore, antibodies were still detectable for a number of weeks



Scheme 2.5 (a) Tn(c)-KLH and (b) TF(c)-KLH clusters.

following the course of treatment, although titer levels eventually did decrease over time. Interestingly, in both trials, the sera obtained were largely unable to effect complement-mediated cell lysis against cells overexpressing the carbohydrates in question. However, in both trials, a decrease in the slope of the log(PSA) as a function of time was observed in some patients following the course of treatment. These results, while far from being conclusive, are supportive of the need to go forward with the program.

Polyvalent Vaccines

The insights garnered from the clinical trials described above, as well as from a number of informative preclinical investigations, led us to consider a more encompassing vaccination approach, through which we hoped to address issues of heterogeneity of tumor cell surface expression. We thus envisioned a polyvalent vaccination strategy, involving simultaneous exposure of the immune system to several different tumor-associated carbohydrate antigens. In theory, this approach should allow for the induction of a more potent and broad-based immunogenic response, culminating in the elimination of a greater proportion of cancer cells than would otherwise be possible through the first-generation monovalent approach.

The first incarnation of this polyvalent strategy involved inoculation of mice with a mixture of individual tumor-associated antigens, each separately conjugated to KLH carrier protein. Antibody titers were then evaluated for their reactivity against each component antigen of the mixture. While this polyvalent approach is surely the most straightforward, and required the least amount of adaptation from a synthetic perspective, it suffers from some potentially serious drawbacks from a translational perspective. Perhaps most significant among these is the fact that this approach requires the use of substantially increased levels of KLH carrier protein, which can over time lead to an actual *decrease* in the level of immunogenicity of the carbohydrate antigen itself. Furthermore, from a practical perspective, the preparation of each monovalent-KLH conjugate requires a low-yielding conjugation reaction as the final step of the synthetic sequence. Finally, the issues of gaining regulatory approval for the various components of the vaccine mixture should not be overlooked.

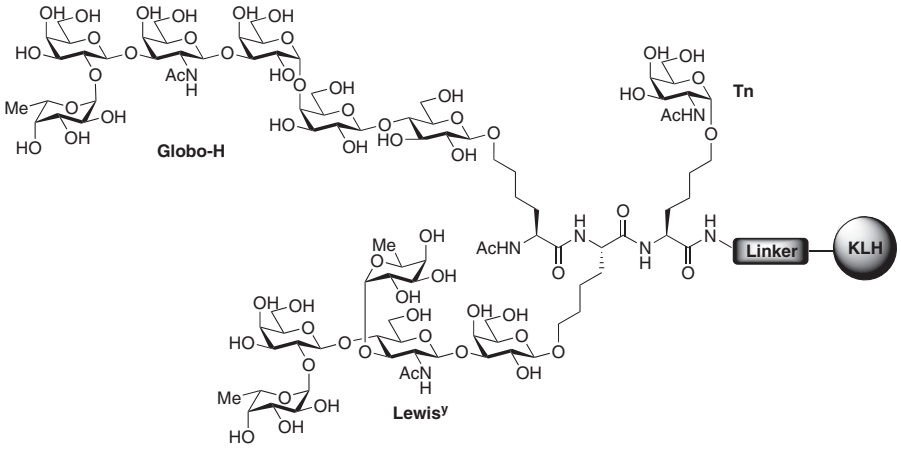
With these limitations in mind, we nonetheless sought to explore the viability of the general strategy of polyvalency of antigens. In one key study, four antigens—GD3, Lewis^y, and the mucin polypeptide core molecules, MUC1 and MUC2—were individually conjugated to KLH carrier protein and coadministered to mice (with QS21 adjuvant). Each individual antigen was also separately administered to control groups of mice. A comparative evaluation between the polyvalent and control groups revealed that equivalent levels of antigen-specific antibodies were produced in the mice injected with the polyvalent vaccine compared with those injected with the individual antigen [21]. A related study in which seven different antigenic constructs were coadministered to mice revealed similar levels of antibody induction against each antigen [22].

These important findings lent experimental support to the notion that an appropriately designed polyvalent vaccine may serve to effectively address some of the challenges posed by the inherent heterogeneity of cell surface carbohydrate expression observed in tumor cells.

Unimolecular Multivalent Vaccines

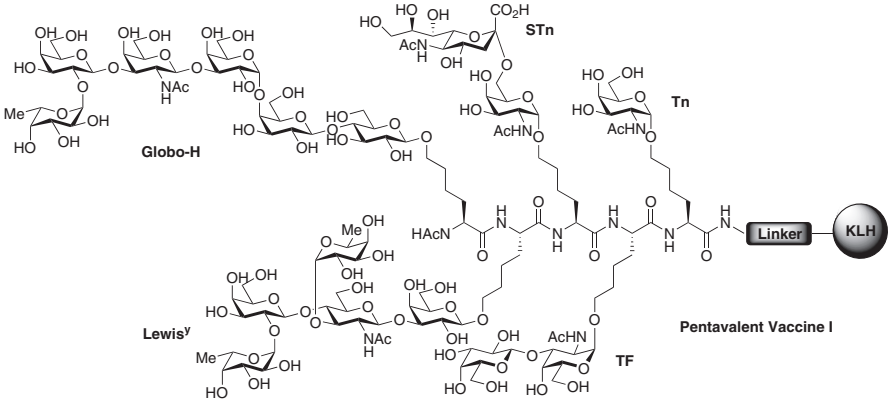
In light of the encouraging results described above, it would be necessary to address some of the issues surrounding the KLH carrier protein levels as well as the synthetic difficulties inherent to the conjugation of the antigen to the carrier protein if we were to truly develop a practical and broadly applicable vaccine of therapeutic value. In this context, the most logical option would be to display each of the component antigens in the framework of a single construct. This multivalent construct would then be appended to the carrier protein in a single conjugation step. In this way, we hoped to overcome each of the perceived obstacles outlined above while effectively addressing issues of heterogeneity of tumor cell carbohydrate expression. It should be noted that, in practice, it was unclear as to whether the individual antigens would retain equivalent levels of immunogenicity in the context of a unimolecular construct such as we had envisioned.

In a proof-of-principle evaluation, we assembled a unimolecular trivalent vaccine construct (Scheme 2.6), incorporating the Tn, Lewis^y, and Globo-H antigens, displayed on a peptide backbone composed of hydroxynorleucine amino acids, and conjugated to KLH. Importantly, mice inoculated with this construct were found to express antibodies against the component antigens, as determined by enzyme-linked immunosorbent assay (ELISA) assay. Furthermore, the antibodies thus obtained were found to react with tumor cells known to express these antigens [23].

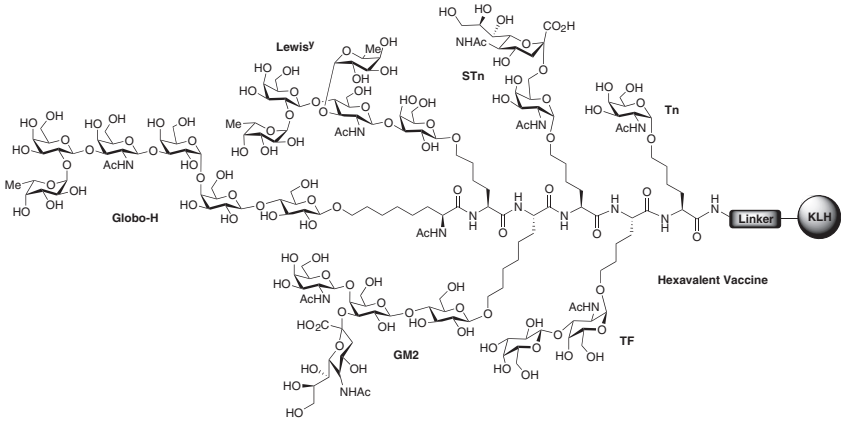


Scheme 2.6 Trivalent vaccine.

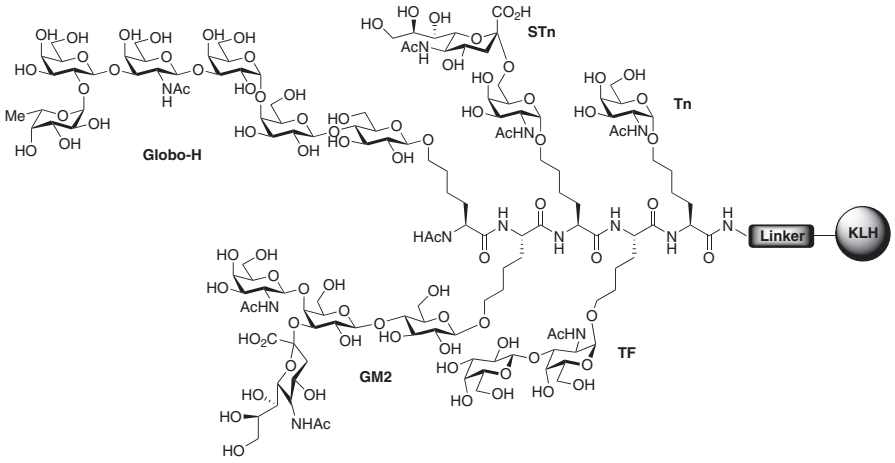
On the basis of encouraging preliminary findings, we prepared unimolecular pentavalent (Scheme 2.7) and hexavalent (Scheme 2.8) constructs containing carbohydrate antigens specifically associated with breast and prostate cancers [24]. The pentavalent vaccine incorporated the breast and prostate tumor-associated antigens, Tn, TF, STn, Globo-H, and Lewis^y, each of which had been previously prepared and evaluated individually in monovalent or clustered settings. More recently, we accomplished a synthesis of GM2 [25], which is overexpressed on both breast and prostate tumor cell surfaces [26]. This antigen was incorporated into our hexavalent vaccine construct. Preliminary immunologic evaluation of these two multiantigenic vaccines in mice provided encouraging results. For the most part, the individual antigens retained their immunogenicity when displayed in the context of the unimolecular system. A notable exception was the Lewis^y antigen. Lewis^y,



Scheme 2.7 Pentavalent vaccine I.



Scheme 2.8 Hexavalent vaccine.



Scheme 2.9 Pentavalent vaccine II.

only moderately immunogenic in monovalent settings because of its high level of endogenous expression, appeared to be incapable of inducing antibody formation when presented on the polypeptide backbone of the unimolecular construct. By contrast, the GM2 antigen was capable of inducing high levels of antibody formation when incorporated into the hexavalent construct.

On the basis of these evaluations, we reworked our synthetic route (which had been designed with a view toward maximal modularity, in order to allow for such modifications), and prepared a unimolecular pentavalent construct incorporating the Tn, TF, STn, Globo-H, and GM2 antigens (see Scheme 2.9). We have recently synthesized ample quantities of this construct for further immunologic investigations as well as phase I clinical trials against breast cancer, which are planned to begin shortly.

2.4 FUTURE DIRECTIONS

The next step in the progression involves bringing these unimolecular multivalent constructs, properly conjugated to carrier protein, into phase I clinical settings. Hopefully the encouraging tolerability and antibody inducement patterns found in the monovalent trials will be sustained in the unimolecular polyvalent setting. The impact of unimolecular polyvalency with respect to various significant tumor markers will be assessed.

Future directions will certainly include a study of the consequences of permuting the antigens along the neopolypeptide backbone. Our previously synthesized affinity columns should prove to be particularly helpful in monitoring progress. Another direction that falls within the scope of our chemistry involves clustering of one or more of the antigens along a lengthened polypeptide framework. Another goal,

which is in the class of "chemically doable," is incorporation into the peptidelike motif, sequences of established tumor associated peptide antigens. Obviously a long-term goal of this program is to mobilize and harmonize all activatable elements of the immune system to the challenge at hand.

In summary, it is well to recall that this program started from initial observations concerning somewhat esoteric issues in organic chemistry. Through close cooperation between the disciplines of chemistry and immunology, the possibility of creating a clinically useful vaccine particularly directed against micrometastasis in adjuvant modes is at hand. At the very least, the progress already achieved speaks to the importance of pursuing fundamental research within disciplines in a context where interdisciplinary cross-fertilization facilitates creative communication between complex specializations.

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3

BACILLUS CALMETTE–GUÉRIN IMMUNOTHERAPY OF GENITOURINARY CANCER

Donald L. Lamm

3.1 INTRODUCTION

Bacillus Calmette–Guérin, first introduced in 1921 as a vaccine against the scourge of tuberculosis, has been steeped in controversy since 1930. Touted as a promising new cancer treatment in the 1970s, it was subsequently discarded as ineffective when controlled trials failed to confirm its benefit. Taking advantage of animal and immunologic studies that defined optimal conditions for BCG response, urologists have developed BCG into the most effective treatment currently available in the treatment of aggressive superficial bladder cancer. This chapter summarizes the history and development of BCG as a modern cancer treatment, reviews current optimal application of BCG immunotherapy in bladder cancer, discusses promising new therapies and approaches closely related to BCG, and briefly explores the possibility that BCG or related treatments may have an application in other malignancies.

Urologists are very familiar with BCG, but many in other fields are unaware of the advantages it provides in the treatment of bladder cancer. Indeed, BCG remains

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largely ignored while immunotherapies using new molecular or immunologic techniques that have response rates far lower than those of BCG are enthusiastically endorsed. The story of the development of BCG as a vaccine against tuberculosis and its subsequent transformation into a cancer treatment provide important lessons in perseverance, observation, and application of the scientific method.

3.2 TUBERCULOSIS

It is estimated that the majority of adults in the eighteenth and nineteenth centuries were infected with tuberculosis and the mortality rate was as high as 25%. As evidenced by the BCG response gene, the prevalence and mortality of tuberculosis make in highly probable that the organism has played a significant role in natural selection [1]. That BCG is a most potent immune stimulant would be expected. Following Kock's demonstration in 1884 that mycobacterium tuberculosis caused tuberculosis, scientists of the day raced to develop an effective vaccine. Nocard isolated mycobacterium bovis in 1904, and Albert Calmette and Camille Guérin, working in the Pasteur Institute in Lille, tamed the bacterium after 231 serial passages over 13 years to produce BCG. After confirmation of avirulence, the vaccine was first given in July 1921 as an oral vaccine to a newborn girl whose mother died with tuberculosis at birth. She was cared for by her grandmother, who had active tuberculosis but remained healthy, and after no side effects were seen, the procedure was started on a routine basis at the Charite Hospital in Paris [2]. Vaccination proceeded uneventfully until 1930, when BCG was implicated in the deaths of 70 infants in Lubeck, Germany. The true cause was later found to be contamination of the vaccine with mycobacterium tuberculosis grown in the same laboratory, but Calmette, who died in 1933, was not to recover from the incident [2]. Fear of BCG continued, and it took several decades to rekindle interest in the vaccine as a possible antineoplastic agent.

3.3 BCG AND CANCER: GENERAL ASPECTS

Coley in the 1890s had successfully used bacterial toxins to treat malignancy, and the concept that immune stimulation from infectious disease reduces the risk of cancer has been confirmed as recently as 1998 [3]. The concept that BCG might have a role in cancer dates to 1929, when Pearl correlated autopsy findings of tuberculosis with a reduced incidence of cancer. BCG was first used to treat cancer by Holmgren in 1935 when successes were reported in a series of 28 patients. In 1936 Rosenthal described the profound stimulation that BCG produces on the reticuloendothelial system, which was recognized at the time to be important in the defense against cancer. These works led Old and Clark in the United States and Halpern in France to animal studies that confirmed that BCG inhibits cancer [4]. In 1972 Rosenthal found that neonates immunized with BCG had a significantly

lower incidence of leukemia, and multiple uncontrolled clinical studies in the 1970s claimed that BCG was effective in the treatment of cancer. In melanoma Morton reported a 92% response with intralesional injection of BCG. Unfortunately, controlled medical oncology trials in the 1970s failed to show significant benefit of BCG therapy in cancer. Most of these studies, however, were in patients with advanced disease who would not be expected to respond to BCG.

3.4 BCG IN BLADDER CANCER

It was with this background that studies of BCG in bladder cancer began. Studies by Coe and Feldman in 1966 [5] demonstrated that the bladder responded to BCG with a delayed-type hypersensitivity reaction such as that observed in the skin, and in 1974 Silverstein reported response of melanoma metastatic to the bladder treated with intralesional BCG [6]. The success in melanoma led us in 1973, at the suggestion of David McCullough, to evaluate BCG in an animal model of bladder cancer [7]. At the same time Morales independently began clinical studies, and published the results of the first successful clinical trial of BCG in nine patients with recurrent bladder cancer in 1976 [8]. In that study six weekly intravesical plus percutaneous administrations of 120 mg of Armand Frappier BCG resulted in a 12-fold reduction in bladder tumor recurrence. This success prompted the NCI to request proposals for controlled clinical trial of Morales' technique, and contracts were given to Lamm at the University of Texas in San Antonio and Pinsky at Memorial Sloan Kettering (MSK) in New York.

The initial controlled BCG trial, published in 1980 [9], showed statistically significant reduction in tumor recurrence with just 54 evaluable patients, an advantage that increased with time [10]. Similar results were reported in much higher-risk patients in the MSK study [11]. Subsequent follow-up of patients in these studies suggested that the benefit of a single 6-week course of intravesical plus percutaneous BCG provided long-term protection from tumor recurrence and even reduced disease progression. The 15-year follow-up of the MSK series, however, illustrated the limitations of BCG without maintenance. While induction BCG reduced recurrence, progression, and mortality at 10 years, by 15 years this advantage was lost, and patients remained at high risk for progression and the development of disease in the prostatic urethra (24%) and upper tracts (25%). Disease in the upper tracts was associated with a mortality rate of 32%, and 44% of patients with prostatic urethral disease died [12].

BCG Immunotherapy Technique in Bladder Cancer

Intradermal Administration

The initial studies of BCG in bladder cancer called for both the intradermal (ID) and intravesical administration of the vaccine. The ID route was later discontinued on

the basis of a study suggesting that the ID administration did not add to the efficacy of the intravesical route [13] but probably served more for patient's and physician's convenience. This deviation of the original protocol may, in fact, have diminished the efficacy of the treatment, as documented by Lamm [14]. Unfortunately, the difficulties in obtaining a Heaf gun for ID administration, the flare-up responses, and the mild dermal reactions have practically eliminated the ID administration. Two independent controlled studies failed to show that intradermal BCG significantly improved the response to BCG, but in both studies groups receiving intradermal in addition to intravesical BCG had lower tumor recurrence [15,16]. Percutaneous BCG induces a positive purified protein derivative (of tuberculin) (PPD) skin test in 90% of patients compared with 60% skin test conversion with intravesical BCG. PPD skin test conversion has been associated with improved antitumor response to BCG. In the SWOG 8507 maintenance BCG study, complete response in patients with carcinoma in situ (CIS) increased from 49% to 77% in patients with a positive PPD (unpublished) and in Badalament's maintenance study PPD skin test conversion was associated with a significant reduction in tumor recurrence [17]. Further study of the role of percutaneous BCG could be productive, especially in patients who fail to respond to treatment.

BCG Compared with Chemotherapy

The successful demonstration that BCG reduced tumor recurrence compared with surgery alone was followed by many controlled comparisons of BCG versus intravesical chemotherapy. As illustrated in Table 3.1, BCG was uniformly found to be superior to chemotherapy with thiotepa, doxorubicin, and epirubicin [18]. Comparisons with mitomycin C, however, showed an inconsistent advantage of BCG. This inconsistent advantage of BCG versus mitomycin C is reflected in the American Urological Association (AUA) management guidelines published in 1999, where BCG or mitomycin C were listed as appropriate for the management of high-grade non-muscle-invasive transitional cell carcinoma. Subsequent work, however, has clearly demonstrated that BCG is superior to mitomycin C in these patients. In the meta-analysis by Bohle [19], six of six comparison studies that used maintenance

TABLE 3.1. Overview of Controlled Trials of BCG versus Chemotherapy for Superficial Bladder Cancer

Treatment	Median Rate of Recurrence, BCG Arm, %	Median Rate of Recurrence, Chemotherapy Arm, %	Number of Positive Trials
BCG versus thiotepa	7	42	3/3
BCG versus doxorubicin	38	64	2/2
BCG versus mitomycin	30	43	3/6

Source: Summarized from Lamm and Torti [18].

BCG schedules found BCG to be superior to mitomycin C, versus only one of five studies that failed to use maintenance BCG. In a subsequent meta-analysis by the Cochrane group, BCG was found to be markedly superior to mitomycin C in patients with high-risk patients, but not in low-risk patients [20].

Carcinoma In Situ

In 1980 Morales reported a complete response rate of 71% in carcinoma in situ of the bladder using 6-week induction BCG [21], and BCG (Theracys) was initially approved by the FDA for bladder cancer in 1990 because of its demonstrated superiority (71% complete response, 48% remaining disease-free for 5 years) over doxorubicin [47% complete response (CR), 18% 5-year no evidence of disease (NED)] in the treatment of carcinoma in situ [22]. The consistency of the efficacy of BCG in CIS is noteworthy, as illustrated in Table 3.2. Complete responses were seen with every strain of BCG despite variations in dose and schedule, and the 70% response rate is remarkably consistent. One controlled study provides important clinical information in the management of CIS. The Southwest Oncology study of 3-week maintenance BCG versus standard 6-week induction without maintenance included 269 randomized patients with CIS. Complete response increased from 58% at 3 months to 68% at 6 months in the induction-only arm without any additional BCG administration, suggesting that while residual CIS is a poor prognostic feature, it may take 6 months for the complete response to occur. With three additional weekly BCG instillations at 3 months, 64% of patients who had residual CIS at 3 months had CR at 6 months, yielding an overall complete response rate of 84% [23]. With continued 3-week maintenance BCG, over 70% of patients with complete

TABLE 3.2. Comparison of Bacillus Calmette–Guérin (BCG) Strains in Treatment of Bladder Carcinoma In Situ

Strain/Series	Total Number of Patients	Complete Responses	Complete Response Rate, %	Range of Response Rates, %
Connaught/8	450	357	79	70–92
Tokyo/4	111	86	77	63–84
Pasteur/7	230	171	74	40–80
Tice/6	277	197	71	56–82
Evans/6	180	117	65	53–88
A. Frappier/6	145	87	60	39–100
South African/1	13	9	69	—
Danish/1	42	28	67	—
Romanian/1	33	21	64	—
RIVM/1	15	9	60	—
Total	1496	1082	72	39–100

response remained free of tumor recurrence for 5 or more years, a remarkable achievement considering that in historical series 54% of patients with CIS were previously destined to develop muscle invasion within that timeframe [24].

Maintenance BCG, Disease Progression, and Mortality

It is generally accepted that BCG is highly effective in reducing disease recurrence, but controversy has persisted regarding the role of maintenance BCG and the ability of BCG to reduce disease progression and mortality. Lamm initially reported a fourfold reduction in the rate of tumor recurrence in patients treated with quarterly single BCG instillations [25], but in 1987 controlled evaluation of this regimen in 42 patients failed to demonstrate significant reduction in tumor recurrence [26]. In the same year Badalament reported a randomized study of 93 patients comparing 6-week induction with monthly mainenance BCG [27]. Again, no significant benefit of maintenance was found. These studies could be criticized for their relatively short follow-up and lack of power, but they had a major impact on clinical practice. Most urologists have used 6-week induction followed by a repeat 6-week course at the time of tumor recurrence. Palou [28] specifically evaluated repeated 6-week maintenance BCG in 126 patients followed for an average of 79 months. A regimen of 6-week maintenance BCG every 6 months for 2 years was not found to be significantly better than induction alone.

With all of these "negative" studies, it would seem ironic that maintenance BCG may be the most important advance in BCG treatment of bladder cancer since the introduction of Morales' 6-week induction regimen. Maintenance immunotherapy without administration of enough BCG to cause immunosuppression is vital. The risk of tumor recurrence and disease progression is lifelong in most patients, and the immune stimulation induced by BCG wanes with time. Our adequately powered controlled trial using an immunologically sound maintenance schedule clearly demonstrated that 3-week maintenance BCG markedly reduces long-term recurrence and disease worsening [23]. While other, presumably inferior maintenance schedules have not achieved statistically significant benefit in randomized controlled trials, a meta-analyses demonstrates that maintenance BCG is superior to induction BCG and may be required for the most important benefit of BCG, reduction of tumor progression. The 6-week induction induces a prolonged protection from tumor recurrence, which may explain the failure of maintenance studies with short follow-up. In animal studies, for example, no benefit of BCG maintenance was seen at 9 months, but highly significant benefit was documented at 15 months. In Herr's series significant reduction in tumor recurrence and disease progression and mortality persisted for 10 years, but was no longer significant at 15 years [12]. The SWOG maintenance study of 384 evaluable patients using a series of up to 3 weekly instillations at 3, 6, 12, 18, 24, 30, and 36 months found a highly significant ($p < .00001$) reduction in tumor recurrence as well as a significant reduction ($p < .04$) in disease worsening (stage progression or the requirement of cystectomy, radiation therapy, or systemic chemotherapy). Bohle's meta-analysis found that six of six studies comparing maintenance BCG with mitomycin C showed BCG to be

significantly superior, compared with only one of five studies that failed to employ maintenance BCG. The most convincing data is in the meta-analysis published by Sylvester [29]. In that analysis of 24 studies consisting of 4863 patients BCG was compared with surgery, chemotherapy, or other immunotherapy and was found to significantly reduce disease progression by 27%. This benefit, however, was limited to studies that used maintenance BCG. In maintenance BCG studies progression was reduced by 37% ($p = .00004$). Bladder cancer mortality was reduced by 19%, but did not reach statistical significance as might be expected with an average duration of follow-up of only 2.5 months.

Improving BCG Immunotherapy in Bladder Cancer

While BCG is arguably the most significant advance in the treatment of superficial bladder cancer since the mid-1970s, one-quarter of patients will fail to respond and more than half eventually become resistant to treatment. New agents and improved BCG strategies are clearly needed. In a double-blind randomized trial bladder cancer patients receiving maintenance BCG received recommended daily allowance vitamins or that plus high doses of vitamins A, B_6, C, E, and zinc [30]. As previously observed in the Veterans Administration randomized comparison of surgery alone versus surgery plus thiotepa, chemotherapy or oral vitamin B_6, no reduction in tumor recurrence occurred for 10 months. High-dose vitamin administration after 10 months was highly effective, cutting recurrence from 80% to 40% overall in high-risk patients at 5 years. The advantage of high-dose vitamins was greatest in those with low-grade, noninvasive tumors—those that have the poorest response to BCG.

Combination Immunotherapy

The use of combinations of chemotherapeutic drugs that have additive efficacy but not toxicity has been a significant advance, and increasing evidence suggests that the same principle applies to immunotherapy. BCG itself might be considered as a combination therapy because it is a complex organism that induces a variety of immune responses, but evidence suggests that the effect of BCG can be enhanced by the addition of cytokines. In the murine bladder cancer model combination immunotherapy with BCG plus interferon, or combination keyhole limpet hemocyanin (KLH) plus interferon, and interferon alpha or gamma plus IL-2 were significantly more effective than single-agent immunotherapy [31]. In a dose-finding phase I study, doses of IFNα2b ranging from 10 to 100 million units (MU) when added to one-half the standard dose of BCG yielded a 92% response rate (75% complete responders and 15% partial responders) in 12 patients with superficial bladder cancer [32]. In high-risk patients who fail to respond to BCG it is reported that 53% can be rescued and remain tumor-free for 2 years with combination BCG plus INFα2b [33]. BCG has also been combined with interleukin 2 with some success, but controlled trials have not been done.

3.5 BCG IN OTHER GU MALIGNANCIES

BCG in Renal Cancer

In an open-label, nonrandomized study Morales compared a group of patients with metastatic renal cell carcinoma (RCC) receiving intradermal BCG with concurrent and historical controls from the same institution [34]. In the control group all patients were dead within 4 years, but 35% of those receiving BCG were alive up to 5 years after treatment and the ratio of observed to expected deaths in the BCG group was 0.59 compared to 1.6 in the controls ($p < .01$). Complete disappearance of metastasis and long-term survival was noted in 2 (10%) patients in the BCG group and none of the 36 controls [35]. In a randomized study of BCG in patients undergoing nephrectomy for renal cell carcinoma in Russia, Mavrichev [36] reported 48% 8-year survival in 23 controls compared with 60% 8-year survival in 23 patients who received postoperative BCG immunotherapy. The results of these trials appear to be as good as those observed with much more complex, expensive, and toxic therapies [37]. Unfortunately, the initial observations did not receive the attention that BCG enjoyed in bladder cancer and, perhaps because of a controlled study in the United States that was widely reported as negative but not published (Paul Lange, personal communication), further research was not pursued in this country. Metastatic RCC has proved to be most recalcitrant to treatment, but with the advent of effective kinase inhibiters such as sunitinib and timsirolimus, further study of adjuvant BCG could prove beneficial.

BCG in Prostate Cancer

Direct injection of BCG into adenocarcinoma of the prostate in men induces a granulomatous response associated with tumor necrosis [38,39], but such approaches have been abandoned because of the risk of fatal septic reactions. Animal model studies have confirmed that BCG inhibits the growth of prostate cancer [40]. In the Dunning R3327 rat model of prostate cancer, BCG plus cryotherapy resulted in cellular and humoral immune responses that prevented growth of transplanted tumor [41]. Using mycobacterial cell wall extracts given by intraperitoneal injection, Morales [42] induced complete remission in 50% of rats with peripherally transplanted Dunning R3327H prostate cancer.

Animal studies clearly demonstrate that immunotherapy with BCG can induce antitumor responses in prostate cancer, but what is the potential clinical application? In a surprising phase II and subsequent phase III clinical trial, Guinan and associates reported remarkable success with what we would now believe is suboptimal BCG immunotherapy. Using percutaneous BCG to the deltoid every 4 months in 28 patients with advanced prostate cancer BCG immunotherapy improved antibody and delayed-type cutaneous hypersensitivity responses, and resulted in an 8-month prolongation in survival when compared with historical controls [43]. In a subsequent randomized controlled trial of 33 patients with advanced prostate cancer

treated with hormonal therapy, survival was increased from a mean of 5.6 months in controls to 8.1 months with BCG immunotherapy [44]. This modest increase in survival might be increased if BCG immunotherapy were improved and tumor burden were minimized.

3.6 IMMUNE RESPONSE TO BCG

It is often said that the mechanism of action of BCG is unknown, but without an effective cellular immune response BCG does not inhibit tumor growth. In fact, a great deal is known about the immune response to BCG. As a complex living organism, the responses induced to BCG infection are broad and highly varied. BCG attaches to bladder tumor cells and urothelial cells by means of specific receptors, fibronectin and integrin [45,46]. Internalization of BCG is correlated with immune response and sensitivity to BCG. In vitro studies suggest that poorly differentiated cell lines, unlike well-differentiated lines, internalize BCG and are sensitive to BCG [47]. Clinical studies similarly suggest that low-grade tumors are relatively less sensitive to BCG [48]. BCG antigens are expressed on the surface of tumor cells, and MHC class II antigen expression is upregulated [49–51]. BCG is a nonspecific stimulant to the reticuloendothelial system and induces a local inflammatory response with the infiltration of granulocytes followed by macrophages and lymphocytes, particularly helper T cells. Phagocytosis and the ratio of helper/suppressor cells are increased [52]. BCG induces a wide range of cytokines, including interleukin 1 (IL-1), interleukin 2 (IL-2), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10), interleukin 12 (IL-12), tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), granulocyte/macrophage colony stimulating factor (GM-CSR) and soluble intercellular adhesion molecule I (ICAM-I) [53]. The cellular immune or Th1 response predominates and is correlated with tumor destruction. More recent studies show that BCG, like other mycobacteria, contains high amounts of CpG oligodeoxynucleotide motifs that induce TNF-related apoptosis-inducing ligand (TRAIL). Following BCG administration urinary TRAIL and TRAIL-expressing neutrophils are found in the urine, and expression is correlated with response to BCG therapy [54]. Using ELISA, urine IFNγ, and TRAIL levels were initially undetectable in BCG therapy patients but were high after later induction treatments. More recent studies have shown CpG oligodeoxynucleotide induce TRAIL expression via IFN production.

3.7 TOXICITY

Most patients tolerate BCG well, but as with most cancer treatments, serious and even fatal toxicity can occur [55]. Irritative bladder symptoms including frequency and dysuria that typically begin after the second or third instillation and last for 1 or 2 days are the result of immune stimulation and cytokine production. These

symptoms, like low-grade fever, may be associated with a more favorable antitumor response to BCG [56]. Severe reactions or infection with BCG typically result from intravenous absorption of the organism, most commonly from traumatic catheterization. While BCG sometimes has to be given in the presence of hematuria, blood issuing from a catheter that has been difficult to insert is an absolute contraindication to instillation of BCG. Patients with continuing symptoms from earlier BCG administrations are best treated with antibiotics rather than forging ahead with more BCG. Logarithmic reduction in the dose of BCG in patients with increasing side effects will typically prevent escalation of toxicity. Since mycobacteria have the propensity to develop resistance to antibiotics that are given singly, combined use of a fluoroquinolone, which promptly inhibits BCG growth, as well as an antitubercular antibiotic such as isoniazid 300 mg daily is recommended. Patients with BCG sepsis require steroids in addition to gram-negative and antitubercular antibiotics to reduce the severe hypersensitivity component that can otherwise be fatal.

3.8 CONCLUSIONS

Bacillus Calmette–Guérin immunotherapy is currently the most effective treatment of superficial bladder cancer and one of the most successful applications of immunotherapy to the treatment of cancer. Once abandoned as ineffective, the success of this old, low-tech treatment should provide lessons for new drug development. This chapter highlights the history of BCG, the principles of BCG immunotherapy, improvements made in bladder cancer treatment, and potential application to other genitourinary cancers.

BCG immunotherapy is a major advance in the management of non-muscle-invasive bladder cancer. While early experience suggested that BCG was much more toxic than intravesical chemotherapy, simple dose reduction, appropriate antibiotics, and knowing not to treat in the face of increasing side effects or traumatic catheterization has greatly increased the safety of BCG. Now the most common intravesical agent used in the treatment of bladder cancer in the United States and Canada, BCG is the primary candidate for the cause of the unexpected reduction in the mortality of bladder cancer in North America. The success of BCG has created an unfortunate problem—although as many as 50% of patients will eventually fail or become intolerant of BCG, no new drug has been approved for the prevention of bladder tumor recurrence since BCG. This needs to be addressed and corrected if we are to continue to improve the management of superficial bladder cancer. Data also suggest that BCG may have efficacy in renal and prostate cancer. With the success achieved in bladder cancer and the relative safety and economy of BCG, further research in other tumors. should be considered.

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4

STIMULATION OF TOLL-LIKE RECEPTOR 9 FOR ENHANCING VACCINATION

Daniel E. Speiser and Arthur M. Krieg

4.1 INTRODUCTION

There has been much interest in improving the efficacy of cancer vaccines by incorporation of new adjuvants that may provide stronger or more effective antitumor T cell responses. Increasing evidence suggests that the strength of the T cell response generated by a vaccine may be critically influenced by the type of innate immune activation that occurs during the earliest phases of the immune response to the vaccine. Several lines of evidence suggest that early production of type I interferons, and induction of costimulatory molecule expression on dendritic cells, may be essential for the generation of durable and effective antitumor immunity. One way to accomplish this goal is through the induction of a Th1-like innate immune response, similar to what is generated in response to intracellular infections. The family of toll-like receptors (TLRs) appears to play a pivotal role in the generation of different classes of innate immune responses, by differential detection of conserved pathogen-expressed molecules. A central task of the innate immune system is to determine whether any pathogen is present extracellularly, in which

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case the "correct" type of immune response to be initiated is a Th2-like response, or intracellularly, in which case the innate immune system should induce a Th1-like immune response, capable of killing infected cells. Recent studies indicate that the innate immune system accomplishes this feat at least in part by making use of the ligand specificity and the cellular site of expression of the TLRs. Specifically, TLR2,4,5,6 are thought to be expressed on the cell surface of certain innate immune cells, where they detect components of extracellular pathogens. Activation of any of these TLRs results in the type of immune response that is most appropriate to resistance to extracellular infection, with relatively low levels of type I IFN production, and weak $CD8^+$ T cell responses. Conversely, TLR3,7,8,9 are expressed within the endosomal compartments of some innate immune cells, where they appear to be "looking inward" to detect nucleic acid components of intracellular pathogens [1]. Activation of these endosomal TLRs can induce strong Th1-like immune activation, with the secretion of type I IFN and strong $CD8^+$ T cell responses. In particular, the targeting of TLR9 has emerged as a powerful tool in the generation of Th1 adaptive immunity, and has shown promise for enhancing the efficacy of cancer vaccination.

4.2 THE ROLE OF TLR9 AS RECEPTOR FOR CpG ODN

TLR9 is used by the innate immune system for detecting unmethylated CpG dinucleotides, which are relatively common in bacterial and viral genomes, but are highly methylated and uncommon in vertebrate genomes [2]. TLR9 appears to be absolutely required for all known CpG-specific immune stimulatory responses to synthetic phosphorothioate (PS) ODN, since there is a complete loss of activity in mice genetically deficient in TLR9 [2–4]. However, native phosphodiester DNA can activate immune cells not only through TLR9 but also through one or more TLR9-independent pathways, if the DNA is delivered into the cells via transfection [5–7]. TLR9 also is not required for the induction of immune responses with DNA vaccines; nearly equivalent antigen-specific immune responses are seen in the presence or absence of TLR9 [8–10]. The review in this chapter focuses only on the adjuvant properties of synthetic PS ODN, which are thought to be solely TLR9-mediated.

Each TLR has a unique pattern of cellular and tissue expression, which presumably has evolved to serve their role in detection of different types of pathogens. Among all of the TLRs, TLR9 has perhaps the narrowest profile of expression—resting human immune cells appear to exclusively express TLR9 in B cells and in plasmacytoid dendritic cells (pDC), (reviewed in Ref. 11). The expression of TLR9 varies significantly between rodents and humans. Mice express TLR9 in B cells, monocytes, and probably all DC subsets, including pDC which produce most of the type I IFN that is made in response to viral infection [12] (reviewed in Ref. 11). This species-specific difference in TLR9 expression makes it difficult at best to predict the effects of TLR9 activation in humans by extrapolating results in mice. Several studies have reported that cellular activation results in a

broader expression of TLR9, including human neutrophils [13], monocytes and monocyte-derived cells [14,15], activated CD4 T cells [16], pulmonary epithelial cells [17,18], and intestinal epithelium [19,20]. Natural killer (NK) cells generally have been reported not to express TLR9, and NK cells appear instead to be activated secondarily to the pDC [21–26]. The literature on TLR9 expression in human cells contains many inconsistent reports, and the interpretation of published studies is complicated by the use of culture conditions that may not recreate the in vivo setting, and by the frequent use of "anti-TLR9" antibodies of uncertain specificity. In addition, many published studies fail to exclude the possible effects of contaminating pDC, which can secrete IFNα, causing secondary activation of other cell types, even when the pDC are present at concentrations of $<0.1\%$. For example, we and others have found that CpG-induced activation of human monocytes was indirect and dependent on the presence of contaminating pDC and IFNα [27,28].

In addition to species-specific variations in the cell types expressing the TLRs, there are species-specific differences in the optimal CpG motif for activating TLR9. Empiric studies defined the CpG motif as a hexamer containing the two bases flanking the CpG dinucleotide to the 5′ and 3′ sides [29]. The optimal immune stimulatory ODN hexamer CpG motif is GACGTT for mice [29–31] but GTCGTT for humans [32] and apparently for many other vertebrate species, including cow, sheep, cat, dog, goat, horse, pig, and nonhuman primates [30,33]. Besides the hexamer CpG motif, the immune stimulatory activity of an ODN is determined by (1) the number of CpG motifs in an ODN (usually two to four are optimal), (2) spacing of the CpG motifs (usually at least two intervening bases are optimal, preferably Ts), (3) the presence of polyG sequences or other flanking sequences in the ODN (the effect depends on ODN structure and backbone), and (4) the ODN backbone (nuclease-resistant phosphorothioate backbone is the most stable and best for activating B cells, but gives relatively weak induction of IFNα secretion from pDC compared to native phosphodiester linkages in the CpG dinucleotide) [29,31,32,34–37].

Most immune cells, but especially DC and B cells, spontaneously take up CpG ODN into one or more acidified endosomal compartments, through pathways that are energy/temperature-dependent, but otherwise not yet well understood. Lymphocyte uptake is greatly increased by cell activation and appears to be receptor mediated [38]. There are likely to be several different receptors responsible for the uptake of different ODN classes, depending on whether the ODN contains polyG sequences, which can bind to scavenger receptors. CXCL16 and possibly other membrane-bound scavenger receptors appear to mediate uptake of class A CpG ODN (which contain polyG motifs, see below), but not other ODN classes [39–42]. TLRs have no known role in ODN uptake. More recent studies suggest that ODN uptake is not sequence-independent, but is selectively enhanced by the presence of a 5′ TCG motif [43].

TLR9 initially is present within the endoplasmic reticulum, but on CpG ODN uptake the TLR9 translocates into the same endosomal compartment as the CpG ODN, thus allowing binding and initiation of signal transduction [44,45]. Inhibitors

of endosomal acidification/maturation completely block the immune effects of CpG ODN, demonstrating an essential role for this compartment in the TLR9-induced signal transduction pathways [46–49]. Immune stimulation by CpG ODN also is blocked by inhibitors of phosphatidylinositol 3 kinase (PI3-kinase), which also appears to have a role in the internalization of the DNA [50].

4.3 IDENTIFICATION OF DISTINCT CLASSES OF IMMUNE STIMULATORY CpG ODN

Three families of immune stimulatory CpG ODN with distinct structural and biological characteristics have been described [51–53]. The class A CpG ODN (also known as *type D*) are potent activators of NK cells and IFNα secretion from pDC, but only weakly stimulate B cells. Class A ODN have polyG motifs at the 5′ and/or 3′ ends that are capable of forming complex higher-ordered structures known as *G tetrads*. These polyG motifs are required for the strong IFNα induction characteristic of this ODN class. For optimal induction of IFNα secretion class A ODN have a central phosphodiester region containing one or more CpG motifs within a self-complementary palindrome (reviewed in Ref. 54). Class B ODN generally have a completely phosphorothioate backbone, do not form higher-ordered structures, and are strong B cell stimulators, but induce relatively little NK activity or IFNα secretion [55]. The class C CpG ODN have immune properties intermediate between the A and B classes, inducing both B cell activation and IFNα secretion [51–53]. The class C ODN have a unique structure, with one or more 5′ CpG motifs, and a palindrome 3′ to this, allowing formation of a duplex. All three ODN classes require the presence of TLR9, as they have no immune stimulatory effects in mice deficient in TLR9 [51]. An important question is how the different classes of CpG ODN can induce such divergent immune effects, since they all appear to act through TLR9. The fact that maximal induction of pDC IFNα secretion requires the ODN to adopt a secondary structure, apparently dimeric in the case of the class C ODN, and multimeric in the case of the class A type, suggests the hypothesis that these higher-ordered structures might either induce TLR9 crosslinking or perhaps promote the recruitment of one or more additional cofactors or adaptor proteins into the TLR9-signaling complex. More recent studies indicate that the class A ODN achieve much of their IFNα inducing effect through interaction with CXCL16, which is expressed on pDC, but not B cells, and which appears to enhance the uptake of the class A ODN, but not class B ODN [39]. The possibility that additional TLR9 cofactors may take part in the recognition of one or more CpG ODN classes has not been excluded. Other studies have also pointed to the possibility that the presence of the different ODN classes in distinct intracellular compartments may contribute to their differential immune effects [56]. Transfection of a class B ODN can induce levels of IFNα secretion similar to those induced by an class A ODN, providing new insights into the biology of these pathways, but also raising new questions about the immune effects of the transfection step [56]. Almost all of the published studies using CpG ODN as vaccine adjuvants have

been carried out with class B CpG ODN, so although the following sections refer to "CpG ODN" generically, reference is actually to class B ODN unless otherwise stated.

4.4 CpG ODN MECHANISMS ACTION IN STIMULATING INNATE AND ADAPTIVE IMMUNITY

In the endosome, TLR9 binding and recognition of a CpG motif leads to the rapid recruitment of the adaptor molecules MyD88, IL-1 receptor-associated kinase (IRAK)-1, and TNFα receptor-activated factor 6 (TRAF6), which appear to form a complex with TLR9 [45,47,57–60]. This signaling complex activates mitogen-activated protein kinases including extracellular receptor kinase (ERK), p38, and Jun *N*-terminal kinase and also the IκB complex, the pathways of which converge on the nucleus to alter gene transcription [32,61–66]. IFNα secretion by pDC is IRF7-dependent, and requires direct interactions between IRF7 and MyD88, TRAF6, and IRAK-1 [67–70].

The immune effects of activating TLR9-expressing cells in humans (B cells and pDC) with CpG ODN are Th1-like and may be considered in two stages: an early innate immune activation, and a later enhancement of adaptive immune responses. Through the signal transduction pathways described above, TLR9-stimulated B cells and pDC show increased expression of costimulatory molecules and TNF-related apoptosis-inducing ligand (TRAIL), resistance to apoptosis, upregulation of the chemokine receptor CCR7 that causes cell trafficking to the T cell zone of the lymph nodes, and secretion of Th1-promoting chemokines and cytokines such as MIP-1, IP-10, and other IFN-inducible genes [55,71]. PDC activated through TLR9 secrete high levels of type I IFN [72].

The innate immune effects of TLR9 activation can promote tumor control or regression through two general routes, including (1) the inducible secretion of Th1-like cytokines and chemokines, some of which are known to have antitumor activity; and (2) the activities of the innate immune cell populations. For example, type I IFN have some activity in the treatment of a few cancers, and IP-10 appears to be important in the migration of T cells into tumors, and for the therapeutic effect of IL-12 in mouse models [73–75]. The CpG-induced secondary activation of innate immune cells exerts antitumor effects through, for example, NK cell–mediated tumor killing [34] and induction of TRAIL expression on activated monocytes due to the pDC-derived IFNα [71]. TLR9-mediated innate immune activation and pDC maturation is followed by the generation of adaptive immune responses. B cells are strongly costimulated if they bind specific antigen at the same time as TLR9 stimulation [29], even if the antigen is presented in the form of an immune complex [76]. This selectively enhances the development of antigen-specific antibodies, suggesting utility of CpG ODN as vaccine adjuvants.

CpG stimulation strongly increases the capability of both B cells and pDC to promote antigen-specific immune responses. When pDC first were studied, they were

thought to be responsible primarily for inducing Th2-type immune responses, and to be relatively ineffective at stimulating primary or recall T cell responses [12]. However, human pDC function is dramatically activated when they are stimulated in vitro through TLR9 within PBMC; both class A and class B CpG ODN increased the frequency of $CD8^+$ T cells with a memory phenotype, while only class B increased the frequency of $CD8^+$ T cells with a naive phenotype [77]. Murine DC activated with CpG ODN show enhanced antigen presentation, stimulating the development of Th1 cells, and resulting in the generation of T helper cell–independent primary effector $CD8^+$ T cells [78–81]. These properties suggest that CpG ODN may be excellent vaccine adjuvants, especially for the induction of strong Th1-biased immunity.

There has been tremendous interest in understanding the role of regulatory T cells (Treg) in determining the outcomes of cancer vaccination and immune therapy [82]. The effects of TLR9 stimulation on Treg are complex, and not yet fully understood. In vitro studies using murine cells suggested that DC activated through TLR9 could overcome the immune suppressive effects of Treg in an IL-6-dependent fashion [83] or in an IL-6-independent fashion [84]. A generally similar type of effect has been observed in studies using human Treg, which have been described to express TLR8 [85]. TLR8 is reportedly activated by the G-rich motifs in class A CpG ODN, resulting in the loss of the Treg suppressive effects and in enhanced antitumor immunity when these TLR8-activated Treg are adoptively transferred into Rag1-deficient mice bearing a human melanoma [85]. In other experimental models the opposite type of effect has been observed; studies using purified human pDC have demonstrated that TLR9 activation enhances the generation of Treg, which suppresses naive T cell responses [86]. Likewise, murine Treg are induced to proliferate when cultured with CpG-activated APC and anti-CD3 [87]. To further complicate matters, the addition of a class B CpG ODN to in vitro cultures of murine DC, Treg, and naive T cells results in the generation of IL-17-producing T cells, suggesting that the net effect of TLR ligation may depend on multiple variables [88]. The in vivo interactions between TLR9 activation and Treg are not well understood, but are likely to be even more complex. Although CpG ODN are excellent vaccine adjuvants, the T cell response to footpad vaccination of mice using a CpG ODN adjuvant was significantly enhanced by prior depletion of Treg, suggesting that CpG ODN seldom overcome Treg influences, and providing a possible approach for further increasing the efficacy of vaccination [89]. Similar results have been reported in a mouse model where Treg-mediated tolerance was induced by transcutaneous immunization through UV-irradiated skin, in which case subsequent vaccination with a CpG ODN failed to induce a strong CTL response, again demonstrating that in at least some models, TLR9 activation does not overcome Treg-mediated immune suppression [90]. The route of administration of CpG ODN may be a critical determinant of the biologic effects; intravenous (IV) but not subcutaneous (SC) administration of a class B CpG ODN results in the induction of indoleamine 2,3-dioxygenase (IDO) expression in the spleen, and suppression of T cell expansion and CTL activity [91,92]. Taken together with the evidence that Treg contribute to the immune suppression of cancer patients, these reports suggest

that the efficacy of CpG ODN as a vaccine adjuvant may be further increased by the prior depletion or inactivation of Treg.

4.5 CpG ODN AS ADJUVANTS FOR INFECTIOUS DISEASE VACCINES

The development of antigen-specific adaptive immune responses is now known to depend critically on activation of the innate immune system. Moreover, the Th1/Th2 character of the adaptive immune response appears to be determined by innate immunity. Since injection of CpG ODN stimulates a strong Th1-like cytokine and chemokine milieu within draining lymphoid tissues [81,93], the ODN are logical candidates for evaluation as vaccine adjuvants. Other factors predicting strong adjuvant activity for CpG ODN in vaccination include (1) a synergy in the presence of antigen between CpG-mediated TLR9 and B cell receptor stimulation, resulting in enhanced B cell stimulation [29]; (2) inhibition of B cell apoptosis, resulting in a prolongation of immune responses [31]; and (3) DC maturation and differentiation, resulting in enhanced activation of Th1 cells and strong $CD8^+$ T cell generation, even in the absence of CD4 T cell help [81,93].

The utility of CpG ODN as a vaccine adjuvant for inducing antigen-specific humoral and cellular responses has been confirmed in studies using a wide variety of antigens, including peptide or protein antigens, live or killed viruses, dendritic cell vaccines, autologous cellular vaccines, and polysaccharide conjugates. In general, CpG ODN are not effective adjuvants for unformulated polysaccharide antigens, but they are quite effective if a protein carrier is conjugated to the polysaccharide [94–96]. Conjugation of CpG ODN directly to the antigen has been used to enhance antigen uptake and reduce antigen requirements [97,98].

CpG ODN has been shown to be an effective and generally well-tolerated vaccine adjuvant when coadministered with numerous viral and bacterial antigens in a range of species, including mice [94,99–102], rats [103,104], rabbits [105], guinea pigs [106], pigs [107,108], cattle [109,110], sheep [111], dogs [112], nonhuman primates [101,113,114] and humans [115–119]. When compared to other adjuvants, CpG ODN appears to induce unsurpassed Th1 responses. For example, in a murine study comparing 19 different immunologic adjuvants with KLH conjugate vaccines containing the two human cancer antigens (MUC1 peptide and GD3 ganglioside), CpG ODN induced the most Th1-biased immune responses of any adjuvant as demonstrated by highest levels of IFNγ secretion in antigen-restimulated splenocytes [120]. Likewise, when compared to other commonly used adjuvants (complete Freund's adjuvant, incomplete Freund's adjuvant, Titermax® Gold, alum, or MPL) for their ability to augment humoral responses to hepatitis B surface antigen (HBsAg) in mice, CpG DNA gave the highest ratio of IgG2a to IgG1 antibodies, an indirect marker for Th1-biased responses, of any adjuvant used alone [78, 121–123]. Moreover, CpG ODN achieve this level of antigen-specific activation without inducing the harsh local inflammatory effects seen with complete Freund's adjuvant.

4.6 CpG ODN SYNERGY WITH OTHER ADJUVANTS

A synergistic effect has been reported between CpG ODN and a broad range of other injectable adjuvants including emulsions (e.g., Emulsigen®, Montanide ISA51 and ISA720 and MF-59), particulate adjuvants (e.g., cationic and anionic microparticles, nanoparticles, virus-like particles, ISCOMs), mineral salts (e.g., aluminum hydroxide), saponins (e.g., QS21, Quill A), liposomes and cationic peptides, polycationic antibiotics (e.g., polymyxin B), polysaccharides, and GM-CSF [124–126] reviewed by McCluskie and Krieg [127]. The synergy between these adjuvants and CpG ODN may result from various factors, including (1) protection of CpG ODN and/or antigen from enzymatic degradation, (2) a depot effect resulting in the sustained release of antigen and CpG ODN to APC, (3) enhancement of antigen and ODN uptake into antigen-presenting cells, and (4) synergy between signaling pathways activated by the other adjuvants and the TLR9 pathway. Full synergy requires careful coformulation of the CpG and the antigen; ideally both should be present within the same endosomal vesicle of the APC [128,129].

4.7 HUMAN CLINICAL TRIALS WITH CpG ODN TO ADJUVANT INFECTIOUS DISEASE VACCINES

In humans, CpG ODN has been used as an adjuvant for infectious disease vaccination either in combination with alum [116] or alone [118]. In addition, CpG ODN has been used with Montanide in a cancer vaccine setting [119]. In a randomized, double-blind phase I/II dose escalation study, healthy individuals received three intramuscular (IM) injections (at 0, 4, and 24 weeks) of an alum-absorbed HBV vaccine (Engerix-B) either in saline or mixed with VaxImmune™ (a class B CpG ODN, CPG 7909) at doses of 0.125, 0.5, or 1.0 mg [116]. HBsAg-specific antibody responses (anti-HBs) were shown to appear earlier and with a higher magnitude at all timepoints up to and including 24 weeks in VaxImmune recipients compared to those individuals who received Engerix-B alone. Moreover, a high proportion of individuals who received VaxImmune as adjuvant developed protective levels of anti-HBs IgG within just 2 weeks of the priming vaccine dose, and there was a trend toward higher rates of positive $CD8^+$ T cell responses in the two higher-dose groups of VaxImmune compared to controls. In this study, VaxImmune was also shown to enhance the late-affinity maturation process, thus increasing the pool of high-avidity antibodies. This CpG mediated effect was antigen-specific, isotype-specific and distinct from the influence on anti-HB production, as avidity did not correlate with anti-HBs IgG titers [130].

In a separate phase I, observer-blinded, randomized study, healthy individuals received two doses 8 weeks apart of a non-alum-absorbed commercial vaccine either alone or mixed with another class B CpG ODN, namely, 1018 ISS [118]. In this study, a dose-dependent enhanced immunogenicity associated with CpG ODN was also seen, albeit at doses (0.3, 0.65, 1.0, and 3.0 mg) [118] higher than the $\leq$1 mg that was used in the VaxImmune study. However, the two studies were

performed at different times, with different CpG ODN, and in the presence or absence of alum, so comparison of the responses across studies is difficult.

The potential of CpG ODN to overcome hyporesponsiveness was first demonstrated in orangutans that were hypo-responsive to the commercial HBV vaccine, Engerix-B, containing alum-absorbed yeast-derived recombinant HBsAg. In this study, coadministration of CpG ODN with Engerix-B greatly enhanced not only the levels of HBsAg-specific antibodies but also the rate at which they appeared [113]. More recently the ability of CpG ODN to overcome hyporesponsiveness has also been demonstrated in a human clinical trial, whereby HIV-positive individuals who were previously nonresponsive to the Engerix-B vaccine rapidly developed seroprotective antibody titers when the same vaccine was administered in combination with CpG ODN (CPG 7909) [115]. Moreover, in those individuals receiving CpG ODN as adjuvant antibody levels were maintained at protective levels for up to 3 years after immunization and significantly enhanced antigen-specific lymphocyte proliferation was observed for at least 2 years compared to those receiving vaccine alone [115]. The Th1 enhancing effect of TLR9 activation in humans was also evident in a clinical trial where coadministration of a 1 mg dose of CPG 7909 as adjuvant with a one-tenth dose of a commercial influenza vaccine restored the full level of antigen-specific IFNγ secretion [117].

4.8 EFFICACY OF CpG ODN AS VACCINE ADJUVANTS IN MOUSE TUMOR MODELS

Vaccine adjuvant comparisons in mouse models have demonstrated CpG ODN to be stronger at inducing antigen-specific Th1 responses than any other TLR agonist or vaccine adjuvant, even including complete Freund's adjuvant [78,79, 120–122,131–136]. CpG ODN have been reported to be the only adjuvants that induce antitumor responses strong enough to eliminate established tumors that have grown to 5–10 mm in size [132,133]. The mechanism of these vaccine enhancing effects of CpG ODN includes both enhanced DC function leading to stronger CD8 T cell responses and the generation of Th1-promoting cytokines such as IFNγ and IL-12. Depending on the antigen, CpG ODN can induce strong $CD8^+$ T cell responses to tumor-derived peptides even when mixed in saline, and even in the absence of other adjuvants [132–134,136]. CpG ODN added to tumor vaccines [132,137] or irradiated neuroblastoma cells expressing GM-CSF [126] can eradicate established tumors in a T-cell-dependent fashion.

4.9 HUMAN CLINICAL TRIALS OF CpG ODN ADJUVANTS FOR CANCER VACCINES

It is generally believed that immune rejection of solid tumors requires antigen-specific (adaptive) immune responses including T cells. Activation of the innate immune system is thought to be critical for the priming of such adaptive T cell

immune responses, but is unlikely to be sufficient alone to generate tumor-specific immunity. Thus, the combination of an innate immune activator with a synthetic tumor antigen should in theory provide the most direct and effective way to stimulate potent anti-tumor immune responses.

T cell vaccination is still in an early phase of development, for protecting against both infection and cancer. Unfortunately, clinically significant therapeutic effects are relatively uncommon with the available vaccines. To provide more information on biological and clinical effects of experimental vaccination, an increasing number of phase I/II clinical trials investigate human immune responses in great detail. One of the aims is to determine whether those immune mechanisms that were established in basic models are actually functional in humans. This is much easier said than done. For example, dendritic cells (DCs) are difficult to assess, because they are infrequent, and it is often impossible to trace activated DCs in vivo. Hopefully, advances in technology will simplify the identification of DCs and the characterization of how they react to various stimuli, and how they subsequently impact on immune responses. By contrast, a field in which great progress has already been made since the mid-1990s is in the investigation of T cells.

T cells are highly "sensitive detectors" of immune stimulation. On natural infection with a virus, antigen-specific naive T cells expand during the first 1–2 weeks to reach high T cell frequencies, with up to 10^5 fold expansion [138,139], thus exceeding the proliferative potential of most other cells in the body! In contrast, ineffective T cell triggering leads to much lower numbers of T cells, which are less likely to protect from disease. Besides these quantitative aspects, the quality of T cell responses is important. Most likely, robust T cell responses should include both effector and memory T cells [140]. Therefore, novel immunotherapies should elicit substantial numbers of T cells, and profoundly impact on T cell differentiation such that effector T cells are capable of destroying tumor cells, and memory T cells ensure long-term maintenance of responses.

To obtain precise information on quantity and quality of immune responses, T cells must be investigated directly ex vivo. This strategy has allowed direct comparison of different adjuvants when combined with tumor antigens for vaccination of melanoma patients, as exemplified by studies with one of the most frequently expressed human tumor antigen, Melan-A [141–143]. After vaccination with Melan-A peptide in saline, T cell frequencies were not increased compared to healthy donors. Similar negative results were obtained with peptide mixed together with QS21 and MPL. In contrast, vaccination with peptide in incomplete Freund's adjuvant (IFA) led to increased frequencies of antigen-specific T cells in about half of vaccinated patients [144], reaching on average 0.1–0.2% antigen-specific cells of circulating $CD8^+$ T cells. Much more strikingly, the addition of the synthetic TLR9 agonist, CPG 7909, yielded strongly and consistently increased T cell frequencies in all patients, reaching a mean of $> 1\%$ of Melan-A-specific cells among circulating $CD8^+$ T cells [119]. Besides triggering quite high T cell frequencies, vaccination with CPG 7909, IFA and peptide was efficient to promote effector cell differentiation, such that effector functions reached high levels comparable to what is found in T cells specific for Epstein–Barr virus (EBV) and cytomegalovirus

(CMV) [145]. Thus, CPG 7909 leads to roughly 10-fold higher T cell frequencies than does vaccination without it [119], indicating that TLR9 agonists, in conjunction with antigen, are the strongest known stimulators for induction of human $CD8^+$ T cell responses. The various other vaccine types induce lower frequencies of antigen-specific $CD8^+$ T cells. This accounts for many different approaches such as peptide pulsed DC [146], recombinant vaccinia viruses expressing peptides and B7 [147] or multiple T cell antigens [148], peptide with IL-12 [149], or peptides with IFA and anti-CTLA4 mAb [150].

Efficient T cell activation is thought to depend on antigen presentation by DCs, primarily so-called myeloid DCs (mDC). A puzzling issue is that human mDC do not express TLR9, so CpG ODN have no direct effect on them, triggering instead pDC, which are generally considered to be inefficient in antigen presentation [151–153]. Why, then, are TLR9 agonists such efficient adjuvants for T cell vaccination? It is likely that pDC (responding to CpG) and mDCs (presenting antigen) are both involved. On CpG triggering, pDC release large amounts of IFNα/β that activate NK cells, which then release IFNγ and TNFα, leading to activation of mDCs [154,155]. A mouse study has shown that CpG triggering also upregulates CD40L on pDC, which interacts with CD40 on mDC, thus mediating a crosstalk between these DC subsets [156]. Through these effects, TLR9 agonists likely promote activation of myeloid DC, which can then activate antigen specific $CD8^+$ T cells. Some evidence also indicates that pDC activated through TLR9 may become competent to induce effective $CD4^+$ and $CD8^+$ T cell responses [157–161]. Finally, besides several other mechanisms not detailed here, some studies suggest that CpG ODN may also directly activate NK cells and T cells [16,83,162–166], but other studies suggest that this activation is indirect [21–26].

4.10 SAFETY OF CpG ODN AS VACCINE ADJUVANT

In mice CpG ODN used as a vaccine adjuvant induce less tissue damage than other tested adjuvants, including Titermax, CFA, IFA, and MPL; when used in combination with other adjuvants, the amount of tissue damage was generally not greater than either adjuvant alone [123]. In sheep, CpG ODN combined with mineral oil, metabolizable oil, or nonoil adjuvants increased antigen-specific immune responses and enabled the use of lower doses of mineral oil-based adjuvants without decreasing the magnitude of immune responses and with significantly reduced tissue damage at the injection site [111]. In humans, the major side effects of CpG ODN vaccine adjuvants has been an increase in the frequency of injection site reactions, and transient flu-like symptoms, which are usually graded as minimal to mild [115–119]. In some studies there has been an increase in the severity of the injection site reactions, but these have still most commonly been mild [167].

Several murine studies [168] have shown that treatment with CpG ODN can lead to production of autoantibodies (e.g. IgG anti-DNA) and of inflammatory cytokines (e.g., IL-6). In addition, activated lymphocytes may develop increased

resistance to apoptosis, which may result in enhanced activity of self-reactive lymphocytes [169–172]. The current status of knowledge is, however, that these CpG ODN-induced changes in the function of the immune system generally are not associated with clinically manifest autoimmune disease, suggesting that other, additional biological pathways crucial to promote systemic autoimmunity are not induced by CpG ODN, and/or that TLR9 activation triggers feedback pathways that prevent this. The immune effects of CpG ODN in mice are qualitatively different from those in humans because of the previously mentioned species-specific differences in the cells expressing TLR9, which makes it impossible to determine whether the results reported in mouse models of autoimmunity are relevant to human therapy. Nevertheless, since tumor-associated antigens may be considered self-antigens, successful tumor vaccination may be considered as a special example of induction of autoimmunity, and further studies into the mechanisms regulating this, and the effects of TLR9 agonism on self-tolerance, will be of great interest.

4.11 CONCLUSIONS

The studies performed to date show great promise for the clinical application of TLR9 activation with CpG ODN for enhancing the clinical outcomes from cancer vaccination. Human clinical trials confirm that this approach leads to a marked acceleration and amplification of both $CD8^+$ T cell and B cell responses. Nevertheless, tumors appear to have multiple means of suppressing or evading antitumor immunity, and it remains unclear to what extent TLR9 activation will be able to overcome these defenses. Most likely, multiple synergistic therapies will need to be used in combinations in order to achieve the full clinical potential of this approach. To date, the safety of TLR9 activation with CpG ODN appears good, and its selective and strong biological effects hold promise for further development and application in larger numbers of patients.

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PART III

ANTIGEN-SPECIFIC THERAPY: NOVEL PRESENTATION OF PEPTIDE AND PROTEIN ANTIGENS

5

POLYEPITOPE VACCINES

Corey Smith and Rajiv Khanna

5.1 INTRODUCTION

Cytotoxic T lymphocytes (CTLs) play an important role in the immunosurveillance and clearance of both intracellular pathogens and malignant cells [1,2]. Following T cell receptor engagement of the peptide–major histocompatibility (MHC) class I complex, CTLs function by inducing lysis of the target cell [3]. The capacity to isolate the peptide determinants presented by MHC class I molecules, typically 8–10 amino acids in length, has resulted in the development of vaccine strategies that employ minimal CTL determinants to target the induction of CTL.

5.2 PEPTIDE-BASED VACCINES AND IDENTIFICATION OF CTL PEPTIDE TARGETS

The discovery that small peptide epitopes encode the specificity for recognition of foreign antigens by CTL provided the impetus for the generation of a range

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of immunization strategies targeting minimal CTL determinants, thus limiting side effects that may be associated with more complex vaccines. The ability to correctly define the appropriate peptide targets, particularly with regard to antigens expressed by malignant cells, is essential in order to generate effective peptide-based vaccines. A number of techniques have been employed to define CTL epitopes, including strategies that directly elute peptides from MHC class I molecules on the surface of cells. More recent strategies typically aim to expand CTL in vitro using whole antigen and subsequently define CTL epitopes using overlapping peptides from the antigens. This second strategy has been particularly useful in identifying CTL epitopes from viruses that are known to be associated with cancer, but it requires knowledge of the antigenic target associated with disease, which may be particularly difficult in malignancies that are not associated with infection, and likely candidates are less obvious. However, strategies have been developed to address these issues, including using whole-tumor lysates to generate CTLs prior to determination of the CTL epitopes. In addition to the employment of correct antigenic targets, the development of vectors and adjuvants that provide the necessary immunostimulatory signals also plays an important role in the activation of effective CTL using peptide-based vaccines.

5.3 VEHICLES FOR DELIVERY OF PEPTIDE-BASED VACCINES

The recent elucidation of the requirements associated with CTL activation, such as the maturation of antigen-presenting cells (APCs) and the production of proinflammatory cytokines, has led to the generation of more effective peptide-based vaccine strategies aimed at eliciting the signals required to generate CTLs. These include recombinant bacterial and viral vectors; recombinant DNA-based delivery systems; and lipid-based delivery systems, such as immunostimulatory complexes (ISCOMs), lipopeptides, and liposomes [4–8]. The most successful of these delivery vehicles are self-adjuvanting, having the capacity to target pattern recognition receptors on the surface of APCs in order to provide the necessary signaling required for maturation of the APC, in addition to the capacity to deliver antigens into the MHC class I pathway. A number of these delivery vehicles, including viral, DNA, and lipid-based vectors, have been shown to be safe for use in humans.

5.4 PEPTIDE-BASED VACCINES FOR TREATMENT OF HUMAN DISEASES

Animal models have thus far provided a platform to model and optimize peptide-based vaccine strategies, typically by analysis of CTL responses of a single specificity presented by a single MHC molecule. Similarly, single-peptide-based vaccines have also been employed in a number of clinical trials to treat human malignancies [9]. However, the development of effective peptide-based vaccines for use in humans will likely be more complex, requiring the presence of peptides presented across a broad range of human HLA types. Although vaccination

with full-length antigens offers an alternative to defining CTL epitopes across all HLA types, the nature of the antigen in question can significantly impact on its suitability as a vaccine candidate. Antigens may be toxic or oncogenic [10], and therefore inappropriate for use as full-length antigens in vaccines. Some antigens are known to be immunosuppressive or nonimmunogenic [11], and vaccination with the full-length antigen may not result in induction of the desired immune response. In addition, the complexity of the disease-causing agent may require the generation of CTLs against a range of antigens, consequently requiring complex expressing systems to produce safe and effective vaccine using full-length antigens. Defining and employing multiple CTL epitopes providing broad specificity offers a viable alternative that avoids possible effects associated with toxicity, oncogenicity, and the poor immunogenicity of some full-length antigens. However, the capacity to produce cost-effective vaccines on the basis of defined CTL epitopes against particular diseases may be limited by the expense of producing individual peptides tailored to different HLA types. In order to effectively employ peptide-based vaccines, a delivery mechanism is required that is cost-effective and can be used to generate responses across a broad range of HLA types. One technique currently under development for the treatment of a number of human diseases involves the construction of a polypeptide that links consecutive CTL epitopes together to produce a polyepitope.

5.5 POLYEPITOPE TECHNOLOGY

There is evidence from animal models that the processing and presentation of CTL epitopes from native antigen can be significantly influenced by the flanking amino acid sequences [12,13]. Modification of these flanking sequences has been shown to completely abrogate the induction of a CTL response. It would therefore seem likely that the generation of effective polypeptide-based vaccines would depend on the presence of appropriate flanking amino acid residues. However, it has become evident from numerous studies that polyepitope-based vaccines can be produced against a range of diseases by simply linking CTL epitopes end-to-end in the absence of appropriate flanking sequences (Fig. 5.1). However, it is as yet not clear why flanking sequences are not required for processing of epitopes from a polyepitope.

The first study to demonstrate that a polyepitope without flanking sequences could be used effectively employed a polypeptide encoded by a recombinant vaccinia virus (VV) vector and expressing nine epitopes derived from Epstein–Barr virus (EBV) that were recognized by six different HLA molecules [14]. The EBV polypeptide was shown to be capable of presenting the epitopes to CTL clones and could be used to expand specific CTLs from peripheral blood mononuclear cells (PBMCs) of EBV-positive individuals. Subsequent studies demonstrated that a single polyepitope could be used to induce CTL responses following vaccination of mice against multiple targets including viruses, a parasite, and a tumor model [15], protective responses of which were shown against both viral and tumor

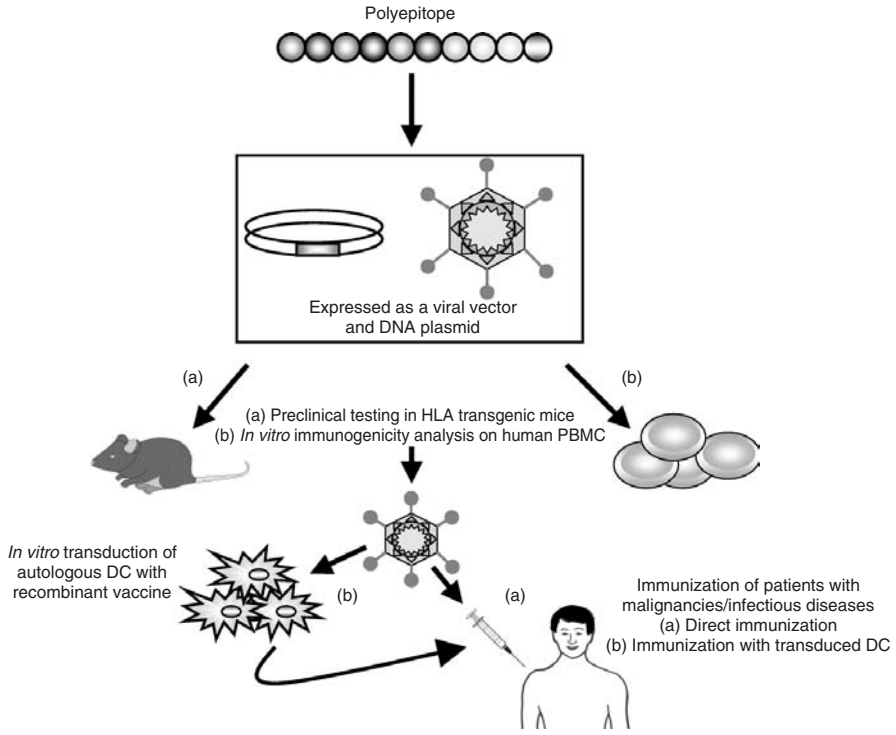

Figure 5.1. Preclinical and clinical development of polyepitope vaccine technology. Polyepitope vaccines can be delivered in either a DNA or viral vector. Preclinical testing of these vaccines in HLA transgenic mice and human PBMC has demonstrated the potential of this technology to induce antigen-specific CTL responses. These vaccine formulations can be delivered either directly of following in vitro transduction of autologous dendritic cells as discussed in the text.

challenges. Other studies have since developed polyepitope constructs directed against diseases caused by foreign agents, including human pathogens such as the human immunodeficiency virus (HIV), human cytomegalovirus (HCMV), and malaria [16,17]; or against malignancies, including those where a foreign agent is present, such as EBV [18,19], or where no foreign infectious agent is present and self-antigens are targeted by CTL, such as in melanoma [20]. Similarly, a range of vehicles have been employed to deliver polyepitope constructs, including genetic delivery vehicles, such as VV vectors, other poxvirus-based vectors, adenoviral vectors and DNA [14,19,21] see Fig. 5.1); and protein-based delivery vehicles such as ISCOMs [22]. One of these polyepitope vaccines, which focuses on generating CTL directed against the latent membrane proteins (LMPs) of EBV, is currently under development for the therapeutic treatment of the EBV-associated malignancies, Hodgkin's lymphoma (HL), and nasopharyngeal carcinoma (NPC).

5.6 POLYEPITOPE-BASED TREATMENT OF EBV-ASSOCIATED MALIGNANCIES

Following the initial lytic stage of infection, EBV becomes latent, resulting in the expression of a limited array of antigens, including the EB nuclear antigens (EBNA) 1–6 and LMP1 and LMP2. In addition to the association of lytic EBV infection with infectious mononucleosis, a number of human malignancies can be associated with latent EBV infection, including posttransplant lymphoproliferative disease (PTLD), which arises in immunosuppressed transplant patients; HL, 30–50% of cases of which are EBV-associated; and NPC, of which the majority of cases are EBV-associated. Evidence gained following treatment of PTLD, using CTL expanded following stimulation of PBMC from EBV-positive individuals with EBV-transformed lymphoblastoid cell lines (LCLs), has demonstrated that some EBV-associated malignant cells are susceptible to recognition and lysis by specific CTLs [23]. However, similar strategies aimed at treating EBV-associated HL and NPC have met with limited success. A possible explanation for the failure of LCL-expanded CTL to offer protection against EBV-associated HL and NPC is the lack of specific T cells in the LCL-expanded CTL that recognize antigens expressed in HL and NPC.

Hodgkin's lymphoma and NPC do not express the full array of latent antigens, as typically occurs in PTLD, and are limited to the expression of EBNA1, LMP1, and LMP2. LCL-mediated T cell expansion predominantly leads to the induction of T cells that are specific for epitopes found in EBNA3 and in most instances generate only a small percentage of cells specific for the LMP antigens or EBNA1. Therefore strategies aimed at using CTL-based adoptive immunotherapy or vaccination to treat EBV-associated HL and NPC need to capable of generating T cells that are primarily specific for the LMP antigens.

Latent membrane proteins 1 and 2 play a significant role in activating and transforming B cells following infection, allowing proliferation and survival of latently infected cells. The LMP antigens are thus oncogenic by nature; the sequences that encode this oncogenic phenotype have been identified. Furthermore, both LMP1 and LMP2 are poorly immunogenic, presumably because of poor processing and the subsequent limited amount of antigen available for presentation by MHC class I molecules. Therefore, vaccines directed against LMP1 and LMP2 are ideally suited to the use of minimal $CD8^+$ T cell epitopes, avoiding any adverse consequences associated with the oncogenicity and poor immunogenicity of the LMP antigens.

One current vaccine under development to treat EBV-associated HL and NPC consists of a modified adenoviral delivery vector encoding a polyepitope containing epitopes from both LMP1 and LMP2 (see Table 5.1). [18,19]. The capacity to expand CTL from the blood of both healthy donors and patients in the acute stages of both HL and NPC, against peptides encompassing the majority of epitopes encoded by the polyepitope, demonstrates that polyepitope technology can be used effectively to activate LMP-specific T cells, including those from patients whose *ex vivo* LMP-specific responses are suppressed. Furthermore, evidence gained following comparison with LCL-mediated expansion clearly demonstrated, in both

TABLE 5.1. Application of Polyepitope Technology to Human Diseases

Disease	Vaccine	Target Antigens	Clinical Stage	Reference
Melanoma	polyMEL	gp100, MAGE-1, MAGE-3, Tyrosinase Melan-A/MART-1,	Phase I/II	www.nrr.nhs.uk/ViewDocument.asp?ID=N0436125613 [24]
HL and NPC	E1-LMPpoly	EBV–LMP1 & 2 and EBNA1	Preclinical	[19]
Breast cancer	polyHER2neu	HER-2	Phase I	www.nrr.nhs.uk/ViewDocument.asp?ID=N0436165563
HIV	EP HIV-1090	Gag, Pol, Env, Nef, Rev, Vif,	Phase I	www.clinicaltrials.gov/ct/show/NCT00141024 [25]
Malaria	ME-TRAP	LSA1, CSP, TRAP, STARP, LSA3, EXP1	Phase I	http://controlled-trials.com/ISRCTN05221133/ISRCTN05221133 [26,27]
Hepatitis B	Undisclosed		Phase I	http://www.innogenetics.com/site/therapeutics.html
Hepatitis C	Undisclosed		Preclinical	http://www.innogenetics.com/site/therapeutics.html
HPV/ Cervical cancer	Undisclosed		Preclinical	http://www.innogenetics.com/site/therapeutics.html

healthy individuals and HL patients that the LMP polyepitope offers a significant advantage by targeting antigen-specific T cells, thus optimizing the production of CTL capable of recognizing malignant cells (Fig. 5.2). The capacity to produce the LMP polyepitope as a single recombinant product, capable of generating specific CTLs against the required epitopes, offers an attractive more cost-effective alternative to the use of single-peptide-based expansion strategies to generate CTL for immunotherapy, which require the production of individual peptides tailored to the HLA-type choice (Fig. 5.3).

In addition to the use of LMP in the expansion of CTL for immunotherapy, therapeutic vaccination with the LMP polyepitope offers an alternative strategy for the treatment of EBV-associated malignancies. With the advent of HLA transgenic mice, the immunogenicity of the LMP polyepitope can be assessed following vaccination. Studies using vaccination of HLA A2/kb transgenic mice have shown that polyepitope-based immunization strategies can be used to generate LMP-specific CTL [18] (see Fig. 5.1), indicating that vaccination with the LMP polyepitope may offer an alternative therapy for treatment of EBV-associated HL and NPC. Evidence gained from studies using the LMP polyepitope clearly demonstrates that the identification of minimal CTL epitopes and subsequence construction of a polyepitope can be employed effectively to specifically target tumor-associated antigens that are both oncogenic and poorly immunogenic, across a broad range of HLA types using a single vaccine vector.

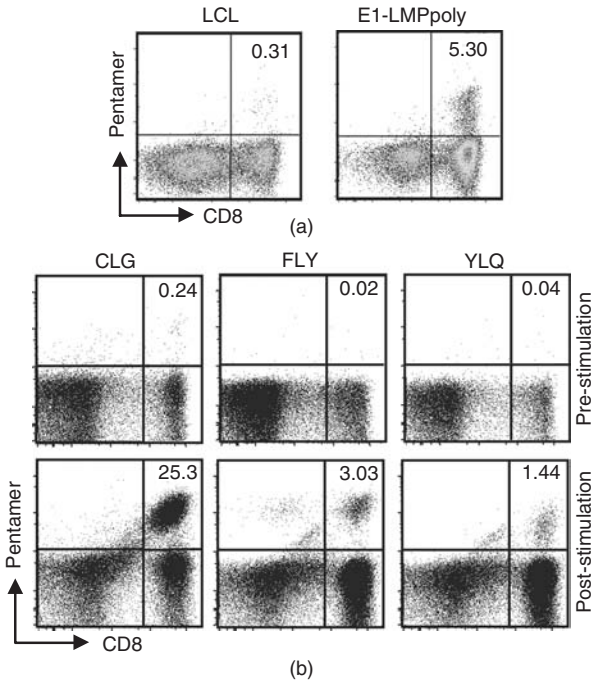

Figure 5.2. In vitro expansion of EBV latent membrane protein-specific T cells using a recombinant polyepitope vaccine: (a) comparison of T cell expansion following stimulation with autologous LCL or a polyepitope vaccine encoding EBV epitopes (referred to as *E1-LMPpoly*), showing MHC-pentamer-positive $CD8^+$ T cells stimulated with LCL or E1-LMPpoly; (b) expansion of multiple epitope-specific $CD8^+$ T cells following E1-LMPpoly stimulation, showing MHC-pentamer positive $CD8^+$ T cells pre- and poststimulation with E1-LMPpoly.

It still remains to be determined whether LMP polyepitope generated CTL have the capacity to provide protection against EBV-associated HL and NPC. However, it has become clear that polyepitope technology can be employed effectively to generate CTL across a broad range of HLA types and targeting antigens that are typically oncogenic and poorly immunogenic. Polyepitope technology should offer an attractive option for the treatment of any number of human malignancies once effective therapeutic strategies that employ CTL, via either vaccination or adoptive immunotherapy, have been developed.

5.7 CLINICAL STUDIES ON POLYEPITOPE VACCINE FOR OTHER HUMAN DISEASES

Having established the concept of polyepitope vaccine in EBV-associated diseases, a number of other groups have extended the use of this technology to other

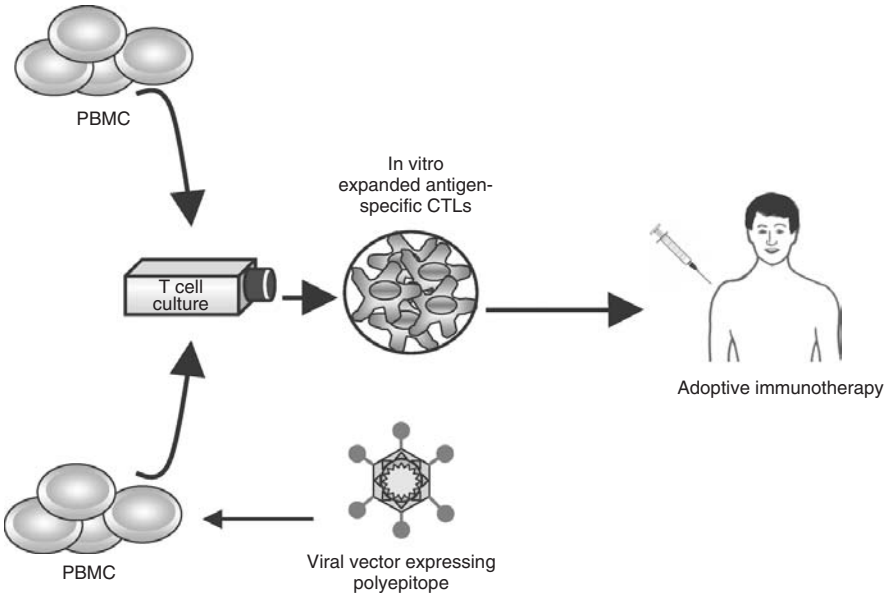

Figure 5.3. T-cell-based adoptive immunotherapy for human malignancies using polyepitope technology. PBMC from the patient are stimulation with autologous PBMC infected with a replication-deficient viral vector encoding the polyepitope. Following stimulation these T cells are assessed for antigen specificity and stored for future adoptive immunotherapy.

human diseases, include infectious pathogens (e.g., HIV, HBV, HCV, malaria) and human malignancies (e.g., melanoma, breast cancer and cervical cancer) (see Table 5.1). Preclinical studies on peripheral blood lymphocytes from melanoma patients have shown that stimulation of T cells with a polyepitope vaccination in combination with autologous dendritic cells induced CTL responses specific for up to six of six HLA-A2 epitopes. These observations were consistent with earlier studies in an HLA-A2 transgenic murine model that showed that 7 of 10 HLA A2-restricted epitopes induced CTL responses after vaccination with a polyepitope vaccine-based melanoma-associated antigen. Smith and colleagues reported that one of the potential limitations of polyepitope technology was that repeated stimulation with melanoma polyepitope vaccine resulted in the expansion of an immunodominant T cell response specific for a single epitope. These authors proposed that the selective expansion of a narrowly focused T cells may be the relative difference in the MHC avidity of each epitope, the effect of precursor frequency, more efficient liberation of dominant epitopes, and suppression of subdominant antigen-specific T cells by those specific for immunodominant epitopes. To overcome this problem we propose that the polyepitope vaccine for human application should include multiple epitopes restricted through different HLA types rather than through a single MHC allele. A phase I study on AJCC stage IV

or inoperable stage III malignant melanoma, based on the melanoma polyepitope vaccine, was recently completed, however detailed results are not yet available (http://www.nrr.nhs.uk/ViewDocument.asp?ID=N0436125613). It is anticipated that the results from this trial will be available in early 2007. Another clinical study currently under way is a phase I trial based on a polyepitope DNA vaccine encoding HER2 epitopes for the treatment of breast cancer. HLA-A2-positive patients with metastatic breast cancer who have failed conventional treatment are being recruited for this study. This study is aimed at finding the maximum tolerated dose of DNA vaccination with HER2 polyepitope and to assess its toxicity and safety. This study is expected to be completed in February 2008 (http://www.nrr.nhs.uk/ViewDocument.asp?ID=N0436165563). Finally, a polyepitope vaccine based on T cell epitopes as an immunotherapy vehicle for HPV in chronically infected patients is being developed by the Innogenetics group (http://www.innogenetics.com/site/therapeutics.html) in collaboration with Pharmexa-Epimmune. This biotech company is also actively working on a preclinical model to determine the most appropriate polyepitope vaccine design for the treatment of cervical cancer.

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6

ANTIGEN-SPECIFIC CANCER IMMUNOTHERAPY: HPV-ASSOCIATED CERVICAL CANCER AS A MODEL SYSTEM

Shaw-Wei D. Tsen, Chien-Fu Hung, and T.-C. Wu

6.1 INTRODUCTION

Cancer remains one of the leading causes of death in developed countries, largely owing to the poor prognosis of late-stage malignancies. Indeed, the development of successful treatments for these late-stage cancers has proved difficult. While conventional treatments such as radiation therapy and chemotherapy are well established, these therapies generally have low specificity for tumors and thus can rarely be used to control advanced or metastatic disease.

Cancer immunotherapy, on the other hand, is an emerging new form of treatment with the potential to selectively and effectively eradicate systemic tumors at multiple sites in the body. Significant advancements in our knowledge of tumor immunology since the mid-1990s have made possible the design of therapeutic vaccines that aim to eliminate precancerous lesions and/or malignant tumors by generating cellular immune responses against neoplastic cells. This approach, termed *antigen-specific cancer immunotherapy*, has become an attractive treatment strategy because the generation of antigen-specific T cells allows for killing of

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tumors with minimal side effects. Strategies for eliciting antigen-specific antitumor immune responses not only are relevant to one cancer system but also can be applied to other systems with known tumor-associated antigens. Thus, a good model for antigen-specific cancer immunotherapy, in particular a model with true tumor-specific antigens such as virus-associated cancers, can potentially be used to generate useful information that may impact treatment strategies for a wide variety of cancers.

In this regard, human papillomavirus (HPV)-associated cervical cancer represents one of the best models for the development of antigen-specific cancer immunotherapy. Cervical cancer is the second most common cancer in women, causing approximately 250,000 deaths worldwide each year. More than 99% of all cervical cancers are caused by infection with high-risk HPV types such as HPV-16 and HPV-18, and cells that are transformed by HPV infection consistently express two viral oncoproteins, E6 and E7, which represent true tumor-specific antigens. Because E6 and E7 are critical for the induction and maintenance of cellular transformation in HPV-infected cells, it is unlikely that the tumor cells can escape immune attack through antigen loss. In addition, since E6 and E7 are foreign proteins, immunization against HPV-associated tumors circumvents some common cancer vaccine–associated problems such as tolerance and autoimmune response. Therefore, therapeutic vaccines targeting E6 and E7 represent an ideal approach for the control of cervical cancer and may have a significant impact on HPV-associated malignancies worldwide. The current approaches for therapeutic HPV vaccine development include peptide- and protein-based, live-vector-based, nucleic acid-based, and cell-based vaccination (see Fig. 6.1) (for review, see Ref. [1]). Table 6.1 summarizes the advantages and disadvantages of the various forms of vaccine.

Of the various approaches, DNA vaccines have become a particularly attractive strategy for therapeutic vaccination against HPV. Plasmid DNA is relatively safe and easy to manufacture, and can be used to sustain high levels of antigen expression in cells. Furthermore, DNA vaccines do not elicit neutralizing antibody production in the patient, and thus can be repeatedly administered to the same patient effectively. Since naked DNA lacks cell-type specificity, it is important to find an efficient route for the delivery of DNA vaccines into target cells. Another concern with DNA vaccines is that they are poorly immunogenic, because DNA lacks the intrinsic ability to amplify or spread from transfected cells to surrounding cells in vivo. The potency of DNA vaccines may be improved by targeting DNA to professional antigen-presenting cells (APCs) and by modifying the properties of antigen-expressing APCs to enhance the antitumor immune response elicited by the vaccines.

It is well known that professional APCs, especially dendritic cells (DCs), are the primary initiators of antitumor immunity. DCs, the most potent professional APCs, can present antigens to naive $CD8^+$ and $CD4^+$ T cells and activate them to become armed effector T cells (see Fig. 6.2). Immature DCs are equipped to capture antigens and subsequently undergo a maturation process in order to efficiently

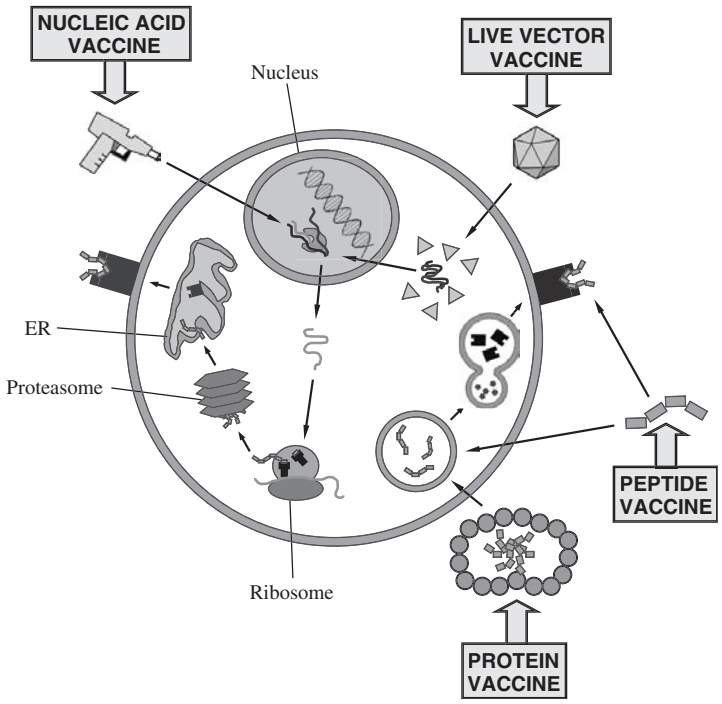

Figure 6.1. **Delivery of the various forms of vaccines into APCs**. Nucleic acid vaccines can be delivered via gene gun directly into the nucleus of APCs. The DNA or RNA is expressed, and the cytosolic protein product is degraded into peptides that are presented via the MHC class I pathway. Live viral vectors, such as adenoviruses, can infect the cell and deliver nucleic acid into the nucleus; the antigen is then processed and presented like a nucleic acid vaccine. Antigenic peptides can either enter the cell in vesicles and be presented via the MHC class II pathway or be targeted for immediate presentation on the cell surface. Protein antigens, which can be encapsulated in liposome delivery systems, enter the cell in vesicles and are degraded into their constituent peptides. These peptides are then presented in association with MHC class II molecules.

present the antigens on the cell surface. These DCs migrate to the lymphoid organs and become potent activators of antigen-specific T cells.

In the context of DNA vaccines, delivery of DNA-encoding tumor antigens to DCs allows for the stimulation of tumor antigen-specific T cells and the generation of tumor antigen-specific immune responses. Thus, to enhance the potency of DNA vaccines we have focused on (1) increasing the number of antigen-expressing DCs; (2) improving antigen expression, processing, and presentation in DCs; and (3) enhancing DC interaction with T cells during T cell priming to augment T-cell-mediated immune responses.

TABLE 6.1. Characteristics of Different Therapeutic HPV Vaccines

Vaccine	Advantages	Disadvantages
Peptide-based	Safe, easy to produce, and stable	Poorly immunogenic and may not be universally effective
Protein-based	Safe, easy to produce, and stable	Poorly immunogenic
Live-vector-based	Highly immunogenic	Potentially toxic, may not be effective in repeat vaccinations, and potency may be influenced by preexisting immunity
DNA-based	Easy to produce, stable, and may be administered repeatedly	Poorly immunogenic and potentially oncogenic
RNA-based	Safe and may be administered repeatedly	Unstable
DC-based	Highly immunogenic	Labor-intensive, costly, and may vary in quality
Tumor-cell-based	Naturally express tumor antigens	Potentially unsafe, labor-intensive, costly, and may vary in quality

6.2 STRATEGIES TO ENHANCE DNA VACCINE POTENCY

Strategies for Increasing the Number of Antigen-Expressing DCs

One method for boosting antigen-expressing DC populations is to find the most effective routes for the delivery of DNA vaccines. Among the different routes of DNA administration, we have concluded that vaccination via gene gun is one of the most potent methods for the delivery of genes of interest into DCs [2]. The gene gun is used to fire DNA-coated gold particles into the epidermis and efficiently transfect intradermal DCs that can mature and migrate to the lymphoid organs for T cell priming. Thus, gene gun delivery of DNA represents a convenient and effective method for the in vivo introduction of naked DNA into DCs.

Another strategy for increasing the number of antigen-expressing DCs is to promote the spread of an encoded antigen between DCs by linking the antigen with proteins capable of intercellular transport. We have investigated the use of DNA encoding HPV-16 E7 fused to herpes simplex virus type 1 VP22 (HSV-1 VP22), a viral protein with intercellular trafficking properties, in a DNA vaccine. In vivo experiments showed that the vaccine dramatically enhanced E7-specific $CD8^+$ T cell responses and generated greater antitumor effects than did DNA vaccines encoding E7 alone [3]. We then generated a vaccine encoding E7 linked to Marek's disease virus type 1 VP22 (MDV-1 VP22), a protein with some homology to HSV-1 VP22, and observed powerful vaccine-elicited antitumor immunity as well [4].

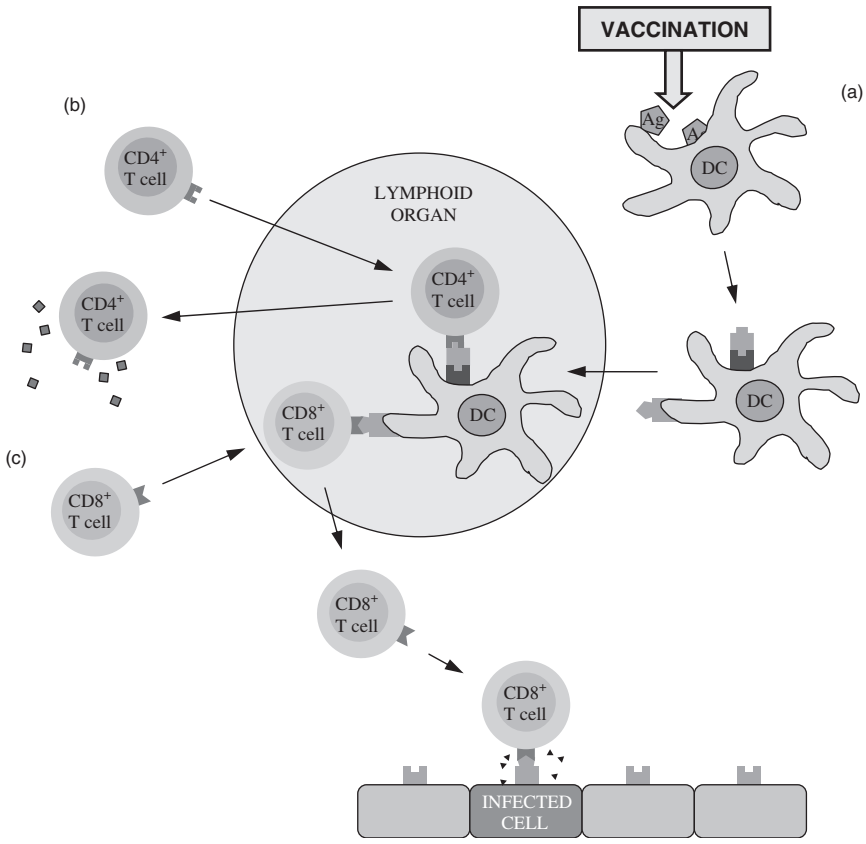

Figure 6.2. **Generation of antigen-specific immune responses**. (a) On antigen uptake, intradermal DCs undergo a maturation process and present the antigen on MHC class I and/or class II molecules on the cell surface. The mature DCs then migrate to the lymphoid organs for T cell priming. (b) Naive $CD4^+$ T cells enter the lymphoid organs from the systemic circulation and are activated through interactions between T cell receptor and MHC class II molecule/antigen complexes. The armed effector $CD4^+$ T cells then secrete cytokines and other immunostimulants to enhance $CD8^+$ T cell function. (c) Circulating naive $CD8^+$ T cells enter the lymphoid organs and are primed through interactions between T cell receptor and MHC class I molecule/antigen complexes. The armed effector $CD8^+$ T cells can then target and destroy infected cells expressing the antigen.

Other methods for improving antigen-expressing DC populations include the linkage of an antigen to molecules that can target the antigen to the surface of DCs, such as Fms-like tyrosine kinase 3 (flt3) ligands that bind to flt3 receptors on DCs [5], and heatshock proteins (hsp) that bind to scavenger receptors on DCs such as CD91 [2].

Strategies for Enhancing Antigen Expression, Processing, and Presentation in DCs

A strategy termed *codon optimization* has been developed for improving antigen expression in DCs. This technique is used to modify antigen gene sequences by replacing rarely used codons with more commonly recognized codons, and can enhance translation of a DNA vaccine in DCs. Codon optimization has been shown to increase levels of HPV E7 expression in transfected cells [6], and mice vaccinated with either codon-optimized E6 DNA [7] or codon-optimized E7 DNA [6] showed better immune responses than did mice vaccinated with wild-type E7 DNA.

Once antigens are expressed, DCs must present the antigens through the MHC class I pathway in order to generate populations of antigen-specific $CD8^+$ T cells. Linkage of an antigen to proteins that target the antigen to the endoplasmic reticulum or facilitate proteasome degradation, for example, can lead to enhanced MHC class I antigen presentation and greater ensuing CTL responses (see Fig. 6.3). We have demonstrated the potent effects against E7-expressing tumors of vaccines encoding E7 linked to various such MHC class I–targeting proteins and protein domains, including *M. tuberculosis* hsp70 [8], γ-tubulin [9], and the translocation domain of *Pseudomonas aeruginosa* exotoxin A [10]. Among these strategies, we found that a DNA vaccine containing E7 linked to CRT, a protein shown to significantly enhance MHC class I antigen presentation, elicited the greatest E7-specific $CD8^+$ T cell responses among all the DNA vaccines that we tested [11]. These findings suggest that the DNA vaccine employing CRT for the generation of potent antigen-specific immune responses may be suitable for further clinical translation.

An effective strategy for circumventing antigen processing and for eliciting stable MHC class I presentation of a peptide encoded by a DNA vaccine is the employment of MHC class I single-chain trimer (SCT) technology. This technique involves the linkage of an antigenic peptide to β2-microglobulin and MHC class I heavy chain, producing a single-chain construct encoding an MHC class I molecule fused to the peptide antigen. It has been shown that immunization of mice with a DNA vaccine encoding a SCT composed of an immunodominant CTL epitope of HPV-16 E6, β2-microglobulin, and H-$2K^b$ MHC class I heavy chain (viz., pIRES-E6- β2 m-K^b) could generate increased E6 peptide-specific $CD8^+$ T cell responses compared to mice vaccinated with DNA encoding wild-type E6 [12].

On the other hand, fusion of an antigen to MHC class II–targeting molecules can redirect the antigen to the class II pathway and result in the generation of greater $CD4^+$ T cell responses (for review, see Ref. 13). These $CD4^+$ T cell responses facilitate the activation of antigen-specific $CD8^+$ T cell immune responses (see Fig. 6.3). We have previously shown that linkage of an antigen to a signal peptide (Sig) and the lysosomal targeting domains of lysosome-associated membrane protein 1 (LAMP-1) can enhance MHC class II antigen presentation of the linked antigen and generate significant antigen-specific $CD4^+$ T cell responses [14]. This construct, namely, Sig/E7/LAMP-1, was tested in the context of a DNA vaccine and produced greater numbers of E7-specific $CD4^+$ T cells and also higher E7-specific CTL activity in mice than did vaccines composed of Sig/E7 or wild-type E7 DNA alone [15].

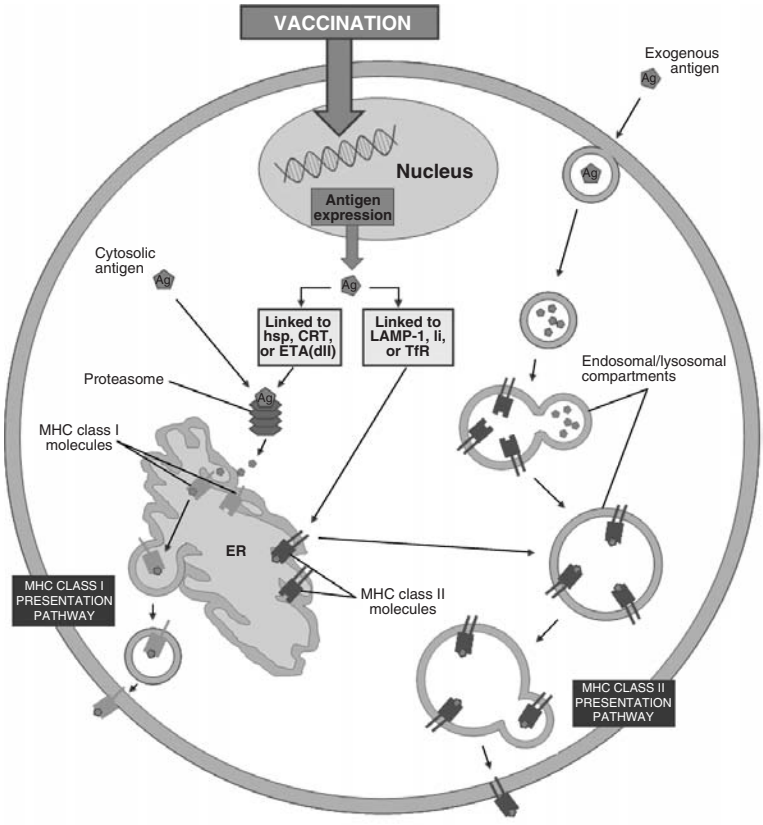

Figure 6.3. **Enhancement of antigen processing and presentation in APCs**. Fusion of an antigen with molecules that target the antigen to the endoplasmic reticulum (ER) can enhance MHC class I presentation, while fusion of the antigen with endosomal/lysosomal-targeting molecules improves class II presentation. *Abbreviations*: hsp—heatshock protein; CRT—calreticulin; ETA(dII)—*Pseudomonas aeruginosa* exotoxin A (domain II); LAMP-1—lysosome-associated membrane protein type 1; Ii—MHC-II-associated invariant chain; TfR—transferrin receptor.

In addition, the MHC class II–invariant chain (Ii) has been employed in the context of a DNA vaccine to effectively enhance class II presentation of antigens in DCs. In the endoplasmic reticulum (ER), Ii binds with MHC class II molecules and the class II–associated Ii peptide (CLIP) region of the Ii chain occupies the class II peptide-binding groove, preventing premature binding of antigenic peptides into the groove. In the endosomal/lysosomal compartments, CLIP is replaced by an antigenic peptide and the MHC class II/peptide complex is presented on the surface of the DC. By substituting the CLIP region of Ii with a desired T helper (Th) epitope such as the pan HLA-DR binding epitope (PADRE), the PADRE epitope can be efficiently presented via the class II pathway in DCs for activation

of PADRE-specific $CD4^+$ T cells. Mice vaccinated with a DNA vaccine encoding Ii-PADRE were shown to generate significant PADRE-specific $CD4^+$ T cell immune responses. Furthermore, coadministration of DNA encoding E7 and DNA encoding Ii-PADRE in mice was shown to elicit potent $CD8^+$ T cell responses compared to coadministration of DNA encoding E7 and DNA encoding unmodified Ii (Hung, personal communications).

Strategies for Enhancing DC–T Cell Interaction

Once an antigen has been processed and presented, the interactions between DCs and T cells become critical for T cell activation. After T cell priming, DCs become targets of activated armed effector T cells and are susceptible to T-cell-mediated apoptosis. To prevent this, DNA encoding antiapoptotic proteins can be used to prolong DC survival and enhance the long-term ability of DCs to prime T cells. In our studies, codelivery of DNA encoding E7 with DNA encoding inhibitors of apoptosis such as BCL-xL, BCL-2, XIAP, and dominant-negative caspases was shown to enhance E7-specific $CD8^+$ T cell responses in mice [16]. In addition, combining strategies to enhance antigen presentation with strategies to prolong DC life was shown to further improve the antigen-specific CTL responses elicited by DNA vaccines [17–19]. The introduction of DNA encoding antiapoptotic proteins into cells, however, raises concerns of oncogenicity; inhibition of proapoptotic proteins using RNA interference (RNAi) may potentially alleviate these problems. We have demonstrated that coadministration of DNA vaccines encoding E7 with short interfering RNA (siRNA) targeting the key proapoptotic proteins Bak and Bax was able to effectively improve DC resistance to apoptotic cell death and enhance antitumor $CD8^+$ T cell responses in mice [20].

The activation of naive antigen-specific T cells is dependent on signals delivered by DCs to T cells, such as costimulatory factors and cytokines. Numerous studies employing strategies to augment these signals have shown enhanced antigen-specific immune responses. Examples include coadministration of DNA vaccines with DNA encoding granulocyte–macrophage colony-stimulating factor (GM-CSF) [21], IL-2 [22], IL-12 [23], or IL-18 [24]. In addition, coadministration of plasmid DNA encoding costimulatory molecules such as B7 with DNA vaccines can also lead to more efficient priming of naive antigen-specific T cells [25].

Clinical Progress in DNA Vaccine Development

With their general success in preclinical testing, several DNA vaccines have translated into human clinical trials. A microencapsulated DNA vaccine, namely, ZYC-101, which encodes multiple HLA-A2-restricted E7-derived epitopes, has been tested in patients with CIN-2/3 lesions [26]. The vaccine was well tolerated, and induced HPV-specific T cell responses in several immunized patients. A new version of the vaccine, namely, ZYC-101a, encodes HPV-16 and HPV-18 E6- and E7-derived epitopes and was shown to resolve CIN-2/3 lesions in a subset of young

women enrolled in the trial [27]. At the Johns Hopkins Hospital, a phase I trial using a DNA vaccine encoding a modified form of HPV-16 E7 (with an abolished Rb binding site) linked to *M. tuberculosis* hsp70 [pNGVL4a-Sig/E7(detox)/hsp70] was performed in patients with CIN-2/3 lesions. The vaccine was well tolerated by all patients, and some of the patients who received the maximum dose of the DNA vaccine (4 mg/vaccination) showed detectable E7-specific $CD8^+$ T cell immune responses (Trimble, personal communications). This DNA vaccine has also been tested in a subset of HPV-16^+ patients with head and neck squamous cell carcinoma. In addition, these investigators have planned to initiate a phase I trial with a DNA vaccine encoding modified HPV-16 E7 DNA linked to CRT in patients with stage 1B1 cervical cancer, using gene gun delivery (Trimble, personal communications). These DNA vaccine clinical trials, as well as other therapeutic HPV vaccine clinical trials, are listed in Table 6.2.

Although HPV DNA vaccines are considered to be safe in comparison to other forms of vaccines such as viral vector-based vaccines, some concerns have been raised that must be addressed before DNA vaccines can be routinely used in the clinical arena. For example, DNA may potentially integrate into the host cell genome, causing genomic instability. To date, however, integration of DNA in treated organs or tissues has not been demonstrated. Vaccination with E6 DNA and/or E7 DNA also has the potential concern of cellular transformation since E6 and E7 are virus-encoded oncoproteins. Modification of E6 or E7 to abolish the transformative capacity of these proteins may alleviate these concerns. Overall, DNA-based vaccination is considered to be a safe and effective means for controlling cervical cancers and other HPV-associated malignancies.

6.3 SUMMARY AND CONCLUSIONS

One of the major challenges in cancer treatment is the selective targeting and killing of tumors. In this regard, antigen-specific cancer immunotherapy represents an attractive approach to cancer treatment. Among the cells of the immune system, T cells are primarily responsible for the control of cancers, and therefore activation of these cells is crucial for generating antitumor immunity. Activation of large populations of tumor-specific T cells allows for effective killing of antigen-expressing tumors with few side effects. Our research focuses on vaccination against human papillomavirus (HPV), the primary cause of human cervical cancer. This cancer system is an ideal model for the development of therapeutic vaccines owing to the clear viral etiology of cervical cancer and the constitutive expression of HPV-encoded tumor-specific antigens, such as E6 and E7, in cervical cancers. Specifically, we have focused on enhancing the potency of DNA-based therapeutic HPV vaccines by developing strategies for antigen delivery to antigen-presenting cells (APCs), improving antigen presentation by APCs, and enhancing dendritic cell (DC)–T cell interactions during T cell priming. These strategies can potentially be applied to other cancer systems with known tumor-associated antigens, and thus this research has broad implications in antigen-specific cancer immunotherapy and may be useful

TABLE 6.2. Therapeutic HPV Vaccine Clinical Trials

Vaccine Type	Developer	Target Subtype(s)	Vaccine Construct	Study Subjects	Reference
Live-vector-based (TA-HPV)	Xenova	HPV-16/18	Recombinant vaccinia vector encoding E6/E7 fusion protein	Phase I in patients with early-stage cervical cancer	[28]
				Phase II in patients with advanced-stage cervical cancer	[29]
				Phase II in patients with high-grade VIN or VgIN	[30]
				Phase II in patients with high-grade VIN	[31]
Live-vector-based (MVA-E2)	Instituto Mexicano del Seguro Social (IMSS)	HPV-16/18	Recombinant vaccinia vector encoding E2	Phase I/II in patients with CIN-1, CIN-2, or CIN-3	[32]
				Phase II in patients with CIN-2 or CIN-3	[33]
Peptide-based	Netherlands National Institute of Public Health and Environmental Protection	HPV-16	E7 epitopes (aa11–22 and aa86–93) and PADRE	Phase I/II in patients with cervical cancer	[34]
				Phase I/II in patients with advanced-stage cervical cancer	[35]
Peptide-based	National Cancer Institute (NCI)	HPV-16	E7 epitopes (aa12–20 or aa12–20 and aa86–93 and helper T cell epitope)	Phase I in patients with CIN-2, CIN-3, VIN-2, or VIN-3	[36]

Peptide-based	NCI	HPV-16	Lipidated E7 epitope (aa86–93)	Phase I in patients with cervical cancer or vaginal cancer	[37]
Protein-based (PD-E7/D16E7)	GlaxoSmith-Kline	HPV-16	E7 linked to *Hemophilus influenzae* protein D (aa1–108)	Phase I/II in patients with CIN-1 or CIN-3	[38]
Protein-based (SGN-00101)	StressGen Biotechnologies	HPV-16	E7 linked to *Mycobacterium bovis* hsp65	Phase I/II in HIV^+ patients with high-grade AIN	[39]
				Phase II in patients with AGW or RRP	[40]
				Phase III in patients with anal dysplasia	[41]
DNA-based (ZYC-101)	MGI Pharma, Inc.	HPV-16	DNA-encoding E7 (aa83–95)	Phase I in patients with anal HSIL	[42]
				Phase I in patients with CIN-2 or CIN-3	[26]
DNA-based (ZYC-101a)	MGI Pharma, Inc.	HPV-16/18	DNA encoding E6 and E7 epitopes	Phase II in patients with CIN-2 or CIN-3	[27]

(*continued overleaf*)

TABLE 6.2. (*Continued*)

Vaccine Type	Developer	Target Subtype(s)	Vaccine Construct	Study Subjects	Reference
DNA-based	NCI	HPV-16	E7 contained in pNGVL4a-Sig/E7 (detox)/hsp70 plasmid	Phase I in patients with CIN-2 or CIN-3	(Dr. C. Trimble, personal communication)
DNA-based	NCI	HPV-16	E7 contained in pNGVL4a-Sig/E7 (detox)/hsp70 plasmid	Phase I in patients with advanced-stage HNSCC	(Dr. M. Gillison, personal communication)
DC-based	N/A	HPV-16	DCs pulsed with HPV^+ tumor lysate (E6 and E7)	Phase Ib in patients with advanced-stage cervical cancer	[43]
DC-based	N/A	HPV-18	DCs pulsed with E7	Case report on a patient with cervical cancer	[44]
Prime-boost (TA-CIN, TA-HPV)	N/A	HPV-16/18	Prime with HPV-16 E6/E7/L2 fusion protein (TA-CIN), boost with recombinant vaccinia vector encoding HPV-16/18 E6/E7 (TA-HPV)	Phase II in patients with VIN-3 or VgIN-3	[45]

[a] *Abbreviations*: aa—amino acid; AGW—anogenital warts; AIN—anal intraepithelial neoplasia; CIN—cervical intraepithelial neoplasia; DC—dendritic cell; HNSCC—head–neck squamous cell carcinoma; HPV—human papillomavirus; HSIL—high-grade squamous intraepithelial lesion; hsp—heatshock protein; RRP—recurrent respiratory papillomatosis; VgIN—vaginal intraepithelial neoplasia; VIN—vulval intraepithelial neoplasia.

for the development of treatment strategies for cervical cancer and for a wide variety of other cancers. Although the synthesis of tumor-specific neutralizing antibodies by B cells also constitutes an important arm of the antitumor immune response, in this chapter we limit our discussion exclusively to T-cell-mediated immunotherapy.

The identification of HPV as the primary etiologic agent of human cervical cancer indicates that vaccination against HPV can help prevent or treat cervical cancer and its precursor lesions. Successful prophylactic vaccines have been developed to prevent the initial viral infection; however, these preventive vaccines may not protect against all HPV infections, and they likely cannot eliminate the established HPV-associated cervical cancers that currently affect millions of women worldwide. Thus, effective therapeutic HPV vaccines are needed for the treatment of HPV-associated cervical cancer and most importantly for the control of lethal late-stage malignancies.

For the development of therapeutic HPV vaccines, we and others have focused on identifying and targeting the most relevant antigens associated with cervical cancer, the E6 and E7 HPV oncoproteins, which represent tumor-specific antigens and are potentially ideal targets for therapeutic HPV vaccines. From our studies and those of others in the field, we conclude that the various current approaches, including peptide- and protein-based, live-vector-based, nucleic acid–based, and cell-based immunization, are each associated with strengths and weaknesses (see Table 6.1), and therefore it is difficult to make a judgment regarding the most ideal strategy for vaccination against HPV.

On the other hand, DNA vaccines represent a relatively safe, stable, and promising strategy for the control of HPV-associated malignancies. Our ongoing efforts to enhance the potency of DNA vaccines include the use of novel delivery systems for the targeting of antigens to DCs, the enhancement of antigen presentation by DCs, and the improvement of cellular interactions between DCs and T cells. These strategies for generating strong immune responses against antigen-expressing tumors are applicable not only to the treatment of HPV-associated malignancies but also to other systems with identified tumor-associated antigens. Thus, this research has broad implications in antigen-specific cancer immunotherapy and may contribute to the development of effective treatments for cervical cancer and for a wide variety of other cancers.

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7

POXVIRAL VECTORS FOR CANCER VACCINES: STATE OF THE ART

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7.1 INTRODUCTION

While the use of viruses for cancer therapy is not a new idea, it was not until recently that viral vectors have been engineered to be more effective for use in clinical trials. While adenoviruses and poxviruses have been used for many years, new approaches using these and other viruses have emerged. While the use of other viruses as cancer vaccines are addressed, here, the focus of this chapter is on the generation of several different poxvirus vectors and their use in clinical disease settings and with current standard-of-care therapies. The review material presented here has been taken from previously published papers where indicated.

7.2 VIRAL VECTORS FOR CANCER THERAPY

Adenoviruses have long been used as a gene therapy vehicle for the treatment of cancer and other diseases [1–3]. Several approaches using adenoviruses encoding

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tumor-associated antigens (TAAs) have been used in clinical trials. A phase I study was conducted with a recombinant adenovirus expressing the melanoma antigens MART1 or gp100, and were administered to patients with metastatic melanoma [4]. Both vaccines were determined to be safe, and one complete response was observed after vaccination. In this study 16% of patients showed a clinical response after vaccination, but it could not be determined whether these responses were due to the vaccine or to the cytokine IL-2, which was also given along with vaccine. Currently, a phase I clinical trial is under way using a recombinant adenovirus engineered to express prostate-specific antigen (PSA) for the treatment of metastatic prostate cancer [5]. One phase II clinical trial used an adenovirus expressing p53 along with radiation in patients with nonmetastatic non–small cell lung cancer [6]. Of the 19 patients, 1 had a complete response, 11 of 19 had a partial response, and 3 of 19 had stable disease. This adenoviral vector is now being used for other tumor types, including a phase III trial for the treatment of head–neck cancer.

More recently, the use of RNA viruses for cancer therapy has been explored. Preclinical models using members of the alphavirus family (Semliki forest virus, Sindbis virus, and Venezuelan equine encephalitis virus) have demonstrated success in cancer therapy [7–11]. For example, it has been shown that intraperitoneal (IP) infection of mice with Sindbis virus results in the regression of tumors grown IP, subcutaneously (SC), intrapancreatically, and in the lungs [9]. While the preclinical results with alphaviruses look promising, their use in the clinic to determine safety and efficacy has not yet been extensively studied, and it is still unknown how these viruses will fare when introduced to patients.

The paramyxovirus Newcastle disease virus (NDV) has been more thoroughly explored as a cancer vaccine. NDV has been used in the clinic directly as a vaccine and also as an oncolysate vaccine, where tumor cells are infected with NDV and then introduced back into the patient as an autologous tumor cell vaccine [12,13]. PV701, an oncolytic strain of NDV, has been used in phase I/II clinical trials with no signs of serious side effects. In a series of three phase I trials in which 95 patients were given PV701, 10 clinical responses were observed [14]. Additionally, in a study using NDV-infected tumor cells as an autologous tumor cell vaccine in patients with advanced head–neck carcinomas, 61% of vaccinated patients were alive 5 years after treatment, which was higher than the expected survival rate of 38%, obtained from a historical cohort [15]. Multiple phase II trials using NDV are currently recruiting patients, as this virus shows promise as an effective cancer vaccine.

Poxvirus Vectors

Poxviruses have been widely used as vaccine vectors for cancer therapy [16,17]. In the clinic, poxviruses have been shown to be safe and effective at inducing immune responses (Table 7.1). While vaccinia virus is the most frequently used vector in the poxvirus family, other viruses such as canarypox and fowlpox have also had success in the clinic. The canarypox vector ALVAC has been well tolerated in several clinical trials and has been shown to increase antigen-specific T cell

responses (Table 7.1) [18,19]. The use of ALVAC is discussed in more detail in a separate chapter. Fowlpox virus has been effectively used as a heterologous boost in certain systems; this will be more thoroughly discussed below.

Vaccinia Virus

Vaccinia virus has been used for many years in the clinic without significant toxicities [16]. Because of its ability to incorporate large quantities of foreign DNA, multiple transgenes have been successfully incorporated into the vaccinia virus genome, making it an ideal vaccine vector. Additionally, proteins expressed by vaccinia virus are highly immunogenic, even more so than the vaccinia proteins themselves [16,20]. Vaccinia virus was successfully administered to over 1 billion people in the campaign to eradicate smallpox [21]. Because of this, vaccinia virus has become widely used in the clinic as a vaccine vector, especially for the purpose of cancer therapy. As such, the rest of this chapter focuses on advances and clinical trials conducted with various recombinant vaccinia vectors.

7.3 TUMOR-ASSOCIATED ANTIGENS

Tumor-associated antigens are proteins expressed specifically on tumor cells, or more frequently, self-antigens that are overexpressed on tumor cells [22,23]. The goal of cancer immunotherapy is to elicit an immune response against these TAAs, triggering T cells to directly attack and kill tumor cells. As such, many viral vectors have been created that express TAAs such as carcinoembryonic antigen (CEA) or prostate-specific antigen (PSA). Several clinical trials have been conducted using a recombinant vaccinia virus expressing a TAA, and have shown that immune responses to the TAA can be elicited in cancer patients. A phase I clinical trial was conducted in which a recombinant vaccinia virus expressing CEA (rV-CEA) was given to patients with advanced carcinoma. While no significant antitumor effect was observed, the safety of giving advanced cancer patients a recombinant vaccinia vector was established, and CTL lines generated from patients were able to lyse tumor cells expressing CEA and CEA peptide-pulsed targets in vitro (Table 7.1) [24,25].

Similarly, a phase I trial using a recombinant vaccinia virus expressing PSA (rV-PSA) was conducted in patients who had received radical prostatectomy but still had recurrent disease. As seen in the rV-CEA trial, minimal toxicity was observed (Table 7.1) [26]. In another phase I study, patients with metastatic androgen-independent prostate cancer were given rV-PSA. Again, toxicity was minimal, and an increase in the proportion of PSA-specific T cells was observed following vaccination. Additionally, T cells obtained from patients after vaccination were able to lyse tumor cells expressing PSA in vitro (Table 7.1) [27]. Thus, it has been demonstrated in several clinical trials that administration of a recombinant vaccinia virus expressing a TAA is safe and can elicit a CTL response capable of lysing tumor cells expressing the TAA.

TABLE 7.1. Cancer Vaccine Trials with Poxviruses

Viral Vector	Tumor	Trial Phase	Comments	Reference
ALVAC-B7-1 ALVAC-IL-12	Melanoma	I	Higher levels of B7-1 mRNA and intratumoral levels of VEGF and IL-10 were observed in vaccinated patients	62
ALVAC-p53	Colorectal	I/II	2 of 15 patients showed enhanced p53-specific T-cell-mediated immunity	63
ALVAC-CEA	CEA-expressing tumors	I	7 of 9 patients showed a significant increase in CEA-specific CTL precursors postvaccination	30
ALVAC-CEA-B7-1	CEA-expressing tumors	I	3 of 18 patients showed stable disease that correlated with an increase in CEA-specific T cell precursors	64
ALVAC-CEA-B7-1	CEA-expressing tumors	I	6 patients showed declines in serum CEA that lasted 4–12 weeks; all 6 showed stable disease after 4 vaccinations; HLA-A-2-positive patients showed increases in CEA-specific T cell precursor frequencies	65
ALVAC-CEA-B7-1 +/− GM-CSF	CEA-expressing tumors	I	CEA-specific T cell precursors were increased in patients receiving vaccine alone, but not vaccine plus GM-CSF	18
ALVAC-miniMAGE-1/3	Advanced cancer (mostly melanoma)	I/II	1 of 30 melanoma patients showed a partial response; 2 melanoma patients showed stable disease; 3 of 4 patients with tumor regression showed a CTL response against MAGE-3	66
ALVAC(2)-gp100 m	Melanoma	I	8 of 18 patients showed a significant increase in gp100-specific CTL frequencies	67
Vaccinia (VV/MUC-1/IL-2)	Prostate	I	1 patient showed an objective clinical response	68
Vaccinia (expressing MUC1 and IL-2)	Breast	I	2 of 28 patients showed a partial response; 15 of 28 patients showed stable disease	49
Vaccinia (rV-CEA(6D)-TRICOM)	CEA-expressing tumors	I	40% of patients showed stable disease for at least 4 months; increased 2-year survival with patients given vaccine + GM-CSF as compared to vaccine alone	48
Vaccinia (rV-CEA and avipox-CEA)	CEA-expressing tumors	I	Greater T cell responses were seen when rV-CEA was given as the prime instead of avipox-CEA; GM-CSF and IL-2 further increased the CEA-specific T cell response	69

TABLE 7.1. (*Continued*)

Viral Vector	Tumor	Trial Phase	Comments	Reference
Vaccinia (rV-CEA)	CEA-expressing tumors	I	T cells from patients shown to lyse tumor cells expressing CEA in vitro	25
Vaccinia (rV-PSA)	Prostate	I	Increase in PSA-specific T cells; T cells from patients shown to lyse tumor cells expressing PSA in vitro	27
Vaccinia (rV-PSA; rV-B7-1)	Prostate	II	13 of 17 patients showed > 3-fold increase in PSA-specific T cells	42
Vaccinia (PROSTVAC™)	Prostate	I	Minimal toxicity; 1 patient showed very low levels of PSA (< 0.2 ng/mL) for > 8 months	26
Vaccinia (rV-PSA)	Prostate	II	78.1% clinical progression free survival; 46% of patients showed an increase in PSA-specific T cells	70
Vaccinia (rV-TRICOM)	Melanoma	I	30.7% objective response rate; 1 complete response for > 22 months	71
Vaccinia (rF-PSA and rF-B7-1)	Prostate	II	Median progression free survival with vaccine and docetaxel was 6.1 months as compared to docetaxel alone	72
Vaccinia (rV-B7-1)	Melanoma	I	1 of 12 patients showed a partial response; 2 patients showed stable disease; increased frequency of melanoma antigen-specific T cells	73
Vaccinia	Bladder	I	3 of 4 patients showed significant immune cell infiltration; these 3 patients were disease-free 4 years posttreatment	74
Vaccinia (rV-PSA) + / − GM-CSF	Prostate	I	14 of 33 patients showed stable disease for at least 6 months; patients showed a PSA-specific T cell response	75
Vaccinia (rV-GMCSF)	Melanoma	I	1 of 7 patients showed a partial response; 1 patient had a complete response	50
Fowlpox-gp100	Melanoma	II	10 of 14 patients showed reactivity to gp100	76
MVA-hTyr	Melanoma	I	Strong response to viral vector, but no measurable immune response to hTyr	77
MVA	Melanoma	I	4 of 7 patients generated CTL specific for a high-affinity melanoma-specific epitope expressed in the vaccine	78
MVA (TG4010)	MUC1-(+) solid tumors	I	4 of 13 patients showed stable disease for 6–9 months; 5 patients showed T cell immune responses	79
MVA-5 T4 (TroVax)	Colorectal	I/II	16 of 17 patients showed 5 T4-specific cellular immune responses; 5 patients showed stable disease from 3 to 18 months; positive correlation between 5 T4 antibody response and patient survival or time to progression	61

Diversified Prime and Boost

To be effective, a vaccine must be administered multiple times in order to sufficiently prime the immune response against the desired antigen. The first vaccine (the prime) should be able to induce a strong T cell response against the antigen, and additional vaccinations (boosts) should further increase the T cell response to the antigen [17]. However, if the same vector is used for the prime and subsequent boosts, the immune response against the vector may prohibit the boosts from being effective. Evidence of this was seen in the clinical trial mentioned above, in which there was no increase in CEA-specific immune responses by the third vaccination of rV-CEA [24,25]. Fowlpox (avipox) virus is an ideal choice for a boost following a vaccinia prime. Although fowlpox is replication-defective in mammalian cells, it can infect these cells and express the transgenes for 14–21 days before the cell dies [28]. In a preclinical study, it was shown that CEA-specific T cells were induced 4 times more when vaccinating with rV-CEA as a prime and boosting with avipox-CEA than when avipox-CEA was given alone 3 times [29]. To determine which prime–boost regimen to use, a clinical trial was conducted in which patients were given either rV-CEA as a prime followed by three avipox-CEA boosts (VAAA) or three avipox-CEA vaccinations followed by an rV-CEA boost (AAAV). Antigen-specific immune responses were better in the VAAA group than the AAAV group (Table 7.1) [30]. Additionally, on further follow-up, five of nine patients in the VAAA arm were still alive, while zero of nine patients were still alive in the AAAV arm; these results correlated with immune responses observed [31].

TRICOM Vectors

Induction of an immune response requires both antigen presentation in a peptide–MHC complex on an antigen-presenting cell (APC; signal 1) and T cell costimulatory molecules on the surface of the APC (signal 2) [22,32,33]. Because TAAs are normally self-antigens that are overexpressed on tumor cells, they are generally considered weak antigens, as the immune system does not respond well to an antigen that it perceives as self [34]. In the presence of a weak signal 1 such as a TAA, T cell costimulation is especially important [35]. It has been hypothesized that tumor cells can evade the immune system by not expressing sufficiently high levels of costimulatory molecules [36–38]. As such, one focus of cancer vaccines is to introduce costimulatory molecules into cancer vaccines along with TAAs to increase antitumor immune responses. B7-1 is one of the most widely studied costimulatory molecules, and preclinical studies have shown that the addition of B7-1 can increase the immunogenicity of a weakly immunogenic tumor [39–41]. Several phase II clinical trials have been conducted using an admixture of recombinant vaccinia virus vectors expressing the TAA prostate-specific antigen and B7-1 (rV-PSA and rV-B7-1, respectively) for the treatment of prostate cancer [42–44]. These trials demonstrated the safety of using these vectors in combination with radiotherapy, hormone therapy, or chemotherapy, and suggest improved clinical benefit when given with the vaccinia vectors over the respective therapy alone.

Two other important costimulatory molecules are intercellular adhesion molecule 1 (ICAM-1) and leukocyte function-associated antigen 3 (LFA-3), both of which are expressed on APCs and are involved in adhesion between the APC and T cell, thereby increasing their interaction time [22]. A recombinant vaccinia virus expressing B7-1, ICAM-1, and LFA-3 was created (designated TRICOM for *tri*ad of *co*stimulatory *m*olecules), in which each molecule primes a unique signaling pathway in T cells [45,46]. In vitro studies comparing T cell activation by cells infected with vectors containing one or all three costimulatory molecules showed that vectors containing all three molecules activated T cells to a much higher degree than that seen with vectors containing any of the molecules alone [46]. When mice were vaccinated with vectors containing CEA and either B7-1 alone (rF-CEA/B7) or TRICOM (rF-CEA/TRICOM), it was found that just one vaccination with rF-CEA/TRICOM elicited a CEA-specific T cell response that was as great as four vaccinations with rF-CEA/B7. Additional vaccinations with rF-CEA/TRICOM further increased the antigen-specific T cell response in mice [47].

The first clinical trial using TRICOM was undertaken in patients with CEA-positive malignancies, the majority of which were colorectal or gastrointestinal (GI) tract cancers (Table 7.1) [34,48]. Patients were vaccinated with a recombinant fowlpox virus expressing an agonist epitope of CEA along with the three costimulatory molecules (rF-CEA(6D)-TRICOM); once safety was established, subsequent patients were vaccinated with an rV-CEA(6D)-TRICOM prime and monthly rF-CEA(6D)-TRICOM boosts. No maximum tolerated dose was reached, and toxicity was minimal. Of the 58 patients enrolled in the trial, 11 showed stable disease initially. Of 25 patients who initially progressed chose to continue therapy through four cycles, 12 showed stable disease, having benefited from the vaccine. After these 12 patients were moved from monthly vaccinations to once every 3 months, all 12 progressed. Interestingly, when 11 of these 12 patients were moved back to the once per month schedule, 6 then restabilized, suggesting that for the vaccinations to be effective, they needed to be given monthly. One patient showed a pathologic complete response. Additionally, in the majority of patients, enhanced CEA-specific T cell responses were observed [48]. The results from this first phase I trial were promising and paved the way for additional phase I/II trials with TRICOM vectors.

7.4 USING VACCINIA VECTORS TO EXPRESS CYTOKINES

Cytokines such as granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin 2 (IL-2) have been used in the immunotherapy of cancer, usually administered as purified proteins. However, it has been demonstrated that another effective method of delivery of these cytokines is through expression by poxvirus vectors. In two separate phase I trials, a recombinant vaccinia virus expressing IL-2 along with the tumor antigen MUC1 was administered to patients. In one of these trials, 2 of 28 patients showed a partial response, while 15 patients exhibited stable disease (Table 7.1) [49].

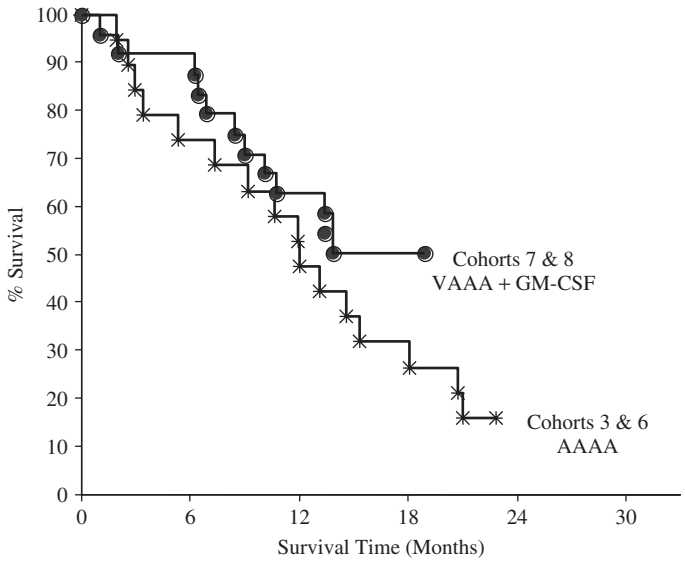

Figure 7.1. Overall survival by cohorts with or without granulocyte–macrophage colony-stimulating factor (GM-CSF). Cohorts 3 and 6 (16 of 19 patients experienced treatment failure) received no Gm-CSF with vaccines; cohorts 7 and 8 (12 of 24 patients experienced treatment failure) received vaccines plus GM-CSF. (Taken from Ref. 48.)

When a recombinant vaccinia virus expressing GM-CSF was given to melanoma patients in a phase I trial, evidence of tumor regression (with one complete remission) was observed (Table 7.1) [50]. When GM-CSF was given along with rV-CEA/TRICOM, it was observed that patients who received GM-CSF along with vaccine showed a longer progression-free survival than did those who received vaccine alone (Fig. 7.1) [48]. Because of these promising results, rF-GM-CSF has been incorporated into the vaccine regimen in current and future trials with TRICOM vectors.

7.5 COMBINATION THERAPIES

Radiation and chemotherapy are the standard of care for many types of cancer. As such, research groups have begun to combine vaccines with one of these treatment modalities in an effort to increase vaccine efficacy. In vitro studies have shown that exposing tumor cells to sublethal doses of radiation alters the phenotype of these cells by upregulating a number of genes such as Fas, MHC class I, ICAM-1, and the TAAs CEA and MUC1 [51–53]. As a consequence, these tumor cells become more susceptible to T-cell-mediated killing. Local irradiation of tumors in mice after vaccination with rV-CEA/TRICOM showed a synergistic increase in antitumor efficacy as compared with either modality alone, as no mice were

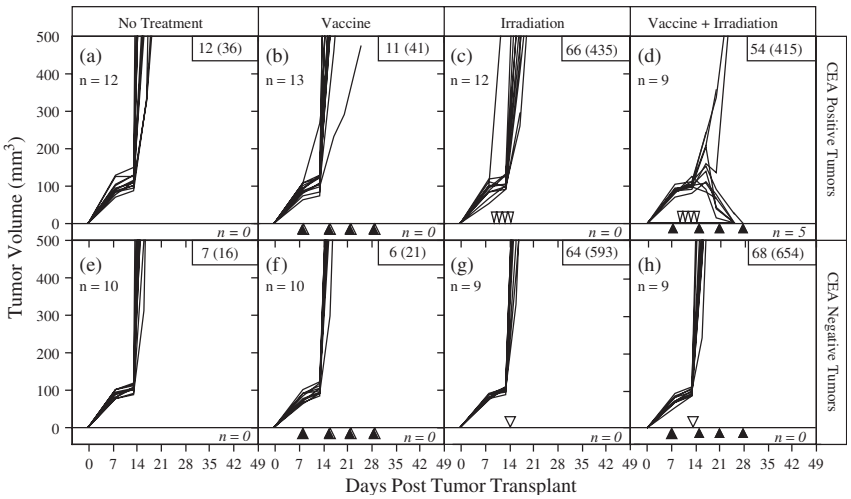

Figure 7.2. The Vaccination regimen consists of poxviral vectors that express a triad of costimulatory molecules called TRICOM (B7-1, ICAM-1, LFA-3), in addition to the tumor-associated antigen CEA. Although either modality alone was ineffective, the combination resulted in tumlor cures in 50% of the mice bearing CEA^+ tumors. This resulted further in protection from subsequent tumor challenge. Interestingly, none of the mice bearing CEA tumors were cured of their tumor masses suggesting that an antigen-specific immune response mechanism was responsible. (Taken from Ref. 22.)

cured after either radiation or vaccine alone, but 50% of mice given both therapies were cured of their tumors (Fig. 7.2) [52]. A phase II clinical trial involving the combination of vaccine and radiotherapy was conducted in patients with localized prostate cancer (Table 7.1) [42]. Patients in the vaccine–radiotherapy arm were given a combination of rV-PSA and rV-B7-1 as a prime and rF-PSA boosts monthly along with local GM-CSF and low-dose interleukin-2 (IL-2). External beam radiation was given between the fourth and sixth vaccinations. Of 17 patients in the vaccine–radiotherapy arm, 13 showed a significant increase in the number of PSA-specific T cells (at least threefold) after therapy, while patients in the radiotherapy-only arm showed no detectable increase in PSA-specific T cells. Additionally, it was found that T cells were generated to other prostate-associated antigens not found in the vaccine, suggesting immune-mediated killing of tumor cells. These results have led to an additional clinical trial using vaccine and radiotherapy in patients with CEA-positive metastatic cancer (`www.clinicaltrials.gov`; keywords TRICOM and radiotherapy).

It has been shown that chemotherapy, like radiation therapy, can also upregulate surface molecules on tumor cells such as MHC class I and multiple TAAs [33,54–56]. Preclinical results showed that when used with a whole-tumor cell vaccine, cyclophosphamide, doxorubicin, and paclitaxel increased the immune response to the tumor and increased the delay in tumor growth over that seen with vaccine alone [57]. A phase II clinical trial combining the use of the

chemotherapeutic agent docetaxel with vaccine was conducted in patients with metastatic androgen-independent prostate cancer. In this trial, patients received either vaccine (an admixture of rV-PSA and rV-B7-1 along with GM-CSF) and docetaxel or vaccine alone, and PSA-specific immune responses were monitored [58]. After 3 months of therapy, the median increase in PSA-specific T cells was 3.33-fold in both arms. As seen in the trial mentioned above using radiotherapy, immune responses were detected against prostate cancer antigens not found in the vaccine. The median progression-free survival was 6.1 months in patients who received docetaxel after vaccine therapy, while a historical control reports a median progression-free survival of 3.7 months after docetaxel therapy alone (Table 7.1) [58,59]. Currently, there are several clinical trials using the combination of chemotherapy (doxorubicin, cyclophosphamide, paclitaxel, docetaxel) and vaccine, primarily in metastatic disease settings (`www.clinicaltrials.gov`; keywords TRICOM and chemotherapy).

These trials will help optimize the combination of vaccine and chemotherapy to improve antitumor responses and hopefully extend survival in a variety of disease settings.

7.6 MODIFIED VACCINIA ANKARA

Modified vaccinia Ankara (MVA) is a replication-defective strain of vaccinia virus that was created by serially passaging vaccinia virus through chicken embryo fibroblast cells. The resulting virus is not able to replicate in mammalian cells, but has retained its ability to infect cells and synthesize viral proteins, thus remaining immunogenic [16]. These properties have made MVA an attractive vaccine vector for cancer therapy, as MVA has been shown to be safely administered to high-risk patients.

In a preclinical study to determine whether MVA was as effective as vaccinia virus at inducing immune responses to tumor antigens, a recombinant MVA expressing the three costimulatory molecules of TRICOM and the tumor-associated antigen carcinoembryonic antigen (CEA; the resulting virus was designated MVA-CEA/TRICOM) was created [60]. Mice transgenic for CEA were primed with either MVA-CEA/TRICOM or a recombinant vaccinia virus expressing the same transgenes (rV-CEA/TRICOM). Because it had previously been shown that priming with vaccinia virus and boosting with an avipox virus elicited a greater T cell response than when avipox was given multiple times [29], mice were boosted 3 times with a recombinant fowlpox virus also expressing these transgenes (rF-CEA/TRICOM). Mice that were primed with MVA-CEA/TRICOM had a significantly greater CEA-specific $CD4^+$ and $CD8^+$ T cell response as compared with mice primed with rV-CEA/TRICOM. To determine antitumor efficacy, mice with established peripancreatic metastases were treated with either MVA-CEA/TRICOM or rV-CEA/TRICOM followed by three rF-CEA/TRICOM boosts, and mouse survival was observed (Fig. 7.3). There was no statistical significance in survival between mice treated with MVA-CEA/TRICOM (71%, or five of seven mice) and those treated with

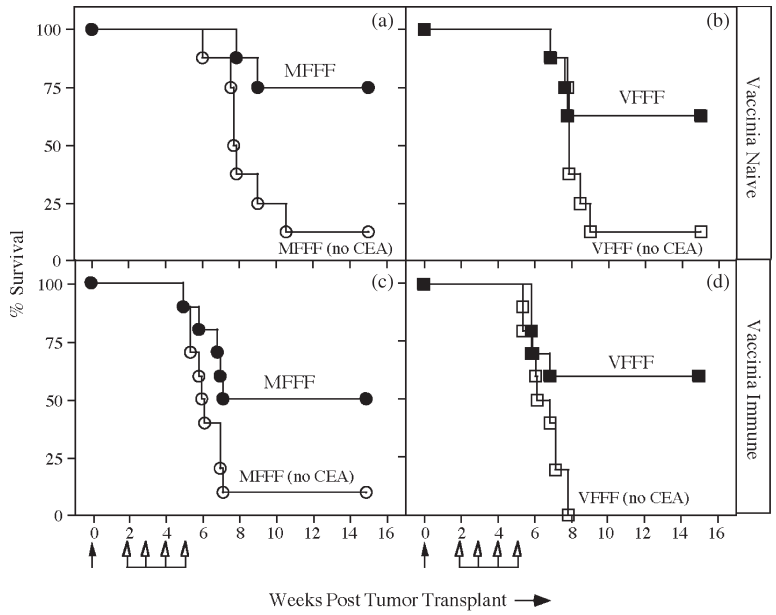

Figure 7.3. Therapy of 14-day established experimental metastases. CEA-Tg mice were transplanted with tumor intrasplencially on day 0 (solid arrow). On day 14 mice received the following doses: (a) MVA-CEA/TRICOM prime vaccination (M) with recombinant GM-CSF and low-dose IL-2 followed by three weekly boosts with rF-CEA/TRICOM (F) admixed with rF-GM-CSF and low-dose IL-2 (closed circles); control CEA-Tg mice received an MVA-TRICOM (no CEA) prime vaccination with cytokines followed by three weekly boosts with rF-TRICOM (no CEA) with cytokines (open circles); rV-CEA/TRICOM prime vaccination (V) with cytokines followed by three weekly boosts with rF-CEA/TRICOM (F) with cytokines (closed squares); control CEA-Tg mice received an rV-TRICOM (no CEA) prime vaccination with cytokines followed by three weekly boosts with rF-TRICOM (no CEA) with cytokines (open squares). Panels (c) and (d) show CEA-Tg mice that were prevaccinated with high-dose V-WT; 6 days later, the mice were transplanted with tumor on day 0 (solid arrow), and 14 days later (c) mice were treated as described in panel (a); (d) mice were treated as described in panel (b). Mice were monitored for survival. (Taken from Ref. 60.)

rV-CEA/TRICOM (57%, or four of seven mice). The results obtained in this study show that MVA is at least as effective as vaccinia virus at eliciting an immune response to tumor antigens and reducing tumor burden, and provides a rationale for the further study of MVA as a cancer vaccine in the clinic.

Several clinical trials using MVA for cancer therapy have been conducted (Table 7.1). A recombinant MVA virus expressing the TAA 5 T4 (TroVax) has been tested in the clinic. Preclinical results were encouraging, with the number of lung metastases reduced by $> 90\%$ after treatment with TroVax as compared with treatment with MVA alone [61]. A phase I/II clinical trial was conducted in patients with metastatic colorectal cancer [61]. In this trial, patients were given TroVax once

every 4 weeks for a total of three vaccinations. Of 17 evaluable patients, 16 demonstrated 5 T4-specific immune responses. Five patients had stable disease for 3–18 months after therapy, and all of these patients showed 5 T4-specific immune responses. There was also a positive correlation between a 5 T4 antibody response and patient survival or time to progression, and the vaccine was well tolerated [61], warranting further clinical trials with TroVax. Currently, there is a promising ongoing phase II trial with TroVax and IL-2 in renal cell carcinoma (RCC) patients, and recruitment is underway for a phase III trial in RCC patients (Oxford Biomedica Website; `http://www.oxfordbiomedica.co.uk/`).

7.7 CONCLUSIONS

While numerous viral vectors have been explored for the treatment of cancer, none have yet been approved by the FDA. By the time most patients are enrolled in many of the previously conducted clinical trials, they have failed other standard-of-care practices, and often present with advanced metastatic disease. More recent clinical studies have shown that poxvirus vectors can be safely combined with the current standard-of-care therapies of radiation and chemotherapy; thus, in future trials it is anticipated that these vaccines will be administered earlier in the disease process, optimizing their efficacy.

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8

IMMUNOTHERAPEUTIC STRATEGIES AGAINST CANCER USING *Listeria monocytogenes* AS A VECTOR FOR TUMOR ANTIGENS

Nicholas C. Souders, Thorsten Verch, and Yvonne Paterson

8.1 INTRODUCTION

Cancer is a leading cause of death worldwide, and for ages humankind has been in desperate need of a therapy that could potentially eliminate all types of cancer. Since there has been renewed interest in harnessing the immune system to combat cancer (as reviewed in the other chapters in this volume), many, including the present authors, have tried to create novel immunotherapeutics to alleviate disease. This chapter covers the use of *Listeria monocytogenes* as a vaccine vector for tumor antigens. We will focus on the requisite background knowledge necessary to understand certain aspects of *Listeria* vaccine development as a whole including a brief review of the major therapies that have used this approach thus far. We include some critical methodology and close with remarks on safety issues and future prospects for this approach against cancer.

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8.2 LIVE RECOMBINANT VACCINE VECTORS

Recombinant vector systems based on viruses or bacteria are a very attractive tool since they induce a powerful innate immune response as well as cellular immunity. Thus passenger antigens are presented in an optimized cytokine environment to the immune system. A disadvantage of viral vectors is the generation of neutralizing antibodies against the vector backbone, which prevent further boosting steps. Intracellular bacteria can potentially address some of the shortcomings of viruses without giving up the intrinsic advantages of live vector systems. Repeated immunizations with intracellular bacteria are possible since neutralizing antibodies do not play a major role in clearing most intracellular bacterial infections [1,2]. Safety is another concern with viral vectors since adverse events cannot be readily controlled, leading in extreme cases even to death of the patient [3]. As bacteria are readily controlled by antibiotics, which can stop vector replication at any given time, their in vivo application is likely to be safer.

Besides the induction of adaptive immunity, bacterial infections result in a strong innate immune response (Fig. 8.1). Conserved antigenic patterns in bacteria such as unmethylated CpG DNA, flagellin, and lipid and carbohydrate structures in the bacterial cell wall serve as danger signals to activate innate immunity, resulting in local inflammation and the influx of macrophages, neutrophils, and dendritic cells. Bacteria can be controlled by both antibodies and cytotoxic T cells depending on the species and the type of bacteria used. These mechanisms can also be used to direct the immune system against tumor antigens resulting in enhanced immunogenicity when delivered by bacterial vectors.

Intracellular bacteria have attracted special interest as vaccine delivery vectors because of their ability to target antigen presenting cells (APCs) directly. While *Salmonella* and *Mycobacteria* remain in the phagosome, which they are able to modify in order to escape lysis, *Listeria* and *Shigella* avoid the antimicrobial environment of the phagolysosome by escaping into the cytosol (Fig. 8.1). This has important consequences on antigen presentation as peptides from the phagolysosome usually are presented in the context of MHC class II, whereas antigens derived from the cytosol are processed in the ER and loaded on MHC class I [4].

Listeria monocytogenes as a Vaccine Vector

Listeria monocytogenes (LM) is a foodborne pathogen capable of colonizing a number of organs, which can result in death for an animal or, more rarely, a person [5,6]. Not only does the *Listeria* bacterium act as a natural adjuvant capable of eliciting a powerful cell-mediated immune response, but in our approach to creating attenuated *Listeria* immunotherapeutics, the bacterium is genetically engineered to secrete the tumor associated antigen fused to a molecular adjuvant that also enhances the overall immune response. *Listeria monocytogenes* infects phagocytic cells such as macrophages, neutrophils, and dendritic cells and will induce a potent CTL response downstream from the initial course of infection [7]. After

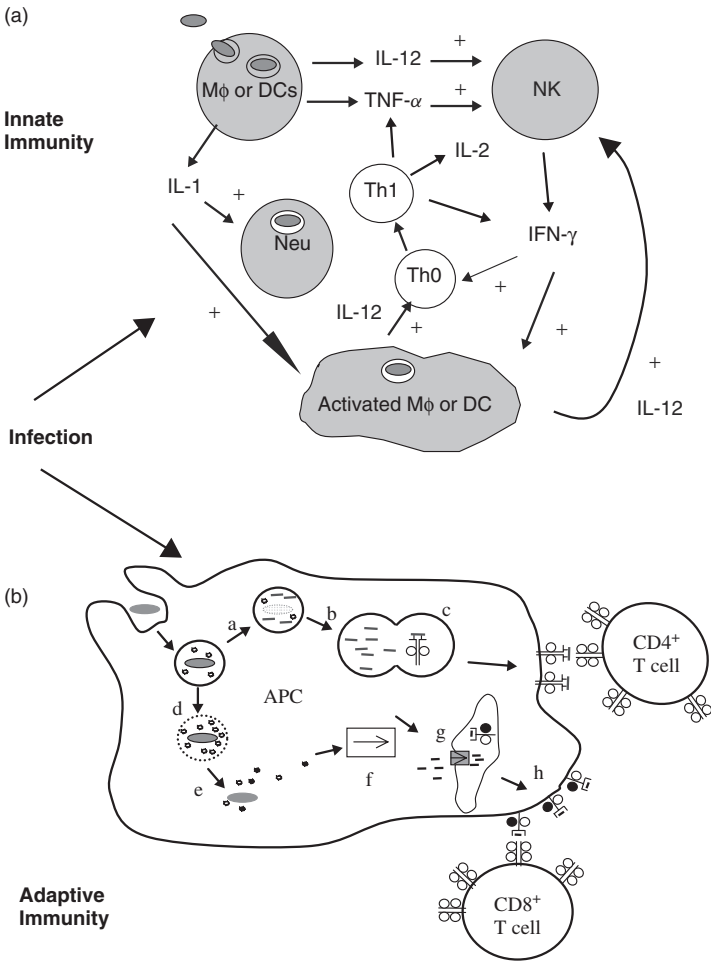

Figure 8.1. Infection with *Listeria monocytogenes* results in innate and adaptive immunity. Panel (a) shows bacteria taken up by phagocytic cells including macrophages (Mϕ), dendritic cells (DCs), and and neutrophils (Neu). Cell wall and other components activate macrophages to produce IL-12 and TNF-α to activate NK cells, and IL-1 to activate neutrophils. Activated NK cells produce IFN-γ, which acts on macrophages to upregulate antigen-processing machinery and increase the further production of IL-12. This burst of inflammatory innate immunity drives the maturation of Th0 cells to Th1 cells. Panel (b) shows that phagocytosed *L. monocytogenes* that are unable to lyse the phagosomal–lysosomal membrane are degraded (a) and their proteins are broken down into peptides (b) that can be loaded onto MHC class II molecules (c) and presented to $CD4^+$ T cells. Alternatively, if the bacteria succeed in lysing the phagosomal membrane (d), they and their secreted virulence factors are released into the cytoplasm (e). These secreted proteins are cleaved by the proteosome (f), and the resulting peptides are transported into the endoplasmic reticulum, where they are loaded onto MHC class I molecules (g). The class I MHC molecules are sent to the cell membrane (h) where they present the peptides to $CD8^+$ T cells.

phagocytosis of *Listeria*, the bacteria can escape from the phagolysome into the cytosol of the APC by producing the hemolytic protein, LLO, which is degraded in the cytosol by the proteosome to prevent host cell lysis [8]. In the cytosol, *Listeria* expresses the actin-polymerizing protein, ActA, which allows it to spread from cell to cell [9]. These virulence factors, LLO, ActA, and others, are under the control of a pluripotential transcription factor, PrfA [10,11]. Once *Listeria* escapes the phagolysome, it can induce the production of IL-12, IL-6, and TNFα and enhance DC maturation [12]. In addition to inducing the production of nitric oxide (NO) by macrophages, *Listeria* can also induce a rare DC subset to produce NO and TNFα [13]. Although antibody responses to *Listeria* can be measured in some model systems, cellular immunity is the key to clearing infections.

We first described the potential of *L. monocytogenes* as a vaccine vector in the early 1990s [14,15], but it has attracted the interest of an increasing number of researchers as a potentially ideal vaccine vector more recently [1,16–26] since its unique lifecycle and low pathogenicity set it apart from *Salmonella* and *Shigella*. Some of the basic principles of the methodology described for *Listeria* later in this chapter can be easily adapted to other bacterial species with some modifications that can be obtained from the literature. The interested reader may also consider several review articles on bacterial vector systems as a guide through the literature [27–32].

For many cancers, immunotherapeutic approaches are focused on inducing a CTL response that can kill tumor cells that consistently express a tumor associated antigen required for maintenance of the malignant phenotype [33]. The generation of tumor-infiltrating lymphocytes, especially tumor-specific $CD8^+$ effector T cells, can be used as a surrogate marker for vaccine efficacy [24,34,35]. As we have shown previously, *Listeria monocytogenes* can be used to eliminate palpable, vascularized tumors in several mouse models because of the vaccine's ability to generate tumor-infiltrating lymphocytes [20,24,25,35].

A drawback to many cancer immunotherapeutics is their lack of applicability to human tumor antigens with homology to self-antigens due to immune tolerance mechanisms, which limit patients' immune responses to their cancers [36,37]. Tolerance allows for the growth of tumors, which have gained the ability to divide very rapidly while being resistant to death, because they are ignored by the immune system. In order for a cancer immunotherapeutic to be effective, it will have to overcome tolerance mechanisms and activate self-reactive T cells to target the tumor. The ability of *Listeria*-based vaccines to break tolerance has not been extensively examined. *Listeria* that secrete the self-melanoma antigen, Trp2, have been tested in transplantable mouse melanoma models both alone [38] and in conjunction with the TLR7 agonist imiquimod [39]. Although, the vector could protect mice against melanoma challenge [38,39], the level of tolerance to this antigen could not be examined because there is no mouse syngeneic to the tumor that lacks this antigen. Attempts to break tolerance to a self-antigen using *Listeria* targeted the endogenous gp70 retroviral gene in the CT26 mouse tumor model; however, the CTL epitope that this strain secreted did not have complete homology with the target gp70 sequence and therefore cannot stringently be considered a self-antigen [16].

Studying novel cancer immunotherapies in a transplantable mouse model is a first approach to evaluating the effectiveness of a therapy. However, if wild-type mice are used as recipients, there may be little or no tolerance unless a self-antigen is expressed by the tumor. A better test of cancer immunotherapies may be to test them in tolerance models, such as transgenic mouse models for cancer [40,41]. Effective treatments in animals that are tolerant to the tumor antigen will theoretically have a greater chance of being effective in human clinical trials. In the paragraphs that follow we will describe our studies using the human tumor antigens, HPV-16 E7 and HER2/*neu* and *Listeria* vectors, which at least partially break tolerance.

Listeria as a Vaccine for Cervical Cancer

Human papillomaviruses 16 and 18 are the primary causative agent of anogenital cancer and have been strongly associated with certain forms of head and neck cancer. In fact, over 50% of both cervical cancer and squamous cell carcinoma of the tonsil are associated with HPV-16 [42–44]. Cervical cancer is the second most common female malignancy-inducing disease in over 400,000 women per year worldwide, and is a leading cause of death when untreated. Of all oral cancers unrelated to tobacco or alcohol usage, 20% are associated with HPV infections [43,44]. Two genes, E6 and E7, are responsible for the induction of cancer by downregulating the tumor repressor genes Rb and p53 and immortalizing the epithelium of the cervix. E6 degrades p53, and E7 binds to and degrades Rb [45–47]. Evidence has accumulated in humans that immune responses can be directed against HPV16 [48,49]. One of the immortalizing proteins in HPV-induced cancers is the Rb-binding protein, E7, which has been well characterized in a number of studies not limited to the ones mentioned below [17,34,50–54].

We created two *Listeria*-based vaccines, LM-LLO-E7 and LM-ActA-E7, as potential vaccines for HPV-16 induced cancers [17,24]. A truncated listeriolysin O (LLO) is fused to E7 in LM-LLO-E7, whereas in LM-ActA-E7, the E7 gene is fused to a truncated version of the ActA protein. We routinely fuse cancer antigens to listerial virulence factors to improve antigen presentation in vivo. Both the truncated LLO and ActA have PEST (proline, glutamic acid, serine, threonine)-like domains within their amino acid sequences, which have been postulated to result in the rapid degradation of proteins in the host cell cytosol [55,56]. A putative PEST-like domain at the *N*-terminus of LLO (amino acids: NKENS ISSMA PPASP PASPK TPIEK KHADE ID) is suspected to degrade this toxic protein by directing it to the proteosome [8], although there has been more recent evidence of the involvement of other mechanisms [57]. The fusion of antigens to LLO or the PEST-like sequence of LLO or ActA seems to enhance the immunogenicity of the fused antigen [17,23,24]. In order to avoid overexpression of toxic LLO, we truncate the protein at AA 441, thereby deleting the hemolytic domain.

LM-LLO-E7 and LM-ActA-E7 have been shown to induce $CD8^+$ T cells that can home to, penetrate, and kill the solid tumor formed by TC-1 cells in a normal C57BL/6 mouse [17,24,35]. The TC-1 cell line is a C57BL/6 immortalized primary

lung epithelial cell line that was transformed with E6, E7, and *c-Ha-ras* oncogenes [52]. TC-1 cells are poorly immunogenic in vivo and form solid tumors when injected subcutaneously into syngeneic host mice, which cannot control the growth of the tumor without some type of intervention [52]. We found that E7 alone delivered by *Listeria*, LM-E7, induced formation of $CD4^+$ $CD25^+$ regulatory T cells in the tumor, whereas LM-LLO-E7 prevented the formation of $CD4^+$ $CD25^+$ regulatory T cells [35]. Both the Lm-LLO-E7 and Lm-ActA-E7 vectors could effectively eradicate established TC1 tumors of a measurable size in the wild-type C57/BL6 mouse.

We created a mouse transgenic for HPV-16 E6 and E7 under the thyroglobulin promoter in order to test the ability of the vaccines to break tolerance. The E6 and E7 genes are expressed in the thyroid and in mouse thymic epithelial cells. We showed that the mouse appeared to demonstrate central tolerance to E7, since the T cells induced in response to these vaccines were of lower avidity than those induced in wild-type mice. Nevertheless, the vaccines were able to eradicate established TC-1 tumors in the E6/E7 transgenic mouse, albeit at a lower frequency than in the wild-type mouse [58].

Listeria Expressing HER2/*neu* as a Vaccine for Breast Cancer

HER2/*neu* is a member of the epidermal growth factor receptor family of tyrosine kinases and has three distinct domains: an extracellular domain, a transmembrane domain, and an intracellular domain [59,60]. The formation of breast tumors can be enhanced by HER2/*neu*, making it a good target for immunotherapy [61]. HER2/*neu*-expressing mouse tumors have been derived from the FVB mouse transgenic for the HER2/*neu* gene under the control of the MMTV promoter [62]. This transgenic mouse exhibits profound tolerance to HER2/*neu* since the transgene is expressed in hematopoietic cells and the thymus [63]. We created five *Listeria* vaccines, each of which expressed and secreted overlapping fragment of HER2/*neu* [25,64,65]. Both the extracellular and intracellular domains were able to be included in this vaccine, and all portions of HER2/*neu* were fused to our molecular adjuvant, the truncated, nonhemolytic LLO. The highly hydrophobic transmembrane domain cound not be secreted by *Listeria* [25]. All five vaccines spanning the length of the extracellular and intracellular portions of HER2/*neu* were equally able to induce the regression of an implantable tumor cell line that expresses HER2/*neu* in the wild-type FVB mouse [25].

In previous studies in the FVB mouse, only one CTL epitope had been described [62]. However, we were able to discover a number of novel epitopes using our five *Listeria* vaccines [64,65]. In addition we have shown that each of these vaccines can control the growth of an HER2/*neu*-expressing transplantable tumor in the FVB mouse transgenic for the HER2/*neu* gene under the control of the MMTV promoter [65], providing further evidence that *Listeria* can overcome tolerance and expand low-avidity T cells for the treatment of tumors expressing self-antigens.

Listeria as a DNA Cancer Vaccine

More recently, a novel bactofection vector using *Listeria* as a DNA-based cancer was generated in which bacteria deliver a plasmid encoding a tumor antigen under the control of a mammalian promoter in an attempt to induce an antitumor immune response [66]. *Bactofection*, or bacteria-mediated intradomain gene transfer, has been proposed as a potential means to develop novel therapeutics to treat a variety of human diseases including cancer [67,68].

There are several potential advantages to using *Listeria* as a bactofection agent to deliver a DNA vaccine as it could circumvent some limitations of the *Listeria*-based protein secreted vaccines. Empirically, we have found that *Listeria* cannot secrete highly hydrophobic proteins, presumably because of their difficulties in translocating across the membrane or cell wall. Additionally, we have observed that there is an upper limit on the molecular weight of the eukaryotic protein to be secreted of about 50 kDa (YP, unpublished observations). The use of a *Listeria*-based DNA vaccine could avoid the complication of soliciting a prokaryote to secrete a eukaryotic protein by diverting the expression of the protein of interest to a eukaryotic host cell.

Utilizing a plasmid release mechanism involving the suicide of the carrier bacteria [69,70], we were able to engineer *Listeria monocytogenes* to induce antitumor immunity to the cervical cancer oncoprotein, E7 [66]. In the TC-1 model, we were able to slow tumor growth and induce a cytotoxic $CD8^+$ T cell response against the RAHYNIVTF epitope for E7. However, although this delivery system was more effective than administering plasmid alone, it was not as effective as LM-LLOE7, which was engineered to deliver the E7 protein in impacting on established tumor growth [66].

Constructing *Listeria monocytogenes* Vectors

Most vector strains have been based on *L. monocytogenes* strain 10403S, a natural streptomycin-resistant strain derived from 10403 [71]. Its sequence is very close to the EGD strain, which can be used as a basis for primer and cloning design. All genetic engineering steps need to be performed in *Escherichia coli* since the transformation efficiency of *Listeria* is very low and requires relatively large amounts of purified plasmid DNA (approximately 1 μg). Several shuttle plasmids have been published, of which we suggest the use of two different types (Fig. 8.2), depending on whether integration of the recombinant gene of interest into the *Listeria* genome is desired. This can be achieved by using pKSV7, which contains a temperature-sensitive origin of replication [72]. This plasmid was modified by introducing approximately 500 bp (base pairs) of *Listeria* open reading frame (ORF) Y and ORF Z interrupted by a multiple cloning site for the recombinant gene in order to allow for integration specifically into the ORF XYZ genome region (Fig. 2a) [73].

The second method for transforming *Listeria*, which is preferred in our laboratory, is to insert a required gene for in vivo bacterial growth on the plasmid to

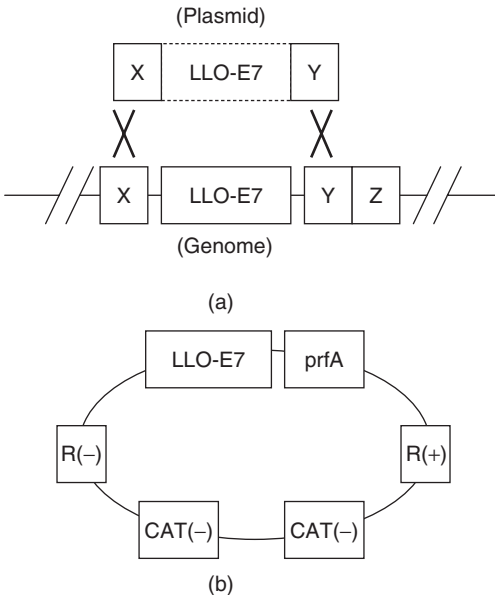

Figure 8.2. Expression of foreign genes in *Listeria* (here as an example: HPV E7 genetically fused to LLO (LLO-E7)): (a) Genome-based gene expression: schematic map of genomic integration. The LLO-E7 expression cassette on a plasmid is flanked by 300–1000 bp of genome sequences from the integration site. The gene of interest will then be inserted into the genome by homologous recombination at these sites. (b) Plasmid-based gene expression: schematic map of a *E. coli–Listeria* shuttle plasmid. The LLO-E7 fusion gene is driven by the LLO promoter. This plasmid is designed to complement a prfA-deficient mutant *Listeria* strain. The prfA gene could be replaced with alternative in vivo selection genes as we discuss in the text. Also included in this specific example are CAT(−), CAT(+)—chloramphenicol transferase genes for in vitro selection in gram-negative and gram-positive bacteria; R(−), R(+)—replication genes for gram-negative and gram-positive bacteria.

avoid plasmid loss on immunization. We have used the pluripotential transcription factor PrfA to complement a mutant strain in which the *prfA* virulence gene has been inactivated [15,17,25]. Plasmid-based gene expression can be achieved using shuttle plasmid pAM401 [74], which contains replication and antibiotic resistance selection genes for both *E. coli* and *Listeria* (Fig. 8.2b). Recombinant gene expression was made possible by introducing the *Listeria* hly promoter and the *prfA* virulence gene resulting in pDP2028 [15]. We have modified this approach by removing the antibiotic-resistant genes and substituting an alanine racemase gene for *prfA* [75]. Using this plasmid to complement a *Listeria* strain, Lm^{DD}, which lacks genes that control the synthesis of the listerial cell wall [76], allows for selection of transformed *Listeria* both in vitro and in vivo. The *p60-dal in trans* complementation system has been developed to allow for bacterial plasmid retention in an antibiotic-free manner [75]. Since the shuttle plasmids are relatively

large, all initial polymerase chain reaction (PCR)-cloning steps are performed in TA-cloning vector pCR2.1 (Invitrogen) prior to moving the recombinant genes into pKSV7 or pAM401.

The expression levels of the recombinant antigen that can be obtained in bacteria containing only a single gene copy are lower than those that can be obtained from a multicopy plasmid. While episomal gene expression could be advantageous due to the presence of multiple gene copies, it requires careful consideration of plasmid stability in vitro as well as in vivo. Since direct cloning into *Listeria* has been tedious in our and others' experience, all genetic manipulations and amplification of plasmid material should initially be performed in *E. coli* prior to electroporation of the final plasmid into *Listeria*. For laboratory research, convenient shuttle plasmids that contain antibiotic resistance genes for in vitro selection of both bacteria have long been available [74]. As these plasmids are rapidly lost in vivo, where no antibiotic pressure is present, our group has used complementation systems for in vivo stabilization. The addition of the *prfA* gene into the plasmid in combination with a *Listeria* strain lacking this gene helped to dramatically improve plasmid stability in vivo [15]. Similar complementation systems using other genes have been described by our group as well as others [70,75].

Genetic manipulations and extended in vitro culture leads to the downregulation of general virulence of *Listeria*. Efficacy can be enhanced by passaging vaccine strains in mice [71,77]. Besides upregulation of bacterial virulence, this results in stronger immune responses against the bacteria and the recombinant antigen [78]. The passaging step needs to be performed only twice in order to establish a cell bank that can be used subsequently to produce fresh immunization material as needed. As most vaccine strains are attenuated either deliberately for safety compliance or because of the recombinant antigen, a larger amount of bacteria can be injected into mice than applicable for wild-type *Listeria*. We routinely start with 10^7-10^8 colony-forming units (CFUs) injected intraperitoneally (IP). On day 3, the spleen is isolated, then homogenized in 5–10 mL of PBS, and 50–200 μL are plated in order to recover single colonies. Four colonies are pooled and expanded in liquid culture. The bacteria are frozen in 20% glycerol, and a dilution series is prepared for CFU counts ranging from $1:10^1$ to $1:10^{10}$ (plate 5–10 μL). A second passage is then performed similarly using the injection stock prepared from previously passaged bacteria. The greatest benefits to bacterial virulence and immunogenicity are observed after two passages in vivo [78]. We routinely prepare vaccine stocks after two passages and determine the virulence of this final stock.

Because, in contrast to viruses, intracellular bacteria such as *Listeria* seldom evoke neutralizing antibodies in the host, multiple vaccination boosts are possible. We have tried up to four immunizations of $0.1 \times LD_{50}$ in mice resulting in increasing vaccine efficacy [1]. Because of the buildup of some immunity against the bacterial vector, higher booster doses can be chosen than those used for primary immunization without increasing the toxicity, specifically, 0.2 or $0.3 \times LD_{50}$. Unless lack of efficacy demands a more extensive immunization protocol, we routinely immunize twice in 1-week intervals with 10^8 CFU (about $0.1 \times LD_{50}$ for most strains).

The route of immunization depends on the type of immune response that needs to be achieved and on practical considerations. As most tumors do not require a mucosal response, we usually inject the vaccine IP or IV, both of which are efficient in the E7 tumor system (unpublished data). Either route of injection has resulted in similar antitumor immunity. Although subcutaneous (SC) immunization may appear to be a logical, convenient, and safe route of immunization for a SC tumor, we found decreased efficacy using this route in tumor regression studies. The oral route is difficult to test since *Listeria* do not readily cross the gut barrier in mice. Guinea pigs are a more suitable study subject [79], but cancer models have not been well established in these animals. Using a less attenuated vaccine strain (LD_{50}: 1×10^7) than usual (LD_{50}: 1×10^9), we were able to immunize orally against challenge with a tumor cell line expressing influenza nucleoprotein [21], and similar results were reported for the E7 model system [19]. Additional attempts with more attenuated strains, however, have not yet been successful in our hands.

8.3 SAFETY CONCERNS

Biologics and live vectors in particular are subject to close scrutiny for patient and environmental safety. Although live attenuated bacteria have been used as vaccines against the pathogenic version of the bacterial strain for several decades, their use as antigen vectors has entered the field only recently (as of 2007) and with limited applications. Therefore, bacterial vectors are often regulated together with DNA vaccines or viral vectors for which extensive guidelines are available. As with other vaccines, oral or subcutaneous application is preferable to IV injection from a regulatory standpoint.

The *Listeria* vector itself should not display pathogenicity. It has to be sufficiently attenuated without the recombinant antigen present. Attenuation caused by the antigen is not considered suitable for safety evaluation. Usually a difference of at least 2–3 log units between the efficacious dose and a pathogenic dose is preferred. The attenuation has to remain stable in a clinical setting to avoid reversion to the wild type during the time of therapy. The attenuation as well as the general geno/phenotype should be well characterized, which was seldom the case for laboratory strains that were transferred between different groups over several years. For a new group embarking on using *Listeria* as a vaccine vector, it is recommended to start out with a strain obtained from a type culture collection such as ATCC.

Any live vector could potentially cause serious adverse reactions in patients. Most of these can be avoided by using stably attenuated strains and careful examination of patients for immune deficiencies. A number of rationally attenuated mutants of *Listeria* have been developed as vaccine strains, which have been previously reviewed [30]. In general, live replicating vectors are thought to result in more profound immunity [14,80]. However, the notion of using bacteria that are unable to replicate in vivo has recently been revisited. One group has developed a new type of killed *Listeria* vaccine with enhanced safety in which the bacteria are killed by psoralens and UV light, but the *Listeria* remains metabolically active and

capable of protein production for some time after exposure to the psoralens [81]. *Listeria* killed by γ irradiation has been shown to activate DCs and prime $CD8^+$ T cells by cross-presentation and protect against live *Listeria* challenge [82]. In our experience, listeriolysin O–negative strains have not been efficient host strains for passenger antigens [15], presumably because of the inability of the bacterium to enter the host cell cytosol and access MHC class I Ag processing machinery. More recently, however, a LLO negative strain of *Listeria* that expresses a $CD8^+$ T cell epitope derived from lymphocytic choriomeningitis virus was found to generate memory, epitope-specific $CD8^+$ T cells. Mice primed with this strain resisted challenge with virulent *Listeria* and lymphocytic choriomeningitis virus [83]. However, whether any strains that cannot replicate in vivo are as competent for the delivery of tumor antigens as attenuated replicating strains has not yet been examined and requires further investigation.

In contrast to virus vectors, bacteria offer an additional possibility for control because of their sensitivity to antibiotics. As the injected bacterial strain is known and characterized, potentially adverse reactions could be immediately addressed by the application of a suitable antibiotic before the bacterial infection is beyond control. For example, most of the safety concerns raised for *Listeria*-based vectors such as the possibility of meningitis can easily be addressed this way. In general, clinical protocols for the application of bacterial vectors should contain an antibiotic dose at the assumed end of the vector infection for safety reasons. Sometimes concerns are also raised for the safety of healthcare workers and patients' families because of a potential spread of the vaccine vectors from person to person. This may, for example, represent a concern in *Shigella* or *Salmonella* vectors based on typhoid strains when not sufficiently attenuated but should not pose a risk for *Listeria* since person-to-person transmission of even wild-type strains does not appear to occur even in clinically confirmed cases of Listeriosis (Bennet Lorber, personal communication).

Besides the safety concerns for caretakers, potential environmental spread of the vector should also not represent a hazard. Therefore, the FDA strongly discourages the presence of any antibiotic-resistance genes in the final vector. The recombinant antigen needs to be maintained stably either on a plasmid or integrated into the bacterial chromosome without antibiotic selection. As these concerns will be raised whenever a bacterial vector is moved into clinical applications, vector design should address them early in the preclinical phase.

8.4 CLOSING REMARKS

Listeria-based cancer vaccines have proved to be relatively easy to create. We have tested numerous strategies over the years and found that *Listeria*-based cancer vaccines, due to the unique lifecycle of *Listeria* and the type of immune response that it elicits, are effective in murine models in eradicating solid, well-vascularized tumors. We are thus optimistic about their potential for treating human disease. The cervical cancer vaccine, LM-LLOE7, is currently undergoing a Phase I clinical trial,

and we expect the vectors to demonstrate no toxicity. Additionally, the finding that *Listeria*-based HER2/*neu* vaccines can elicit a therapeutic immune response even in mice for which this is a self-antigen [25,64,65] encourages us to begin to translate our HER2/*neu* vaccines from the mouse and into the human.

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9

COUPLING INNATE AND ADAPTIVE IMMUNITY WITH YEAST-BASED CANCER IMMUNOTHERAPY

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9.1 INTRODUCTION

Many different strategies for the generation of immune responses using cancer vaccines or cancer immunotherapy are being employed for the treatment and/or prevention of human tumors. The two major elements of cancer immunotherapy are the selection of the antigens used to focus the specificity of the immune response and the approach or vehicle employed to stimulate the immune system against these antigens to eliminate target antigen-bearing tumor cells.

Recombinant nonpathogenic brewer's yeast, *Saccharomyces cerevisiae*, which we call Tarmogens™ (acronym for *tar*geted *mo*lecular immuno*gens*), are attractive vectors for cancer immunotherapy for the following reasons: (1) yeast trigger both innate and adaptive immune responses and therefore do not require additional adjuvants; (2) yeast deliver polypeptide antigens that are effectively processed into a

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full complement of appropriate-sized peptides competent for presentation by MHC class I and class II pathways, irrespective of HLA type; (3) yeast elicit potent T cell immune responses against tumor cells expressing target antigens; (4) yeast are not neutralized by the host immune system, even on repeated administration; (5) yeast-based immunotherapy is not a custom-manufactured, patient-specific vaccine, yet multiple tumor antigens may be targeted, so that treatment may be customized with a yeast "cocktail" dependent on a patient's tumor genotype profile; and (6) recombinant yeast are simple to manufacture.

This chapter discusses methods for engineering yeast to express tumor antigens and the unique properties of recombinant yeast in the activation of innate and adaptive immune responses. The broad applicability of the yeast-based immunotherapy to elicit protective T cell immune responses has been demonstrated in preclinical studies with numerous foreign, mutated, and overexpressed antigens. For instance, the therapeutic administration of yeast expressing mutated Ras protein triggered the complete ablation of carcinogen-induced mutant Ras-bearing tumors in mice. The safety and immunogenicity of yeast Tarmogens in cancer patients are also discussed.

9.2 YEAST-BASED IMMUNOTHERAPY: MOLECULAR MECHANISMS OF ACTION

Yeast delivering tumor antigens activate innate immunity as well as the adaptive antigen-specific immune responses. The dual contributions to immune activation arise from the inherent "adjuvant-like" properties of yeast combined with direct delivery of the expressed tumor antigen for processing and presentation to the immune system. Yeast components activate the innate immune system by transmitting the "danger signals" of microbial infection through distinct surface receptors on APCs that recognize particle-associated molecular patterns (PAMPs) by pattern recognition receptors. These pattern recognition receptors include toll-like receptors (TLR1,2,4,6) and *phagocytic* receptors, such as dectin, mannose, and glucan receptors [1–3]. The inherent recognition of yeast component patterns as a "foreign" infection is not restricted to *S. cerevisiae*, but is shared with other fungi [1,4,5].

Saccharomyces cerevisiae yeast cells are avidly phagocytosed in vitro by murine bone-marrow-derived dendritic cells DCs, human myeloid DCs and human plasmacytoid DCs, macrophages, and neutrophils, as well as human EBV-immortalized B cells [6,7] (See also Munson S, Parker J, Franzusoff A, unpublished observations). The uptake of multiple yeast per phagocytic cell has been observed within a few (0.5–6) hours after exposure, as determined by flow cytometry and fluorescence microscopy. The uptake of yeast by DCs triggers DC activation, maturation (including upregulation of cell surface receptors and secretion of various cytokines), and the presentation of peptides from yeast-expressed tumor antigens on MHC-I and MHC-II receptors, leading to an antigen-specific response by CD8 and CD4 T cells in the immunized hosts [8]. The elements of DC activation and antigen processing triggered by yeast exposure are elaborated in the following sections.

DC Activation and Maturation

The interaction of innate signaling receptors TLR1,2,4,6, plus phagocytic receptors on the surface of APCs, appears to be a combinatorial process that enhances and modulates the innate responses that would otherwise be achieved by binding individual innate receptors [9,10]. The consequences of TLR recognition of yeast molecular patterns by DCs results in the expression of genetic responses that are distinct from the profile elicited in response to bacterial components, viral components, or individual TLR-ligand agonists [11]. TLR2 interacts with zymosan (preparations of *S. cerevisiae* yeast cell walls) in conjunction with TLR1 and TLR6 [12,13]. TLR4 interacts with mannan on the surface of *Candida* yeast, and presumably on the surface mannan of *S. cerevisiae* [12,14]. Other receptors apparently involved in *S. cerevisiae* recognition and response are the β-glucan receptor (dectin-1 in the mouse), mannose receptor, complement receptor 3 (CR3), and the scavenger receptor CD36 [14–19]. The consequences of these multiple receptor interactions with yeast are the initiation of endocytic and phagocytic mechanisms in DCs, the activation of phagolysosomes that process the internalized yeast cells, degradation of the yeast in the endosomes and phagolysosomes, presentation of exogenous yeast-associated peptides into the MHC-II receptor pathway (within specialized endosomes), and activation of the proteasome pathway for cross-presentation of yeast-associated peptides into the MHC-I receptor pathway. As shown in Figure 9.1, the incubation of yeast also triggers upregulation of cell surface molecules, such as CD80 (B7.1) and CD86 (B7.2) and CD83 [6,7] (Borges V, unpublished data). Stimulation of DCs by yeast also induces secretion of a proinflammatory panel of Th1-type cytokines, such as IL-6, IL-12, and TNFα [6,7,20,21].

Cross-Presentation of Exogenous Yeast-Associated Antigens into the MHC-I Pathway

The classical view of MHC-restricted antigen presentation is that peptides for stimulating CD8 T cell responses via the MHC-I pathway must be derived from polypeptides endogenously expressed by the APCs. Further, this model holds that exogenous antigens are delivered exclusively by endocytosis into the MHC-II presentation pathway. More recent work has provoked revision of the classical view of MHC-I antigen presentation, since it was discovered that certain types of exogenous delivery methods were effective at loading peptides into the MHC-I pathway, and thereby stimulating activation of CD8 T cell immune responses, in a process known as *cross-priming* or *cross-presentation* [20–26].

Yeast are capable of inducing cross-priming, as shown by a variety of in vitro and in vivo studies [6,27]. In vitro, recombinant yeast expressing a model antigen, namely, ovalbumin, when incubated with DCs, were able to activate OVA-specific MHC-I restricted T cell responses. Immature murine DCs were incubated with either soluble ovalbumin alone, yeast lacking foreign antigens (YVEC),

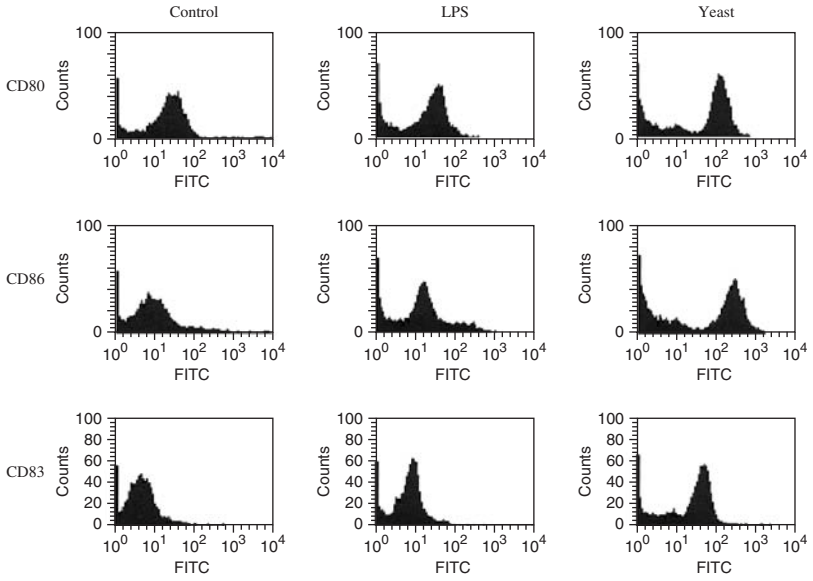

Figure 9.1. Human DC activation and maturation by bacterial LPS and yeast. Peripheral blood mononuclear cells from healthy human donors were incubated with GM-CSF and IL-4 for 5 days followed by 2 days of GM-CSF + IL-4 and incubated with saline (control), incubated with bacterial lipolysaccharide (LPS), or incubated with yeast. Flow cytometry of surface staining on DCs with CD80 (top row), CD86 (middle row), or CD83 (bottom row) antibodies is shown.

or yeast expressing chicken ovalbumin (OVAX). The pulsed DCs were then incubated with naive OVA-specific $CD8^+$ T cells (obtained from OT-1 T cell transgenic mice). As expected, DCs pulsed with soluble ovalbumin or YVEC were unable to activate ovalbumin peptide-specific $CD8^+$ T cells. In contrast, DCs incubated with OVAX activated the ovalbumin-specific $CD8^+$ T cells and the amount of proliferation was improved in a yeast dose-dependent manner by increasing the yeast to DC ratio during pulsing [6]. Surprisingly, DCs pulsed with soluble ovalbumin mixed with YVEC also stimulated $CD8^+$ T cell proliferation [6]. However, 40,000-fold more protein is required when provided as soluble protein plus empty yeast (YVEC), compared to the amount of ovalbumin that efficiently triggers antigen-specific $CD8^+$ T cell responses when delivered inside the yeast. This result indicates that innate receptor agonists such as yeast are several orders of magnitude more effective at eliciting adaptive immune responses when the innate receptor agonists are physically associated or linked with the tumor antigens.

Tumor challenge models in immune-competent or CD8-knockout mice, or inactivating CD8 T cells with specific anti-CD8 antibodies are in vivo examples demonstrating that immunization with recombinant yeast primes tumor-targeted CD8 T cell responses [6] (Duke R, unpublished observations). Immune-competent mice immunized with OVAX were protected against challenge

with ovalbumin-expressing EL-4 tumor cells, but not to challenge with EL-4 cells that did not express ovalbumin (6) (see also Duke R, unpublished observations). The protection mediated by immunization with OVAX yeast was abolished in mice deficient in $CD8^+$ T cells (6). Thus, while not all immune responses that protect animals from tumor challenge or spontaneous carcinogen-induced tumors are CD8 T cell-restricted, this study and others have demonstrated that immunization with yeast are potent inducers of CD8 T cell responses. Furthermore, the incubation of immature DCs with yeast expressing ovalbumin, but not YVEC yeast or mock treatment, was sufficient for activating tumor protective immune responses when the OVAX-pulsed DCs were introduced back into mice challenged with ovalbumin-expressing tumors [6]. These results suggest that yeast activation of APCs and antigen delivery by yeast to APCs is necessary and sufficient to explain the mechanism of yeast-based immunotherapy in immunized hosts.

9.3 MUTATED Ras AND THERAPEUTIC ANIMAL TUMOR MODELS FOR YEAST-BASED IMMUNOTHERAPY

The selection of tumor antigens for targeted immunotherapy is typically based on (1) mutated proteins that arise during tumorigenesis [e.g., Ras, adenomatous polyposis coli (APC)], (2) overexpression of *self-proteins* specific to tumor cells [e.g., epidermal growth factor receptor (EGFR)], (3) differentiation, neo- and self-antigens whose expression is activated or restricted to tumor cells [e.g., melanoma antigens, mesothelin, carcinoembryonic antigen (CEA)], or (4) proteins expressed in the stroma that support tumor survival and proliferation (e.g., PSMA). However, given the multiple mechanisms by which tumor cells alter protein expression or trigger escape mutations under selective drug or immune pressure, the optimal antigens for initial therapeutic targets should be those that are critical for tumorigenesis or metastasis. Some of these targets have been identified as "antigens of addiction" because of their essential role in the cancer phenotype.

Mutated Ras protein was selected as an ideal target antigen for yeast-based immunotherapy because of its essential role as an engine driving tumorigenesis in multiple types of human cancer. The three Ras protein members, K-, H- and N-Ras, are GTPase-switch proteins important for cell proliferation acting downstream of receptors that respond to external stimuli for cell division, such as EGFR. Single amino acid mutations in at least two domains in Ras (amino acids 12 or 13, and residues 59 or 61) are commonly associated with cancer as these mutations cause constitutive, signal-independent cell growth and tumorigenesis [28]. Mutations in K-Ras are present in 90% of human pancreatic adenocarcinomas, 35–40% of colorectal tumors, and 30–35% of non-small-cell lung cancer adenocarcinomas [29,30].

The mutated Ras proteins commonly found in cancer were expressed as tumor antigens in yeast (the GI-4000 series of yeast strains). A carcinogen-induced lung tumor model was used to test the effect of the GI-4000 yeast (31). In this system, one intraperitoneal injection of urethane in A/J mice spontaneously triggers 25–50

individual lung tumors per mouse, each driven by single amino acid mutations in the Ras oncoprotein. Most of the individual tumors are driven by K-Ras mutated at codon 61 (i.e., Q61R or Q61L). The genotype of some urethane-induced tumors harbor mutations at K-Ras codon 12 (i.e., G12V or G12D), while a minority of the 25–50 tumors do not appear to express mutated Ras proteins [32,33] (see also Lu Y, Franzusoff A, unpublished observations). Mice display pulmonary alveolar hyperplasia by 2 weeks post-urethane exposure, adenomas by 5 weeks, and adenocarcinomas by 14 weeks post-injection. The animals expire within 12 months from tumor-associated respiratory distress.

Yeast strains were engineered to express the mouse K-Ras protein with the Q61R mutation [strain GI-4000 (Q61R)], or the K-Ras protein with the Q61L mutation [strain GI-4000 (Q61L)]. After culturing the yeast and inducing expression of the mutated K-Ras proteins, the yeast were harvested, heat-killed, washed, and resuspended as intact cells in PBS. Dosing of animals is based on the number of yeast administered (1 YU $= 10^7$ yeast cells) with a known quantity of mutated Ras protein expressed per YU yeast at the time of administration.

The therapeutic efficacy of yeast-based immunotherapy in the urethane-induced lung tumor model was examined by initiating dosing 2–5 weeks post-urethane exposure, a time at which 25–50 tumors per mouse are already present as hyperplasias or adenomas. Mice were administered 5 YU yeast per injection site. Animals received total 6 doses (i.e., every other week), 10 total doses (i.e., each week), or 6 injections at each of two sites every other week (i.e., 12 total doses) of buffered saline (PBS), control yeast that harbored no heterologous protein (YVEC/GI-1001) or yeast harboring the mutated Ras proteins [e.g., GI-4000 (Q61R) yeast or GI-4000 (Q61L) yeast]. At 14 weeks post-urethane exposure, where untreated tumors would be macroscopically visible, the tumors were excised, counted, and measured with a caliper to calculate the volume of each individual tumor, and the average tumor burden per mouse was determined. Compared with the saline-treated group, the tumor burden in mice that received 6 doses of yeast vaccine GI-4000 (Q61R) led to an average reduction in total tumor volume per mouse of 39%, mice that received 10 doses showed an average reduction in tumor burden of 55%, and mice that received 12 doses exhibited 52% reduction in tumor burden (Fig. 9.2) [33].

To assess the impact of targeted yeast-based immunotherapy, the *K-ras* sequences in the residual tumors were genotyped. The tumor-sequencing results revealed that while 56% tumors from saline or mock-yeast-treated mice harbored K-Ras Q61R mutations, the administration of increased numbers of doses of GI-4000 (Q61R) yeast led to the reduction in number of Q61R-Ras-bearing tumors to the point where 12 doses resulted in complete ablation of all tumors driven by Q61R-mutated K-Ras (Fig. 9.2). Conversely, when mice were administered GI-4000 (Q61L) yeast, the tumors bearing Q61L-mutated K-Ras were targeted for destruction by the yeast-mediated immune response [33]. Interestingly, when the two different yeast were injected in urethane-treated mice at different sites, or mixed

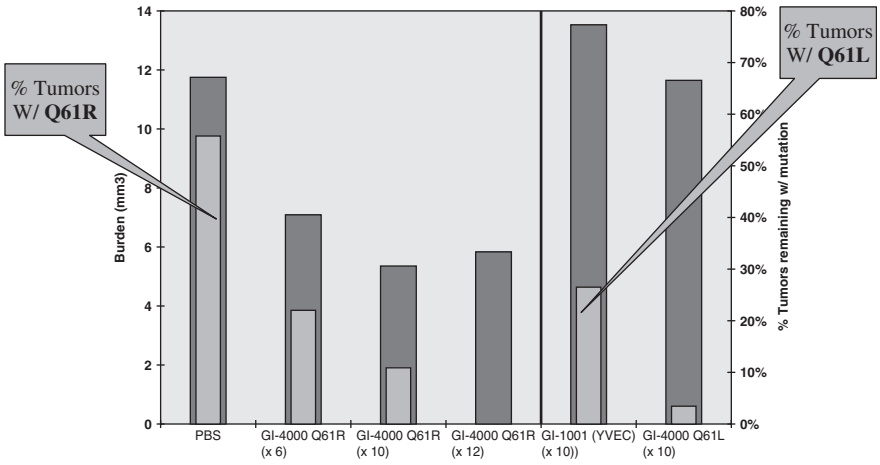

Figure 9.2. Mutation-specific tumor ablation observed when yeast vaccine administered contains the Q61R or Q61L mutation. A/J mice received a single injection of urethane, then 2 weeks later received either (1) 6 doses every 2 weeks of GI-4000(Q61R) or GI-4000(Q61L), (2) 10 doses of GI-4000(Q61R) weekly, (3) 12 doses of GI-4000(Q61R) every 2 weeks, (4) mock treatment of saline, or (5) 6 doses of GI-1001(YVEC, empty yeast vector) every 2 weeks. Then 14 weeks post-urethane exposure, tumors were excised, counted, and measured, and the total tumor burden was calculated. In total, 10–30 single tumors/group were used to prepare DNA and the *ras* sequence determined for each. Dark bars designate the total tumor burden (left axis), whereas light bars designate the percentage of tumors sequenced that bear Q61R or Q61L mutations (right axis). The light bars left of the line represent % tumors bearing Q61R-mutated Ras, whereas light bars right of the line represent % tumors bearing Q61L mutated Ras. (Adapted from [33] and Lu Y, Franzusoff A, unpublished.)

in the syringe for injection at a single site, improved protection compared to individual yeast administration was observed, and some animals had eliminated all of the tumors, supporting the premise of combinatorial treatment by cocktails of yeast targeting different antigens (Lu Y, Fiolkoski V, Bellgrau D, Franzusoff A, unpublished observations).

Taken together, these results showed that therapeutic immunization with yeast bearing mutated Ras proteins was able to activate the immune system to eliminate all tumor cells driven by a single amino acid Ras mutation with yeast-based targeted immunotherapy. Tumor-targeted immune responses were enhanced with repeated yeast immunizations, even when administered weekly. Furthermore, although humans typically harbor cancers arising from a single tumor source, these results underscore the importance of genotyping human tumors for the specific Ras mutation driving the cancer, so that the appropriate mutated Ras-bearing yeast will be administered.

9.4 EXPANDING THE CATALOG OF YEAST-EXPRESSED TUMOR ANTIGENS

Besides the GI-4000 yeast targeting individual Ras mutations, several GI-4000 yeast were engineered to express multiple Ras mutations as fusion proteins. All three different yeast strains currently being tested in the clinic harbor one of the three most common amino acid mutations at position 12 (G12V, G12 C or G12D), in addition to both Q61R and Q61L mutations described above. Other yeast strains have been engineered to express in one yeast the most common Ras mutations found in human cancer. These strains, namely, RastaFAR GI-4000 yeast, express fusion proteins with multiple cassettes of Ras polypeptide harboring different Ras mutations. Each of these polypeptide cassettes is sufficiently long so that epitopes for MHC-I and MHC-II presentation may be randomly generated in all different frames and lengths by antigen-presenting cells to support the diversity of HLA binding specificities found in humans.

Table 9.1 lists additional yeast strains that have been engineered to express different tumor antigens. GI-3000 yeast express EGFR as an intracellular protein to target tumors overexpressing EGFR. The administration of GI-3000 yeast expressing EGFR has resulted in the breaking of immune tolerance to gliomas overexpressing EGFR (Lu Y et al, manuscript in preparation). The MUC1 and CEA proteins expressed in GI-6000 Tarmogens have been characterized as overexpressed differentiation or self-antigens in several ductal cancer types, including breast, pancreatic, and colorectal carcinomas. The apparent role of these tumor proteins in metastasis combined with the availability of well-defined transgenic systems featuring them makes these antigens especially attractive targets for yeast-based immunotherapy. Because of the apparent role of these tumor-associated proteins in metastasis, and

TABLE 9.1. Tumor Antigens Expressed in Yeast for Cancer Immunotherapy

Yeast Product Name	Tumor Antigen	Immunogenicity	Tested in Tumor Protection Model	Source[a]
GI-3000	EGFR (human or rat)	Yes	Yes	1
GI-4000	Common mutations in human, mouse or rat K-, H-, or N-Ras	Yes	Yes	Refs. 34,35
GI-6000	Muc1 (human)	No	Yes	2
	CEA	Yes	NT[b]	3
GI-7000	Mart-1 (human)	Yes	Yes	4

[a] *Sources:* (1) Lu Y et al, manuscript in preparation; (2) Borges V, Bellgrau D, Lu Y, Franzusoff A, unpublished observations; (3) Hodge JW, personal communication; (4) Fujita et al, in press.
[b] Not tested to date.

because of the availability of well-defined tumor models, including transgenic animal tumor models, these proteins are attractive target tumor antigens for yeast-based immunotherapy (Borges V, Bellgrau D, Lu Y, Franzusoff A, unpublished observations and Hodge JW, personal communication). The melanoma-associated MART1 protein expressed in GI-7000 yeast has elicited antigen-specific immune responses and tumor protection in transplant and transgenic animal tumor models [43]. Interestingly, since MART1 is not fundamental to the malignant phenotype (unlike EGFR or mutated Ras), eventually cells from treated animals shed the MART1 antigen under immune pressure [43], as would be predicted from the discussion above. The breadth of results demonstrating the immunogenicity of multiple antigens and tumor protection against these different targets illustrates the potential of yeast as a vector for therapeutic cancer vaccines. All of the tumor antigens expressed in yeast tested to date have been immunogenic and have promoted antitumor activity, even in transgenic or homologous model systems.

Yeast-based immunotherapy has also been used to target immune responses against foreign antigens for infectious disease targets such as HIV, HCV, influenza, and fungi. A yeast Tarmogen, GI-5005, expressing an HCV NS3 and Core fusion protein is currently being tested in a phase II clinical trial. Further discussion of yeast-based immunotherapy for infectious disease indications is, however, beyond the scope of the current review.

9.5 YEAST-BASED VACCINE VECTOR DESIGN AND STRATEGIES

Results with the urethane-induced tumor model showed that increasing the number of doses of yeast-based immunotherapy improves efficacy in animal tumor models. Studies performed with a variety of target antigens have revealed that increasing the amount of antigen expressed per yeast cell is also important for enhancing antigen-specific immune responses [21]. Thus, the number of yeast administered and the amount of antigen per yeast are two critical elements of yeast-mediated immune responses. In this section, the methods employed to increase the amount of heterologous antigen expressed while culturing yeast will be described. There are many parameters within vector design that likely impact heterologous protein expression. For this discussion, the focus will be on two key elements of yeast expression vector engineering: plasmid copy number and the yeast promoter used to express the heterologous protein.

Controlling Plasmid Copy Number

Gene expression cassettes may be integrated into the yeast genome as single or several units, or may be maintained as extrachromosomal, or episomal, plasmid elements at low, intermediate, or high copy number. Episomal plasmids are inherently unstable because of the extra metabolic cost required by the cell to replicate the episomal plasmid for cell division. To overcome this metabolic price tag, the

plasmids encode an essential yeast gene product that allows the transfected yeast to survive and grow in media lacking specific nutrients. Thus, yeast that harbor genomic mutations in one or more biosynthetic gene products are considered auxotrophic for a particular biochemical pathway. The standard auxotrophies used for yeast engineering include mutations in the synthesis of individual amino acids, such as histidine (*his3* mutations) or leucine (*leu2* mutations), or pathways for nucleic acid precursors such as uridine (*ura3* mutations) or adenine (*ade2* mutations). A parent yeast that harbors these mutations can be "rescued" by addition of the end product to the media, or by introduction of the wild-type gene for that pathway on an episomal plasmid element to convert the cell from auxotrophy to prototrophy. Therefore, a plasmid engineered to express a heterologous protein also encodes the protrophic gene so that the plasmid will be retained by the cell under selective growth conditions. When the selection is no longer applied, then the yeast will no longer need to replicate the plasmid for survival and the episomal plasmid will be diluted by cell division until new daughter cells will be produced that no longer possess any episomal plasmids.

The advantage of integrating the heterologous expression cassette into the yeast genome is the inherent stability of replicating chromosomes with each cell division. This means that yeast can be cultured in rich, nonselective media that allow for shorter generation times and the density of yeast number per liter of culture is typically an order of magnitude higher than that of yeast grown in minimal selective media. However, the number of copies of the desired gene product is then limited to the number of copies integrated into the yeast genome.

Episomal plasmids may be engineered to encode replication regulatory sequences that dictate how many plasmid copies will be maintained in yeast cells with each cell division. One category of vectors encodes an yeast origin of DNA replication, the autonomously replicating sequence (ARS) plus a centromeric DNA element (CEN) that binds the yeast mitotic spindle, enabling accurate plasmid segregation. This class of plasmids is commonly referred to as "low copy" because accumulation is limited to approximately two to three copies per cell. The other major regulatory element, known as the *origin of replication*, is derived from a naturally occurring circular DNA found in yeast strains, and is also called the *2-μm circle*. This 2-μm origin or replication drives plasmid high-copy accumulation to $\sim 40-60$ molecules per cell. These vectors maintain chromosome-like stability by signaling the recruitment of specialized plasmid-partitioning machinery [35,36]. Thus, the highest possible heterologous protein expression is typically achieved by employing 2-μm-based vectors for expression cassette gene copy numbers higher than those that could be obtained with either chromosomal integration or expression from the low-copy *ARS-CEN*-based vectors.

One additional strategy for amplifying plasmid copy number is that a particular mutation in the *LEU2* gene product, known as the *leu2d* mutation, may also be recruited as a selectable marker for replicating plasmids [37]. The defective leu2d protein is unable to support leucine biosynthesis unless the protein is present in high numbers in the cell (i.e., mass action to overcome the biochemical deficiency). Thus, yeast prototrophy achieved with the *leu2d* gene encoded on the vector results

in very high copy numbers—100–200 copies per cell—when cells are grown in absence of added leucine to the culture media. In practice, though, selection using *leu2d* alone is more complex for initial clone isolation, presumably because of the requirement to achieve immediate high copy number of the episomal plasmids to support auxotrophic growth in the absence of added leucine. Hence, most vectors that utilize *leu2d* for selection also include another prototrophic selection, such as *URA3*, so that the clones harboring the desired expression vector may be selected by growth in the absence of added uridine and once established, the copy number may be enhanced by eliminating added leucine to the culture media. Our studies have indicated that target genes expressed from *URA3* + *leu2d* based vectors generate two- to five-fold more heterologous antigen in growth medium lacking both leucine and uracil than in medium lacking uracil alone (King T, Lu Y, Guo Z, Kelley V, Franzusoff A, unpublished observations).

Promoter Choice: Inducible, Constitutive, or Repressible

The second key element for engineering high-level heterologous protein expression is the choice of promoter for regulating transcription of the heterologous gene (factors that regulate the efficiency of translation initiation or transcription termination are not reviewed here). The type of promoter employed will be influenced by several interrelated factors, such as the need to control the timing of antigen expression, the impact of foreign protein expression on yeast cell health, and whether multiple antigens are produced from one or multiple plasmids within the yeast. The ability to control the rate of mRNA (and protein) accumulation is useful in cases where the antigen is toxic to the yeast cell and/or prone to aggregation. For such proteins, the investigator may need to have control over the rate and timing of mRNA synthesis. This may be achieved through the use of so-called rheostatic promoters—those that can be regulated by the addition of chemical compounds to the cell growth medium. By lowering the transcription rate (e.g., by adding a repressor compound) or initiating transcription late in the yeast culturing process, a heterologous protein that interferes with normal yeast growth may be better produced to higher levels, in addition to yielding higher-density cell harvest. For aggregation-prone proteins, slow or delayed synthesis is sometimes the key to avoiding these unproductive complexes because the protein molecules of interest are allowed to completely fold before encountering other partially folded proteins. From the process development standpoint, some of these controls are achieved by growing yeast at lower- or higher-than-optimal temperatures. Wild-type yeast strains grow most rapidly at 30°C, but can be cultured at 4–37°C. A second advantage of rheostatic promoters is the ability to evaluate the consequences of antigen content independent of the number of yeast administered. Some useful rheostatic promoters for use in *S. cerevisiae* include those that can be induced (e.g., *CUP1*), repressed (*MET25*) or tuned in either direction (e.g., *GAL1–10*, tetracycline-inducible or -repressible variants) [38–42].

The use of constitutive promoters may circumvent limitations of rheostatic promoters and can be useful for simpler proteins that are not prone to aggregation or

that cause toxicity to the yeast. The compounds used to regulate rheostatic promoters are in some cases toxic and need to be removed from bulk yeast drug substance, which may complicate manufacturing and testing for clinical applications. These substances must be added at a defined point in the cell growth cycle, which imposes the need to closely monitor cell growth rate during the production run. The use of a constitutive promoter can mitigate or even bypass these problems because no promoter-activating compounds need to be added. Furthermore, transcription rates from constitutive promoters are frequently higher than those for rheostatic promoters, resulting in higher total levels of heterologous protein. Examples of strong constitutive promoters in *S. cerevisiae* include *ADH1* (alcohol dehydrogenase), *ENO2* (enolase), *TEF1*, *TEF2* (translation elongation factors 1 and 2), and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase). The endogenous products of these promoters are among the most abundant in the yeast cell under routine culture conditions (e.g., glucose plus nitrogen source). Promoters normally used to make components of the protein synthesis machinery are excellent choices for the present application because they generally drive transcription of essential, single-copy genes that must be activated to achieve protein synthesis. Thus, heterologous protein expression driven by a constitutive promoter that is normally used in ribosomal biogenesis will correlate directly with bulk cellular protein synthesis.

Finally, promoter choice may be influenced by the need to create multiantigen products. Yeast have been engineered for immunotherapy to express two different antigens at the same or different levels, raising the question as to whether both genes should be expressed from the same or different promoters. The *CUP1* promoter recruits a transcription factor (Ace1p) whose amount is limiting for maximal transcription of one mutated Ras protein, like that engineered for the GI-4000 strains. Overexpression of the *ACE1* gene on a 2-μm-based plasmid was observed to result in doubling the quantity of *CUP*-driven expression of mutated Ras protein produced on copper addition (King T, Lu Y, Franzusoff A, unpublished observations). This example illustrates that a promoter can have limited capacity to drive transcription because of a limited supply of a promoter-specific transcription factor (or factors). If two different antigens should be highly expressed, the best expression strategy may therefore be to utilize two strong promoters, such as *TEF2* and *GAPDH*, that use independent regulatory factors.

This discussion illustrates that there are numerous ways to express tumor antigens in yeast. It is also important to point out that antigens may be localized to different compartments in yeast and still be immunogenic, specifically, as cytosolic proteins or membrane proteins, and even secreted into the periplasm, as long as the protein remains behind the cell wall or is somehow retained by yeast after administration [6]. Success can be achieved with various strategies, and the optimal system will be empirical, depending on the number of tumor antigens being expressed and how their biochemical properties affect protein accumulation and yeast cell metabolism. In the end, the optimal yeast expression system will result in high-level heterologous protein levels with a simple manufacturing protocol that does not involve many post-culturing steps to reduce the potential for toxic by-products of manufacturing.

Parameters of Dose Responses with Yeast-Based Immunotherapy

Four parameters of dosing with recombinant yeast have been investigated: (1) the amount of antigen expressed per yeast cell, (2) the number of yeast administered per dose, (3) the number of doses administered over time, and (4) the route of administration. The importance of these four parameters to efficacy of yeast-based immunotherapy have been elucidated with in vitro and in vivo studies of T-cell-dependent immune responses and tumor protection, respectively [21]. These studies were performed with yeast expressing several different tumor or infectious disease antigens, so the identity of the antigen does not seem to influence the reproducibility of observations related to the parameters of dosing efficacy.

Increasing the number of yeast and the amount of antigen expressed per yeast appears to improve the relative abundance of CTLs in immunized animals, particularly for foreign antigens and mutated self-antigens. As described in Section 9.4, the amount of antigen per yeast may be modulated by using different promoters and plasmids to express the same antigen at different levels in different yeast strains, or to modulate the activity of an inducible promoter by adjusting the amount of inducer (e.g., copper) added to the cultures for activating antigen expression in yeast. Thus, for foreign antigens (HIV-Gag, HCV NS3 and core proteins, influenza HA and M1 antigens) and mutated self-antigens (mutated Ras), a "more is better" paradigm appears to be operative, namely, more yeast administered plus more antigen expressed per yeast. Immune efficacy with recombinant yeast expressing lower amounts of antigen may still be achieved by increasing the number of yeast administered per dose. Similarly, fewer yeast are needed to exceed the threshold of immune efficacy if the heterologous antigen is expressed at very high levels in yeast, specifically, when the antigen is expressed as high as 5–10% of total yeast cell protein [21] (Franzusoff A, Duke R, unpublished observations). Surprisingly, for targeting self-antigens such as wild-type EGFR and CEA, the results from preliminary studies suggest that the administration of fewer yeast, albeit with high levels of heterologous antigen expressed per yeast, may be more efficacious than dosing with higher numbers of yeast (Hodge JW, personal communication; Lu Y, Franzusoff A, manuscript in preparation). The reasons for this alternate paradigm are currently being investigated.

Activating T cell responses by immunization via multiple different routes of administration has been studied with in vitro and in vivo models and with different yeast-expressed antigens. Immune responses have been assessed by multiple parameters, namely, by lymphocyte proliferation, CTL, ELISpot (enzyme-linked immunospot assay) and intracellular cytokine secretion assays, to compare the impact of administering yeast by subcutaneous, intranasal, oral, intraperitoneal, and intravenous routes. Each of these routes of administration were effective for activating antigen-specific immune responses with recombinant yeast. Interestingly, more profound differences were observed when animals were challenged with tumors implanted in different locations. Subcutaneous and intranasal administration of yeast-based immunotherapy were equally effective against challenge

with tumors implanted subcutaneously. However, when tumors were implanted intracranially, administering recombinant yeast by the intranasal and oral routes achieved significantly better protection against tumor challenge than did subcutaneous dosing (Lu Y et al, manuscript in preparation).

Thus, the choice of administration route may be important for specific disease indications, since T cells that become activated by one route of administration are likely to recirculate in the related lymphoid tissues for surveying the appearance of target antigens presented by diseased cells. For instance, the intranasal and oral routes are effective for activating mucosal-tissue-associated lymphoid surveillance. Hence, some diseases that are associated with mucosal tissues, such as lungs, intestines as well as head and neck cancers, may receive more attention of T cells activated by the mucosal route. The relative benefit of route of administration for specific indications is admittedly speculative.

In summary, yeast-based immunotherapy surprisingly exhibits dose–response parameters commonly associated with dosing small-molecule drugs. In this regard, the amount of antigen in the yeast, the number of yeast given, the number and schedule of immunizations, and the route of administration are all factors contributing to the extent of the immune response and its effectiveness in a given model system. These factors may contribute different levels of importance depending on the antigen and model used, and thus represent an empirical guide for testing. These preclinical findings continue to be evaluated with more antigens engineered into the yeast and with cancer models of spontaneously induced tumors or infectious diseases that involve different organs. The more conclusive tests of these hypotheses will require randomized controlled trials with patients harboring these different diseases.

9.6 PRECLINICAL SAFETY STUDIES AND CLINICAL TRIALS IN CANCER WITH YEAST-BASED IMMUNOTHERAPY

Preclinical Safety Studies with Yeast-Based Immunotherapeutics

Yeast-based vaccines expressing three different antigens have been tested in five separate toxicity studies in rabbits conducted under Good Laboratory Practices (GLP). Rabbits were injected subcutaneously with 0.5–100 YU per injection site, and up to 13 weekly injections before histopathological analysis. Aside from occasional minor injection site reactions that resolved after 2 weeks' recovery, none of the rabbits showed signs of abnormality, aside from increased levels of circulating neutrophils. The equivalence of the safety profile from the five GLP toxicity studies implies that the yeast vector, not the expressed antigen, has the greatest influence on the reactions to immunization. Indeed, the investigational new drug (IND) application for the three GI-4000 series of products that are being tested in the clinic in patients with cancers harboring mutated Ras was filed with a single chemistry manufacturing and controls (CMC) section, by agreement with the FDA. However, because of the mechanism of action elucidated by the study with

urethane-induced tumors in mice (described in Section 9.2), only one of the three GI-4000 yeast is administered to cancer patients. The selection of which GI-4000 yeast to administer is governed by the identification of the Ras mutation genotype in the patient's tumor to match the yeast expressing the relevant mutated Ras polypeptide.

GI-4000-01 Phase I Safety and Immunogenicity in Metastatic Cancer

In a phase I clinical trial of GI-4000 series yeast, 33 subjects with advanced pancreatic or colorectal cancer were enrolled. Greater than 90% of the patients enrolled in the trial had metastatic disease at the time of enrollment, and subjects had received an average of three previous therapy regimens prior to participation in the GI-4000-01 trial.

The design for this "first time in human" study was a dose escalation trial, in which cohorts received either 0.1, 1, 5, 10, 20, or 40 YU of the mutation-matched GI-4000 yeast administered subcutaneously for 5 weekly doses. Subjects were monitored for safety, local injection site reactions, complement cascade activation post-injection, and antigen-specific immunoloGI-c responses. No dose-limiting local or systemic toxicities were observed at any of the doses tested. The majority of subjects exhibited antigen-specific responses as demonstrated by lymphocyte proliferation and/or intracellular cytokine staining assays. Several subjects continue to be followed for survival. A manuscript describing the results of this study is in preparation.

GI-4000-02 Phase II Testing in Resected Pancreas Cancer

GI-4000-02 is a multicenter, double-blind, placebo-controlled, adjuvant trial comparing GI-4000 in combination with a gemcitabine regimen versus a gemcitabine regimen alone in patients with successfully resected *ras*-mutation-positive pancreas cancer. Another enrollment criterion is that the surgery have a tumor resection status of R0 or R1, indicating that post-resection, the pathology shows a resection margin that is free of even microscopic disease (R0), or that there is evidence of microscopic disease at the resection margin, but no macroscopic disease (R1).

Subjects will receive three doses of GI-4000 yeast after resection, but prior to initiation of gemcitabine. Monthly doses of GI-4000 or saline placebo are administered after the start of gemcitabine during the drug holiday between gemcitabine cycles. The primary endpoint of the trial is median overall survival at 15 months after resection.

9.7 CONCLUSIONS

This chapter summarizes key aspects of using the recombinant yeast Tarmogen platform technology for cancer immunotherapy. The danger signals from recombinant yeast immunization trigger a multifactorial innate immune response. This

innate immune response profoundly impacts and activates the antigen-presenting cells, which promotes specific T cell responses to the yeast-delivered tumor antigen. The yeast-based immunotherapy platform has been used for immune responses against a variety of foreign, mutated, or self-antigens.

The expression of polypeptide tumor antigens in yeast promotes the processing of a comprehensive assortment of peptides in these yeast-activated APCs. The delivery of this broad catalog of peptides into antigen presentation pathways increases the probability that MHC receptors from diverse HLA genotypes will be able to bind peptides derived from yeast-expressed antigens. As a result, even tumors bearing single amino acid mutations in Ras, or overexpressing self-antigens, are capable of being targeted for destruction by yeast-mediated immune responses in a broad range of immunized hosts. The specific targeting and ablation of tumor cells that is highly desirable for the immune protection against cancer may be achieved without off-target side effects.

Through the many studies with yeast-based immunotherapy in animals and the phase I clinical trial in cancer and the phase Ib trial in chronic hepatitis C infection, numerous significant (including some surprising) observations about the use of recombinant yeast for activating innate and antigen-specific immune responses have emerged:

Antigen delivery and yeast-mediated immune responses

- Dosing with recombinant yeast drives a Th1-type cytokine response profile, including TNFα, GM-CSF, and IFNγ.
- Recruitment and activation of APCs does not require the addition of artificial adjuvants.
- Yeast-expressed antigens are delivered into the MHC-I pathway by nonclassical "cross-presentation", in addition to classical MHC-II presentation of yeast-delivered antigens.
- Orders-of-magnitude improvement in antigen-specific $CD8^+$ T cell responses when target antigen is expressed in yeast compared to mixing soluble protein with empty yeast.

Vaccine efficacy

- Recombinant yeast are immunogenic by multiple routes of administration.
- Yeast-based immunotherapy is able to break immune tolerance to self-antigens.
- The potential for administering "cocktails" of yeast expressing different antigens may be adopted for customizing the immunotherapy according to the target tumor genotype.
- The yeast may be immunized repeatedly without eliciting neutralizing antibodies that interfere with improvements in antigen-specific immune response.
- The clean safety profile observed with dosing recombinant yeast in animals and humans.

Vaccine manufacturing

- Heat-killing yeast does not destroy the immunogenicity of yeast-expressed antigens (although this must be confirmed for each new antigen tested).
- The simplicity and scalability of the Tarmogen manufacturing process.

In closing, immunotherapy represents an attractive strategy for the treatment of cancer. Nevertheless, the challenge of dealing with high-burden tumors in late-stage cancer patients should not be underestimated. The phase II trial design that we have adopted for testing yeast-based immunotherapy in pancreas cancer specifically avoids the complications of high-burden cancer (see Section 9.6, on GI-4000-02 phase II testing in resected pancreas cancer). The range of potential obstacles that may emerge with treating high-burden tumors include immunosuppressive factors such as TGFβ, amplification of regulatory T cells, as well as the potential for reduced numbers of nascent immune cells to promote antigen-specific immune responses in patients with advanced cancer. Fortunately, cancer immunotherapy may be combined with other strategies that target these hurdles contributed by high tumor burden, such as targeted therapy with monoclonal antibodies or small-molecule drugs and chemotherapeutic regimens. We are currently exploring the impact of combining yeast-based immunotherapy with approaches that are predicted to reduce or overcome these hurdles.

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PART IV

CELL-BASED THERAPY: USING CANCER CELLS AS A MEANS TO INDUCE SPECIFIC TUMOR IMMUNITY

10

ALLOGENEIC WHOLE-CELL VACCINES

John Copier and Angus Dalgleish

10.1 INTRODUCTION

There is evidence that allogeneic whole-cell vaccines can raise immune responses and have clinical efficacy in prostate cancer and in melanoma [1,2]. Much effort has been applied to the discovery of tumor-specific antigens, resulting in tried and tested vaccination strategies involving peptide, whole protein, and cDNA. In contrast, whole-tumor cells have been used in a "polyvalent" approach that does not require knowledge of tumor antigens but instead assumes that target antigens are present in the vaccine cells themselves. In this chapter we will deal with the mechanisms by which whole-cell vaccines work and discuss the ways in which modification of vaccine cells may improve their efficacy. Particular emphasis will be given to those vaccines with demonstrable clinical outcomes.

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10.2 HOW WHOLE-CELL VACCINES ELICIT EFFECTIVE T CELL RESPONSES

Preclinical studies in mice have demonstrated robust antitumor responses to whole-cell vaccines that are dependent on a variety of immunologic mechanisms, including T cell (both $CD4^+$ and $CD8^+$) and NK responses, and the presence of macrophages and eosinophils depending on the formulation of the vaccine [3–10]. How these vaccines work is poorly defined and remains an area of controversy. The majority of studies indicate a substantial requirement for cytotoxic T cell responses that are elicited through one or both of the following mechanisms: either (1) the vaccine cells directly prime T cells or (2) vaccine antigens are taken up by professional antigen-presenting cells (APCs), which then stimulate a T cell response, an effect known as *cross-priming*. Evidence for direct priming is scant, but a few studies have indicated that access of tumor cells to the T cell areas of the lymph node are a requirement for tumor rejection, indicating the likelihood of a direct interaction between the vaccine and the T cells [11–13]. Dendritic cells are capable of taking up and presenting tumor antigens [14,15]. Moreover, several studies have shown that cross-priming through presentation on host APCs is of greater importance than direct priming by the tumor cell since a match between the tumor MHC and the host was irrelevant [15,16]. Whether direct priming or cross-priming is the central mechanism for stimulating cytotoxic responses against whole-tumor vaccines remains controversial, and at least one study has suggested that both mechanisms are required [13]. It is clear that in some tumor models depletion of $CD4^+$ cells abrogates the responses to vaccination. Although initial data implied that MHC class II–positive tumor cell vaccines directly primed $CD4^+$ T cells [17,18], more recent data show that dendritic cells are absolutely required for this process [19]. Furthermore, there is startling evidence that MHC class I and class II molecules can be transferred (presumably along with their peptides) from the tumor vaccine to dendritic cells, a mechanism called "cross-dressing" [19].

10.3 ADVANTAGES OF WHOLE-CELL VACCINES

There are a number of advantages to using whole-cell vaccines. As mentioned above, there is no specific requirement for knowledge of the nature of the target antigen. In addition, cellular vaccines can be modified in ways that modulate immune responses (see discussion below). The polyvalent approach assumes that vaccine cells have an antigenic profile identical to, or overlapping with, the patient's tumor. In the case of autologous tumor cell vaccines, prepared directly from resected tumor tissue, this is probably the case, and clinical benefit has been shown using such vaccines in randomized trials of colorectal and renal cancers [20,21]. However, autologous vaccines have a number of disadvantages when translated to the clinic. Resected autologous tumor's must provide enough cells for a vaccine schedule, and where this is not possible, they must be amenable to culture, which is often not

the case. Moreover, lengthy culture periods can allow sufficient time for a patient's disease to progress. Since allogeneic cell lines are already established in culture, this is not a problem and vaccine is relatively easy to prepare. Furthermore, allogeneic cells can be prepared as a "modified" vaccine (i.e., transduced to express cytokines or costimulatory factors). Autologous cell lines modified in this way are difficult to establish and similarly have the problem of lengthy culture time.

In order for allogeneic vaccines to work, it is necessary for them to share an antigenic profile with the patient's tumor. Several preclinical studies have demonstrated that selection of the source of tumor cell line is important to determine its effectiveness as a vaccine. Allogeneic cell vaccination was successful in a B16-F10 melanoma model when allogeneic cells of a melanoma background were used but not when the vaccinating cells were derived from an irrelevant carcinoma [22,23]. However, not all allogeneic melanoma cell lines were able to protect against challenge with B16-F10 [22]. These studies suggest that very careful selection of tumor cell lines must be made in order to match the patient with the vaccine. Cell lines derived from secondary tumor sites require careful screening, as it has been demonstrated that expression levels of known tumor antigens vary considerably depending on their origin [24,25]. Furthermore, there are variations in other factors that may influence immunogenicity, such as expression of LMP and TAP, although treatment with IFNγ was shown to upregulate these molecules, indicating that the immunogenicity of the cell lines can be manipulated in vitro. To ensure an adequate antigenic profile, most cellular vaccines incorporate several different cell lines from the same background as the patient's tumor [1,2].

10.4 EFFECTIVENESS OF ALLOGENEIC WHOLE-CELL VACCINATION

Proof of principle for the effectiveness of allogeneic whole-cell vaccination has been established in mouse tumor models where both prophylactic protection against tumor challenge and the presence of tumor-specific CTLs was demonstrated [22,26,27]. In a rat Lobund–Wistar model of prostate cancer, prophylactic vaccination with an allogeneic cell vaccine was effective in protecting 80% of the treatment group against challenge with a PAIII tumor [28]. When this was repeated in a Copenhagen rat model (where the cancer was poorly immunogenic and more aggressive), no protection was elicited by treatment with allogeneic vaccine. However, this and other studies have shown that addition of adjuvant to ineffective allogeneic vaccines leads to protection against tumor challenge [27,28].

Clinical trials have been quick to follow, and while encouraging clinical results have been shown in melanoma and prostate cancer, there remain difficulties in monitoring immunologic responses to such vaccines. Unlike monovalent vaccines, where the antigen or even the epitope is known, polyvalent vaccines rely on unknown antigens. It is therefore difficult to apply immunologic tests that measure the specificity of the response. Most investigators look for DTH responses (against the vaccine), CTL/IFNγ responses or cytokine responses (raised in vitro

against either the vaccine or autologous tumor). However, it is difficult to interpret these data unless there is a clear correlation with a clinical response.

A vaccine composed of three allogeneic cell lines (OnyCap23, LncaP, and P4E6) has been used in clinical trials for the treatment of hormone-resistant prostate cancer with encouraging results [2]. Patients treated with the vaccine had a median time to progression of 58 weeks, compared to 26 weeks for historical controls, and there were prolonged decreases in PSAV (a prognostic biomarker used to assess the response of prostate cancer to therapy) in 11 of 26 patients. Immunologic responses were monitored and analyzed using artificial neural networking (a powerful multiparametric analytic tool that can be used when standard statistical methods fail to show patterns in the data). This analysis revealed that positive clinical outcome correlated with a reduced PSAV and a T_H1 response compared to nonresponsive patients who had a mixed T_H1/T_H2 response. A phase IIB trial began in August 2006.

Clinical trials in melanoma have also been encouraging. Vilella and coworkers used a mixture of 10 allogeneic melanoma cell lines in combination with BCG (as an adjuvant) to vaccinate metastatic melanoma patients and showed clinical benefit in 6 of 23 patients: three complete responses, two partial responses, and one mixed response. Patients had a median survival of 20.2 months [29]. A series of trials for Canvaxin (a mixture of three allogeneic melanoma cell lines plus BCG) showed clinical benefit and immunologic responses in advanced melanoma patients. In phase II trials the vaccine significantly prolonged survival in the treatment arm when compared with a nonvaccinated control arm [30,31]. A preexisting T_H2 cytokine profile in peripheral blood $CD4^+$ T cells was associated with better prognosis following vaccination and, moreover, DTH and humoral responses to vaccine were shown to correlate with clinical outcome [30,31]. However, a phase III trial in which Canvaxin was compared to a control arm (BCG alone) was stopped early as there was no evidence that the two arms were diverging. The BCG arm itself did better than expected and may account for the differences between this and earlier trials. Despite the failure of this trial, earlier results were convincing enough to warrant some optimism since differences between the phase II and phase III trial results may be due to differences in the trial design or patient selection [32]. Moreover, melanoma studies often result in disappointing stage III results after encouraging phase II trials, perhaps reflecting the chaotic nature of melanoma, which is characterized by rapid division and widespread metastasis.

10.5 MODIFICATION OF ALLOGENEIC CELL VACCINES WITH COSTIMULATORY MOLECULES

Tumor cells are generally thought to be poorly immunogenic, although immunogenicity varies considerably between tumors of different backgrounds. A variety of strategies have arisen that are aimed at improving the immunogenicity of the vaccine including modification of vaccine cells to express costimulatory molecules or cytokines. Modification of allogeneic cell lines is often difficult as transfection

often fails and it is necessary to use viral vectors to introduce the relevant cDNA, which itself carries certain implied health risks to patients. Nevertheless, a certain degree of success has been obtained in preclinical and clinical studies using modified allogeneic cells as cancer vaccines.

Poor immunogenicity is due at least partly to the heterogeneous expression of MHC and costimulatory molecules [33,34]. The absence of costimulation raised concerns that vaccine cells might induce tolerance, and consequently the introduction of B7.1/CD80 costimulatory molecules into vaccine cells was investigated in preclinical models. Several studies showed that expression of CD80 on autologous vaccine cells elicited CTL responses and improved protection against tumor challenge [35–38]. However, results from one study suggested that CD80 expression is effective only when the vaccine cells are themselves immunogenic [39]. In allogeneic models effective protection against tumor was also demonstrated [40].

Studies using human cells have shown that introduction of costimulatory molecules results in a large alloresponse, but despite this finding, tumor-specific CTLs could also be demonstrated [41,42]. Furthermore, HLA-$A2^+$ allogeneic breast cancer cells modified to express CD80 were shown to stimulate $CD8^+$ T cell responses from PBMCs of an HLA-$A2^+$ patient [43]. In a phase I clinical trial to treat 19 patients with relapsed metastatic NSCLC, an allogeneic vaccine modified to express CD80 and a patient-matched HLA-A molecule (either A1 or A2) was used [44]. In the majority of patients, IFNγ release from $CD8^+$ cells was seen (as measured by ELISpot) in response to stimulation with mock transfected vaccine cells ($CD80^-$/HLA-A1/$A2^-$). Patients had a 32% response rate (one partial response and five stable disease) with a median overall survival of 18 months compared to the previously reported median survival of less than one year for metastatic lung cancer.

The apparent effectiveness of modification of vaccine cells with costimulatory molecules raises further questions concerning the mechanism by which whole-cell vaccines stimulate T cell responses. Introduction of costimulatory molecules into vaccine cells presumes that direct priming forms a component of the mechanism for T cell stimulation. Work by Cayeux and colleagues showed that, in mouse models, allogeneic cellular vaccines caused rejection of tumor via a predominantly cross-presentation pathway [40]. However, direct priming occurred when CD80 was introduced into the vaccine, suggesting that introduction of costimulation may influence the mechanism of T cell stimulation. It is interesting to note that many allogeneic cell vaccines used in humans are matched for at least one HLA allele, leaving the possibility that such vaccines work, at least in part, through direct presentation of antigen.

10.6 MODIFICATION OF ALLOGENEIC VACCINES TO SECRETE CYTOKINES

Cytokines have been used to treat advanced cancers, including renal cell carcinoma and metastatic melanoma with some success [45,46], but systemic administration

is associated with marked toxicity. Recombinant cytokines expressed by modified tumor cells can be used to target the cytokine to tumor or vaccination sites where they can induce a proinflammatory environment, with the additional benefit of lowering the levels of circulating cytokine and therefore circumventing the toxicity related to high systemic doses.

Cytokine-secreting tumor cells have been tested extensively in mouse models for their efficacy in reducing tumorigenicity, protecting against tumor challenge and raising immune responses. To date a range of cytokines, including IL-2 [6,47,48], IL-4 [8,49,50], IFNγ [51,52], GM-CSF [53], IL-12 [54,55], IL-15 [56], IL-21 [57–60], IL-23 [61–63], and IL-27 [64–66], have been investigated in models of autologous tumor cell vaccination with a considerable degree of success. Of these only IL-2 and GM-CSF have been shown to have potential in allogeneic tumor cell vaccination models.

Interleukin 2 is produced by $CD4^+$ T cells of the T_H1 type. It supports recruitment, differentiation, and proliferation of T and NK cells and is frequently used systemically for the treatment of renal cell carcinoma and melanoma. In autologous IL-2-secreting cell vaccination models there is a requirement for $CD8^+$ T cells and/or NK cells to establish effective protection against tumor challenge [4,5,47]. In a study where allogeneic cell vaccines were shown to be protective against tumor challenge in a prophylactic treatment model, IL-2-secreting vaccine was more effective than unmodified cells [67]. Furthermore, vaccination with IL-2-secreting allogeneic cells gave rise to a cytotoxic response that recognized syngeneic tumor. This study also showed that a mixture of IL-2-secreting autologous cells and allogeneic cells gave complete protection in a therapeutic model. In human studies limited immunologic responses have been demonstrated after IL-2-modified allogeneic cell vaccination. Two studies in melanoma patients showed that $CD8^+$ T cell responses could be mounted against vaccine cells in a small proportion of the patients [68,69]. Another study demonstrated that IgG responses could be stimulated using the allogeneic melanoma cell line Mel 4932 [70]. These sera stained autologous tumor and could elicit ADCC responses against Mel 4932, although no killing of autologous tumour was seen. Trials using allogeneic IL-2-secreting tumors have generally been clinically disappointing [68,69]. However, one notable study showed clinical benefit when patients were vaccinated with a mixture of allogeneic IL-2-secreting cells and autologous cells [71]. Of 30 patients with metastatic RCC, objective responses were seen in 14 (1 complete response, 4 partial responses, 9 with stable disease). There was improved median survival in the vaccination group compared to control subjects (18.9 months and 12.2 months, respectively; $p = .01$).

Although GM-CSF has highly pleiotropic effects, it is involved principally in stimulation of proliferation and activation of granulocytes and monocytes. GM-CSF stimulates dendritic cell maturation and is able to augment APC function [72]. Protective immunity is induced by vaccination with allogeneic GM-CSF-secreting cells, and stimulation of cytotoxic responses to tumor cells has been demonstrated [26,73,74]. Furthermore, antitumour responses were shown to be dependent on both $CD4^+$ and $CD8^+$ T cell subsets. GM-CSF-secreting tumor cell vaccines appear to elicit responses through a cross-priming mechanism [40], which is consistent

with the data showing that GM-CSF secreting allogeneic cell vaccine leads to an accumulation of dendritic cells at the vaccination site [75], a process that is dependent on MIP-1α (a chemokine secreted by macrophages).

In clinical trials a GM-CSF-secreting allogeneic vaccine composed of two pancreatic cell lines (PANC-10.05 and-6.03) exhibited a trend toward disease free survival in patients with pancreatic adenocarcinoma, although patient numbers were too small to make this study statistically significant [76]. More recent studies have used a combination of GM-CSF-secreting cells (PC-3 and LNCaP) for the treatment of advanced hormone-resistant prostate cancer. Presentations at ASCO suggest that in 7 of 10 patients there was a trend toward increased survival [77]. Several other phase I/II trials using this allogeneic vaccine are ongoing for prostate cancer.

These studies demonstrate that, in principle, immunologic responses can be raised to IL-2- and GM-CSF-secreting allogeneic tumors and that some clinical benefit can be demonstrated. However, the clinical efficacy of these vaccines (and in particular GM-CSF) have not borne out the promise of preclinical trials, and further work is required to optimize these treatments for humans.

10.7 FUTURE RESEARCH

Improvements in our understanding of the underlying biology of tumours and the nature of antitumour responses may help us to improve the efficacy of whole-cell vaccines. It is well known that tumors provide an immunomodulatory environment in which T cell responses may be suppressed. One key factor in the modulation of antitumor responses is the presence of T-regulatory cells (Treg), a subset of $CD4^+$ T cells that suppress immune responses and are found in many tumor environments [78]. Ablation of Tregs prior to immunotherapy has resulted in improved protection indicating that antitumour T cell responses are effective when not suppressed by Tregs. Several preclinical studies have shown that treatments that affect immunomodulation by Tregs, such as CTLA-4 blockade or Treg depletion, result in improved effectiveness of cytokine-secreting tumor cell vaccines [59,62,79,80]. These studies have been performed with autologous vaccines, and work has yet to be done to determine the efficacy of Treg modulation in combination with allogeneic vaccines.

There is a growing movement in the field of immunotherapy toward the combination of vaccines with other forms of intervention, including standard chemotherapy and radiotherapy. GM-CSF-secreting autologous vaccines, have been studied in combination with chemotherapy, principally doxorubicin, docetaxel, paclitaxel, and cyclophosphamide [81–83]. These preclinical studies demonstrated improved protection against tumor challenge when vaccine was administered in combination with chemotherapy, but that efficacy was dependent on correct timing of administration of the drug and the vaccine. Further work is required to demonstrate the effectiveness of vaccine–chemotherapy combinations in the allogeneic setting. Clinical

trials are currently under way to test the allogeneic GM-CSF vaccines in combination with docetaxel for hormone-resistant prostate cancer (GVAX, Cellgenesys) and with cyclophosphamide and doxorubicin.

10.8 CONCLUSIONS

Whole-cell allogeneic vaccines have shown promising results in preclinical and clinical settings. Other polyvalent vaccine strategies, beyond the scope of this chapter, using allogeneic tumor cell lysates have been more clinically successful in prostate cancer and melanoma [20,84], suggesting that the antigens required to raise an antitumour response are present but that the method of presentation of the vaccine is important. Furthermore, dendritic cells loaded in vitro with whole allogeneic cell lysates of tumor cells have been investigated in melanoma with some encouraging results [85,86]. These strategies effectively mimic the first steps of cross-presentation that occur in vivo and allow tumor antigen presentation by professional APCs. However, the requirements for optimal loading of dendritic cells with lysate remains elusive.

Ultimately, in vitro loading of dendritic cells will provide indications of the requirements for cross-priming in vivo and give clues to the improvement of whole-cell vaccines.

Further work is required to determine the best strategy for allogeneic whole-cell vaccination. Parameters such as number of cells, route of injection, timing of vaccinations, and presence of adjuvant still require optimization. The potential for use in combination with other therapies adds a further layer of complexity. However, results from unmodified allogeneic cell vaccines are very encouraging for prostate cancer [2] and possibly melanoma [1].

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11

JUMP-STARTING TUMOR IMMUNITY WITH BREAST CANCER THERAPEUTICS

Leisha A. Emens

11.1 INTRODUCTION

Breast cancer stands out among the solid tumors as a model for advances in cancer care. The past few decades have produced multiple incremental improvements in local management with surgery and radiation, and in systemic treatment with endocrine manipulation and chemotherapy. In the aggregate, these improvements have decreased the morbidity and mortality of breast cancer in a clinically meaningful way, but up to 40% of patients diagnosed with the disease continue to ultimately succumb to it [1]. More recently, the genomic revolution has opened the door for designing therapy individualized to the patient's tumor biology as reflected by gene expression patterns rather than anatomic staging. This advance will build on the longstanding success of tamoxifen as an effective treatment for tumors that express the estrogen receptor α (ERα), as it will facilitate the incorporation of novel therapeutics that target the critical regulatory pathways underlying breast cancer biology. The clinical success of the HER2/*neu*-specific monoclonal antibody trastuzumab illustrates the power of this customized approach to disease

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management [2]. Despite considerable progress and the promise of novel targeted therapies, fixed drug resistance remains a major limit to the efficacy of traditional drug-based systemic breast cancer therapy, arguing for an innovative approach to the management of mammary cancer.

Tumor vaccines offer a unique alternative that recruits the patient's own immune system to the therapeutic effort, altering the host–tumor relationship rather than directly targeting the tumor itself. A number of breast cancer vaccines have already been tested in the clinic, with some evidence of vaccine-induced immunity [3]. However, there has been little evidence of clinical benefit to date. Multiple factors likely explain this lack of success: (1) breast cancer vaccines have frequently been tested in advanced disease, where the disease overwhelms the immune response by both its physical magnitude and tumor-associated networks of immune suppression; (2) most breast cancer vaccine formulations are relatively weak immunogens, with suboptimal platforms, tumor antigens, or both; and (3) many trials have simply added a breast cancer vaccine to standard systemic therapy with little regard for how they might interact. Consideration of each of these factors should facilitate the future design of robust clinical vaccine trials that yield important mechanistic and clinical information, thereby hastening the development of breast cancer vaccines and bringing them to clinical fruition.

11.2 BARRIERS TO EFFECTIVE BREAST TUMOR IMMUNITY

As suggested above, barriers to effective breast tumor immunity include the sheer physical burden of tumor cells in advanced disease, well-established mechanisms of systemic immune tolerance, and immunosuppressive regulatory pathways specific to the tumor microenvironment itself. Therefore, strategies for combining breast cancer vaccines with standard cancer therapeutics can be developed that minimize the impact of tumor burden by cytoreduction, lift layers of systemic immune tolerance to allow tumor immunity to develop, augment T cell activation, and groom the tumor microenvironment to facilitate the activity of infiltrating immune effectors. Ideally, combinatorial immunotherapy will incorporate each of these strategies to produce a synergistic antitumor response that is effective and uniquely durable by virtue of the immunologic memory response.

11.3 CHEMOTHERAPY

Tumor Immunity and Cytoreduction

Therapeutic doses of chemotherapy can enhance the activity of tumor vaccines in at least three ways: (1) by achieving a state of minimal residual disease prior to vaccination, (2) by augmenting vaccine-induced immunity through treatment-related apoptosis, or (3) by skewing the T cell repertoire during lymphopenia-induced homeostatic proliferation.

The magnitude of both the spontaneous and therapeutic antitumor immune response is frequently outmatched by the sheer physical burden of the tumor cells it must contend with, particularly in the setting of advanced, disseminated disease [4]. In this case, it may be prudent to use standard cancer therapy (including surgery, radiation, and chemotherapy) to debulk existing disease, thereby achieving a state of minimal residual disease prior to or after therapy with a tumor vaccine. In this case, cytolytic doses of chemotherapy are used. Whether the impact of chemotherapy on the vaccine-induced immune response is positive or negative depends on the drugs, the dose given, and the timing of chemotherapy in relation to immune-based therapy [5]. At least two studies have shown that standard-dose chemotherapy can inhibit the activity of tumor vaccines if given in close proximity or as multiple cycles [6–8]. A distinct study showed that the vaccine-induced immune response can be restored with appropriate vaccine boosting schedules [6,8]. Interestingly, several studies have shown that standard-dose chemotherapy given after vaccination in patients with various types of advanced cancer correlates with clinical benefit and (in some cases) with enhanced antitumor immunity [9,10]. This observation is likely due to the boosting of preexisting immunity by chemotherapy-induced tumor apoptosis. In human breast cancer, neoadjuvant paclitaxel (PTX) chemotherapy induces both tumor apoptosis and the accumulation of tumor-infiltrating lymphocytes (TILs) at the tumor site, with the extent of first-dose apoptosis predicting both increased numbers of TIL and clinical benefit [11]. Finally, vaccinating during lymphopenia, such as occurs during bone marrow transplantation or after aggressive chemotherapy, can direct the reestablished T cell repertoire toward a desired antigenic specificity [12–15]. This concept has been evaluated in clinical trials testing the adoptive transfer of T cells after myeloablative chemotherapy [16–18], with a correlation seen between tumor regression and significant levels of peripheral tumor-specific T cells in some cases [17,19–21].

Immune Tolerance and Activation

Chemotherapeutic agents can also modulate immune tolerance in novel ways (Fig. 11.1), depending on the dose and timing in relation to an antigen exposure [5,22,23]. Cyclophosphamide (CY) and doxorubicin (DOX) have been the mainstay of adjuvant chemotherapy for breast cancer. Both can modulate immunity, particularly when given at low doses. For example, CY given at low doses 1–3 days prior to antigen exposure can break immune tolerance, facilitating both humoral and cellular immunity. Conversely, CY given at the time of or after antigen exposure induces immune tolerance. CY augments tumor immunity by eliminating the influence of $CD4^+CD25^+$ regulatory T cells [24–27], reversing immunologic skew (favoring T helper type I immunity over T helper type 2 immunity) [23] and promoting the evolution of a memory T cell response [28]. DOX augments $CD8^+$ T cell responses when given after chemotherapy in some systems [22,23] and prior to chemotherapy in others [29]. PTX and DTX are also key components of systemic therapy for breast cancer, and both have immunomodulatory

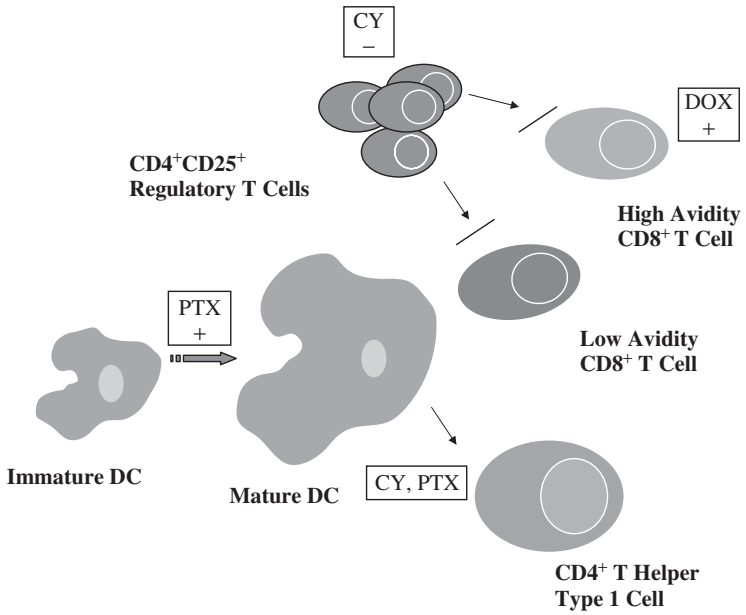

Figure 11.1. Chemotherapy modulates immune tolerance. Standard breast cancer therapeutics can modulate immune tolerance. Paclitaxel (PTX) can facilitate dendritic cell (DC) maturation by binding to toll-like receptors. Both PTX and cyclophosphamide (CY) promote the differentiation of T helper type 1 cells relative to T helper type 2 cells. CY can promote the recruitment of high-avidity $CD8^+$ T cells to the immune response by abrogating the suppressive influence of $CD4^+CD25^+$ regulatory T cells; the impact of this influence on recruitment and activation of low-avidity $CD8^+$ T cells remains unclear. Doxorubicin (DOX) can augment the activity of effector $CD8^+$ T cells; the influence of DOX on T cell avidity remains unclear.

potential beyond the ability to induce apoptosis [30]. PTX mimics the activity of lipopolysaccharide (LPS) by binding to the toll-like receptor 4 (TLR4) expressed by murine dendritic cells (DCs) [31,32]; in humans these two drugs have a similar activity that is dependent on Myd88 and likely occurs via a distinct TLR [33]. Like CY, PTX also favors the evolution of T helper type 1 responses; this immunodulatory activity of PTX occurs only at low doses, and only when the drug is given prior to antigen exposure [23]. The time dependence of the idiosyncratic adjuvant activity of these drugs is clear, and suggests synergy at the time of immune priming for CY ($CD4^+CD25^+$ regulatory T cells) and PTX (DC modulation/tumor cell apoptosis), and at the time of effector activity for PTX and DOX (tumor cell apoptosis, and increasing with direct priming, cross-priming, or both).

Immunosuppressive Pathways in the Tumor Microenvironment

Chemotherapy can also modulate the tumor microenvironment, rendering it more receptive to a productive antitumor immune response. CY, PTX, DOX, and vinblastine given at low doses frequently (metronomic chemotherapy) preferentially target the tumor vasculature over transformed tumor cells [34]. This may increase both tumor cell apoptosis and vascular access early in treatment. Chemotherapy with drugs like 5′-aza-2′-deoxycytidine and 5-fluoruracil can upregulate the expression of molecules involved in antigen processing and presentation, thereby rendering tumor cells more sensitive to lysis by cytotoxic T lymphocytes (CTL) [35,36]. This has been demonstrated in tumor cell lines in vitro. Other chemotherapeutics may upregulate costimulatory molecules within the tumor microenvironment, thereby enhancing T cell activity [35–39].

11.4 MONOCLONAL ANTIBODY AND ENDOCRINE THERAPY FOR BREAST CANCER AND TUMOR IMMUNITY

Monoclonal Antibody Therapy

Therapeutic monoclonal antibodies that are specific for the tumor cell are promising for combination immunotherapy because they provide a strategy for passively restoring the antigen-specific humoral immune response in the context of activating the cellular immune response with cancer vaccines. Trastuzumab is a humanized monoclonal antibody specific for HER2/*neu* [2], a protooncogene overexpressed by up to 25% of human breast cancers [40,41]. HER2/*neu*-overexpressing tumors are more aggressive, relapse earlier, and are associated with a higher mortality than the majority of breast cancers. Trastuzumab is now in widespread use for the treatment of breast cancer at every stage except ductal carcinoma in situ. This antibody has the capability of potentiating tumor immunity in several ways:

1. Trastuzumab interferes with signaling pathways that promote tumor cell growth and metastasis [42].
2. Like standard-dose chemotherapy, trastuzumab induces significant tumor cell apoptosis in human breast tumors even after only one dose [43].
3. Trastuzumab induces the ubiquitination and degradation of internalized HER2/*neu* molecules [44,45], thus increasing proteasome-dependent antigen presentation and augmenting the lytic activity of MHC class I–restricted CTLs against HER2/*neu*-positive target cells [46,47].
4. Trastuzumab recruits innate immune effectors to the tumor microenvironment to facilitate antibody-dependent cellular cytotoxicity (ADCC) [48,49].

The pleiotropic nature of these immunomodulatory activities is striking, as trastuzumab in combination with tumor vaccine has the potential for augmenting innate, humoral, and cellular immunity in a highly concerted fashion.

Bevacizumab is a humanized monoclonal antibody specific for the vascular endothelial growth factor (VEGF), a cytokine that both promotes the growth of the tumor-associated vasculature and suppresses tumor-associated immune responses. Although not yet FDA-approved for the management of breast cancer, phase III clinical trial data are compelling enough to warrant the widespread use of bevacizumab in combination with chemotherapy in the management of metastatic breast cancer. The reported data support its use as first-line therapy for metastatic breast cancer in combination with standard-dose PTX. VEGF itself can cause thymic atrophy, inhibit T cell development, and diminish DC function [50–52]. Importantly, monoclonal antibodies specific for VEGF can increase the number and function of DCs in tumor-bearing mice, thereby facilitating the effectiveness of DC-based immunotherapy [53]. Together, these observations suggest that VEGF blockade may augment the activity of tumor vaccines, or the function of adoptively transferred tumor-specific lymphocytes.

Cetuximab is a humanized monoclonal antibody specific for the *epidermal growth factor receptor* (EGFR), a transmembrane tyrosine kinase receptor overexpressed by a variety of epithelial tumors, including colorectal, aerodigestive, prostate, ovarian, and breast cancers. Notably, the EGFR protein has emerged as a prominent molecular feature of the basal-like subset of breast cancers. This breast cancer subset is "triple negative"; that is, it does not express the estrogen receptor (ER), the progesterone receptor (PR), or HER2/*neu*. Therefore, no targeted therapies exist to complement chemotherapy for the treatment of this breast cancer subtype, and it poses an unmet medical need. Like trastuzumab, cetuximab inhibits the signaling pathways that promote tumor growth and progression, and also induces tumor apoptosis. In particular, cetuximab can synergize with PTX to reduce angiogenesis and induce tumor cell apoptosis [54]. Immunomodulatory activity for cetuximab has not been reported.

Endocrine Therapy

There is a well-defined subset of breast cancers with a high likelihood of response to endocrine therapy. Frequently, these tumors are much more responsive to manipulating the hormonal milieu than they are to systemic chemotherapy. Endocrine therapy for breast cancer includes the selective estrogen receptor modulators and destroyers (SERMS and SERDS: tamoxifen and raloxifene, and fulvestrant), the aromatase inhibitors (anastrozole, letrozole, and exemestane), and ovarian ablation. Despite a clear role for estrogen in the growth and development of lymphocytes, there is little information available about the potential impact of these therapies on tumor immunity. Tamoxifen and raloxifene can inhibit the differentiation and LPS-induced maturation of DCs, antagonizing the ER and maintaining the DCs in an immature state in vitro [55,56]. Aromatase inhibitors can sensitize tumor cells to monocyte-mediated ADCC [57]. While no immunomodulatory effects of

ovarian ablation have been reported, it is interesting that androgen ablation in murine models of prostate cancer can diminish immune tolerance [58]. These findings warrant further investigation of these interactions in clinically relevant breast cancer models.

11.5 THE *neu* TRANSGENIC MOUSE MODEL

A Stringent Model for Human Breast Cancer Immunotherapy

The *neu* transgenic mouse model is a highly selective, clinically relevant laboratory model for discriminating the most active multimodality immunotherapeutic regimens for testing in breast cancer patients. Studies have shown that *neu* mice stochastically develop mammary carcinomas that develop in a histologic sequence that recapitulates the progression of human breast cancers [59]. They develop spontaneous mammary carcinomas that overexpress the protooncogene HER2/*neu*, and represent an informative model system for the biology of HER2/*neu*-positive breast cancers. Moreover, they display a profound immune tolerance to the HER2/*neu* oncoprotein [60] and develop very low levels of HER2/*neu*-specific antibodies and T cells that fail to prevent tumor progression, thereby recapitulating the immunobiology of breast cancer patients [60–62].

Granulocyte–macrophage colony-stimulating factor (GM-CSF)-secreting cell-based vaccination is an active vaccination platform for cancer immunotherapy [63]. This immunotherapeutic strategy capitalizes on the ability of GM-CSF to recruit and activate DCs, widely regarded as the most effective professional antigen-presenting cell (APC) [64,65]. The human vaccine is typically composed of one or more tumor cells that are genetically modified to secrete GM-CSF, and is inoculated intradermally [66,67]. There, the paracrine secretion of GM-CSF recruits and activates host DCs, which take up and process tumor antigens shed by the vaccinating tumor cells for presentation to the immune system. This approach has two major advantages: (1) the cross-priming mechanism of immune activation obviates the need to match the vaccine cells to the MHC haplotype of the host, and (2) the use of cells to deliver pertinent tumor antigens allows the delivery of multiple tumor antigens in an unbiased way, activating a multitargeted immune response to antigens that are both known and unknown. Therefore, the vaccination strategy utilizes an optimal mechanism that maximizes the specificity and diversity of immune priming.

GM-CSF-Secreting Vaccines and *neu* Transgenic Mice

The Jaffee group has interrogated the immunobiology of *neu* transgenic mice using GM-CSF-secreting vaccines. *Neu* transgenic mice were derived from FVB/N mice by introducing the rat *neu* protooncogene into the germline DNA under the control of the mammary-specific promoter MMTV [59]. They spontaneously develop HER2/*neu*-positive breast cancers that can metastasize to the lung, and less commonly to other sites. Vaccinating tumor-free *neu* mice with a GM-CSF-secreting,

HER2/*neu*-specific cell-based vaccine can delay the outgrowth of both spontaneous and transplanted HER2/*neu*-positive tumors [60]. In contrast, vaccinating tumor-bearing *neu* transgenic mice with the same vaccine has absolutely no impact on tumor growth [23]. These observations highlight the impact of tumor burden on the efficacy of cancer vaccines in the setting of immune tolerance.

Comparing the immune response of parental FVB/N mice and *neu* transgenic mice to HER2/*neu*-targeted vaccination highlights the immense impact of immune tolerance itself on vaccine efficacy. Vaccinating tumor-bearing (>0.5 cm), nontolerant, parental FVB/N mice produces a complete and durable tumor rejection. This tumor rejection response is associated with an immune response characterized by robust HER2/*neu*-specific antibody titers, and significant numbers of active, HER2/*neu*-specific CTLs [23,60]. These CTLs are almost exclusively specific for the immunodominant epitope of rat HER2/*neu*, $RNEU_{420-429}$ (p50) [68]. In contrast, vaccinating tumor-bearing (nonpalpable), tolerant *neu* transgenic mice is completely ineffective in delaying tumor outgrowth [23]. The immune response induced by vaccination is tepid, with very low titers of HER2/*neu*-specific antibodies, and low numbers of HER2/*neu*-specific T cells. Notably, the T cell population that does develop contains a paucity of T cells specific for the immunodominant epitope p50 [23,25]. This immune response is so weak that the tumor outgrowth curves of mice vaccinated with cells that express HER2/*neu* and secrete GM-CSF overlap completely with those of mice vaccinated with cells that are HER2/*neu*-negative and secrete only GM-CSF. By combining vaccination with established breast cancer therapeutics, it is possible to partially overcome this immune tolerance, enhancing vaccine activity in tolerant *neu* mice.

Combining GM-CSF-Secreting Vaccination and Chemotherapy in *neu* Transgenic Mice

The Jaffee group has conducted a systematic analysis of chemotherapy in sequence with HER2/*neu*-directed, GM-CSF-secreting vaccination in *neu* transgenic mice [23]. In this study, low doses of CY and PTX were found to augment vaccine activity if given prior to vaccination, but not if given after vaccination. In contrast, low doses of DOX were found to augment vaccine activity if given after vaccination, but inhibited vaccine activity when given prior to immunization. Notably, combining both CY and DOX with vaccination produced the greatest antitumor effect, curing up to 30% of *neu* transgenic mice of preexisting tumors. Studies to elucidate the mechanism by which chemotherapy augments vaccine activity revealed several important findings:

1. The $CD4^+$ T helper type I response is facilitated by both CY and PTX pretreatment; giving these drugs after vaccination actually inhibits this response [23]. This observation supports the reports that CY and PTX can reverse the immunologic skew that antagonizes effective tumor immunity in the setting of established tumor burdens.

2. CY abrogates the immunosuppressive impact of cycling $CD4^+CD25^+$ regulatory T cells (Tregs) on the induction of effective $CD8^+$ CTL in tumor-bearing, tolerized *neu* transgenic mice [25]. Importantly, latent, high-avidity p50-specific T cells are recruited to the immune response of cured tumor-bearing *neu* transgenic mice when the influence of these cycling Tregs is alleviated. This is an important observation, because it demonstrates that high-avidity, tumor-specific T cells can exist in the periphery, and can be recruited to the antitumor response with the appropriate vaccination regimen. Moreover, it is consistent with observations by other groups that CY can mitigate the influence of Tregs. Another group has shown that CY can induce type I interferons, thereby promoting the evolution of a $CD44^{hi}$ memory T cell response [28].
3. Our preliminary data suggests that low-dose PTX administered prior to GM-CSF-secreting vaccination in *neu* transgenic mice augments tumor immunity by signaling through TLR4 at the time of immune priming (Jaffee EM, unpublished data). The immunomodulatory activity of DOX in *neu* transgenic mice has not been evaluated, but the drug promotes the evolution of tumor-specific $CD8^+$ T cells when given after GM-CSF-secreting vaccination in the nontolerant CT26 mouse model of colon cancer [22].

GM-CSF-Secreting Vaccination and Monoclonal Antibody Therapy in *neu* Transgenic Mice

Our group has also shown that the combination of HER2/*neu*-specific monoclonal antibodies and vaccination are more effective than either alone in *neu* transgenic mice [45]. In vitro studies have demonstrated that HER2/*neu*-specific monoclonal antibodies can enhance the lytic activity of MHC class I–restricted HER2/*neu*-specific CTLs against HER2/*neu*-positive targets [46,47]. Consistent with this concept, the passive administration of both HER2/*neu*-specific antibodies and HER2/*neu*-specific CTLs induces a more effective antitumor response than either alone in severe combined immmunodeficient mice [69]. Building on this observation, the passive administration of HER2/*neu*-specific monoclonal antibodies in combination with vaccination in *neu* transgenic mice has a greater antitumor effect than does either alone, with combined therapy curing about 40% of tumor-bearing mice [45]. Combination therapy augments the number of vaccine-induced, tumor necrosis factor (TNF)-secreting, HER2/*neu*-specific CTLs as measured by ELISPOT. Notably, the monoclonal antibodies alone induce new $CD8^+$ T cell immunity specific for HER2/*neu*. These observations are consistent with observations that humoral HER2/*neu*-specific immunity induced after peptide vaccination in patients with breast cancer can inhibit tumor cell growth and signaling [70]. We have more recently shown that mitigating the influence of Tregs with low-dose CY in the setting of vaccination and weekly therapy with HER2/*neu*-specific monoclonal antibodies is the most effective, curing almost 60% of *neu* transgenic mice of preexisting tumors (Emens LA, unpublished data). This is associated with both increased tumor cell apoptosis as determined by immunohistochemical staining of

tumors for cleaved caspase 3, and by both increased numbers of HER2/*neu*-specific $CD8^+$ T cells and higher HER2/*neu*-specific antibody titers.

Clinical Trials of GM-CSF-Secreting Vaccination with Standard Breast Cancer Therapeutics

We have developed a human allogeneic, GM-CSF-secreting breast tumor vaccine for clinical administration [67]. The vaccine itself is composed of two cell lines, SKBR3 and T47D, both of which have been genetically modified to secrete human GM-CSF by plasmid DNA transfection. The vaccine is designed to account for the majority of breast cancers that are seen clinically, with T47D representing ER-positive and HER2/*neu*-negative breast cancers, and SKBR3 representing ER-negative and HER2/*neu*-positive breast cancers. We are currently accruing to two clinical trials that are testing a fixed dose of 5×10^8 cells of this GM-CSF-secreting breast tumor vaccine in sequence with standard breast cancer therapeutics in patients with metastatic breast cancer. The first study tests this fixed dose of vaccine in sequence with a range of doses of CY (250–450 mg/m^2) and DOX (15–35 mg/m^2) given 1 day prior to and 7 days after vaccination, respectively, in patients with stable metastatic breast cancer [71]. The goal of this study is to assess the bioactivity of the vaccine by measuring immune responses to HER2/*neu* (delivered by the SKBR-3 component of the vaccine), and to determine which doses of sequential chemotherapy produce the greatest immune response. Thus far, the vaccine has been quite safe, with side effects largely limited to local injection site reactions consisting of erythema, induration, and pruritis. Analysis of HER2/*neu*-specific immune responses is just beginning. We have just opened a second study that tests this same fixed dose of 5×10^8 vaccine cells with CY modulation (300 mg/m^2) in the setting of weekly trastuzumab therapy in patients with HER2/*neu*-overexpressing metastatic breast cancer. This second study is distinguished from the first in that it aims to augment vaccine activity by (1) enhancing antigen processing and presentation (trastuzumab) and (2) mitigating the influence of Tregs (CY). Importantly, both of these studies allow the concomitant administration of endocrine and bisphosphonate therapy as appropriate.

11.6 THE FUTURE: MANIPULATING IMMUNOLOGIC CHECKPOINTS WITH NOVEL IMMUNE-BASED THERAPIES

It is clear that standard breast cancer therapeutics can exert an immunomodulatory effect that can be harnessed for immunotherapeutic benefit. The next generation of breast cancer vaccine studies will surely combine breast tumor vaccines with agents that manipulate immunologic checkpoints. These checkpoints represent critical control loci for upregulating or downregulating the antitumor immune response

[72,73]. During immune priming, the strength of T cell activation is determined by the primary signal conferred by the recognition of the antigen–MHC complex by the T cell receptor, and the secondary signals contributed by the summation of positive and negative accessory signals for T cell activation. It is possible that the antitumor T cell response can be facilitated by amplifying positive costimulatory signals for T cell activation, or by blocking negative accessory signals for T cell activation. For breast cancer, the positive regulatory pathway with the most promise to date is the OX40 pathway [74]. OX40 signaling supports the activation, expansion, and survival of both $CD4^+$ and $CD8^+$ T cells, and can abrogate immune tolerance in vivo. The treatment of tumor-bearing mice with monoclonal antibodies specific for OX40 alone resulted in prolonged $CD4^+$ and $CD8^+$ T cell-dependent tumor-free survival [74]. Furthermore, we have shown that HER2/*neu*-targeted vaccination combined with OX40 monoclonal antibody therapy in tumor-bearing *neu* transgenic mice could overcome immune tolerance, augmenting tumor-free survival and the HER2/*neu*-specific T cell response in about 20% of *neu* transgenic mice [75]. Importantly, analysis of vaccine-induced immune responses specific for the immunodominant epitope showed that vaccination alone induced a small and transient increase of p50-specific T cells that were largely ineffective in delaying tumor growth, whereas the addition of therapy with a monoclonal antibody specific for OX40 resulted in the sustained induction of long-lived p50-specific T cells of high avidity. Single-agent monoclonal antibody therapy targeting OX40 is just now entering human clinical trials.

The negative regulatory pathway with the most promise to date is the CTLA4 counterregulatory signaling pathway [76]. CTLA4 signaling serves as a check on the process of T cell priming, both raising the threshold for T cell activation and limiting T cell expansion. Notably, CTLA4 is also expressed at significant levels by Tregs, and modulating this pathway can affect the primary T cell response as well as the complementary Treg response. Although little data exist for breast cancer, several clinical trials have tested monoclonal antibodies specific for CTLA4 as a single agent, either subsequent to or in active sequence with vaccination in other tumor types [77–80]. In the aggregate, these clinical trials have been quite promising, with evidence of increased tumor-specific immune responses and tumor regression. Notably, bioactivity has tracked with symptoms of significant autoimmunity, further supporting the potency of this drug as an immunomodulator with promise. Currently (as of 2007), CTLA4-specific monoclonal antibody therapy is being tested in combination with endocrine therapy in patients with metastatic breast cancer.

A multitude of immune checkpoint modulators might be tested for activity in combination with breast cancer vaccines in the future. Those that are currently available for clinical use include monoclonal antibody specific for CD25 (daclizumab) and denileukin diftitox (Ontak), both of which would be used to abrogate the negative influence of regulatory T cells. These two drugs target the interleukin 2 (IL-2) receptor, which is overexpressed on Tregs. Supporting its use, denileukin

diftitox has been demonstrated to deplete Tregs, resulting in a marked reduction of tumor growth in *neu* transgenic mice [81]. It has also been shown to deplete Tregs in humans [82,83]. Treg modulators that might be of future use include those that target the glucocorticoid-induced tumor necrosis factor receptor (GITR) and lymphocyte-associated globulin 3 (LAG3) [83]. Other promising agents include monoclonal antibodies or other drugs specific for the B7 family of positive and negative accessory molecules of T cell activation, agents specific for the CD40 pathway, and drugs that target the 4-1BB pathway [72].

11.7 SUMMARY AND CONCLUSIONS

Improvements in the management of breast cancer have long led revolutionary shifts in cancer care. Targeted therapy, exemplified by the use of tamoxifen to treat breast cancers that overexpress the estrogen receptor α (ERα), has been a mainstay of breast cancer therapy since the mid-1970s. Increasing numbers of molecularly targeted drugs are entering the treatment armamentarium for breast cancer as well as other cancer types. More recently, insights elucidated by the genomic revolution have opened the door for individualized therapy based on fundamental tumor biology rather than anatomic staging. Breast cancer genomics also represents a framework for the application of new targeted therapies designed to disrupt the key regulatory pathways that underlie the biology of distinct breast tumor types. However, continued improvements will likely continue to be limited by the specter of fixed drug resistance, arguing for innovative treatment strategies that work by a completely different mechanism. Tumor vaccines represent a unique therapeutic strategy that aims to retool the host–tumor interaction to facilitate tumor rejection. Importantly, multiple established and novel breast cancer therapies have immunomodulatory potential. Breast cancer thus represents a powerful model system for elucidating mechanisms of therapeutic synergy between breast tumor vaccines and both standard and novel breast cancer therapeutics. Capitalizing on this opportunity will ensure that breast cancer morbidity and mortality continue to decline.

Breast cancer vaccines have great promise for further improving clinical outcomes in the management of breast cancer. They function by a mechanism completely different from that of any breast cancer therapy to date, and have potential for breast cancer prevention as well as treatment. It is clear that their clinical development will be accelerated and optimized by taking into account how the immune system interacts with the disease, and how the vaccine-induced immune response interacts with established breast cancer therapeutics. The clinical success of trastuzumab illustrates the potential of immune-based therapy for this disease. Close collaboration between laboratory and clinical researchers will ensure that immune-based therapy continues to impact breast cancer outcomes in profound ways.

Conflict of Interest

This chapter describes work using granulocyte–macrophage colony-stimulating factor-secreting tumor vaccines. Under a licensing agreement between Cell Genesys, Incorporated and the Johns Hopkins University, the university is entitled to a share of royalty on sales of products described in this chapter. The terms of this agreement are being managed by the Johns Hopkins University in accordance with its conflict-of-interest policies.

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12

T-REGULATORY CELL MANIPULATION IN TUMOR IMMUNOTHERAPY

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12.1 INTRODUCTION

Established malignant tumors express a variety of tumor-associated antigens (TAA), yet rarely elicit an endogenous immune response that effectively eradicates tumor. Much evidence suggests that specific immune surveillance can eliminate tumors at early stages, whereas established tumors generally induce immune tolerance [1]. It has now been demonstrated that numerous tumor-derived factors contribute to immune tolerance and immunosuppression in the tumor microenvironment [2], helping explain the relative lack of effective immune surveillance at later stages of tumor development.

Earlier concepts that simply boosting the numbers or function of effector cells alone would be therapeutically useful have given way to the sobering realization that active tumor-mediated mechanisms can defeat antitumor effector functions. Thus, effective cancer vaccines must overcome the immunosuppressive tumor microenvironment to help stimulate a useful antitumor immune response.

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Engendering strong antitumor immunity may involve breaking Treg-mediated peripheral tolerance to tumor-associated antigens (TAAs). Tregs are emerging as important mediators of tumor microenvironmental immunosuppression in patients with cancer, including suppression of TAA-specific immunity. Consistent with this concept, experimental depletion of Tregs in tumor-bearing mice improves endogenous immune-mediated tumor clearance [3], improves TAA-specific immunity [4], and enhances the efficacy of tumor immunotherapy such as vaccination [5] or CTLA4 blockade [6]. Nonetheless, as Tregs also control self-reactive T cells present in all individuals, including those mediating homeostatic peripheral tolerance, the therapeutic benefit of attempting to deplete TAA-reactive Tregs may be limited by the development of pathologic autoimmune phenomena if homeostatic Tregs maintaining normal peripheral tolerance are also depleted. Further, more recent data suggest that cancer vaccines may also expand existing pools of tumor microenvironmental Tregs [7], which has potential to exacerbate immunosuppression further. Certain subsets of dendritic cells can also expand various Treg subsets [8,9], which may also contribute to this problem.

Although $CD4^+$ Tregs have been studied and characterized extensively (reviewed in Refs. 10 and 11), many questions regarding their origins and functions remain. The best known express the $CD4^+CD25^+Foxp3^+$ phenotype and are referred to as *regulatory T lymphocytes, T-regulatory cells*, or *Tregs* [12–14], as we refer to them here. Tregs can suppress the function of tumor-specific $CD4^+$ [15] or $CD8^+$ [16] T effector cells by mechanisms that are not fully understood, but most likely include soluble factors and cell-to-cell contact [10,11,16–18]. Functional Tregs circulate in increased numbers in peripheral blood of patients afflicted with many types of human cancers [3,10,11,19–26] and in the solid tumor mass [20]. Human $CD4^+CD25^+$ Tregs inhibit TAA-specific immunity, allow tumor growth in the presence of TAA-specific immunity and predict poor survival in human ovarian cancer [20]. Foxp3 expression also correlated with poor survival in ovarian cancer [27]. Thus, reducing tumor microenvironmental Treg function is emerging as a means to improve the efficacy of tumor immunotherapy. We have defined four general strategies to reduce Treg function for potential use in immunotherapy. The first strategy, killing of Tregs, which can be effected using monoclonal antibodies, targeted toxins, selected chemotherapeutic agents, or other molecules. Alternative strategies include blockade of Treg function, trafficking, or differentiation. Some of these strategies have already been tested clinically, while others are under preclinical investigation. Each strategy is discussed below as it relates to $CD4^+CD25^+$ Tregs, although such strategies could also be applied to other types of regulatory cells.

12.2 T-REGULATORY CELL DEPLETION

Monoclonal Antibodies against CD25

CD25, the α-chain of the IL-2 receptor, is constitutively expressed on many tumor-associated Tregs and is thus a candidate molecule to target them for depletion. Injection of the anti-CD25 monoclonal antibody PC61 mediated an apparently

rapid and efficient depletion of $CD4^+CD25^+$ Treg function [4] and promoted tumor injection in mice [3]. However, more recent results suggest that the mechanism of action of PC61 monoclonal antibody treatment is functional inactivation, not depletion of Tregs [28]. Nonetheless, the net effect is reduced Treg activity with concomitantly improved antitumor immunity.

Two antihuman CD25 antibodies, daclizumab (Zenapax) and basiliximab (Simulect), are approved for human use in transplantation, autoimmunity, and cancers including HTLV1-induced adult T cell lymphoma/leukemia (reviewed in Ref. 29). There are no published reports to our knowledge on the efficacy of these anti-CD25 antibodies to deplete Tregs in humans, but anecdotal information suggests only very limited, if any, such activity. A definitive report describing the effects of these antibodies on tumor-related Tregs would be very useful.

Ontak

Ontak, DAB_{389}IL-2 (denileukin diftitox), is a recombinant diphtheria toxin conjugate consisting of the active domain of diphtheria toxin and human interleukin 2 (IL-2). It is approved by the FDA for the treatment of cutaneous T cell leukemia/lymphoma [30]. It targets cells expressing the IL-2 receptor [31,32] and is proposed to be internalized into $CD25^+$ cells by endocytosis, where it inhibits protein synthesis, leading to apoptosis. More recent evidence demonstrates that Ontak also targets cells through components of the IL-2 receptor in addition to CD25 [32,33] (also Curiel and coworkers, unpublished results).

Because of the phenotypic similarity between $CD4^+CD25^+$ cutaneous T cell leukemia/lymphoma cells, for which Ontak is approved for treatment, and $CD4^+CD25^+$ Tregs, we hypothesized that Ontak would deplete $CD4^+CD25^+$ Tregs in humans. We undertook a clinical trial demonstrating that in blood of human patients with cancer, Ontak depleted functional Tregs, correlating with improved measures of immunity [34] and therapeutic benefit [35]. A clinical trial [36] showed that pretreatment with Ontak improves the immunogenicity of vaccination with RNA-transfected dendritic cells, enhancing the stimulation of tumor-specific T cells in patients with renal cell cancer compared to vaccination alone. This report also demonstrated that Ontak depleted functional Tregs in blood, and the authors speculated that as the mechanism of action. Ontak depletes tumor-associated Tregs and improves immune-mediated rejection of tumor in a mouse model of breast cancer [37]. Nonetheless, a clear link between Treg depletion and any immunologic finding or clinical improvements in any reports in humans to date remains to be determined.

In another clinical study [38], treatment with Ontak did not induce any objective response in human melanoma or renal cell carcinoma and evinced no reduced suppressive effect of $CD4^+CD25^+$ cells in vitro. Why Ontak did not reduce Treg function in this study is unknown, but could relate to the dose or schedule of Ontak used, differences in the immunobiology of different cancers, prior treatment of patients with IL-2, or other factors. For example, IL-2 can increase numbers of $CD4^+CD25^+Foxp3^+$ Tregs in blood of patients with renal cell carcinoma or

melanoma [39] and pediatric sarcoma [40]. Furthermore, IL-2 signaling through the IL-2 receptor on Tregs increases their survival and activates their suppressor function [41].

As recently activated T cells also express high-level CD25, Ontak could also deplete $CD25^+$ effector cells. We observed effector cell depletion in some of our patients following three or more weekly Ontak infusions at 12 μg/kg (Curiel and coworkers, unpublished results), which has also been suggested by in vitro work [36]. An optimal balance between effector and Treg depletion may be achieved using different Ontak doses or schedules. We are currently testing monthly Ontak infusion in this regard.

Cytotoxic Chemotherapy

Cyclophosphamide

Cyclophosphamide is an alkylating agent widely used in various chemotherapeutic regimens. Low-dose cyclophosphamide can reduce the number of Tregs and thus may be beneficial as an immune-modulating agent, even in the setting of cyclophosphamide-resistant tumors [42,43], although the mechanism is not well understood [44]. Depletion of Tregs by cyclophosphamide in a rat tumor model potentiates the efficacy of an active tumor-specific immunotherapy [45]. Treg depletion by low-dose cyclophosphamide in humans has not yet been convincingly demonstrated [46]. Further investigations of cyclophosphamide actions apart from its direct tumor cytotoxicity are warranted, especially since low-dose cyclophosphamide has also been shown to be antiangiogenic [47].

Fludarabine

Fludarabine is a purine analog used to treat chronic lymphocytic leukemia [48]. It has been to reduce or abolish the suppressive activity in the $CD4^+CD25^+$ T cell population of patients with chronic lymphocytic leukemia [49]. As Tregs may play a role in the dysfunctional immune system of patients with chronic lymphocytic leukemia, the effect of fludarabine on Tregs in this and possibly other malignancies merits further investigation.

12.3 FUNCTIONAL BLOCKADE OF T-REGULATORY CELLS

Anti-GITR Antibodies

The GITR (glucocorticoid-induced tumor necrosis factor receptor-related gene) is a cosignaling antigen expressed on murine [50] and human [51] T lymphocytes. This antigen is expressed constitutively at high levels on Tregs and is further upregulated on T cell activation [52]. Stimulation of GITR by monoclonal antibodies in vitro abrogates the suppressor function of murine Tregs [52,53], but a similar

effect has not been demonstrated convincingly in human Tregs [54]. Treatment of tumor-bearing mice with an agonistic anti-GITR monoclonal antibody provoked potent tumor-specific immunity and eradicated established tumors without eliciting overt pathologic autoimmunity [55]. The molecular mechanisms of this effect were not characterized in detail but likely involve attenuation of Treg-mediated suppression in the presence of antigenic stimulation [55]. This strategy has not yet been tested in a human clinical trial to our knowledge.

TLR Signaling

Approximately 15 toll-like receptors (TLRs) are ubiquitously expressed on various mammalian cells, including human Tregs and dendritic cells [56]. They bridge the innate and the acquired immune system by recognizing a set of bacterial and viral pathogen-associated molecular patterns, and initiate important steps in generating a specific immune response, such as dendritic cell maturation [56]. The suppressive function of Tregs can be directly abrogated in vitro by TLR-derived signals [57]. The importance of TLR signaling for tumor immunotherapy is demonstrated by in vitro experiments showing that only virus-based vaccines provide TLR signals required for reversing Treg-mediated tolerance. Conversely, dendritic cell (DC)-based vaccines lack TLR signaling, and can break tolerance of $CD8^+$ cells only after removal of Tregs or with the coadministration of another TLR ligand [58]. These experiments suggest that functional Treg inhibition by TLR ligation (such as with TLR9 ligation by CpG oligonucleotides) may be a novel means to boost efficacy of vaccine-based cancer treatments. Combining dendritic cell-based vaccines with Treg depletion is another alternative strategy, as discussed above.

Anti-CTLA4 Antibodies

Cytotoxic T lymphocyte antigen 4 (CTLA4) is strongly expressed on Tregs [10,11,59,60] as well as on certain other $CD4^+$ and $CD8^+$ T lymphocytes [61]. As this molecule downregulates T cell responsiveness [62,63], CTLA4-mediated inhibition may restrict T cell activation during the initiation and progression of the antitumor immune response [61]. CTLA4 blockade by monoclonal antibodies elicits a strong antitumor response in a murine melanoma model [6]. A human anti-CTLA4 monoclonal antibody has been tested in various different cancers in phase I clinical trials [64] with promising results, although pathologic autoimmune phenomena are significant side effects. CTLA4 is also expressed on effector T cells.

An analysis of the cellular mechanisms of CTLA4 blockade suggests that the antitumor effects of CTLA4 blockade are due to a direct effect on $CD4^+$ and $CD8^+$ effector T cells, and not to an inhibition or depletion of Tregs [65]. The significant symptoms of pathologic autoimmunity observed with anti-CTLA4 treatment [65] may possibly be controlled by using a different antibody clone. Thus, whereas CTLA4 blockade is a promising candidate for immunotherapy, it may not significantly manipulate Treg function. On the other hand, raising the threshold of effector cells to Treg suppression (such as with anti-CTLA-4 antibody) is an additional strategy to combat immunopathology.

Treg Trafficking Blockade

Chemokine Signaling

Chemokines are a family of chemotactic molecules that modulate trafficking of immune cells through generation of a concentration gradient between normal and pathologic tissues [66]. While various normal tissue types produce chemokines, cancers also secrete them [67]. We have identified the tumor-secreted chemokine CCL22 as a candidate mechanism for mediating Treg migration into the microenvironment of human ovarian cancer [20]. We also found that CCR4 (a receptor for CCL22) is expressed on the majority of Tregs in ovarian cancer. In vivo blockade of CCL22 with a monoclonal antibody significantly reduced Treg migration into ovarian tumors in an immunodeficient murine xenograft model, improving immune rejection [20]. Thus, blocking Treg trafficking is a potential strategy for treating human cancers. However, CCL22 signals may also regulate the trafficking of effector T cells. Therefore, the net benefit of CCL22 blockade, or effects of interrupting other trafficking signals in immunocompetent hosts requires further investigation as to potential therapeutic utility.

Ovarian cancer cells also produce large quantities of the chemokine CXCL12 [also known as *stroma-derived factor 1* (SDF1)], which can attract dysfunctional plasmacytoid dendritic cells [75], which in turn can induce differentiation of $CD8^+$ regulatory T cells [68]. Thus, blocking CXCR4 signals may be therapeutically useful to reduce Treg numbers or function in ovarian cancer, in addition to prior proposals to use it to block metastasis. More recent data suggest that IL-2 promotes Treg cell migration into tumors by augmenting their CXCR4 expression, a receptor for CXCL12 [69], suggesting an additional level of regulation that may be exploitable therapeutically. Various chemokines and chemokine receptors have been targeted successfully in animal models of arthritis [70], and selective small-molecule chemokine receptor antagonists have entered phase I clinical trials [71], including CXCR4 signaling antagonists. However, therapeutic success of chemokine or receptor antagonism may be limited by the binding diversity of chemokine receptors for multiple ligands, the redundancy of the individual chemokine–ligand pair, and the role played by chemokines in normal tissue homeostasis or antitumor defense. These additional effects of chemokine–receptor antagonism must be considered in chemokine–receptor blocking strategies.

12.4 INTERACTIONS BETWEEN T-REGULATORY AND OTHER CELLS

Interactions between Dendritic Cells and Treg Cells

Dendritic cells are a heterogeneous group of antigen-presenting cells with a variety of differences in anatomic localization, cell surface phenotype, and immunologic function [72]. Human dendritic cells are typically divided into two main populations: myeloid (MDC) and plasmacytoid (PDC) [72]. While it is well established

that dendritic cells are responsible for initiating or upregulating immune responses, it has also been shown more recently that they are also capable of inducing immune tolerance [2].

We have identified several mechanisms whereby dendritic cells may induce Tregs that contribute to impaired tumor immunity [2,8,9,73]. Thus, it may be possible to modulate the suppressive activity of Tregs by blocking dendritic cell trafficking or function in the tumor environment and thereby subsequently enhance tumor immunity as discussed further below.

Interactions between PDCs and T Cells

Plasmacytoid dendritic cells are a subtype of dendritic cells responsible for producing type I interferons in peripheral blood in response to viruses and other stimuli [74]. In contrast to normal blood PDCs, tumor environmental PDCs promote tumor vascularization [75], and induce differentiation of IL-10-expressing T cells [9]. Tumor PDC-activated T cells include $CD8^+CCR7^+CD62L^+IL\text{-}10^+$ T cells, which suppress effector T cell function through IL-10 production [9] and are functional $CD8^+$ Tregs. Thus, interactions between PDCs and $CD8^+$ T cells may contribute to the differentiation of $CD8^+$ Tregs in the tumor microenvironment. This differentiation could potentially be inhibited by blocking tumor PDC–T cell interactions.

The chemokine CXCL12 induces ingress of PDCs into the tumor environment through CXCR4 signals in ovarian cancer [73]. We showed that blocking CXCR4 with specific antibodies reverses tumor-mediated protection of PDCs from apoptosis, chemotaxis, and adhesion/transmigration in vitro [73]. Various bicyclam compounds with specific antagonistic activity for CXCR4 (AMD3100 [76] or AMD3465 [77]) are effective in various clinical settings such as stem cell mobilization and human immunodeficiency virus infection [78]. They may also be useful in the local tumor environment by blocking PDC ingress, thereby preventing PDC–T cell interactions (in addition to blocking tumor metastasis through CXCR4 signals, or other pathologic activities).

An alternative approach is to deplete PDCs selectively. Relatively specific surface antigens on human PDCs (such as BDCA2 or BDCA4) [79] have been described. However, a PDC-depleting antibody has not yet been tested in humans. Thus, while the manipulation of PDC–T cell interactions in the context of tumor immunity represents an attractive target for tumor immunotherapy, more research must be done to establish its clinical efficacy and a suitable means to effect it.

Interactions between MDCs and T Cells

The B7-H1 molecule is member of the B7 family of cosignaling molecules. It is upregulated on MDCs in ovarian cancer [8]. Signaling through B7-H1 facilitates tumor growth [80] by inducing apoptosis of effector T cells [81]. Furthermore, B7-H1 signals in the tumor environment induce IL-10 production by T cells, leading to immune suppression [8]. Blockade of B7-H1 enhances MDC-mediated T cell

activation accompanied by downregulation of T cell IL-10 production and upregulation of T cell IL-2 and interferon γ (INFγ) [8]. It has been shown that B7-H1 signals on endothelial cells induce $CD4^+CD25^+FOXP3^+$ Tregs [82]. Thus, blockade of B7-H1 signals on MDCs (and perhaps other cells) in the tumor environment may inhibit development or function of tumor-associated Tregs. This mechanism most likely contributes to the improved tumor immunity after B7-H1 blockade in vivo [8].

12.5 VASCULAR LEUKOCYTES

A novel subset of antigen-presenting cells named *vascular leukocytes* that accumulate in human and murine ovarian cancer and other tumors have been described [83,84]. These myeloid lineage cells exhibit features of both dendritic cells as well as endothelial precursor cells. Vascular leukocytes are endowed with vasculogenic potential, because they can autonomously build blood vessels in vivo, and promote tumor growth [83,84]. Human vascular leukocytes are actually dendritic cells, as >97% are $CD11c^+$ and express class II antigens among other molecules. They also express endothelium-specific markers. Vascular leukocytes are distinct from bona fide endothelial cells, which express no leukocyte markers and are also distinct from bona fide MDCs or PDCs. The immune function of vascular leukocytes in tumors remains to be fully characterized, but they are tolerogenic, induce IL-10-secreting T cells, and promote tumor growth. Thus, vascular leukocytes resemble tolerogenic dendritic cells that drive the development of Tregs. New evidence now indicates that vascular leukocytes also contribute to Treg expansion in cancer (Coukos G and others, unpublished data). Thus, means to prevent T cell–vascular leukocyte interactions could reduce Treg numbers or function, which could be exploited therapeutically.

12.6 T-REGULATORY CELLS AND TUMOR VACCINES

Tumor vaccines have enjoyed some limited successes, but their utility remains modest. Although some vaccines induce TAA-specific immunity, significant, longlasting clinical benefits from these vaccines have seldom been observed. The prior discussions of tumor-associated Tregs now help provide mechanisms for the failure of at least some vaccines.

Effective cancer vaccines must overcome the immunosuppressive tumor microenvironment to help stimulate a potent antitumor immune response. However, more recent data suggest that cancer vaccines may contribute to immunosuppression by expanding tumor microenvironmental Tregs [7]. These data are consistent with other recent data showing that certain subsets of dendritic cells can also expand various Treg subsets [8,9]. Taken together, these data imply that the capacity of cancer vaccines to induce effective antitumor immunity may be impaired by the simultaneous expansion of vaccine-induced Tregs.

As we begin to combine Treg depletion with active cancer vaccination, additional issues must be dealt with for optimal effectiveness. TAA-specific $CD4^+CD25^+$ Tregs have been identified [85]. Treg depletion should attempt to deplete TAA-specific Tregs and not other Tregs so as to perturb homeostatic immunity the least. Treg depletion could also induce pathologic autoimmunity. In this regard, Treg depletion may be necessary, but not sufficient to induce autoimmunity [86]. The timing of Treg depletion in relation to active vaccination in a mouse model for colon cancer greatly influenced vaccine-engendered immunity. Treg depletion (using PC61 antibody) was most effective when given at the time of active vaccination [87]. Timing of Treg depletion likely will vary depending on factors related to the balance of TAA-specific effector cells versus Tregs in an individual, the agent used to deplete Tregs, the specific tumor type, and other factors. The potential for depletion of bystander cells was demonstrated using weekly Ontak dosing (Curiel and others, unpublished results). Future strategies to deplete Tregs should focus on mechanisms to target Tregs selectively, which will be facilitated by identification of Treg-specific molecules.

Finally, our work and that of most investigators in both mice and humans have all focused on effects of $CD4^+CD25^+$ Tregs. Nonetheless, additional regulatory cell populations exist, including $CD8^+$ Tregs [68], immature myeloid cells [88], and B7-$H4^+$ myeloid cells [89]. Additional regulatory populations such as NKT cells [90,91] have been proposed to be immunopathogenic in cancer, and likely will also be shown to play roles in immune suppression and the lack of efficacy of tumor vaccines.

12.7 SUMMARY AND CONCLUSIONS

Malignant tumors express associated antigens that should make them objects of immune attack. Nonetheless, spontaneous immune clearance of established cancer is rare. Evidence demonstrates that cancers utilize active mechanisms to block host antitumor immunity. Significant evidence implicates $CD4^+CD25^+$ T-regulatory cells (Tregs) as important mediators of active immune evasion in cancer. Animal models for cancer demonstrate that Treg depletion improves endogenous antitumor immunity and the efficacy of active immunotherapy. Thus, inhibiting Treg function could improve human cancer immunotherapy, where success has been modest to date. We propose four means to block Treg activity: depletion of Tregs, interference with Treg trafficking, inhibiting Treg differentiation, or blocking Treg function. The fusion toxin denileukin diftitox (Ontak) can reduce Treg numbers in blood of some patients with cancer. We have discussed strategies to block Treg activity and presented some of our preliminary data in this regard. Combining Treg depletion with active vaccination and other approaches poses additional challenges that must be addressed. Disrupting tumor-mediated mechanisms hindering host immunity is a novel approach to tumor immunotherapy.

More recent evidence suggests that $CD4^+CD25^+$ T regulatory cells (Tregs) significantly impair tumor-specific immunity and contribute to poor survival in

some cancers. Consequently, inhibiting Treg function could improve cancer therapy, including immunotherapy, which is corroborated by studies in animal models for cancer and Treg depletion. We proposed four general strategies to inhibit Treg function: (1) reducing their numbers, or interfering with their (2) function, (3) trafficking, or (4) differentiation. Reports from small human clinical trials demonstrate that killing Tregs is associated with augmented endogenous or vaccine-induced immunity, including tumor-specific immunity in some cases. Thus, Treg depletion may be a useful adjunct to some therapies. Which of these means to inhibit immunopathologic Treg function will prove most effective remains to be established. For example, Treg depletion might encourage rapid local Treg regeneration. Blocking Treg trafficking might inadvertantly reduce ingress of beneficial effector cells.

The fusion protein denileukin diftitox (Ontak) has received attention as a potential agent to deplete functional Treg. As Ontak theoretically depletes any T cell bearing IL-2 receptors (including effector T cells), its utility may be limited in some settings. Additional agents to deplete Tregs are now under study, including cyclophosphamide or agents specifically targeting Tregs. Additional issues to address when to consider combining Treg depletion with active vaccination include the timing of Treg depletion versus vaccination, consideration for depletion of TAA-specific Tregs, collateral damage to newly induced TAA-specific effector cells, and potential for development of pathologic autoimmunity. The role of additional suppressor cell populations in tumor immunopathology aside from $CD4^{+}CD25^{+}$ Tregs also merits further study.

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13

TUMOR VACCINATION AFTER AUTOLOGOUS HSCT: WHAT HAS BEEN LEARNED FROM EXPERIMENTAL MODELS

Weiqing Jing and Bryon D. Johnson

13.1 INTRODUCTION

The use of vaccines for the treatment of cancer has been a clinically challenging endeavor for several reasons, including the fact that unlike infectious agents that contain foreign antigens capable of inducing relatively strong immune responses, cancer cells are of self-origin and therefore may be capable of inducing only weak immunity. Since cancer is a disease of self-origin, and immune regulatory networks are in place to prevent self-reactivity [1], cancer vaccines need to induce a response that is strong enough to overcome the immune regulation. Another problem is that unlike the use of vaccines for infectious agents, which are given to prevent infection, cancer vaccines are administered in the face of active disease. Cancer cells themselves may produce factors that suppress infiltrating immune effectors [2], or the growing cancer may activate immune regulatory cells capable of suppressing the immune system [3]. Therefore, unless used in the context of minimal disease and altered immune regulation, vaccines for cancer may be destined to fail. High-dose therapy followed by hematopoietic stem cell transplantation (HSCT) is an attractive

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platform for cancer vaccine administration since it can achieve both of these goals (reduction of disease to minimal levels and altered immune regulation). Clinical and experimental data have also suggested that the lymphopenic state occurring early after HSCT might allow for better induction and skewing of immunity toward tumor reactivity [4–7].

Vaccinating early after HSCT has become of particular interest since the mid-1990s as researchers and clinicians strive to improve the efficacy of cancer vaccines. This chapter briefly explores what has been learned from mouse syngeneic HSCT studies regarding early posttransplant vaccination for cancer.

13.2 ANIMAL MODELS OF AUTOLOGOUS HSCT: WHAT WE HAVE LEARNED

The primary goal of autologous HSCT for cancer has been to eliminate disease using high doses of chemotherapy and/or radiotherapy. Unfortunately, although tumor cell numbers can be reduced to undetectable levels, patients often relapse from residual microscopic disease since there is a lack of graft-versus-tumor immune reactivity in the autologous HSCT setting. Results from experimental mouse studies have shown that cancer vaccines can elicit tumor immunity when administered after syngeneic HSCT [4,8–17], but a number of factors can influence the effectiveness of vaccination, including the level of immune reconstitution, the presence of immune regulatory cells, and tumor burden (i.e., numbers of remaining tumor cells). Each of these factors has been or is currently being examined in mice administered syngeneic HSCT. Information learned from these experimental studies is briefly discussed in the following sections.

13.3 IMMUNE RECONSTITUTION

T lymphocytes are important effectors for the generation of vaccine-induced antitumor responses. While data from investigators have suggested that it may be easier to skew T cell immune reactivity toward cancer after HSCT [4,5], the ability to generate an immune response immediately after HSCT may be difficult because of the severe lymphopenia that typically occurs after high-dose conditioning. When mice are conditioned with lethal total-body irradiation (TBI), there is a period of profound lymphopenia that lasts for approximately 3 weeks after HSCT (Fig. 13.1a). T cell numbers are severely reduced during this time period, and their numbers remain below pretransplant levels even 50 days after transplant (Fig. 13.2). T cell reconstitution early after transplant occurs primarily through thymus-independent mechanisms, and since mouse bone marrow (BM) contains relatively few T cells ($\sim$1–4%), the first 3 weeks after HSCT are dominated by small numbers of residual host (or recipient) $CD4^+$ T cells. Since thymic reconstitution does not occur until around 2 weeks after transplant (Fig. 13.1b), relatively few BM-derived T cells

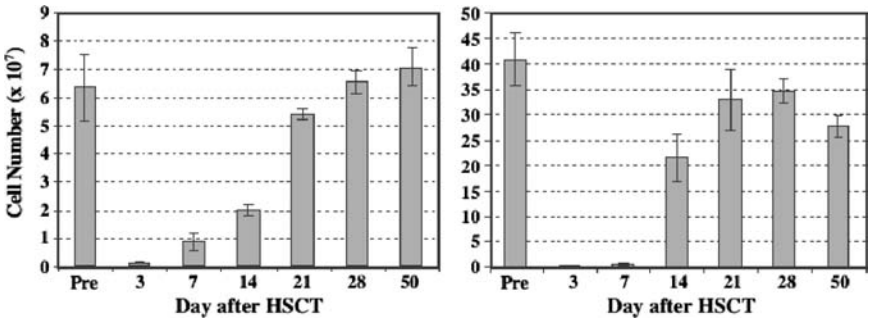

Figure 13.1. Lymphoid tissue reconstitution after syngeneic HSCT: (a) splenic reconstitution; (b) thymic reconstitution. AKR/J recipients were lethally irradiated and transplanted 24 h later by intravenous injection with 10^7 syngeneic AKR/Cum bone marrow cells. Cell counts were done on spleens (a) and thymuses (b) collected before HSCT (Pre) or on days 3, 7, 14, 21, 28, and 50 after HSCT. The data represent the averages and standard deviations of three mice analyzed at each timepoint.

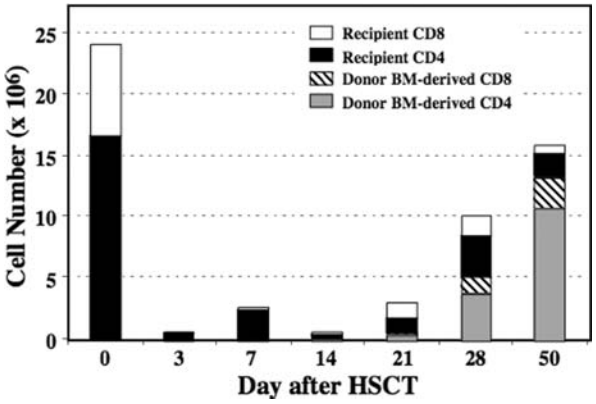

Figure 13.2. T cell reconstitution after syngeneic HSCT (bone marrow only). Lethally irradiated AKR/J recipients (Thy1.1$^+$) were transplanted with 10^7 syngeneic AKR/Cum (Thy1.2$^+$) bone marrow cells. Phenotypic analysis of spleens was done before HSCT (Pre) or at the indicated timepoints after HSCT. Recipient T cells could be distinguished from bone marrow–derived (donor) T cells because of the Thy1 allelic difference. The data represent the averages of three mice analyzed at each timepoint.

can be detected in the spleen until 3–4 weeks after HSCT. Other investigators [4,9] have similarly reported this pattern of cellular reconstitution in mice. In order to prevent posttransplant tumor relapse using cancer vaccines, one might hypothesize that vaccination should be initiated as soon as possible after HSCT. However, the data in Fig. 13.2 suggest that T cell numbers during the first 3 weeks following

HSCT may be suboptimal for the generation of effective vaccine-induced tumor immunity. To support this, Teshima and coworkers [16] found that immune reconstitution was critical for the induction of tumor immunity elicited by tumor vaccination. These observations suggested that if T cell reconstitution could be accelerated, then the efficacy of early posttransplant tumor vaccination could be improved.

Our laboratory has examined the impact of supplementing mouse BM with a source of T cells (splenocytes), or infusing purified T cells early (3 days) after HSCT, in an effort to improve the efficacy of tumor vaccination early posttransplant. When we examined T cell reconstitution in lethally irradiated mice given BM supplemented with 20 million splenocytes (containing $\sim$6 million T cells), we found that although total splenic T cell numbers are not dramatically altered during the first 3 weeks after transplant (cf. Fig. 13.3 vs. Fig. 13.2), the majority of T cells during this time period are from the infused splenocytes (Fig. 13.3). Importantly, when a vaccine for neuroblastoma is administered to syngeneic A/J mice on days 7 and 14 after HSCT, only those mice that receive an infusion of syngeneic T cells are able to resist a challenge of live neuroblastoma cells 21 days after HSCT (Fig. 13.4). Thus, for this particular tumor and tumor vaccine, there is an absolute requirement for T cell adoptive transfer in order to induce tumor-protective immunity early after HSCT. Interestingly, as

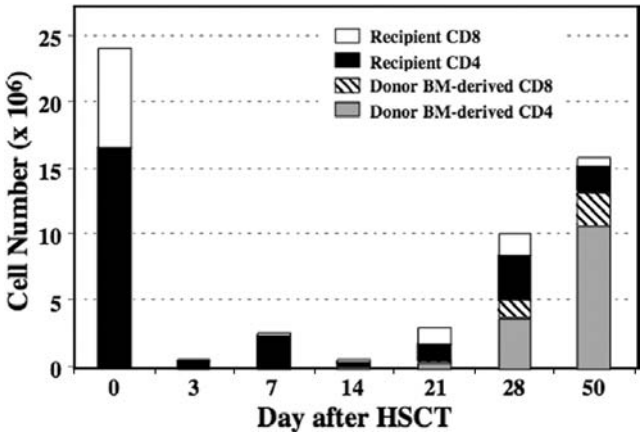

Figure 13.3. T cell reconstitution after syngeneic HSCT (BM + splenocytes). C57BL/6 (Thy1.2$^+$CD45.2$^+$) mice were lethally irradiated and 24 h later injected with 10^7 bone marrow cells from congenic B6.PL-*Thy1a* mice (Thy1.1$^+$CD45.2$^+$) plus 2×10^7 splenocytes from congenic B6-CD45.1 mice (Thy1.2$^+$CD45.1$^+$). Mice were killed at the indicated timepoints and the spleens collected for analysis. Nucleated cell numbers were counted, and phenotypic analysis of spleens was done before HSCT (Pre) or at the indicated timepoints after HSCT. Recipient, donor bone marrow (BM)–derived, and donor spleen (Spln)–derived T cells could be distinguished from one another by the Thy1 and CD45 allelic differences. The data represent the averages of six mice analyzed at each timepoint.

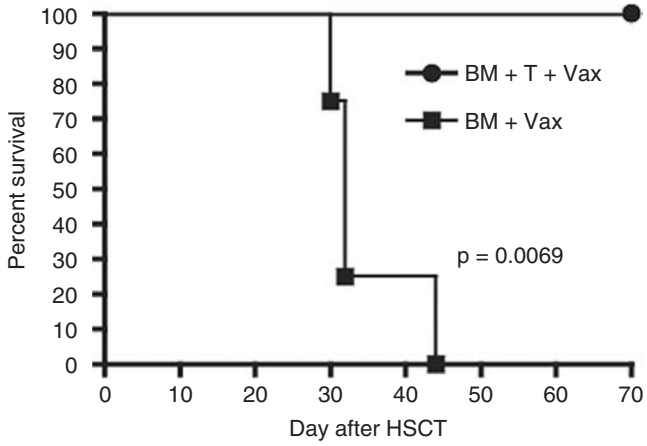

Figure 13.4. Protective tumor vaccine-induced immunity requires the presence of T cells in the graft. Lethally irradiated A/J mice were transplanted with 10^7 syngeneic BM cells, and 3 days later given or not given 5×10^6 purified T cells. One week after HSCT, recipients were vaccinated twice weekly with a cell-based neuroblastoma vaccine consisting of tumor cells that had been genetically modified to express CD54, CD80, CD86, and CD137 L. One week after the last vaccination, the mice were challenged with 10^4 live parental AGN2a neuroblastoma cells and followed for tumor development. The data ($n = 4$ mice/group) are representative of two separate experiments.

noted by Borrello and colleagues [4], recovery of lymphoid cell numbers toward pretransplant levels is not required for generation of protective vaccine-induced immunity.

In an effort to examine whether early post-HSCT tumor vaccination might facilitate better tumor-specific T cell responses as compared to later vaccination, we administered two weekly neuroblastoma vaccines at different times after HSCT (started on days 7, 14, 21, or 28), and tested purified immune T cells for neuroblastoma reactivity in interferon-gamma (INFγ) ELISPOT assays. The highest frequencies of tumor-reactive $CD4^+$ and $CD8^+$ T cells in these assays were observed in mice vaccinated on days 7 and 14 (Fig. 13.5), and as compared to historical data from nontransplanted/vaccinated mice, frequencies of tumor-reactive T cells were higher at all post-HSCT vaccine timepoints. These data are in agreement with observations by other investigators who have shown that altered T cell homeostatic mechanisms early after HSCT allow for the skewing of T cell immune responses toward tumor antigens [4].

While it is clear that T cells are critical for the generation of effective vaccine-induced antitumor immunity, experimental data have indicated that other immune cells can contribute to, or play an important role in, antitumor immune responses depending on the nature of the target antigens and the type of vaccine used. Earlier experimental HSCT studies focused on generation of antiidiotypic immunoglobulin (Ig) responses to tumor-derived surface Ig as a strategy to treat

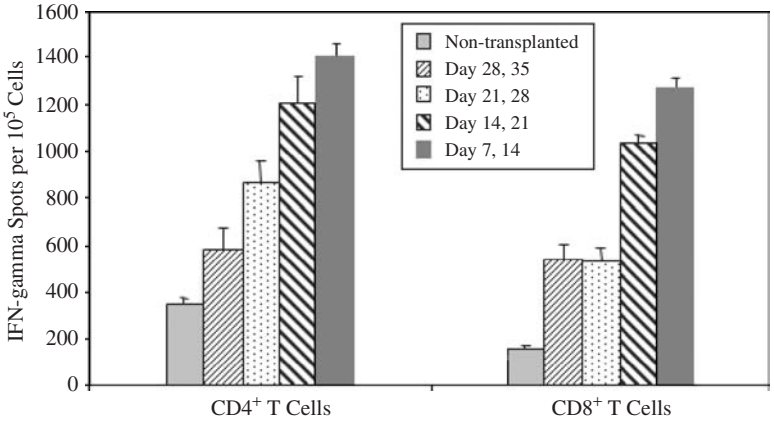

Figure 13.5. Tumor vaccination early after HSCT induces the highest frequencies of tumor-reactive T cells. Lethally irradiated A/J mice were transplanted with syngeneic bone marrow cells plus 2×10^7 syngeneic splenocytes. At the indicated timepoints after HSCT (see legend in the diagram), the mice were vaccinated with a cell-based neuroblastoma vaccine consisting of tumor cells that had been genetically modified to express CD54, CD80, CD86, and CD137 L. Five days after the last vaccination, $CD8^+$ and $CD4^+$ splenic T cells were isolated and analyzed for frequencies of neuroblastoma-specific IFNγ-secreting cells by ELISPOT. CD4 cell reactivity was assessed using a MHC class II^+ neuroblastoma cell line (AGN2a-CIITA) that was created by transfecting AGN2a neuroblastoma cells with a plasmid encoding CIITA. A group of nontransplanted/vaccinated mice was included for comparison. The data represent the averages and standard deviations of triplicate wells.

B cell lymphoma [12,13]. These studies showed that tumor-protective Ig responses could be generated as early as 3 weeks after HSCT by vaccination [13], demonstrating that Ig responses can play a role in post-HSCT immunity to cancer if appropriate antigenic targets are present on the tumor. Chen and colleagues [10] showed that early post-HSCT vaccination with tumor-derived chaperone-rich cell lysates, which are composed of heatshock proteins, could induce immunity to a mouse leukemia. Natural killer (NK) cells as well as T cells were involved in the antitumor immunity. These results point out that both adaptive and innate immunity can be induced by early post-HSCT vaccination, and since there is crosstalk between these two arms of the immune system, efforts to activate both might provide additive or synergistic effects.

In summary, despite the lymphopenia and depressed T cell numbers early after myeloablative HSCT (Figs. 13.1 and 13.3), experimental animal data have shown that if sufficient numbers of mature T cells are present in the graft, cancer vaccines given early post-HSCT (within the first 2 weeks) can induce tumor-protective immunity. Furthermore, early post-HSCT vaccination may allow for the skewing of T cell immunity in favor of tumor reactivity.

13.4 PRESENCE OF IMMUNE REGULATORY CELLS

With increasing numbers of studies indicating that regulatory T (Treg) cells play a role in the suppression of immunity to cancer [3], investigators are examining whether inhibiting these cells can increase the efficacy of cancer immunotherapies. While different populations of Treg cells have been described, the best-characterized population consists of $CD4^+$ cells that coexpress the transcription factor, Foxp3, and the alpha chain of the IL-2 receptor, CD25 [18]. One consequence of the myeloablative treatment used in preparation for HSCT is the elimination of Treg cells in addition to elimination of potential immune effector cells. Our laboratory has examined $CD4^+Foxp3^+$ cells in lethally irradiated mice given syngeneic HSCT with BM and 20 million added splenocytes. Only small numbers of $CD4^+Foxp3^+$ cells survive the lethal conditioning (Fig. 13.6a), and numbers remain low until 3 weeks after HSCT. Notably, the majority of $CD4^+Foxp3^+$ cells during the first 4 weeks after HSCT are derived from the infused splenocytes, and new thymus-derived $CD4^+Foxp3^+$ cells do not appear until days 21–28 (not shown). Despite the depressed numbers of $CD4^+Foxp3^+$ cells during the first 2 weeks after HSCT, increased percentages of $CD4^+$ cells express Foxp3 (Fig. 13.6b), in large part due to increased percentages of $Foxp3^+$ cells originating from the infused splenic $CD4^+$ cells (not shown). On the basis of these data, we speculate that $CD4^+Foxp3^+$ cells might have some impact on vaccine-induced immunity early post-HSCT. More recent preliminary results suggest that depletion of $CD25^+$ cells from T cells (splenocytes) added to BM may increase vaccine efficacy against a mouse neuroblastoma (Fig. 13.7), but these experiments will need to be repeated to see if the results are statistically significant.

There is some concern that manipulation of Treg cells will increase the risk for development of autoimmune disease, particularly in the HSCT setting where Treg cells are already being impacted by the conditioning regimens. However, despite the relatively low numbers of $CD4^+Foxp3^+$ cells early after HSCT, development of autoimmune disease has not been an issue following syngeneic HSCT in mice. Perhaps a small number of radioresistant Treg cells are adequate to prevent development of autoimmune immune disease, as has been suggested by Benard and colleagues [19]. In addition, since the time interval between transplant and thymic reconstitution in mice is relatively short, small numbers of Treg cells early post-HSCT may be sufficient to blunt autoreactivity until new thymus-derived Treg cells emerge. Future studies using Treg-deficient, thymus-deficient mice as recipients of Treg cell-depleted HSCT should be able to address these possibilities.

The role of Treg cells in cancer immunity early after HSCT is just beginning to be explored. Whether Treg cells impact vaccine-induced immunity early after HSCT, and what the magnitude of their impact might be, can be examined using appropriate experimental animal models. While myeloablative therapy might allow for improved vaccine efficacy in part due to reduction in Treg cell numbers, it will be important to know whether further inhibition of Treg cells increases vaccination efficacy and/or increases the risk for development of autoimmune disease.

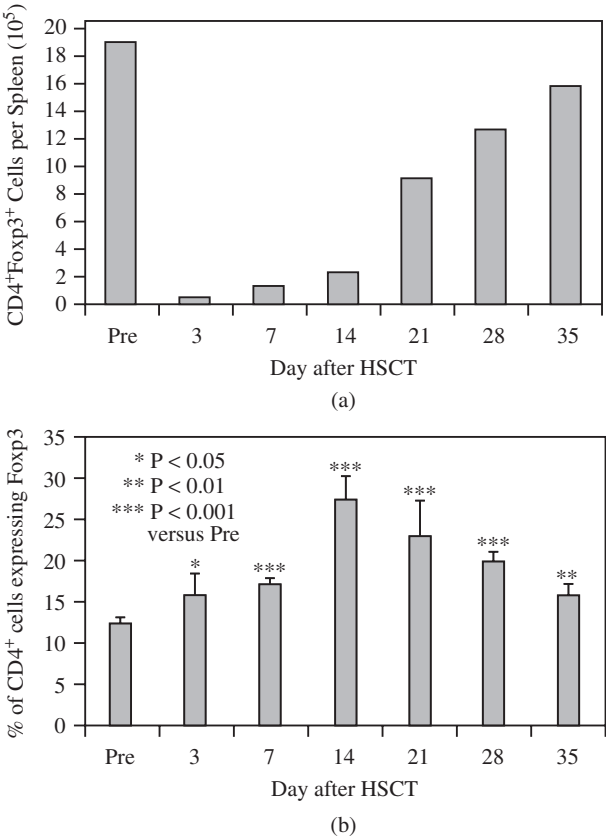

Figure 13.6. $CD4^+Foxp3^+$ T cells after HSCT. C57BL/6 mice were lethally irradiated and 24 h later injected with syngeneic bone marrow cells plus 2×10^7 splenocytes. Mice were killed at the indicated timepoints and the spleens collected for analysis. Nucleated cell numbers were counted, and the remaining splenocytes were analyzed by flow cytometry to assess absolute numbers of $CD4^+$ cells coexpressing Foxp3 (a) and the percentages of $CD4^+$ cells coexpressing Foxp3 (b). The data represent the mean values of six mice combined from two experiments ($n = 3$ mice analyzed in each experiment). Standard deviations and p values are shown in panel (b).

13.5 TUMOR BURDEN

Vaccination for infectious disease has taught us that vaccines, even for potent non-self-antigens, are likely to fail in the face of established disease. The lack of clinical success using cancer vaccines to treat patients with "bulky" disease confirms this. Using experimental models, investigators have shown that vaccination against "established disease" can be effective if given early after disease inoculation, while vaccination at later timepoints only delays growth [20]. The

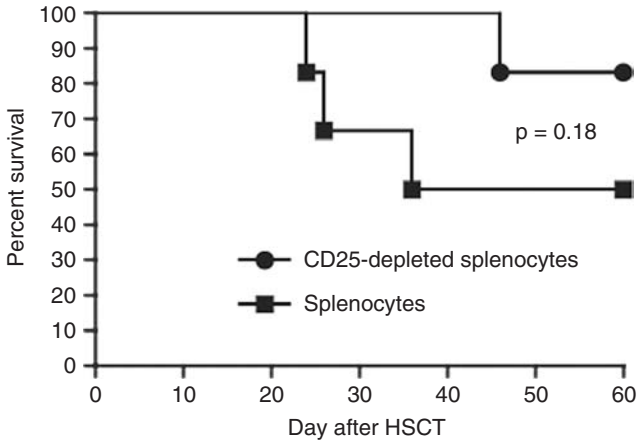

Figure 13.7. Depletion of $CD25^+$ cells from T cells added to BM appears to increase vaccine efficacy. Lethally irradiated A/J mice were transplanted with syngeneic bone marrow cells plus 2×10^7 nonseparated splenocytes or splenocytes depleted of $CD25^+$ by immunomagnetic depletion. The transplanted mice were vaccinated on days 7 and 14 after HSCT with irradiated neuroblastoma cells that had been genetically modified to express CD54, CD80, CD86, and CD137 L. All mice were then challenged 3 weeks after the last vaccination with 5×10^6 live neuroblastoma (AGN2a) cells. The groups consisted of six mice per group.

ability of myeloablative therapy to reduce tumor burdens to nondetectable levels, or nearly nondetectable levels, is why the early post-HSCT time period is attractive for vaccinating against cancer. While animal studies have provided data showing that vaccines can induce protective tumor immunity early after syngeneic HSCT, studies have shown that even in the post-HSCT setting it can be difficult to eliminate established disease by vaccination [8,10]. Part of the difficulty in interpreting the results of these studies is that mouse tumor cell lines are sometimes highly aggressive when inoculated in vivo, providing a narrow "window of opportunity" in which to vaccinate tumor-bearing animals. However, a clear message from the experimental observations is that vaccination should be initiated as early as possible after HSCT.

Chen and colleagues [10] found that the most effective early post-HSCT vaccination of mice with established leukemia required transplant of "immune" BM and splenocytes from prevaccinated syngeneic donors. Mice that had been administered nonimmune HSCT showed no benefit from tumor vaccination during the first week after transplant [10], confirming similar observations by other investigators [4]. These results suggest that a multifaceted approach combining myeloablative therapy, HSCT, adoptive immunotherapy (with vaccine-primed T cells collected pretransplant), and vaccination may be required to effectively eliminate residual disease for some tumors. It is noteworthy that this type of multifaceted approach is already being explored clinically, with interesting results [21].

13.6 CONCLUSIONS AND FUTURE CONSIDERATIONS

Experimental mouse studies have provided information regarding the ability to vaccinate early after HSCT setting, have shown that the presence of T cells is crucial to the success of early post-HSCT tumor vaccination, and have demonstrated that tumor burden can impact on vaccine efficacy. Lessons learned from the experimental work are being applied in the clinic, but there is a need for additional preclinical work. Some important questions that still need to be addressed include

1. What is the frequency of vaccination needed to induce optimal antitumor immunity early after HSCT? Are repeated vaccinations needed to maintain optimal tumor immunity?
2. Can post-HSCT vaccination be optimized by using vaccine preparations that activate both innate and adaptive immunity?
3. Can further perturbation of immune regulatory mechanisms improve vaccine efficacy without toxicity?
4. How can adoptive immunotherapy be most effectively combined with post-transplant vaccination, and what mechanisms are involved?
5. Is the generation of T cell memory important to vaccine-induced immunity post-HSCT, and if so, how can memory be most effectively generated?

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14

VACCINES IN THE SETTING OF HEMATOPOIETIC STEM CELL TRANSPLANTATION

Ronald Gress and Claude Sportes

14.1 INTRODUCTION

Effective treatment of metastatic solid tumors remains a clinical challenge. Escalating doses of available chemotherapeutic agents have not, in general, produced high cure rates in metastatic breast cancer [1], for example, and much of the current emphasis is placed on the introduction of more targeted agents. It is in this setting that interest exists in developing alternative, immune-based strategies such as tumor vaccines, adoptive transfer of in vitro generated effector T cell populations, and allogeneic hematopoietic stem cell transplantation (HSCT). While the limitations of standard chemotherapeutic agents are recognized, those that provide efficacy in disease control also typically deplete cells of the immune system. On initial consideration, this side effect may seem undesirable for the development of new treatment strategies that are immune-based, but characterization of the regenerating immune response has provided insights indicating that immune-depleting chemotherapy might act as a platform for immunotherapies such as those employing tumor-specific vaccines or adoptive T cell transfer. That perspective is based

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on the understanding and derivative hypotheses that dose-intensive chemotherapy can lengthen the progression-free survival period [2–4], thus allowing a "time window" for a slow-acting therapy such as vaccination to be effective, that maximally decreasing the patient's tumor burden may increase the effectiveness of immunotherapy [5,6] by mechanisms including decreases in tumor-induced immune suppressive effects [7–9], and that providing tumor antigen exposure following immune depletion in the form of repeated immunizations may take advantage of the pattern of immune reconstitution at early timepoints (antigen-driven peripheral expansion of T cells) and allow for development of a T cell repertoire biased toward tumor antigens. In addition, unaltered T cells in the form of reinfused lymphocytes collected prechemotherapy can be used to augment T cell responses to antigen following immunoablative chemotherapy, perhaps especially in the setting of primed T cell transfer.

14.2 TRANSPLANTATION, TUMOR MASS, AND TIME

Several animal models of immune-mediated tumor eradication have shown that preventing the establishment of a tumor is much more readily achievable than eradicating an established tumor. This suggests that a lower tumor burden increases tumor susceptibility to immune effector cells. The advantage of a lower tumor burden in immunotherapy has also been demonstrated clinically in the postallogeneic HSCT setting using donor lymphocyte infusions (DLIs) in patients with hematologic malignancies. Chronic myelogenous leukemia (CML) patients with cytogenetic or hematologic relapse have a significantly higher complete response rate and improved outcome with DLI compared to those with greater tumor burden [5,6]. A second consideration is immune suppression induced by the tumor itself. Such suppression may in part be directly related to tumor bulk and thus may be improved with a greater reduction in tumor mass through high-dose chemotherapy. Experimental evidence points to a specific immunosuppressive effect of large tumor burdens, including decreases in specific antitumor T lymphocyte activity as the tumor burden increases [7]. Depressed specific antitumor responses have been described in tumors expressing the MUC1 antigen, via either direct suppression [8] or induction of lymphocyte apoptosis [9]. It is likely that some of these findings may also be relevant to other tumor antigens.

Immune strategies may require a prolonged period for effectiveness. This was illustrated in CML patients following allogeneic HSCT who showed a progressive conversion from cytogenetic to molecular remission over a period of months [10]. A more recent study in patients with renal cell carcinoma also demonstrates the need for prolonged exposure to immune effectors for in vivo tumor eradication [11]. A dose-intense regimen that can result in a prolongation of the time to tumor progression may increase the time window in which an immune-based strategy can have its effect. It may be advantageous to decrease the tumor burden with dose-intense therapy, not only to decrease the tumor mass but also to potentially increase the efficacy of the subsequent immune intervention by decreasing

antitumor immune suppression and by widening the time window for a slow-acting immune therapy. Therefore, dose-intense chemotherapy followed by immune depletion may serve most importantly as a platform for subsequent immune-based therapies.

14.3 TRANSPLANTATION AND A WINDOW OF SURVIVAL: AN EXAMPLE

In 1996 we opened a clinical protocol seeking to improve the survival of patients with metastatic breast cancer. Treatment consisted of three to seven cycles of continuous 72-h infusion of paclitaxel (160 mg/m^2) and daily cyclophosphamide (900 mg/m^2 $\times$ 3) (to maximum clinical response). The second cycle was used for stem cell mobilization purposes. For patients not previously treated with an anthracycline, four cycles of doxorubicin (60 mg/m^2)/cyclophosphamide (600 mg/m^2) were administered, and local control was approached using surgery and /or radiation therapy as clinically indicated. The high-dose chemotherapy consisted of melphalan (160 mg/m^2)/etoposide (1800 mg/m^2) followed by autologous peripheral blood stem cell transplantation. In order to proceed with the high-dose chemotherapy, patients had to at least achieve a partial remission (defined as 50% or more reduction of measurable disease). Hormonal therapy and/or bisphosphonate therapy were given as clinically indicated. At the time of last analysis [12], progression-free and overall survival probabilities at 24 months posttransplant for stages IIIA, IIIB, and IV transplanted patients were 82/81/42%, 100/94%, and 68%, respectively. The median progression-free and overall survival times from entry on study for stage IV patients were 15.3 and 38.1 months, respectively. For stage III patients, the median progression-free survival has not been reached. In both groups, the window of survival was sufficient to allow introduction of immune-based therapies in an attempt to develop a curative therapy.

14.4 ALTERNATIVES TO HIGH-DOSE CHEMOTHERAPY FOR CONTROLLING DISEASE: T CELL DEPLETION OUTSIDE THE SETTING OF HIGH-DOSE CHEMOTHERAPY

In other allogeneic HSCT protocols, the combination of fludarabine and cyclophosphamide (Flu-Cy) has been used successfully by our group to induce a state of profound immune depletion prior to nonmyeloablative T-cell-depleted HSCT in patients with recurrent metastatic breast cancer [13,14]. One or two cycles of fludarabine (30 mg/m^2) and cyclophosphamide (600 mg/m^2) for 4 consecutive days were used with the specific goal of depleting lymphocytes prior to administering the preparative regimen for transplantation. Patients received one or two courses of the Flu-Cy regimen on the basis of peripheral blood CD4 counts such that those patients with a CD4 count >50/mm^3 after neutrophil recovery, 18–24 days after the first course, went on to receive a second course of Flu-Cy before progressing to the preparative regimen for transplant.

The results comparing the cumulative immune depleting effect of the standard chemotherapy doses in the autologous transplant protocol described in the previous section (first 25 patients), and the immune-depleting effects of one course of Flu-Cy (first 12 patients) showed that the most striking differences involved the $CD4^+$ T cell population. The absolute postdepletion counts were significantly lower after Flu-Cy than after several cycles of chemotherapy ($44/mm^3$ vs. $161/mm^3$, $p < .0001$), although the pretreatment CD4 counts were initially higher in the former group ($612/mm^3$ vs. $436/mm^3$, $p = .023$). Notably, the relative CD4 depletion ("Post"–"Pre"/"Pre") obtained after one cycle of Flu-Cy was significantly greater with Flu-Cy (88% vs. 78%, $p = .003$). Thus, the biology of T cell regeneration, which contributes to the strategy of tumor vaccine administration in HSCT, also applies to T-cell-depleting nonmyeloablative chemotherapy.

14.5 ANTIGEN EXPOSURE POSTIMMUNE DEPLETION: T CELL REPERTOIRE RECONSTITUTION

Experimental and clinical studies [15–21] of posttransplantation immune reconstitution have defined two pathways of T-cell regeneration following T-cell-depleting chemotherapy and autologous HSCT (Fig. 14.1). In murine models, in which the

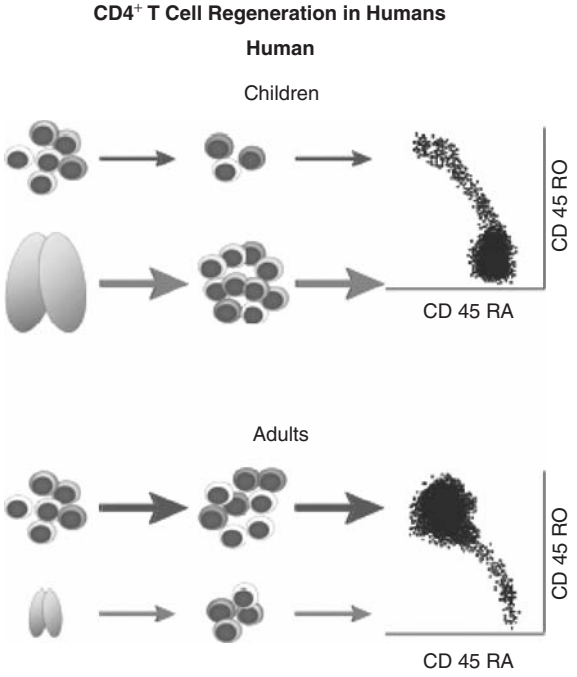

Figure 14.1. $CD4^+$ T cell regeneration in humans.

existence of two primary (and only two primary) pathways of T cell regeneration following acute loss of peripheral T cell populations was demonstrated, it was shown that a thymus-dependent pathway was dominant in young animals, but that in the absence of a thymus, appreciable populations of competent T cell were generated by peripheral expansion of mature T cells; thus, mature T cells served as progenitors for reconstituting peripheral T cell populations by clonal expansion. Further, it was shown that this thymus-independent peripheral expansion pathway was influenced by antigen, and therefore was prone to skewing. The thymic pathway, on the other hand, was a source of T cell repertoire diversity. Because there is no known pathway for generation of appreciable T cell receptor diversity outside the thymus, identification of diversification is evidence of thymic activity. Perhaps most important in these early murine studies was the identification of CD45 isoform expression as a means to study T cell regeneration in humans in that the pathway gives rise to reconstituted T cell populations with characteristic phenotypes; the thymus-dependent pathway gives rise to naive T cell populations that primarily have a CD45RA isoform phenotype, while the peripheral expansion, thymus-independent pathway obligatorily gives rise to memory T cell populations with a CD45RO isoform phenotype.

When CD45 isoform expression was used to study T cell regeneration in humans, it was the thymus-dependent pathway was found to predominate for regenerating lost $CD45RA^+CD4^+$ T cells in children and young adults (Fig. 14.1), and that it allowed the reconstitution of a widely diverse repertoire of naive T cells. In initial studies in adults, it was found that peripheral expansion was the predominant pathway of T cell recovery, and no evidence for effective thymopoiesis was observed. In a later, long-term study involving multiple parameters of thymic activity (thymic size by imaging, identification of recent thymic emigrants by TREC analysis, assessment of T cell receptor diversity, and CD45 isoform expression by reconstituted T cell populations), it was found that thymopoiesis can contribute to reconstitution of lost T cell populations in a subset of adults with age as a controlling variable for its occurrence and timecourse—the younger the adult, the more likely that thymic recovery occurred, and that it occurred in these younger adults more quickly after cessation of T-cell-depleting chemotherapy [12,19]. The variation in thymic recovery by age reached its limit at approximately age 50, in that very few individuals beyond this point were observed to have numerically significant recovery of lost T cell populations even over 5 years of observation. This longer-term study confirmed the T cell repertoire skewing associated with T cell recovery via the peripheral expansion pathway. It also revealed a fundamental difference in the patterns of regeneration by $CD4^+$ versus $CD8^+$ T cells in that the latter underwent more profound peripheral expansion in some individuals and contributed long term to the numerical recovery of $CD8^+$ T cell populations, whereas the long-term numerical recovery of $CD4^+$ T cells populations was dependent on thymic activity (Figs. 14.2a and 14.2b).

The difference in recovery patterns between $CD4^+$ and $CD8^+$ T cells finds its basis in the distinctly different homeostatic regulation of the two T cell subsets. Peripheral T cell populations exist in homeostatic cytokine-defined niches: IL-7

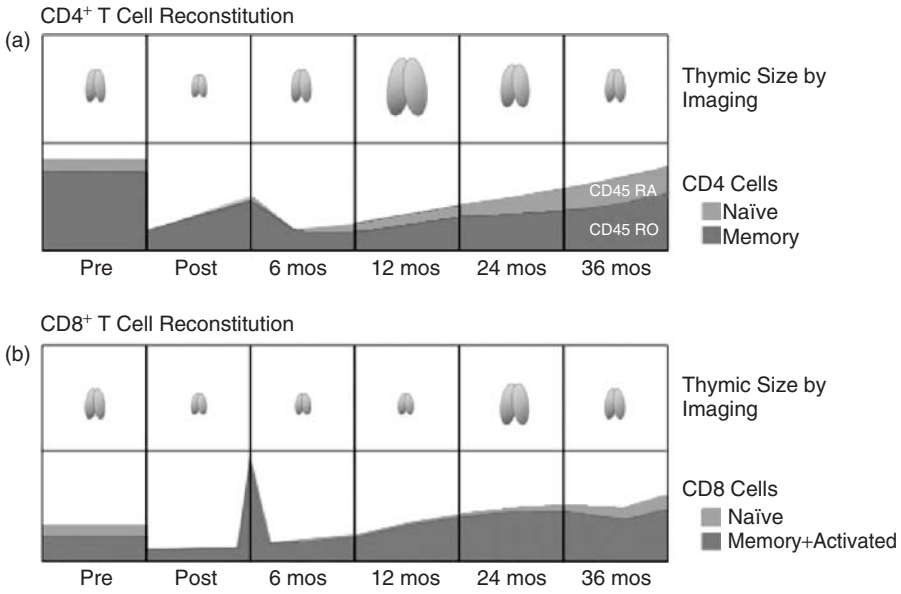

Figure 14.2. T cell reconstitution timecourse: (a) $CD4^+$ T cell reconstitution; (b) $CD8^+$ T cell reconstitution.

is the cytokine predominantly defining the niche for naive $CD4^+$ and $CD8^+$ T cell populations, while IL-15 predominantly defines the niche for central memory and activated effector $CD8^+$ T cells. While IL-7 levels are not dynamically regulated to an appreciable extent (although they vary with $CD4^+$ T cell number as a homeostatic regulator), IL-15 is a component of the inflammatory cascade, and therefore does vary, and is able to support large expansions of activated $CD8^+$ T cells. At least in the mouse, homeostatic control of the central memory $CD8^+$ T cell pool appears to be stabilized by TGFβ in that animals containing T cells incapable of responding to this cytokine have ever-expanding populations of $CD8^+$ T cells with a central memory phenotype [23]. These studies of T cell reconstitution have thus identified three strategies for optimizing or exaggerating T cell responses to vaccine antigens in the setting of cancer therapy: (1) early vaccination postcessation of chemotherapy to take advantage of antigen-driven T cell reconstitution (with the antigen consisting of tumor specific vaccines), (2) adoptive infusion of T cells presensitized to specific antigens during the "early" vaccination time period to augment the progenitor pool available for peripheral expansion, and (3) "Late" vaccination to take advantage of the newly repaired T cell repertoire that emerges with thymic recovery in younger adults.

While the rationale for administration of tumor vaccines during T cell regeneration after HSCT applies to both the autologous and allogeneic settings, certain unique features apply to the latter. One feature is that the immunotherapeutic effects inherent to allogeneic HSCT (i.e., graft–tumor reactivity) may be enhanced

by administration of tumor antigens in the posttransplant period. Another unique feature regarding allogeneic HSCT is availability of the transplant donor to supply T cell populations that have been unaffected by tumor exposure, disease burden, or chemotherapy that can be administered as DLI, thereby providing a source of lymphocytes that can be coadministered with repeated vaccinations. A third feature is the immunosuppression associated with graft-versus-host (graft–host) disease and its therapies. Therefore, while vaccine strategies may be developed for autologous and allogeneic HSCT that have common elements, distinct considerations apply to the latter setting.

14.6 OPTIMIZING T CELL REGENERATION

Use of Homeostatic Cytokines

As outlined, the size of T cell populations, or T cell "space," is defined by homeostatic cytokines. Current evidence favors IL-7 as the principal homeostatic cytokine for naive $CD4^+$ and $CD8^+$ T cell populations, and IL-15 as the important homeostatic cytokine for memory $CD8^+$ T cell populations. IL-2 has been identified as a homeostatic cytokine for regulatory $CD4^+$ T cells [24]. Homeostatic cytokines affecting the peripheral, or thymus-independent, pathways of T cell regeneration have not yet been tested clinically in the setting of immunotherapy. IL-7 has now reached initial phase I trials and shows activities in humans similar to what has been described in basic and preclinical investigations (Ref. 25 and manuscript in preparation). IL-15 has yet to be tested in phase I trials, but its presence at high concentrations in the posttransplant period provides a setting for directed expansion of in vitro–derived memory/effector $CD8^+$ T cell populations that have been adoptively transferred [26]. As a homeostatic cytokine, IL-7 is also increased in the same time period, thereby creating a favorable cytokine milieu for multiple T cell subsets.

Enhancing Thymic Reconstitution

Thymic regulation is poorly understood. Some strategies for enhancing thymic activity have been proposed for clinical testing including administration of growth hormone or keratinocyte growth factor (KGF), or blockade of androgen signaling. The last of these strategies has already been investigated in humans as well as in mice [27].

14.7 VACCINE STRATEGIES: TARGET ANTIGENS AND TOLERANCE

Vaccine approaches to the therapy of solid tumors have been extensively investigated for over fifteen years. Two critical components (in addition to elements of immune reconstitution) that must be considered for a cancer vaccine therapy to

be successful are the judicial choice of target antigen(s) and strategies addressing ways to overcome immune tolerance or boost responses to weak immunogens. There are numerous reviews summarizing the current knowledge regarding these issues [28–39], and some of these are covered in other chapters in this book. Several mechanisms of tumor-induced suppression of immune responses have been demonstrated. They include expression of IL-10 [40,41] or TGFβ [42–44], and presence of various immune suppressive tumor-related molecules [8,45–47]. As noted, it is reasonable to hypothesize that certain of these limitations may be lessened in parallel with reduction in tumor burden associated with HSCT, and that repair of the defective T cell repertoire may occur in the course of T cell regeneration in the same setting.

14.8 CONCLUSIONS

In conclusion, the combination of conventional chemoradiotherapies with immune interventions can become a therapy paradigm that can be tailored to a variety of clinical cancer settings depending on circumstances and stage of disease with the following principles:

- The combination of dose-intensive chemotherapy followed by immune-depleting chemotherapy provides a platform for subsequent immunotherapy by
  - Lengthening the progression-free survival period, thus allowing time for more slowly acting immunotherapies such as vaccination to be effective
  - Decreasing tumor-induced immune suppression associated with bulky disease
  - Allowing for repeated immunizations to more effectively skew T cell reactivity toward tumor at both "early" (through antigen-driven peripheral expansion of T cells) and "late" (activation of new thymus-derived T cells) timepoints
- Owing to the postimmune depletion defects and delay in immune reconstitution, an adequate immune response to vaccines may not occur unless the patient is coadministered T cells in the form of tumor antigen(s)-sensitized lymphocytes collected prechemotherapy.
- The late recovery of thymic function (18–24 months), resulting in reappearance of naive T cells, may play a determinant role in the prevention of later disease progression, providing rationale for prolonged immune interventions, such as a late series of reimmunizations. This late recovery may be enhanced by cytokines.

"Appropriate" immunotherapeutic strategies (vaccines and/or adoptive lymphocyte transfer) will be required to overcome the low antigenicity of some tumors and tumor-associated immune tolerance mechanisms.

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15

INTRATUMOR GENERATION OF VIGOROUS ANTITUMOR IMMUNE RESPONSES

Ping Yu and Yang-Xin Fu

15.1 INTRODUCTION

Given the vast number of genetic changes associated with carcinogenesis, it would not be unexpected that tumors express many neoantigens. Lines of convincing evidence also exist to indicate that many cancers are antigenic [1]. Even unchanged germ cell proteins ectopically expressed in tumors are recognized by the T cells of cancer patients [1]. Mere recognition by adaptive immunity, however, is insufficient, as even antigenic cancers rarely regress spontaneously. An effective antitumor immune response not only depends on the presence of precursors of tumor-reactive lymphocytes in the tumor-bearing host but also requires at least three additional conditions: (1) naive tumor-specific T cells must encounter the tumor antigen in an environment of sufficient quality to permit expansion and maturation to effector cells, (2) the effector T cells must be capable of reaching the site of the malignancy, and (3) lymphocytes at the tumor site must be able to appropriately execute effector functions to destroy cancer cells. Multiple mechanisms employed by tumors to hinder the immune response at each of these steps have been identified.

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Overcoming tumor-associated immune-suppressive mechanisms to induce potent antitumor immunity is the goal of cancer immunotherapy.

15.2 PRIMING OF TUMOR-SPECIFIC T CELLS

Impeded by Tumor Stroma

All solid tumors are composed of malignant cells that are embedded in a stroma composed of a mixed population of nonmalignant cells: bone marrow (BM)-derived macrophages, granulocytes, non-BM-derived endothelium, fibroblasts, and cells of the vasculature. Priming of tumor-specific T cells is impeded by this tumor stroma. Although still poorly understood and infrequently considered a target for immunotherapy, this nonantigenic stroma may represent an important immunologic barrier that prevents immune recognition and destruction of tumors. Early experimental evidence demonstrated that immunologic rejection is relatively easy to achieve during the first few days following tumor cell inoculation, but these same therapies become entirely powerless against established tumors entrenched in stroma [2]. Inoculations of cancer cells embedded in stroma are 10–100-fold more tumorigenic than cancer cells alone in suspension [3,4]. More recent experiments have analyzed spontaneously arising primary tumors that developed from single transformed precursor cells embedded in nonmalignant stroma. In this more physiological model, nascent primary solid tumors, even when highly antigenic in vitro, fail to stimulate the proliferation of antigen-specific T cells during the initial stages in vivo. Tumor-specific T cells are nonfunctional when they proliferated at a later stage, the reasons for which are not fully understood [5]. Tumor antigen-loaded stromal cells [6] or tumor cells themselves [3] usually transport antigen to the draining lymph nodes (DNLs), the site of immune initiation. With low levels of tumor antigen, delivery may be inefficient. Thus, cancer cells embedded in stroma may be ignored altogether [3] if antigenic molecules are not expressed at high levels by the tumor cells [6]. Other experiments also suggest that tumor stroma may act as a barrier to antigen presentation and immune recognition [7,8]. It is thus critical to penetrate and bypass the nonantigenic stroma to allow priming of tumor-specific T cells and start a strong antitumor immunity.

15.3 CURRENT STRATEGIES FOR CANCER IMMUNOTHERAPY

Active immunization and adoptive T cell transfer therapy are the main strategies used thus far for cancer immunotherapy. Both of these strategies are designed to overcome the deficiency in the priming of tumor antigen-specific T cells in the cancer-bearing hosts. Cancer vaccines rely on immunization of patients with antigenic peptides, proteins, or DNA expressed by the tumor directly or delivered by DC, virus, or another vehicle. Despite its relative simplicity and safety, vaccine

treatments have shown very limited success [9]. Although the generation in vivo of antitumor T cells in vaccinated patients could be demonstrated by techniques such as tetramer or ELISPOT assays [10–12], clinical responses observed from these trials were few [9]. This was consistent with the finding in murine models that the presence of even large numbers of antigen-specific T cells is insufficient to mediate tumor regression [8,13]. There are a multitude of explanations for this, including the relatively inadequate numbers or avidity of the immune cells, the inability of the tumor to recruit or activate quiescent or precursor lymphocytes, shortlived effector cells, tolerance mechanisms including the lack of costimulation, anergy, and active suppressive influences produced by the tumor or the immune system itself. These obstacles must be overcome if cancer vaccines are to be effective in mediating cancer regression. Adoptive transfer therapies, in which tumor-infiltrating lymphocytes (TILs) have to be isolated from the tumor, or T cells from patients' peripheral blood in some cases, to be expanded in a relatively antigen-specific way for adoptive transfer [14], have shown promise in a small number of highly selected melanoma patients [15]. However, the requirement for knowledge of the antigens and the potential inability to isolate or expand T cells against tumor likely limit their application to only a minority of cancer patients in at least the near future.

15.4 THE LESSON FROM AUTOIMMUNITY

The Role of Tertiary Lymphoid Structure

Although cancer cells express mutant or unique proteins that the immune system can recognize as foreign [16], malignant cells are surrounded by nonmalignant stroma to form a complex multicellular "organ" resembling self. The induction of immunity against normal, nonmutated differentiation antigens expressed by tumors resembles autoimmunity. Comparable to antitumor immunity, it is often observed in murine autoimmune models that the presence of autoreactive cells alone is not sufficient to cause tissue destruction [17]. It has been reported that the organized tertiary lymphoid structure (TLS) is necessary and sufficient to induce autoimmune destruction of pancreatic islets [18]. Indeed, de novo organization of TLS is known to precede the development of a number of human autoimmune diseases as well as in the animal models [19,20]. These observations suggest that lymphoid neogenesis within the target tissue may have a critical role in initiating and maintaining immune responses against persistent antigens. TLSs are not supplied by afferent lymph vessels and are not encapsulated, which implies that they are directly exposed to signals such as stimulating antigens and cytokines from the environment. This incomplete development of TLSs could potentially result in unrestricted access of dendritic cell (DC) lymphocytes to the TLS, favoring immune activation. Although disruption of established TLS or prevention of TLS formation may be advantageous in treating autoimmune diseases, initiation of intratumor TLS formation may facilitate the eradication of tumors. Considering the T cell repertoire may be less responsive against the self-antigens involved in autoimmunity than

against the unique antigens involved in tumor immunity, the destruction of cancers would be further facilitated by the availability of high-affinity T lymphocytes in the presence of TLS inside the tumor.

15.5 THE INTERNAL TUMOR ENVIRONMENT

Creating Lymphoid Tissue inside the Tumor to Improve Recognition

Clues to understanding the signals that lead to TLS formation come from the study of signaling pathways involved in secondary lymphoid tissue organogenesis. Studies in mutant mice and blocking experiments have identified a key requirement for TNF family members, mainly lymphotoxin $\alpha 1\beta 2$ ($LT\alpha 1\beta 2$), and to some extent TNF, in the development and organization of lymph nodes and spleen microarchitecture [21–24]. Binding of $LT\alpha 1\beta 2$ and TNF to their respective receptors, $LT\beta R$ and TNFR1, induce the expression of chemokines and adhesion molecules, which directly mediate lymphocyte migration and homing [25]. The first evidence that TLS could involve the same signaling pathways that regulate lymphoid organogenesis came from studies of transgenic mice [20], in which the ectopic expression of $LT\alpha$ and $LT\beta$ in pancreatic islets induced the formation of in situ TLS [26–29]. Extrapolating from this, stimulation of $LT\beta R$ or TNFR expressed by tumor stromal cells might promote the formation of lymphoid-like structure inside the tumor tissues for its destruction. Systemic TNFR signaling is, of course, too toxic, as evidenced by other systemic TNF treatments [30]. Soluble $LT\alpha$ can signal through the TNFR, resulting in the upregulation of chemokines. To avoid systemic toxic effects, recombinant $LT\alpha$ has been conjugated with tumor-specific antibody to be delivered specifically to the tumor tissue [31]. Targeting of recombinant $LT\alpha$ to the tumor elicits an efficient immune response associated with induction of peripheral lymphoid-like tissue containing L-selectin$^+$ naive T cells and MHC class II$^+$ antigen-presenting cells [31]. Secondary lymphoid tissue chemokin (SLC or CCL21) is among the chemokines controlled by $LT\beta R$ and TNFR signaling [25]. It is normally expressed in high endothelial venules and in T cell zones of spleen and lymph nodes and strongly attracts naive T cells and DCs [32]. The expression of SLC inside the tumor resulted in a substantial, sustained influx of T cells within the mass as well as retention of DCs at the tumor site. By recruitment of T cells and DCs, SLC in the tumor may lead to extranodal priming and inhibition of tumor growth [33].

Besides $LT\alpha 1\beta 2$, $LT\beta R$ is activated by another member of the TNF family, LIGHT [name derived from: homologous to *l*ymphotoxins, shows *i*nducible expression, and competes with herpes simplex virus *g*lycoprotein D for *h*erpesvirus entry mediator (HVEM), a receptor expressed by *T* lymphocytes] [34]. LIGHT is a ligand that signals through two receptors, $LT\beta R$ expressed on stromal cells and HVEM expressed on T cells [34,35]. LIGHT is expressed predominantly on lymphoid tissues, especially on the surface of activated DCs and T cells. Signaling via its receptor HVEM, LIGHT acts as a strong costimulatory molecule

for T cell activation [34,36,37]. Our data indicate that the interactions between LIGHT and LTβR restore lymphoid structures in the spleen of $LT\alpha^{-/-}$ mice [38]. In vivo data demonstrate that LIGHT, signaling through two receptors, mediates a microenvironment sufficient to break immunologic tolerance to self-antigens. First, ectopic expression of LIGHT in the pancreatic islets resulted in the formation of lymphoid-like structures and was necessary and sufficient for pancreatic islet destruction [18]. In addition, sustained expression of LIGHT on T cells leads to their activation, migration into peripheral tissues, and the establishment of lymphoid-like structures in situ [38,39]. LIGHT also acts as a strong costimulatory molecule for T cell activation, possibly by binding to the HVEM on T cells [36]. Therefore, LIGHT is an ideal candidate to be delivered inside the tumor to create TLS to recruit more T cells and subsequently expand antigen-specific ones inside the tumor. In addition to providing a cross-presentation pathway, tumor-reactive T cells can be activated via a direct-presentation pathway in the presence of antigens and costimulation. Indeed, LIGHT ectopically expressed in the tumor can effectively recruit and activate naive T cells. The expression of LIGHT in the tumor environment induces an infiltration of naive T lymphocytes that correlates with an upregulation of both chemokine production and expression of adhesion molecules inside the tumor [40]. Activation and expansion of these infiltrating T cells, likely via both cross- and direct-presentation pathways, leads to the rejection of established, highly progressive tumors at local and distal sites [40]. These experiments demonstrate that introduction of the lymphoid-like structure into the tumor stroma can be highly effective in enhancing antigen recognition and may be an effective strategy for cancer immunotherapy.

The ability to directly prime naive T cells within the tumor itself permits several advantages:

1. The efficiency and specificity of priming will be increased because of the higher tumor antigen load in situ relative to that collected in the draining lymphoid tissues [6].
2. A broader repertoire of tumor-specific naive T cells is recruited to the site of tumor antigens, leading to a more comprehensive response [31,41]. It has been shown that some tumor antigens may not be efficiently cross-presented because of the antigen bias in T cell cross-priming [42]. In these instances, we have demonstrated in our experimental system using the tumor cell line $Ag104L^{d}$-LIGHT that certain antigens are presented to and activate naive T cells within the tumor via a direct-presentation mechanism [42]. The same tumor would have been otherwise missed by the host immune response had it relied on solely draining lymphoid tissues and cross-presentation.
3. No additional migration steps are required for CTL to reach the site of effector function, which leads to the appearance of activated tumor-reactive T cells in a short period of time.
4. T cell responses may react more readily to the shifting tumor antigen expression profile in situ.

T cell stimulation in the absence of costimulation can induce anergy and apoptosis of antigen-specific T cells [43–45]. Costimulation has also been shown to greatly enhance tumor-specific T cell function during the effector phase [46]. Earlier studies show that LIGHT provides CD28-independent costimulation [36], which may be essential for the selective and effective activation, expansion, and maintenance of tumor-specific T cells among infiltrating naive T cells in $Ag104L^{d}$-LIGHT tumors. It appears that the introduction of lymphoid-like milieus, such as the combination of chemokines, adhesion molecules, and costimulatory property, inside tumor tissues may allow LIGHT to become a potent molecule for better immune response against established tumor.

The Immunosuppressive Environment inside Tumors

Even with immune recognition of cancer, which can occur in cancer patients and is evidenced by the frequent observation of T cell infiltration into cancerous tissues [47–50], it is rare for such tumor-infiltrating T cells to induce the spontaneous rejection of established tumors. Accumulating evidence indicates that the tumor environment contains cells and cytokines that actively suppress primed effector T cells [51,52]. High concentrations of transforming growth factor β1 (TGFβ1) are frequently found in solid tumors and production of TGFβ1 by cancer cells or tumor stromal cells interferes with effective immune rejection of transplanted solid tumor fragments [53]. TGFβ1 is a cytokine essential for the generation and survival of $CD4^{+}CD25^{+}$ regulatory T cells [54,55], which may themselves produce TGFβ1 and IL-10 to reinforce the immune suppressive loop that exists in the tumor environment. While active TGFβ1 does not inhibit lysis by CTL, it can inhibit maturation of T cells to that effector state [56]. This explains, at least in part, why $CD8^{+}$ T cells resistant to TGFβ1 can mediate tumor rejection [57]. Highly antigenic tumors may yet fail to regress in the host owing to an accumulation of regulatory T cells (Tregs) within the tumor microenvironment [58]. These $CD4^{+}CD25^{+}$ Tregs seem not only to inhibit developing tumor-specific $CD8^{+}$ T cells from gaining full effector function, but also can even block transferred, fully activated tumor-specific T cells from mediating tumor rejection in vivo [59]. There is little question that tumors can develop, establish, and persist while retaining their full antigenicity in normal hosts [5]. Therefore, neutralizing anti-TGFβ1, anti-CD4, or anti-IL-10 antibodies injected into established solid tumors may be effective in counteracting the immunosuppressive environment that prevents tumor-specific T cells from acquiring full effector function [58]. We have demonstrated that the local intratumoral depletion of these Tregs changes the cytokine milieu of the tumor, unmasks the immunogenicity of tumor, and reverses CTL tolerization, leading to the rapid rejection of well-established tumors [58]. Our data support the idea that Tregs inhibit not only the early priming events but also the effector function of T cells inside tumors, which has been shown by a more recent study [59].

Established progressive tumors cause splenomegaly and increased $Gr1^{+}$ $CD11b^{+}$ immature myeloid cells [60,61]. These cells can promote angiogenesis [62] and stimulate growth of cancer cells in vitro [63]; they also have powerful

immunosuppressive effects in vitro. Splenic $Gr1^+$ immature myeloid cells differentiate into suppressive tumor-associated macrophages (TAMs) [64,65]. Treatment of mice with anti-Gr1 suppresses tumor growth in T-cell-deficient mice [66] and allows normal mice to reject otherwise lethal tumor challenge [67]. This treatment may reduce $Gr1^+$ splenic precursors of TAM, abolish a neutrophil-dependent macrophage recruitment mechanism in the tumor by eliminating $Gr1^+$ granulocytes [68], and/or eliminate $Gr1^+$ tumor growth-promoting cells within the tumor microenvironment. Again, it is not clear whether myeloid suppressor cells or TAM would interfere with tumor rejection In vivo when fully activated tumor-specific T cells are transferred.

Changing the Tumor Environment

In addition to stromal cells and T cells, LIGHT also interacts with other cells, such as natural killer (NK) cells and dendritic cells (DCs) [69,70]. LIGHT can activate NK cells via HVEM, which produce substantial amounts of IFNγ, leading to further activation of effector $CD8^+$ T cells inside the tumor [69]. LIGHT also stimulates DCs and promotes their expansion and/or survival [70]. This may lead to more efficient priming of T cells. In addition to costimulation of T cells, LIGHT alters the phenotype of macrophages to one producing an abundance of IFNγ, possibly through an indirect pathway (unpublished data). The expression of LIGHT inside the tumor switches the environment to a proinflammatory one [40], which seems to facilitate further accumulation of effector T cells and tumor rejection. These results are consistent with more recent studies using CpG oligodeoxynucleotides [71], which suggest that the induction of a proinflammatory microenvironment in autochthonous tumors enhances extravasation of effector cells and cancer destruction. It remains to be determined whether and how a LIGHT-conditioned microenvironment can also prevent in situ activated T cells from succumbing to tumor-mediated immunosuppression.

Generation of CTL in the LIGHT-Mediated Tumor Environment for Treatment of Metastases

Micrometastases can establish early in heterogeneous primary tumor development and seed distal sites prior to clinical detection [72]. Therefore, at the time of diagnosis many cancer patients already have microscopic metastases, an observation that has led to the development of postsurgical adjuvant therapy for patients with solid tumors. Despite advances in early detection and modifications to treatment regimens, success has been limited and optimal treatment of metastatic disease continues to pose a major challenge in cancer therapy.

We have shown that expression of LIGHT in the tumor can lead to rejection not only at a local site but also at the distal sites. To develop more clinical relevant approaches, adenovirus vectors that express LIGHT (Ad-LIGHT) have been constructed to deliver LIGHT into the tumor tissue. The advantages of adenovirus are (1) high production of nonreplicable virus, (2) easy manufacture, (3) activation of

innate immunity, (4) an ability to express its carrying gene in undividing cells, and (5) ready expression in most tumor cell lines. Administration of Ad-LIGHT into the tumor tissue leads to the complete rejection of an aggressive fibrosacoma $Ag104L^d$ and retards the growth of other tumors, such as melanoma B16, colon cancer MC38, and breast cancer 4 T1 in mice [76]. The poorly immunogenic 4 T1 mammary carcinoma closely mimics human breast cancer in its anatomic site, immunogenicity, growth characteristics, and more importantly, metastatic properties [73–75]. We have demonstrated in this model that generating immune responses in primary tumor tissues prior to surgical resection can produce tumor-specific effector T cells sufficient to eradicate distant metastases in a CD8-dependent fashion. Local treatment with Ad-LIGHT initiated priming of tumor-specific $CD8^+$ T cells directly in the primary tumor, with subsequent exit of CTL, which homed to distal tumors to elicit immune-mediated eradication of spontaneous metastases [76].

Conventional treatment such as surgical removal of tumor followed by radiation and chemotherapy may prevent effective immune recognition of cancers due to the loss of a major source of antigens, and damage to preexisting CTL by radiation and chemotherapy. An alternative strategy would be to utilize the primary tumor as the site of CTL priming prior to surgical resection. Delivery of LIGHT expression within the tumor environment recruits naive T cells and generates tumor-specific CTLs that can survive and exit the microenvironment to patrol peripheral tissues and eradicate disseminated metastases (see Fig. 15.1 for model).

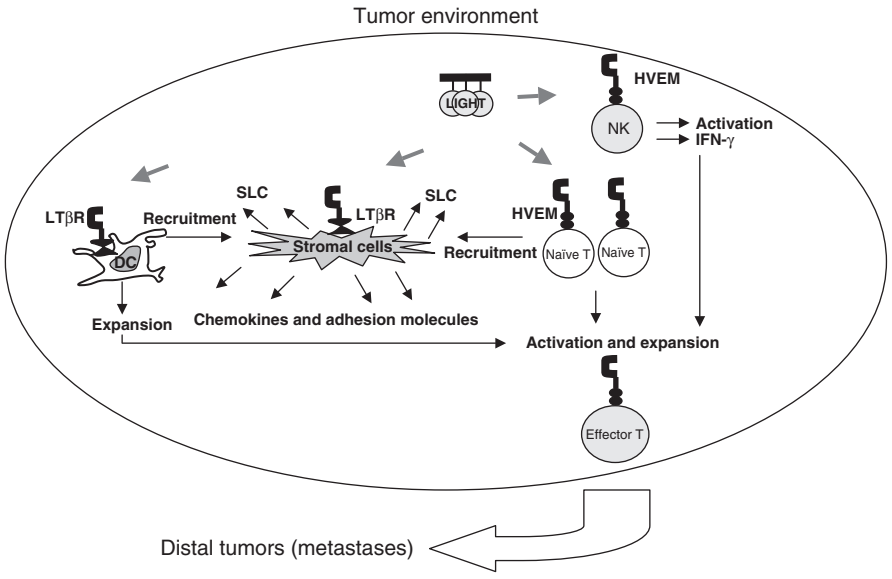

Figure 15.1. Tumor environment.

15.6 CONCLUSIONS

Understanding the balance of antitumor effectors versus suppressors may be important in determining the outcome of immune responses inside tumors. Our more recent studies have demonstrated that creation of lymph-like structures within the tumor leads to rapid recruitment of naive lymphocytes and expansion of $CD8^+$ T cells. This may be a means of generating a predominantly proinflammatory environment, resulting in the rejection of both primary tumor and metastases [40] (Fig. 15.1). We further demonstrated that the depletion of regulatory T cells inside the tumor is an efficient means of converting the antiinflammatory environment inside tumor to a proinflammatory one [58]. The ideal combination treatment would more rapidly expand the effector cells at the tumor site, while blocking local suppressive factors, including depletion of regulatory cells. This could prove a potent strategy for enhancing antitumor immunity and permitting a clinically desirable outcome for cancer patients. Therefore, by overcoming the suppressive mechanisms in the cancer microenvironment, tumor tissue might be used to generate a stronger immunity against itself. It is likely that such immunotherapy, followed by surgical removal of primary tumor or local radiation, which increases antigen load to improve cross-priming of T cells, allows CTL, previously generated inside tumor to eradicate residual or undetected metastatic tumor cells. How to design immunotherapy and avoid immune suppression induced by chemotherapy and radiation might be the key to reduce metastases in patients.

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16

CANCER IMMUNOTHERAPY: UNTAPPING THE POTENTIAL OF COSTIMULATORY MOLECULES BEYOND CTLA-4

Mathew Augustine and Lieping Chen

16.1 INTRODUCTION

Over 200 years have passed since Edward Jenner ushered the world into the field of immunology and immunoprophylaxis by treating healthy individuals with cowpox and observing the protection that his strategy of vaccination provided against the fatal human disease smallpox. Later, William Coley reported his cogent observation of tumor regression in patients harboring microbial organisms within malignant tissue. Coley's observation, along with Paul Ehrlich's theory on the role of the immune system in controlling tumor growth, ignited a contentious debate that has raged since the early 1900s on the nature of tumor cell "foreignness" and the immune system's capacity to recognize and eradicate neoplastic tissue. Naturally, out of these ideas evolved the concept of vaccination as an immunotherapeutic strategy to eradicate cancer. Studies performed in the mid-1950s revealed for the first time that the immune system recognizes tumor-associated antigens and indicated that the immune system could, on some level, sense tumor as nonself [1].

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More recent experiments have not only validated Ehrlich's hypothesis, but have implicated all levels and specific components of the immune system (both innate and adaptive) in influencing antitumor immune responses [2]. However, clinically detectable cancers have invariably either escaped tumor recognition altogether or devised mechanisms to outwit their opponent [3], ultimately rendering the immune system impotent in tumor control.

In T cell signaling, at least two signals are considered integral in defining the fate of naive T cells and their competence to combat disease. Major histocompatibility complex (MHC)–peptide interactions with T cell receptor (TCR) provide the first signal in immune responses. However, while the MHC–peptide interaction with TCR is essential in determining specificity and initiating T cell activation, this process is not sufficient to determine the fate of T cells [4]. Costimulatory and coinhibitory molecules, which are cell surface glycoprotein molecules, supply additional signal or "second signal" to T cells through antigen-presenting cells (APCs), providing context to the state of activation or inactivation that develops within the T cell [5]. The second signal delivered is most likely a product of both costimulatory and coinhibitory influences. Positive signals result in T cell proliferation, expansion, and survival; specific cytokine release; and cytotoxic effector responses. Negative signaling results in T cell death, reduced memory responses, suppressor T cell induction, and immunosuppressive cytokine secretion. An example of a signaling system with both positive and negative output is the B7/CD28/CTLA4 pathway, the most thoroughly studied cosignaling pathway [6,7]. CD28 ligation on T cells results in costimulatory signals delivered by B7-1 (CD80) and B7-2 (CD86). CTLA4 provides coinhibitory signals also through interactions with B7-1 and B7-2. CTLA4-deficient mice exhibit severe lympoproliferative disease and autoimmunity [8,9]. Considering the range of immune responses that can be elicited by these molecules, it is not surprising that tumor cells have enlisted costimulatory/coinhibitory molecule to help in their armamentarium to render them poorly immunogenic or nonimmunogenic.

The influence of costimulatory/coinhibitory molecules on the state of T cell activation and their expression on cancer cells has made these molecules attractive agents for immunotherapy. While anti-CTLA4 antibody therapy [10] is the first in this class of tumor immunotherapy to reach clinical trials and exhibit efficacy, its association with the development of grade 3/4 autoimmunity supports investigation into the therapeutic potential of other costimulatory/coinhibitory molecules, as they may grade the potency of the immune response on a different contextual scale. The molecules that are currently being brought to phase I clinical trials for therapeutic applicability are members of the PD-1/B7-H1/B7-DC pathway and the CD137/CD137L pathway. These molecules are discussed in this chapter, detailing the fundamental studies that have illuminated their potent influence on the state of immune tolerance and activation, with a focus on manipulation of this pathway as an immunotherapeutic strategy toward tumor clearance.

16.2 PD-1/ B7-H1/ B7-DC PATHWAY

Programmed death 1 (PD-1), a member of the immunoglobulin gene superfamily, was initially identified by subtraction hybridization in an attempt to discover genes expressed during classical cell death progression in two cell types, a murine T cell hybridoma (2B4.11) stimulated with PMA and ionomycin, and interleukin 3 (IL-3)-deprived LyD9, a lymphoid/myeloid progenitor cell line [11]. Initially, PD-1 mRNA expression was identified in the thymus and was increased with enhancement of in vivo thymic apoptosis by anti-CD3 antibody. Despite the apparent connection between cell death and expression of PD-1, clear evidence to support the role of PD-1 in apoptosis was unavailable, as LyD9 cells transfected with PD-1 mRNA showed neither increased apoptosis nor defects in proliferation compared to the parental line. Instead, subsequent studies provided evidence that activation of the Jurkat T cell leukemia line as well as peripheral blood mononuclear cells (PBMCs) resulted in the upregulation of human PD-1, and that this expression did not correlate with apoptosis [12]. PD-1 expression was also identified on thymic, splenic, and lymph node T cells after either anti-CD3 or concanavalin A (ConA) treatment as well as on B cells after in vitro stimulation with anti-IgM antibody [13]. Similar to earlier studies, cell deprivation did not result in increased PD-1 expression. These results offer further evidence against a role for PD-1 in apoptosis and supported a possible link between activation of T and B lymphocytes and PD-1 expression.

The genes PD-1, CD28, CTLA4, and ICOS, are member of the same costimulatory family and share approximately 20% sequence identity. The mature form of murine PD-1 consists of 268 amino acids and is a type I transmembrane glycoprotein exhibiting 60% sequence identity to its human counterpart [14]. PD-1, in contrast to CD28, CTLA4, and ICOS, does not contain interchain disulfides known to mediate covalent dimerization and was found to be a stable monomer both in solution and on the cell surface [15]. Notably, PD-1 contains two tyrosine residues in its cytoplasmic domain. One is an immunoreceptor tyrosine–based inhibiting motif (ITIM) that is generally associated with inhibiting immune responses [16]. The second has been identified as an immunoreceptor tyrosine-based switch motif (ITSM) and is thought to be the actual domain through which inhibitory signals are relayed by PD-1 ligation [17]. The inhibitory activity was initially proposed to result from association of SHP-2, a cytoplasmic tyrosine phosphatase, with the ITIM [18]. Further experiments, however, demonstrate that the ITSM, and not the ITIM, mediates the inhibitory effects of PD-1 as selective mutations of the ITSM resulted in the inability of PD-1 to abrogate T cell activation [19].

The PD-1 gene is known to associate with two ligands, B7-H1 (designated as PD-L1) and B7-DC (also designated as PD-L2). B7-H1, a member of the immunoglobulin superfamily, is a type I transmembrane protein consisting of 290 amino acids and was identified through a homology search of a human cDNA

expressed sequence tag (EST) database using the sequences for B7-1 and B7-2 [20]. The mRNA transcript for B7-H1 was identified in the majority of tissues including heart, skeletal muscle, placenta, lung, thymus, spleen, kidney, and liver tissues. In contrast to this broad distribution of mRNA, antibody against the B7-H1 protein revealed its constitutive expression on limited tissues/cells and the expression is associated largely with tissue macrophages. However, surface expression of B7-H1 could be detected in virtually all the cells tested so far on induction with IFNγ [21,22]. B7-DC, a 247-amino-acid member of the immunoglobulin superfamily, was identified as a second ligand that binds PD-1 [23,24]. It was initially discovered by its differential expression between dendritic cells (DCs) and macrophages. However, expression of B7-DC on macrophages was subsequently reported after incubation with GM-CSF, IL-4, and IFNγ [25].

With accumulating evidence confirming the expression of PD-1 on activated T and B cells, and the constitution of its cytoplasmic domain with both an ITIM and ITSM, knockout studies were performed to identify potential phenotypes indicative of immune dysregulation. PD-1-deficient mice were produced in two different backgrounds and presented with distinct forms of autoimmunity. PD-1-deficient C57BL/6 (B6) mice spontaneously developed lupus-like glomerulonephritis and progressive arthritic lesions along with deposition of IgG3 and C3 complement protein within the renal glomeruli. This phenotype presented earlier and with greater severity when the Fas gene mutant, lpr, was introduced [26]. Noticeably, PD-1 gene disruption in Balb/c mice resulted in the development of severe dilated cardiomyopathy with subsequent premature death as a result of congestive heart failure [27]. This phenotype did not develop in PD-1, recombinase activating gene (RAG) double-knockout mice, indicating that the effect was mediated through T and B cells. The sera of the affected mice contained high titers of autoantibody against a heart-specific protein, which was subsequently identified as cardiac troponin I [28]. Further supporting an association between autoimmunity and PD-1, human genetic studies showed an association between the presence of type 1 diabetes and systemic lupus erythematosus with single-nucleotide polymorphisms (SNPs) in the intronic region of the PD-1 gene [29,30]. Therefore, these findings support a general role for signaling through PD-1 via PD-1 ligands in controlling T and B cell responses, with disruption of this pathway resulting in distinct autoimmune outcomes in different mouse strains. The role of PD-1 in moderating autoimmune responses was further exemplified by utilizing anti-PD-1, anti-B7-H1, and anti-B7-DC antibodies in a murine model of experimental autoimmune encephalitis (EAE) [31]. Blockade of PD-1 accelerated the onset of disease, induced greater EAE severity score, increased anti-MOG antibodies, and increased IFNγ-secreting T cells. Surprisingly, while expression of both PD-1 and B7-H1 (but not B7-DC) on spinal cord sections of mice with EAE was exhibited, blockade of B7-DC, but not B7-H1, resulted in more severe disease.

While the aforementioned studies established a mediating role for PD-1 in inhibiting autoimmune pathogenesis, the mechanism by which the PD-1 ligands signal through PD-1 has been less clear. Molecular modeling of B7-H1 and B7-DC indicates significant structural heterogeneity at the interface where the two

molecules interact with PD-1, supporting unique binding features of the two ligands [32]. In addition, ligand mutants could still perform costimulatory functions with increased cytokine production in vitro, supporting previous experiments indicating a stimulatory role for B7-H1 binding. Interestingly, these studies also suggest the possible existence of an unknown second receptor that delivers stimulatory signals distinct from PD-1.

Early studies on the function of B7-H1 provided conflicting results supporting both costimulatory and coinhibitory properties. B7-H1 was shown to promote in vitro proliferation of T cells as well as production of IL-10, IFNγ, and GM-CSF in the presence of suboptimal concentrations of anti-CD3 through the use of a B7-H1:Ig fusion protein construct. Both processes were shown to require IL-2. In addition, B7-H1 was shown to augment T cell proliferation in the absence of CD28, but was unable to generate cytolytic T cell responses after transfection of the P815 tumor line [33]. Supporting a negative role, B7-H1 was identified on monocytes and dendritic cells and was capable of inhibiting proliferation of T cells through PD-1 as well as the cytokine production of IFNγ and IL-10 [34]. B7-H1 was also shown to inhibit proliferation and IL-2 production with reversal in the presence of soluble agonistic anti-CD28 antibody [35]. However, over prolonged activation, B7-H1:PD-1 inhibition predominates by decreasing IL-2 production through $CD4^+$ T cells, which partially influences $CD8^+$ T cells because of their intrinsic inability to secrete IL-2 on activation. Furthermore, a study utilizing a blocking antibody against B7-H1 on DCs could reverse anergized T cells [36]. These latter results support a model in which B7-H1 modulates the immune response by tuning down activation and proliferation of T cells, rendering them unresponsive.

Much like B7-H1, B7-DC has exhibited a role in both positive and negative signaling. B7-DC was reported to costimulate T cell proliferation and increase IFNγ production from naive T cells [24]. Crosslinking B7-DC with antibody resulted in increased MHC:peptide presentation, IL-12 production, promoted DC survival, improved DC migration to draining lymph nodes, and induced antigen-specific T cell immune responses [37,38]. A subsequent study reported a role for B7-DC in inhibition of T cell proliferation, $CD4^+$ T cell cytokine production, and B7/CD28 signaling by the PD-1:B7-DC pathway [23]. A B7-DC knockout mouse was generated and provided both in vivo and in vitro evidence of a role for B7-DC in tuning Th1 responses as knockout mice displayed reduced $CD4^+$ T cell IFNγ production and IFNγ-dependent IgG2a humoral responses [39]. $CD8^+$ T cell responses and CTL activity were also reduced. Related to this finding was evidence of accelerated hepatic tumor growth in the knockout mice compared to wild-type mice. These results indicate an important role for B7-DC in fine-tuning the adaptive immune response. In support of an inhibitory role, monoclonal antibodies against B7-DC on dendritic cells resulted in increased T cell proliferation and increased cytokine production of IFNγ and IL-10 [40]. Recently, a second study utilizing a B7-DC knockout mouse was described [41]. $CD4^+$ T cells, in the presence of knockout APCs, were more sensitive to anti-CD3 antibody stimulation, even in the absence of CD28, and produced higher IFNγ and IL-4 levels. $CD8^+$ T cells also displayed

higher proliferation and increased IFNγ and TNFα secretion. Spleen cells recovered from mice immunized with ovalbumin and CFA and stimulated in vitro with peptide resulted in enhanced proliferation and increased Th1 and Th2 cytokine production. Furthermore, B7-DC knockout mice failed to induce oral tolerance. These results support a role for B7-DC in negative regulation of T cells and maintenance of oral tolerance.

Adding to the complex relationship between peripheral B7-H1 expression and tolerance or activation, allografts of pancreatic islet β cells from transgenic mice bearing B7-H1 exhibited accelerated rejection [42]. The transgenic mice showed evidence of elevated glucose levels and periinsulitis that was not seen within the wild-type control group. The presence of B7-H1 increased $CD8^+$ T cell proliferation and transfer of antigen-specific T cells with concomitant administration of B7-H1:Ig fusion protein resulted in increased cell division. B7-H1 was also found to play an important role in regulating a murine form of colitis [43]. Colonic samples taken from SCID mice transferred with $CD4^+$ CD45RBhi T cells were found to express high levels of PD-1, B7-H1, and B7-DC. When given anti-B7-H1 antibody, these same mice showed no evidence of overt experimental colitis and had reduced IL-2 and Th1 cytokine production (IFNγ and TNFα). These results provide further support toward the potential involvement of a second receptor for B7-H1-positive signaling. The possibility of "reverse signaling" on T cells through B7-H1 was also described, as rheumatoid arthritis patients possess autoantibodies to B7-H1, and the presence of this autoantibody correlates with active status of disease [44]. On activation, human $CD4^+$ T cells were shown to augment the expression of B7-H1. Antibody to B7-H1 was shown to enhance the proliferation of $CD4^+$ T cells in vitro in the presence of suboptimal concentrations of anti-CD3 antibody, although the effect was less than that produced by anti-CD28 antibody. These studies support a role for B7-H1 in both forward and reverse signaling involving T cell activation and autoimmune pathogenesis, although the number and nature of target receptors are undefined.

Despite evidence suggesting a stimulatory role for B7-H1 in both forward and reverse signaling, data have accumulated supporting a predominant role for this molecule in mediating inhibitory immune signals and peripheral tolerance through binding to PD-1. Female NOD mice administered anti-PD-1 or anti-B7-H1 antibodies exhibited rapid development of diabetes with worse insulitis scores [45]. B7-H1 was found expressed on the islets of NOD mice that developed diabetes. The same effect could not be elicited with anti-B7-DC antibodies. Interestingly, mice given anti-CTLA4 antibodies developed disease only when the antibody was given as a neonate, while anti-PD-1 and anti-B7-H1 antibodies could induce disease regardless of age, suggesting that CTLA4 mediates its effects at an early stage of development, while PD-1:B7-H1 influences progression of disease at all developmental stages. This may be related to the presence of B7-H1 within the peripheral tissue, where MHC class I is expressed, supporting an important role for this molecule in maintaining peripheral tolerance to autoreactive $CD8^+$ T cells.

In a recent set of experiments, B7-H1/B7-DC (PD-L) double-knockout mice were crossed onto the NOD mouse and exhibited clear evidence of an earlier onset

of autoimmune diabetes with complete penetrance [46]. The transfer of splenocytes from prediabetic WT NOD mice into WT or PD-L-deficient NOD SCID mice resulted in earlier autoimmune diabetic progression in the PD-L knockout mice, indicating that the presence of these molecules on nonlymphoid cells suppresses autoimmune progression. Furthermore, the presence of PD-L on hematopoietic cells was not sufficient to prevent diabetes. Finally, B7-H1 and not B7-DC was shown to influence autoimmune progression. These studies in combination suggest that B7-H1 expression on pancreatic β-islet cells is important in delivering inhibitory signals that maintain peripheral tolerance and prevent autoimmunity.

To show that B7-H1 exhibits an important negative regulatory role in vivo, mice lacking B7-H1 were generated [47]. B7-H1 knockout $CD4^+$ and $CD8^+$ T cells, on stimulation, produced elevated levels of IFNγ compared to WT controls. For $CD4^+$ T cells, this occurred at suboptimal anti-CD3 antibody concentrations, while for $CD8^+$ T cells, this occurred at higher anti-CD3 concentrations. This effect could be delivered by APCs as B7-H1-deficient dendritic cells showed augmentation of IFNγ secretion by $CD4^+$ T cells. B7-H1 knockout mice developed worse EAE and increased IL-2 and IFNγ. Human endothelial cells exposed to IFNγ could also be induced to express B7-H1 [21,22]. Blocking the B7-H1:PD-1 interaction enhanced IL-2 and IFNγ expression. These results support a negative role for B7-H1 on both DC and endothelial cells in T cell activation and effector function, thereby supporting its role in the induction of tolerogenic states.

The augmented expression of PD-1 on activated T cells suggests an important role for PD-1 during a distinct phase of the T cell response. In addition, the relationship between PD-1 and autoimmune disease implicates PD-1 and its ligands in mechanisms related to peripheral tolerance. Peripheral tolerance is a critical aspect of normal liver immunity exemplified by the fact that allogeneic liver transplantation can occur without the utilization of immunosuppressive regimens as well as antigen-specific tolerance induction by antigen fed through the portal vein. With consideration toward these associations, B7-H1 was identified on nonparenchymal cells within the liver, namely, sinusoidal endothelial cells and Kupffer cells [48]. Coculture of PD-1-deficient T cells with liver nonparenchymal cells augmented their proliferative responses and had prolonged IFNγ production compared to wild-type T cells. The use of an anti-B7-H1 mAb in this same system resulted in increased proliferation comparable to that of PD-1-deficient T cells. These results were consistent whether Kupffer cells or liver sinusoidal endothelial cells were used. Moreover, PD-1-deficient mice infected with adenovirus exhibited higher numbers of proliferating $CD4^+$ and $CD8^+$ T cells in the liver as compared to WT mice. The inflammation was more severe over a shorter timecourse, resulting in rapid clearance of the virus from the liver. These results support an important role for the PD-1:B7-H1 pathway in augmenting hepatic immune tolerance. Subsequently, B7-H1-deficient mice were generated and shown to have increased selective accumulation of antigen-specific $CD8^+$ T cells in the liver [49]. Apoptosis, not enhanced cell proliferation, was identified as the primary cause of this accumulation. Furthermore, experimental autoimmune hepatitis caused by elevated $CD8^+$ T cells could be rapidly induced in these mice. T cell responses could also

be inhibited by activated hepatic stellate cells on B7-H1 induction [50]. These stellate cells induced a state of hyporesponsiveness in allogenic or anti-CD3-induced T cell activation, attributed to enhanced apoptosis and not decreased cytokine production. These results provide further evidence of a critical role for B7-H1/PD-L1 on various nonparenchymal cells within the liver in deleting activated intrahepatic $CD8^+$ T cells and furthering its claim as a mediator of peripheral tolerance.

Previously, chronic infection with LCMV was shown to result in the development of specific "exhausted T cells" with reduced cytokine production, proliferation, and capacity to lyse target cells [51,52]. In an attempt to identify genes in "exhausted T cells" that might be preferentially upregulated during chronic viral infection, PD-1 was culled from a genomewide microarray analysis [53]. Fluorescence-activated cell sorter (FACS) analysis confirmed these results as chronic LCMV infection resulted in antigen-specific, PD-1^+, $CD8^+$ T cells. Persistently infected mice that received anti-B7-H1 blocking antibody exhibited higher antigen-specific $CD8^+$ T cells expressing IFNγ and TNFα on peptide stimulation and cleared virus more effectively from multiple anatomic locations than did control mice. Removal of $CD4^+$ T cells, rendering "exhausted" $CD8^+$ T cells "helpless," did not alter this outcome as these mice displayed higher antigen-specific $CD8^+$ T cells in multiple compartments, increased proliferation, contained more IFNγ-producing cells, and functionally exhibited more effective specific lysis and viral clearance. Importantly, these "exhausted" cells persisted for several weeks after blockade. This result was due to reactivation of PD-1^+ cells on blocking the B7-H1 pathway rather than generation of new responses from naive T cells. Three papers extended these results by examining the role of the PD-1 pathway in HIV infection [54–56]. Utilizing MHC class I tetramers associated with common HIV epitopes, PD-1 expression, and not CTLA4, was found to be highly expressed on $CD8^+$ T cells from chronically infected HIV patients in comparison to seronegative controls. An inverse correlation was observed between the percentage of proliferating cells and expression of PD-1. Moreover, PD-1 expression correlated with viral load and inversely correlated with $CD4^+$ T cell count, indicating an association between PD-1 expression, antigenic load, and disease progression. The use of antiretroviral treatment coincided with a decrease in PD-1 expression on $CD8^+$ T cells. Inhibition of the PD-1:B7-H1 pathway with blocking antibody resulted in the expansion of tetramer-positive cells with increased IFNγ, TNFα, and lymphotoxin-α expression. PD-1 expression was once again associated with influencing apoptosis. These intriguing results further illuminate a critical role for PD-1:B7-H1 interactions in influencing T cell function and the role that directed therapy against this pathway might play in the rejuvenation of "exhausted" $CD8^+$ T cells in persistently infected hosts with chronic viral infection or malignant disease. The administration of antiretroviral therapy suggests an important relationship between antigen level and PD-1 expression. Most tantalizing is the potent response that can be rescued in mice lacking $CD4^+$ T cell help as these results have potential applicability in disease states such as HIV infection, where unresponsiveness or progressive depletion of $CD4^+$ T cells occurs.

Considering the influence of the PD-1, B7-H1, and B7-DC pathways in modulating immune responses, cancer cells were examined to identify both expression and functional relevance of B7-H1 and B7-DC on the tumor cell surface. As for B7-DC, one report indicated that primary mediastinal B cell lymphoma could be discriminated from diffuse large B cell lymphoma by its expression. B7-DC was also identified on Hodgkin's lymphoma cells [57]. While most normal solid tissues lack B7-H1 expression, FACS analysis of tumor cell lines, including lung and ovary, showed surface expression of this molecule [58]. On treatment with IFNγ, lung, colon, ovary, duodenum, leukemia, lymphoma, placenta, and melanoma cancer cell lines also upregulated expression of B7-H1. A $CD8^+$ T cell clone generated against a melanoma cell line transfected with B7-H1 resulted in a 62% increase in apoptosis compared to controls. Antibody against B7-H1 or the delivery of PD-1:Ig fusion protein inhibited apoptosis and increased the number of viable T cells. A similar result was also found using a breast cancer cell line that constitutively expresses B7-H1. Interestingly, these CTLs lack PD-1 expression, and this finding further supports the possibility of a non-PD-1 receptor mediating this apoptotic effect. Furthermore, in vivo delivery of antigen-specific $CD8^+$ T cells into $RAG^{-/-}$ mice with B7-H1-transfected P815 cells was compared to transfer of $CD8^+$ T cells into $RAG^{-/-}$ mice with mock-transfected P815 cells. T cells from the mice that harbored B7-H1-transfected P815 tumor cells showed decreased numbers and underwent apoptosis while blockade of B7-H1 resulted in inhibition of growth of the B7-H1-transfected P815 cells. These results indicate that B7-H1 functions in inducing apoptosis of activated T cells as a mechanism for immune evasion. This study was supported by a subsequent report linking B7-H1 to tumor immune evasion by using a similar model system. P815 cells transfected with B7-H1 were more resistant to $CD8^+$ T cell killing than were nontransfected cells, with abrogation of this effect on exposure to anti-B7-H1 antibody [59]. Subcutaneous injection of tumor cells expressing B7-H1 into syngeneic hosts exhibited progressive local tumor growth and systemic tumor invasion, and succumbed to malignancy earlier compared to mice inoculated with nontransfected P815 cells. These events were partially reversed in mice receiving anti-B7-H1 antibody. Furthermore, inoculation of tumor cells expressing B7-H1 into PD-1 knockout mice in comparison to PD-1^+ mice resulted in tumor clearance in the knockout mice, suggesting that signals delivered through the B7-H1:PD-1 pathway confer on tumor cells a mechanism to evade immune responses and that blocking this pathway could be a rational immunotherapeutic strategy.

In a study of human glioma cell lines, cell surface expression of B7-H1, but not B7-1 and B7-2, was identified and increased in response to IFNγ exposure [60]. Coculture of glioma cells with $CD4^+$ or $CD8^+$ T cells and anti-B7-H1 antibody resulted in increased IFNγ and IL-2 cytokine secretion as well as increased expression of the T cell activation marker CD69. Similarly, blockade by anti-B7-H1 antibody of a B7-H1-transfected squamous cell carcinoma cell line enhanced adoptive T cell immunotherapy and improved survival when tumor cells were injected in vivo [61]. These studies provide strong supportive evidence of the role of B7-H1 in

the induction and maintenance of immune tolerant states, and more importantly, the utilization of this molecule by tumor cells to evade antitumor immune responses.

The pervasiveness of B7-H1 expression within the tumor microenvironment was illustrated by the expression of B7-H1 on monocyte-derived myeloid dendritic cells (MDCs) within draining lymph nodes from ovarian cancer as well as within the tumor itself [62]. MDCs, exposed to tumor macrophages and IL-10, which upregulate B7-H1, exhibited impaired capacity to stimulate T cell proliferation. Similar to other studies, the use of antibody against B7-H1 resulted in increased T cell proliferation and increased IFNγ and IL-2 production with decreased IL-10 production. Tumor-bearing mice, transferred with MDC-activated T cells in the presence of anti-B7-H1 antibody, showed impaired tumor growth over a defined time period as compared to untreated mice. Increased IFNγ-expressing T cells were recovered from the tumor treated with anti-B7-H1 antibody; however, the numbers of $CD8^+$ T cells and apoptotic T cells were equivalent, suggesting that the effect on tumor control regulated by B7-H1 blockade in this study was related to a qualitative shift in IFNγ-expressing T cells.

With the reports of B7-DC expression limited to a small subset of lymphomas, more recent experiments have shed light on the potential role for B7-DC modulation in immunotherapy. As was previously mentioned, a crosslinking antibody against B7-DC was shown to induce IL-12 production and enhance T cell responses [37,38]. Concomitant transfer of this anti-B7-DC antibody with transfer of a poorly immunogenic melanoma cell line to mice rendered a significant number of these mice tumor-free [63]. Furthermore, this effect appeared to be mediated through dendritic cells exhibiting enhanced pinocytotic function without increased expression of B7-1 or B7-2 both in vitro and in vivo. Both $CD4^+$ and $CD8^+$ T cells, but not NK cells, were necessary for the antitumor effect seen by crosslinking B7-DC in vivo and were resistant to secondary challenges of tumor cells. This response was shown to be tumor-specific as secondary challenge with unrelated tumor did not provide a protective effect. The results of this study support a role for B7-DC in activation of dendritic cells, acquisition of antigen for presentation to $CD4^+$ and $CD8^+$ T cells, enhancement of CTL responses, and subsequent antitumor immunity that is potent and selective.

The role of B7-DC on tumor cells was assessed by transfecting J558 plasmacytoma cells with B7-DC [64]. Mice given B7-DC-transfected J558 tumor cells showed evidence of immediate tumor growth, but then complete regression. The response was mediated by $CD8^+$ T cells as depleting anti-CD8 antibodies, but not anti-CD4 antibodies, resulted in persistent tumor growth. Mice that received the B7-DC-transfected J558 tumor cells cleared tumor and exhibited memory response as rechallenge with tumor 2 months after primary inoculation resulted in tumor clearance. Mice given B7-DC-transfected J558 cells also elaborated production of antigen-specific $CD8^+$ T cells that exhibited faster division. Adoptive transfer of antigen-specific T cells into mice containing B7-DC-transfected J558 tumor cells presented with higher numbers of these T cells one month after transfer as compared to mock-transfected J558 tumor cells. Interestingly, these studies also raised the possibility of a second receptor for B7-DC that might facilitate costimulation

as a B7-DC:Ig fusion protein bound both PD-1-positive and PD-1-negative T cells. Both PD-1^+- and PD-1-deficient T cells could lyse B7-DC-transfected tumor cells. Furthermore, a blocking antibody to B7-DC reduced PD-1:B7-DC interaction, while the same blocking antibody did not prevent B7-DC binding to PD-1-negative cells, raising the possibility that B7-DC uses a different epitope to bind PD-1 positive and negative cells. These results support long-term antitumor immune responses enhanced by expression of B7-DC with T cell killing effect mediated by a PD-1 and/or a non-PD-1 receptor.

The abovementioned studies provide provocative insight into the role that the PD-1:B7-H1 pathway performs in regulating immune responses and specifically antitumor immune responses. A more recent study attempted to weigh the relative role performed by different signaling pathways using blocking antibody as a therapeutic strategy. Mouse tumor cells were classified on the basis of B7-H1 expression, transplanted into syngeneic mice, and treated with agonistic anti-CD137 (4-1BB) antibody, a costimulatory molecule known to enhance the antitumor immune response [65]. Interestingly, anti-CD137 antibody treatment failed to induce tumor regression in tumor cells displaying B7-H1 but did so in tumor cells without B7-H1. The addition of anti-B7-H1-blocking antibody to anti-CD137 treatment relieved the inhibition, resulting in tumor clearance. B7-H1 on the tumors inhibited tumor lysis under extended periods of incubation, although the CTLs were functionally normal. The blockade of B7-H1 or PD-1 could relieve this inhibition. These results indicate an overarching umbrella effect conveyed by B7-H1 on tumor cells, shielding them from select immune assault under conditions of strong activation.

16.3 CD137 AND CD137 LIGAND

The CD137 protein (also designated as 4-1BB), a member of the tumor necrosis factor/receptor (TNF/TNFR) superfamily is a 30-kDa protein initially identified by a modified differential screening process [66]. The murine form is located on chromosome 4; consists of 256 amino acids; and contains a signal peptide sequence, a cysteine-rich region, a region containing multiple serines and threonines, a hydrophobic transmembrane domain, and a cytoplasmic domain. The human analog was subsequently identified from activated human T cell leukemia virus type I–transformed human T lymphocytes and was found to be located on chromosome 1 [67,68]. It is a 27-kDa protein consisting of 255 amino acids containing extracellular, hydrophobic transmembrane, and intracellular domains and showing 60% amino acid identity to the murine form [69]. The cytoplasmic domain contains five regions of sequence conservation between mice and humans, implicating these regions in CD137 function [70]. CD137 was found to be expressed as both a monomer and a dimer under nonreducing conditions [71].

CD137 mRNA was identified in unstimulated peripheral blood T cells, and on stimulation with phytohemagglutinin (PHA), phorbol myristate acetate (PMA), and ionomycin was expressed in the T cell lines, CEM and Jurkat [69,72]. In addition, B lymphocytes stimulated with PMA and cell surface Ig antibodies as well as blood

monocytes stimulated with LPS, IL-1β, and PMA induced gene expression [72]. In mouse splenocytes, CD137 mRNA expression appears approximately 3 h after stimulation and surface expression peaks around 60 h [70,71]. In human T cells, CD137 mRNA expression was detected at 1.5 h, peaked at 8 h, and declined to baseline levels by 48 h. Under unstimulated conditions, CD137 mRNA expression was not detected in murine nonlymphoid cells. However, upon IL-1β stimulation, human CD137 could be detected in hepatoma and epithelial cells. FACS analysis showed evidence of CD137 expression on activated $CD4^+$ and $CD8^+$ T cells as well as B lymphocytes. These results indicate that CD137 expression occurs promptly after lymphocyte activation and its presence on activated T and B lymphocytes supported a role for the molecule in modulating immune responses. Subsequent to these findings, CD137 was found expressed on murine natural killer (NK) cells and dendritic cells (DC) [73–75]. Activation of bone marrow (BM)-derived DCs through CD137 resulted in increased production of IL-6 and IL-12 and enhanced dendritic cell stimulation of T cell proliferation [76]. CD137 expression was also identified on $CD4^+CD25^+$ regulatory T cells [77]. Human CD137 expression was separately identified on follicular DCs, blood neutrophils, monocytes, and a significant proportion of blood vessels in malignant tissues [75–80]. Interestingly, a soluble form of CD137, generated by differential splicing in activated lymphocytes, was isolated in the sera of patients with rheumatoid arthritis, although the functional significance of this remains unclear [81].

To identify binding partners for CD137, a CD137/Fc fusion protein construct was generated, culminating in the identification of the CD137 ligand (CD137L) in an EL4 lymphoma cell line. Screening of an esophageal carcinoma (EC1) cDNA expression library led to cloning of the CD137L gene [82]. Murine CD137L maps to chromosome 17 and is composed of a 309–amino acid type II membrane protein. The human homolog was subsequently identified on activated $CD4^+$ T cells also using a CD137/Fc fusion protein chimera. It contains 254 amino acids and exhibits 36% amino acid identified with the murine form and maps to chromosome 19. In a separate study, a fusion construct was created utilizing the extracellular domain of CD137 linked to alkaline phosphatase and was shown to bind preferentially to primary B cells and B cell lines [69]. CD137L is expressed predominantly on professional antigen-presenting cells (APCs) including dendritic cells, macrophages, and B cells [68,71,74,82,83]. However, the ligand's expression has been identified on tumor cells and activated T lymphocytes as well [84]. CD137L has been isolated in a soluble form in patients with various hematological malignancies, including myelodysplastic syndrome (MDS), non-Hodgkin's lymphoma (NHL), and acute myeloblastic leukemia (AML) [85]. Soluble CD137L, which was also identified in the sera of LPS-treated BM-derived DCs [76], was increased on activation of leukocytes and was capable of activating T cells through the increased production of both IL-2 and IFNγ, suggesting a potential functional role in vivo.

CD137-deficient mice were generated by homologous recombination and exhibited no overt phenotypic defects [86]. Myeloid progenitors were found to be increased in bone marrow, spleen, and blood and underwent rapid turnover, indicating a role for CD137 in regulating BM progenitor development. These mice

had decreased IgG2a and IgG3 production. However, overall neutralizing antibodies were similar, and the mice could produce specific antibodies. Interestingly, anti-CD3 antibody or mitogen-induced cell proliferation was assayed and found to be increased in CD137-deficient mice compared to wild-type. Despite the proliferative enhancement, CTL activity was reduced and production of IL-4, IL-2, and IFNγ was decreased in CD137 knockout mice.

Considering the association between the activation of T cells and inducible expression of CD137, along with the expression of CD137L on professional APCs, a possible role for the CD137:CD137L pathway in modifying the immune response was investigated. Utilizing the fusion protein construct CD137:Fc, a soluble competitor to CD137, proliferation was partly inhibited when splenocytes were activated with anti-CD3. An optimal blocking effect was observed between 48 and 72 h [87]. In addition to decreased proliferation, IL-2 production was reduced by 70%. They [87] were able to show that proliferative responses of $CD4^+$ T cells could be blocked through the use of the soluble fusion chimera in the presence of a CD137L-expressing lymphoma cell line, indicating a role for CD137 signaling in influencing both cellular and humoral immune responses. Another study demonstrated a role for the CD137/CD137L pathway in $CD8^+$ T cell activation [88]. Monoclonal antibodies to CD137 that were costimulatory in the presence of anti-CD3 stimulation were isolated and demonstrated stimulation not only of $CD4^+$ T cell proliferation, but also enhancement of $CD8^+$ T cell proliferation and IFNγ production at lower anti-CD3 concentrations. Their results also indicated that activation through the CD137:CD137L pathway was biased at costimulating $CD8^+$ T cells, in contrast to CD28, which was apparently more effective at enhancing $CD4^+$ T cell proliferation. Agonistic anti-CD137 mAbs also enhanced CTL activity and enhanced cardiac allograft rejection, indicating a possible therapeutic target for preventing graft rejection. One of the first studies attempting to establish an in vivo role for CD137 utilized staphylococcal enterotoxin A (SEA), a superantigen that binds to MHC class II molecules on APCs [89]. SEA activates T cells bearing the Vβ3 portion of the TCR. Mice receiving both SEA and agonistic CD137 antibody resulted in reduced depletion of superantigen-activated T cells, indicating an important role for CD137 in enhancing T cell survival.

The aforementioned studies focused on the role of CD137 at the priming phase of the T cell response. A subsequent analysis illustrated an important role for CD137 in restimulation [90]. By blocking previously primed splenic T cells with CTLA4:Ig fusion construct, which binds B7 receptor and thereby blocks B7-mediated costimualtory signals, CD137 was sufficiently capable of inducing a proliferative response suggesting an independent role for CD137 in secondary T cell activation. Furthermore, anti-CD137 was shown to inhibit activation-induced cell death (AICD) and prevent DNA fragmentation in reactivated T cells, a signal of apoptotic cell death. In support of a role for CD137 in T cell survival, $CD8^+$ T cells stimulated with anti-CD3 and agonistic CD137 antibody resulted in enhanced survival and increased expression of bcl-xL and bfl-1, antiapoptotic genes considered critical mediators of cell survival [91–93]. These results support an important role for CD137 in maintaining the survival of T cells on repeated activation.

With evidence of a role for CD137 in modulating immune responses and previous studies on the role of CD28 in costimulation, experiments were performed to address the relative impact of each on activation and memory responses. Insight into the roles of CD137 and CD28 came from studies utilizing CD137L- and CD28-deficient mice [94]. CD28-deficient mice showed significantly impaired early responses to influenza virus infection as evidenced by a lower proportion of tetramer-positive $CD8^+$ T cells and fewer IFNγ-positive $CD8^+$ T cells on days 5 and 7 postinfection. Between 21 and 38 days postinfection, tetramer-positive $CD8^+$ T cells in CD137L-deficient mice drop significantly in comparison to WT mice, indicating that CD137L plays an important role in maintaining the size of the memory population, while apparently having little to no role in initial expansion. CD137L-deficient mice had a slight defect at 21 days postinfection in CTL activity as compared to WT mice. With regard to the secondary response, CD137L-deficient mice showed defects in $CD8^+$ T cells, in percentages of both tetramer-positive and IFNγ-positive cells. $CD4^+$ T cell and antibody responses to secondary responses appeared normal in relation to WT mice, suggesting that the defects seen in CD137L-deficient mice are focused on its capacity to influence survival and/or responsiveness by $CD8^+$ T cells.

$CD4^+$ and $CD8^+$ T cells express CD137 during antigen-specific primary responses [95]. CD137L was capable of enhancing CD28-positive and CD28-negative antigen-specific T cell proliferation as well as increased cytokine production. Both responses were inhibited when soluble CD137:AP was added. Target cell killing was improved in CD137L-expressing, antigen-specific EL4 cells. Responses of purified $CD4^+$ and $CD8^+$ T cells differed according to whether they were stimulated by agonistic anti-CD137 antibody or CD137 L. $CD4^+$ T cells released IL-2 and IL-4, with little IFNγ in response to CD137L-expressing APC. $CD8^+$ T cells produced no IL-4, little IL-2, and more IFNγ than did $CD4^+$ T cells. Interestingly, both $CD4^+$ and $CD8^+$ T cells exposed to anti-CD3 and anti-CD28 antibody stimulation resulted in increased early proliferation compared to anti-CD3 antibody and CD137L stimulation. However, over longer periods, the effect was comparable. These results suggest that $CD4^+$ and $CD8^+$ T cells are affected by CD137 activation, at both the priming phase and effector phases. One explanation for why the priming response is stronger to CD28 may be due to its constitutive expression. Inducible CD137 may be more important in sustaining the cell populations. These results were comparable in a separate study involving human T cells except that $CD8^+$ T cells exhibited diminished expansion attributed to reduced IL-2 production [96].

The presence of CD137 on activated T cells stimulated further interest in understanding the costimulatory molecule's role in influencing B cell responses. Administration of agonistic CD137 antibody within one week to mice immunized with T-cell-dependent antigens resulted in poor antibody-specific responses [97]. This result could not be replicated in humoral responses that do not require T cell–B cell interactions. $CD8^+$ T cells were shown not to mediate this inhibition. It is also unknown whether direct binding of antibody to B-cell-associated CD137 is required for this effect. Whether this regulation is due to $CD4^+$ T cells or CD4/CD8

double-negative T cells was not ascertained. To further clarify the role of CD137L in vivo with the humoral response, a transgenic mouse expressing the CD137L under the control of the MHC class II I-Eα promoter was generated, producing exclusive expression of this gene on APCs [98]. Grossly, these mice displayed evidence of splenomegaly, with no significant changes observed within the thymus or lymph nodes. Despite the presence of splenomegaly, the mice showed no consistent differences in total immune cell numbers in comparison to nontransgenic littermates. However, aged mice (4–12 months old) displayed undetectable levels of B cells in both the spleen and lymph nodes with no B cell follicles present, and low levels of total serum IgG both at baseline and after immunization. Aged transgenic mice also showed poor capacity to stimulate proliferation and alloreactivity against the EL4 target cell line. These results indicate that activation of CD137 can negatively impact humoral immune responses while CD137L expression on APCs negatively influences the humoral immune response over time through reduced survival of B cells.

Initial therapeutic strategies involved the use of an agonistic monoclonal antibody to CD137 in a tumor transplant system [99]. The use of anti-CD137 antibody against the poorly immunogenic Ag104A sarcoma cell line, which were well established in syngeneic mice, resulted in tumor regression as compared to control antibody administration. Mice that showed evidence of tumor clearance were rechallenged with subcutaneous tumor at 3 months and exhibited longlasting specific immunity to Ag104A. Tumor clearance via anti-CD137 antibody treatment was shown to be dependent largely on $CD8^+$ T cells as depletion of $CD8^+$ T cells resulted in tumor progression. Specific CTL activity was enhanced with anti-CD137 mAb treatment. These results indicate that basal activation of tumor-specific $CD4^+$ and $CD8^+$ T cells occurs, enabling CD137 augmentation of the antitumor response and subsequent clearance of tumor.

Using a gene therapy approach, cell-bound single-chain Fv fragments from the agonistic anti-CD137 antibody (1D8) were expressed in the poorly immunogenic, low-expressing MHC class I K1735 melanoma cell line [100]. Mice inoculated with transfected tumor showed clear tumor regression as compared to K1735 cells alone. Depletion of either $CD4^+$ T cells or NK cells resulted in tumor expansion of transfected K175 cells, supporting a role for these cell types in this specific antitumor response. Specific recall response could be induced as a result of rechallenge of mice with wild-type (WT) K1735 tumor. Importantly, mice given WT tumor and subsequently incoluated with 1D8-K1735 exhibited tumor regression, indicating this method as a potential vaccine strategy. Splenocytes from 1D8-K1735 mice exhibited increased proliferation as well as increased fraction of IFNγ-producing cells. These results indicate that this specific type of gene therapy approach may be useful as a vaccine strategy in those tumors that are poorly immunogenic and lack robust expression of MHC class I, enhancing tumor regression through Th1 $CD4^+$ T cells and NK cells.

Evidence indicating an important role for CD137 in enhancing the antitumor immune response led to further experiments to dissect the role of specific cell types that might directly or indirectly mediate this effect. DBA/2 mice were injected

with anti-asialoGM1 antiserum to selectively deplete NK cells in vivo and subsequently inoculated with P815 tumor cells followed by agonistic anti-CD137 antibody [73]. In comparison to mice treated with control antibody, NK-cell-depleted mice displayed progressive tumor growth. Since anti-asialoGM1 antiserum could potentially deplete T cell populations, anti-NK1.1 antibody was administered in DBA/2 $\times$ C57BL/6 mice along with tumor and control or anti-CD137 antibody. This experiment confirmed the previous one, indicating an important role for NK cells in mediating the antitumor effect. However, a subsequent experiment showed that in T-cell-deficient mice, the NK cell effect was abrogated, suggesting that NK cells may be necessary, but not sufficient, for this response. Furthermore, agonistic anti-CD137-antibody-treated NK cells could not lyse P815 cells and did not result in increased IFNγ production. These results implicate NK cells in the antitumor effect, although its role may be immunoregulatory rather than by direct effector function.

Considering the role that the CD137L pathway performs in modulating immune responses, studies were carried out to assess the presence and functionality of this molecule in tumor cells. FACS analysis performed on cancer cell lines, tumor cells inoculated into athymic mice, and tumor cells derived from patient tumors revealed the presence of the CD137L through the use of a CD137:Ig fusion protein construct as well as anti-CD137L antiserum [84]. Treatment of cancer cells expressing CD137L with the CD137:Ig fusion construct resulted in IL-8 production (a cytokine considered important in chemoattraction and the acute immune response), indicating that CD137L on tumors is functional. Subsequently, the group was able to show that CD137L expression on cancer cells could signal back to T cells activated with anti-CD3, resulting in IFNγ production. This response was markedly reduced by the use of a neutralizing anti-CD137 antibody. Their work identifies CD137L at the tumor cell surface and implicates CD137:CD137L in bidirectional immune responses, which could potentiate T cell activation and function.

In an effort to understand and enhance DC-mediated tumor immune responses, a strategy utilizing tumor-associated antigen (TAA) delivery via replication-deficient adenoviral vector and CD137L was adopted [101]. In this study, the human papillomavirus-16 E7 oncoprotein was recombined into the adenovirus, and infected DCs were shown to direct both effector and memory CTL responses secreting IFNγ. Both responses were enhanced when CD137L was coinfected. This augmentation was revealed only at low levels of antigen availability unless another costimulatory molecule was added. Furthermore, their results indicated that the presence of CD137L resulted in a reduction of nonspecific T cell activation and induced increased expression of B7-1 and B7-2 on dendritic cells. These results indicate that CD137L expression can enhance selective immune responses where low antigen availability exists, supporting previous evidence of the CD137:CD137L pathway in enhancing immune responses to suboptimal T cell activation. In addition, they support expression of CD137L in DC-based vaccination strategies as they help generate a more favorable specific response, reducing the probability of inducing nonspecific responses to intrinsic antigens that could result in autoimmunity.

In a subsequent study incorporating a colorectal tumor hepatic metastasis model, gene therapy of replication-defective adenovirus with IL-12 and CD137L was utilized [102]. Previous reports documented an enhanced tumor clearance with the use of intratumoral injection of adenovirus-mediated IL-12 in a colorectal tumor hepatic metastasis model [103]. However, this treatment strategy resulted in only 25% of mice surviving beyond 70 days. The protection provided was attributed to both NK and T lymphocytes [104]. Poorly immunogenic MCA26 tumor cells were injected into the left lateral lobe of the liver, allowed to mature, and then injected with various adenovirus vectors. The combinatorial administration of IL-12 and CD137L resulted in statistically significant long-term survival. This survival advantage was lost with the depletion of NK cells or $CD8^+$ T cells. More importantly, memory responses were generated as evidenced by protection against subcutaneous tumor challenge. These results further support the role of CD137L interactions in mediating enhanced primary and memory antitumor responses, and corroborate previous studies illustrating an important role for NK cells and $CD8^+$ T cells in the antitumor effect. A subsequent experiment utilized the same metastatic model; however, this time agonistic antibody to CD137 was administered, replacing CD137L gene transfer [105]. In addition to showing a potent tumor regression response with the administration of both IL-12 and anti-CD137 Ab, the dose of IL-12 could be substantially reduced with the same effect. The treatment was effective in both liver and lung metastasis. Once again, both NK and T cells were implicated in tumor regression as depletion of either resulted in reduced target cell lysis. Both cell subtypes were also shown to be instrumental in mediating protective effects on subcutaneous rechallenge.

In a study associating the role of CD137 with $CD4^+$ T cell help in modulating $CD8^+$ CTL responses, antigen-specific $CD4^+$ T cells were shown to be important in directly supporting the expansion and lytic activity of CTL [106]. Moreover, this response was enhanced by costimulatory molecules such as CD137 and abrogated by antibodies to CD137. $CD8^+$ T cells cultured with irradiated $CD4^+$ T cells execute in vitro antitumor immune responses, lysing melanoma cells. Their results suggest a direct cell–cell communication between $CD4^+$ and $CD8^+$ T cells through CD137 in productive CTL responses and subsequent tumor immunity.

To further dissect the roles of CD28 and CD137L in primary and recall responses to tumor cells, A20 B cell lymphoma cells were transfected with either B7-2 or CD137L and transferred to Balb/c mice [107]. Mice that were able to reject tumor were also capable of rejecting secondary systemic challenge of parental tumor. The same experiments were then performed in mice deficient in CD28 [108]. Mice with A20 expressing CD137L were able to reject primary tumor inoculation, although mice transferred with A20 expressing B7-2 had progressive tumor growth. However, secondary challenge of mice that rejected the CD137L-transfected A20 was unsuccessful. Recovered splenocytes showed reduced CTL activity against A20 with reduced IL-2. Their results indicate that the tumors expressing CD137L can induce a priming immune response even in the absence of CD28. However, recall responses are attenuated in the absence of CD28, possibly as a result of poor IL-2 production and weakened CTL.

Other studies have focused on the combined strengths of CD28 and CD137 in mounting a strong antitumor immune response [109]. In an adoptive therapy strategy, mice were inoculated with poorly immunogenic A9P melanoma cells and draining lymph node T cells were isolated and stimulated with IL-2 and combinations of anti-CD3, anti-CD28, and anti-CD137. Mice receiving T cells stimulated with all three antibodies showed improved long-term survival. This phenomenon could be attributed to neither changes in the $CD4^+$ or $CD8^+$ T cell populations nor differential expression of IL-2, IL-4, or IFNγ, all previously shown to be influenced to some extent by CD137 interactions. In another study, tumor-draining lymph node (TDLN) T cells were shown to upregulate the expression of CD137 by 70% [110]. The activation of TDLN T cells by both agonistic anti-CD137 antibody in addition to anti-CD3 and anti-CD28 resulted in enhanced T cell proliferation with reduced AICD as evidenced by less propidium iodide and annexin V staining. Cytokine production was skewed toward a Th1 phenotype with increased IFNγ and GM-CSF production and decreased IL-10 production in vitro. Transfer of anti-CD137, anti-CD28, and anti-CD3 stimulated TDLN T cells into mice receiving tumor cells resulted in less metastatic pulmonary nodules and improved survival as compared to TDLN T cells that were stimulated with anti-CD28 and anti-CD3 only. Neutralization of IFNγ abrogated the antitumor immune response, while anti-IL-10 improved tumor regression. These results support an enhancement of the CD3/CD28 antitumor immune response by CD137 as a result of augmented Th1 promotion and improved T cell survival.

In another study investigating the roles of CD28 and CD137, P815 tumor cells transfected with CD137L and inoculated into syngeneic mice resulted in substantial tumor rejection [111]. However, transfection of the poorly immunogenic sarcoma cell line AG104A with CD137L or CD28 resulted in no rejection. The cotransfection of both genes resulted in tumor clearance in both the primary and secondary phases. In vivo depletion of $CD8^+$ T cells indicated that this cell type is critical in mediating CD137L-expressing P815 antitumor immune response. These results support a synergistic effect mediated by both CD137 and CD28 in promoting antitumor immunity. Utilizing a murine squamous cell carcinoma (NRS1) cell line that expresses high levels of the costimulatory molecule B7-1, CD137L was shown to be required to induce tumor rejection [112]. These results suggest that the presence of B7-1 may not be sufficient to generate adequate tumor immune responses and directed therapy at CD137 signaling might augment the antitumor effect.

The previous reports strongly support the significance of incorporating CD3, CD28, and CD137 in generating effective antitumor immune responses. Armed with this knowledge, an ex vivo strategy was developed to optimally expand CTL for in vivo use [113]. An artificial APC (aAPC) was engineered utilizing a K562 erythromyeloid cell line (which lacks HLA proteins and therefore reduces allogeneic responses) stably expressing CD137L and CD32, a human low-affinity Fcγ receptor. CD32 was used as a substrate for coating anti-CD3 and anti-CD28 antibodies on the cell surface, bringing into close proximity CD3, CD28, and CD137 L. aAPCs incorporating CD137L, anti-CD3, and anti-CD28 promoted enhanced long-term

proliferation and survival of $CD8^+$ T cells. Antigen-specific T cells could be propagated effectively using this technique and displayed specific cytolytic activity. The presence of CD137L appeared to enhance the capacity for preservation of the diverse T cell repertoire that was initially provided to the aAPC system. Their results further a synergistic role for CD3, CD28, and CD137 in mediating potent antitumor immune responses and add this system to the armamentarium of adoptive therapeutic strategies focused on antitumor immune responses.

Another study investigated the role of both the anatomic site of tumor inoculation and the immunogenicity of the tumor with antitumor immune responses [114]. Mice given intracranial MCA205 sarcoma cells and GL261 glioma cells and provided with agonistic CD137 antibody manifested prolongation of survival and cure of disease in some mice with the development of systemic and memory responses. However, only a partially therapeutic effect was seen in mice given tumors subcutaneously or intravenously. No beneficial effect was seen when the tumor was a weakly immunogenic B16/D5 melanoma tumor. Mice that were able to clear tumor revealed a requirement for both $CD4^+$ and $CD8^+$ T cells. Interestingly, the administration of anti-CD137 during lymph node sensitization by tumor, while stimulating hypertrophy of the nodes, did not result in enhancement of the antitumor immune response, but rather had an adverse effect. In addition, adoptive transfer of TDLN cells in the presence of CD137 mAbs resulted in poor antitumor reactivity. The results of this study demonstrate that the level of tumor immunogenicity as well as the site of tumor inoculation influence the effectiveness of CD137 to deliver meaningful therapeutic effect, although the mechanisms related to these context-dependent effects remain unclear.

16.4 CONCLUSIONS

Despite advances in our understanding of the immune system during the past two centuries, vaccination and immunoprophylaxis continue to be at the forefront of immunotherapeutic strategies directed at the control and eradication of disease. While some forms of cancer will be treated from a prophylactic standpoint, a significant proportion of patients will still present with well-established tumor burden. Surgery, chemotherapy, and radiotherapy can provide some level of tumor control. However, none can potentially provide the level of selectivity and potency that could be harnessed by the immune system. Costimulatory molecules, such as PD-1/B7-H1/B7-DC and CD137:CD137L, provide attractive alternatives to anti-CTLA-4 antibody therapy in the antitumor armamentarium. Each has been shown in various model systems to influence and enhance productive antitumor immune responses. The evidence indicating that the immune system "sees" tumor and responds by the presence of tumor-infiltrating lymphocytes indicates that tumor cells develop strategies to tune down immune cells and induce tolerance, possibly exhausting T cells from their intended goal. One of the mechanisms utilized is most undoubtedly the expression of costimulatory/coinhibitory molecules that provide a

shielding effect on the tumor and the tumor microenvironment. Immunotherapeutic strategies aimed at manipulating the PD-1/B7-H1/B7-DC pathway have the capacity to not only rejuvenate T cells but also push T cells into an activated state when other forms of "help" cannot be provided. Furthermore, signaling systems such as the CD137:CD137L pathway may improve the antitumor response by augmenting not only selective responses but also responses in low-antigen-level states. However, all of these potential future benefits must be weighed against previous evidence of deleterious effects from immunotherapy, including autoimmunity and cellular depletion. Finally, immunotherapeutic strategies will have to be tailored to the type of tumor, the level of immunogenicity of the tumor, as well as the location of the tumor, as each of these factors will most likely have significant bearing on the clinical benefit. Nevertheless, there continues to be much to gain from manipulating "signal 2" in the hopes of eradicating cancer.

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PART V

DEFINING EFFECTIVE CLINICAL RESPONSES

17

ADVANCES IN IMMUNE MONITORING STRATEGIES FOR CANCER VACCINES AND IMMUNOTHERAPY

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17.1 INTRODUCTION

The discipline of immune monitoring for cancer vaccine and immunotherapy trials is experiencing an era of unparalleled growth because of an increased understanding of the fundamental basis of human cancer and host–tumor interactions. Knowledge gained in basic cancer immunology and molecular biology has helped identify a growing list of tumor antigens. The ability to identify specific HLA-restricted peptide epitopes within these antigens has led to development of highly targeted cancer vaccines [1–5]. The availability of such epitopes has also allowed a more sophisticated approach to immune monitoring, although the antigenic epitopes responsible for tumor recognition and rejection are not always known. Nonetheless, careful monitoring of clinical vaccine trials can provide important clues to the mechanism of a particular vaccine or immunotherapy strategy. Furthermore, as monitoring assays become more sensitive, they may be able to serve as surrogates for clinical response or for comparing the potency of different vaccines. Thus, developing

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standardized methods and validating individual assays is a major priority for tumor immunologists.

This chapter focuses on current state of the art immune monitoring techniques for cancer vaccine and immunotherapy trials. The essentials of commonly used in vitro and in vivo assays are described with pertinent examples of how these assays have been used in monitoring clinical vaccine trials. Despite several attempts to standardize these assays, there is currently no universal agreement as to which assays and validation criteria should be used, and limited data exist on how particular vaccines mediate tumor regression in humans [6]. The challenge to develop a set of universal standard criteria is further complicated by the need to use ex vivo assays to determine in vivo status of the immune response, especially across a range of tumor types and stages. The future of this field will likely depend on refinements to the current set of assays and continued monitoring of clinical trials. These trials should be scientifically sound and include plans for appropriate statistical analysis of the data.

17.2 CURRENT STATUS OF TUMOR IMMUNOTHERAPY

Current immunotherapy approaches include adoptive transfer of T cells, peptides, recombinant viruses, autologous or allogeneic tumor cells, and dendritic cell (DC)-based vaccines [7]. All of these strategies have shown therapeutic responses and evidence of systemic immunity in preclinical murine models and in selected patients enrolled on early-phase clinical trials [8–11]. There is emerging evidence that measurement of immune responses can be used to predict clinical responses with these agents. In studies using recombinant poxviruses expressing T cell costimulatory molecules with or without tumor antigens, objective clinical response and disease stabilization have been associated with an increase in antigen-specific T cell precursors detected by ELISpot assay [8]. Adoptive T cell immunotherapy has shown great promise, especially after nonmyeloablative but lymphodepleting chemotherapy, with objective clinical responses in 50% of patients [12,13]. Adoptively transferred T cell clones persisted in responding patients and preferentially localized to tumor sites and mediated antigen-specific immune responses that could be detected by ex vivo T cell analysis [14]. Perhaps the best example is the correlation of anti–human papilloma virus (HPV) antibody titers in women receiving an HPV virus-like particle (VLP) vaccine with prevention of cervical cancer [15,16]. These examples suggest that immune monitoring might be useful in predicting clinical responses for cancer patients receiving immunotherapy.

Despite intense efforts to increase the frequency of tumor antigen-specific T cells by various vaccines, a strict correlation has not always been seen and clinical responses are rare [17]. Careful monitoring, however, has provided critical insight into the reasons for the low responses in some trials [19,20]. For example, in cancers such as ovarian and melanoma, the presence of $CD4^+CD25^+$ regulatory T (Treg) cells in tumor is a predictor of poor patient survival [18]. These Tregs have also been shown to inhibit T cell responses following vaccine treatment in

murine studies [20]. The role of Tregs in blocking effective antitumor immunity was based on careful immune monitoring and has led to proposals for combining vaccines with Treg blockade to improve the therapeutic activity of tumor vaccines [19]. Furthermore, combining immunotherapy with chemotherapy, radiation, and/or antiangiogenic therapy are currently being actively pursued in the clinic the basis of on immune monitoring data supporting an improved response with specific combinations of these strategies [20,21].

While the future of immunotherapy for the treatment of cancer is promising, it is difficult at present to directly compare the various immunotherapeutic approaches because monitoring assays have not been standardized with respect to technical or analytic methodology. Most of the currently used assays have focused on monitoring adaptive immune responses, such as antibody titers and T cells, although innate immune responses may also be important in immunosurveillance and therapeutic responses in cancer patients. Nonetheless, the focus on T cell immunity is probably important in terms of data from preclinical studies and analyses of the tumor microenvironment in cancer patients. The ideal T cell assay should be sensitive, specific, reliable, and reproducible. The assay should be easy to perform and reflect the status of the immune response in vivo. In addition, the assay should demonstrate a close correlation with clinical outcomes. The assays currently used to measure immune response can be divided into those assays that are performed directly in patients and those that are performed in vitro using patient-derived blood or tissue samples. Examples of direct in vivo assays are the delayed-type hypersensitivity response, imaging of lymph nodes and tumors, and whole-body scans. The more commonly employed in vitro T cell assays include the enzyme-linked immunospot assay (ELISpot), tetramer assay, intracellular cytokine flow cytometry (CFC), quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), microarray/proteomics, proliferation assay, and standard cytotoxicity assay. Humoral immunity is typically determined by enzyme-linked immunosorbant assay (ELISA). The choice of an assay for monitoring of tumor vaccines needs must be based on a complete understanding of the characteristics and limitations of the assay, the proposed mechanism of action for the vaccine or regimen under investigation, the availability of pretreatment and posttreatment samples, and the skill of the immunologists doing the assays.

Immune Monitoring Assays

A large number of tests are available for immune monitoring of cancer vaccine and immunotherapy trials. The primary objective of this monitoring is to determine the effects of a vaccine or regimen on priming or augmenting specific humoral or cellular immune responses. The prevalence and functional characteristics of tumor-specific B and T cell responses can be quantitated and monitored in vitro by various methods as summarized in Table 17.1. While ELISA has become the standard for detecting antibody responses, there are many assays for monitoring T cell responses. Although all of the T cell assays in Table 17.1 are currently in use, three assays—ELISpot, tetramer staining, and CFC—have emerged as

TABLE 17.1. Characteristics of In Vitro Immune Monitoring Assays

Assay Methods	Target	Detection Limit	Quantification	Sensitivity	Assay Parameters
ELISpot	Cellular	+++	+++	+++	Functional
Tetramer analysis	Cellular	++	+++	++	Nonfunctional, phenotypic
CFC	Cellular	++	+++	++	Functional, phenotypic
qRT-PCR	Molecular (DNA/RNA)	+++	+++	+++	Nonfunctional
Microarray	Molecular	+	++	+	Nonfunctional
Proteomics	Molecular	N/A	+	+	Nonfunctional
Proliferation	Cellular	N/A	N/A	N/A	Functional
Cytotoxic assay	Cellular	N/A	N/A	N/A	Functional
ELISA	Molecular (protein)	+++	+++	+++	Nonfunctional

[a] *Abbreviations*: ELISpot—enzyme-linked immunospot; CFC—cytokine flow cytometry; qRT-PCR—quantitative reverse transcriptase polymerase chain reaction; ELISA—enzyme-linked immunosorbant assay; N/A—not applicable; the number of + signs depicts the level.

first-line methods for monitoring T cell responses in most clinical trials. These assays are relatively reliable and sensitive and allow ex vivo T cell analysis at the single-cell level. In addition to these standard assays, other approaches, such as flow cytometry, proliferation, and cytotoxicity assays may provide additional functional information. Other more complex methods, such as quantitative real-time PCR, microarray analysis, and proteomics have been useful for gene and protein profiling of the tumor microenvironment and as complements to first-line monitoring assays [22]. More recently, efforts at in vivo imaging have been evaluated, and these may provide additional and complementary information to the more familiar in vitro assays.

Enzyme-Linked Immunospot Assay (ELISpot)

The interferon-γ (INFγ) ELISpot assay is a commonly used procedure and has been extensively validated for the detection and quantification of functional T cell responses in patients treated with cancer vaccines. ELISpot is an attractive and powerful assay that allows tracking of antigen-specific T cells in the peripheral blood of patients who receive vaccines provided enough T cells are available for pre- and postimmunization sampling. ELISpot also permits quantitative measurement of cytokine secreting cells at the single-cell level directly ex vivo or after in vitro stimulation, which can be used to expand low-frequency T cells. The function of cells can be inferred from the types of cytokines secreted by cells in response to antigenic stimuli. ELISpot assay is often the first choice in vaccine evaluation

since high-throughput screening using a microtiter plate format is efficient and can accommodate large numbers of samples and multiple antigens. In general, the ELISpot assay has the lowest limit of detection and can often detect as few as 1 in 100,000 T cells [23].

The ELISpot assay is conducted by pretreating a nitrocellulose-coated 96-well microtiter plate with an anticytokine antibody. While INFγ is commonly used, as its secretion represents a functionally active T cell, other cytokines can be used as well. The antibody-coated plates are then used to plate a defined number of T cells derived from peripheral blood lymphocytes, usually through Ficoll gradient separation. The T cells are activated by coculture with autologous or artificial antigen-presenting cells (APCs) pulsed with a peptide or other antigenic material. While autologous DCs are ideal for this purpose, they are not always available. Alternative sources of APC for ELISpot assay include allogeneic or xenogeneic APC; human lymphoid hybrid T2 cells, artificial HLA-A2 dimers, and human chronic myelogenous leukemia K562 cells transfected with the HLA-specific genes [24]. On activation of the T cell, cytokines are released and captured by the antibody on the nitrocellulose membrane. The cells are washed away and a secondary cytokine-specific antibody is added to the plate and a color reaction linked to the secondary antibody will result in a "spot" at the site of a functional T cell. The number of spots can be counted in each well, and because the number of T cells plated is known, the precursor frequency of the tumor-reactive T cells in vivo can be estimated. The assay can be used to run cryopreserved pre- and posttreatment samples reducing the interassay variability. For situations where the T cell frequency may be very low, T cells can be expanded by repeated exposure to antigen in a process known as *in vitro stimulation* (IVS). While IVS may help detect low frequencies of reactive T cells, prolonged IVS may bias the response and not reflect the actual in vivo responses. The ELISpot can be performed with bulk T cells or on separated populations to define individual $CD4^+$ and $CD8^+$ responses, provided that MHC class II and class I antigens are available. The cell specificity can also be determined by repeating the assay in the presence of class I and class II blocking antibodies. Although the ELISpot assay is often most sensitive when using a single peptide for stimulation, the assay can be used to define T cell reactivity against multiple peptides or even whole-cell lysates. This may be advantageous in situations where the peptides or antigens are not defined.

There has been a concerted effort to validate the ELISpot assay in order to achieve superior reliability and reproducibility of performance [25]. While the definition of a positive immune response for the ELISpot assay varies with the affinity and avidity of the antigen(s) being tested and the inter- and intraassay variability, there is general consensus on how to define a positive immune responses by ELISpot. Most laboratories use a two- to threefold increase in spot-forming units (SPU) for T cells exposed to an experimental antigen compared to nonantigen or irrelevant antigen-stimulated T cells as a positive response [26,27]. An additional criterion is an increase in T cell frequency at least 2 standard deviations above the mean frequency [23,28]. For specific antigens, one or both of these parameters may be used and the assay usually requires validation for individual antigens using a

normal pool of donor T cells to establish baseline levels of response. Many clinical tumor vaccine trials have been monitored by ELISpot assay, and evidence for a correlation between positive ELISpot data and clinical responses has been reported [45–53].

In addition to cytokines, which define one functional aspect of T cell activation, other markers that more closely represent the cytotoxicity profile of activated T cells have been used to detect functional T cells in vaccinated patients. Shafer-Weaver and colleagues reported that T cell reactivity in a granzyme B ELISpot assay was more closely associated with functional status of cytotoxic T cells than in other assays, including an INFγ ELISpot [29]. Other functional readouts for ELISpot assays have included IL-10, IL-4, IL-5, GM-CSF, and perforin. This highlights an important advantage of ELISpot assays in providing both a quantitative assessment of T cell precursor frequency and functional data on the quality of the response.

The major limitations of the ELISpot assay include the need for sufficient numbers of cells, the skill of the technician, and the inter/intraassay variability. Viable T cells can be obtained from fresh and frozen peripheral blood lymphocytes. When pretreatment and posttreatment samples are evaluated at the same time, there is a reduction in the interassay variability. In previous studies, the variability of ELISpot assays due to blood processing was determined by testing leukopheresed PBMC shipped overnight in medium versus cryopreserved PBMC from matched donors. There were no major differences in ELISpot results in fresh or cryopreserved T cells, suggesting that with proper handling in cryopreservation does not reduce the sensitivity of the assay [30]. Intraassay variability can be reduced by technical training of lab personnel and routine validation on normal donor samples to establish reproducibility and reliability data.

Tetramer Analyses

Tetramer analysis is a flow-cytometry-based technique that replaces monoclonal antibodies with folded peptide–MHC complexes attached to a color substrate. Initially four complexes were used (hence the term *tetramer*), and the tetramer binds to the cognate T cell receptor because of the specificity provided by the peptide. Today, other complexes, including dimers and pentamers, have all shown promise in the identification of monoclonal T cells. The assay can be used to quantitate the number of antigen-specific T cells in samples with as few as 10,000 cells. Thus, the availability of sufficient cells for the assay is rarely a problem for tetramer analysis. The interaction of T cell receptors on T lymphocytes with tetrameric MHC–peptide complexes in vitro mimics the specific interaction of T cells with APCs in vivo. Tetramers consist of four biotinylated HLA–peptide epitope complexes bound to streptavidin conjugated with a fluorescent dye. The nature of the multivalent binding increases the affinity and enables staining and analysis of cognate T cells with the sensitivity of detection as low as 0.02% of the total T cell pool (i.e., approximately 1 in 50,000 lymphocytes) [28,30]. In contrast to many other methods, such as the ELISpot assay and the ^{51}Cr-release assay, analysis by tetramers does not depend on the functionality of the T cells, that is, the secretion of cytokines on activation

and cytolysis of targets. Thus, a major disadvantage of tetramer analysis is that all T cells recognizing the specific antigenic peptide embedded in the proper MHC complex will be labeled and recognized regardless of functional status [28,30,31]. Currently, tetramer staining in combination with costaining by antibodies against other cell surface or intracellular cytokines has become a frequent practice in order to add functional information to the assay. This combination enables the direct assessment of antigen-specific T cells for lineage differentiation, activation status, and cytokine production in a single assay, and thus offers the advantages of speed and the ability to identify subsets of reactive cells.

There are several other advantages to the tetramer assay. Once monoclonal T cells are identified by tetramer analysis, tetramer-labeled T cells can be cloned by cell sorting for further characterization and study. The tetramer approach not only is useful for identifying T cells in peripheral blood but can also be used to identify T cells in tissue through in situ hybridization. For example, combinational tetramer analysis of $CD3^+CD8^+$ $tetramer^+$ cells from tumor, lymph node, and PBMC obtained from HLA-$A2^+$ patients with head and neck cancers was used to define the frequency and functional status of the T cells [32]. The $CD3^+CD8^+$ $tetramer^+$ cell frequency was significantly higher in tumor-infiltrating lymphocyte (TIL) than autologous PBMC. Furthermore, the $CD4^+CD25^+$ TIL population was enriched with cells expressing Foxp3, GITR, and CTLA4, hallmarks of Treg cells. Finally, $CD8^+$ TIL had low TCR zeta-chain expression and produced little IFNγ after ex vivo stimulation indicating the functional anergy of these cells in the suppressive tumor microenvironment [32]. This study nicely demonstrates the wealth of information that can be attained with tetramer analysis. One limitation is the need for properly folded tetramers, which may not always be available, and the need to have defined antigens as targets for analysis.

Tetramer analysis can also be used to monitor the number and activity of adoptively transferred T cells. In a clinical trial of adoptively transferred $CD8^+$ T cell clones with specificity for MART1 and gp100 in patients with metastatic melanoma, tetramer analysis indicated that persistence of transferred T cell clones in vivo correlated with positive clinical outcomes [14,33,34]. There are, however, limitations with tetramer assays since functional information is not inherent in the assay. For example, in a trial evaluating p53 peptide-specific $CD8^+$ T cells in the circulation of patients with squamous cell carcinoma of the head and neck (SCCHN), there was no correlation between clinical response and p53-specific T cell accumulation in the tumor. These results highlight limitations of this assay, in addition to the complexity of interactions between the tumor and the host immune system [35].

Another limitation of tetramer assays is the need for different complexes for each distinct HLA allele and peptide, rendering it difficult to apply to rare HLA haplotypes and across large outbred populations. The reagents for tetramer assays are also relatively expensive and require expertise in flow cytometry for data acquisition and analysis. Nonetheless, advances in the biochemistry of tetramer formation and advances in flow cytometric analyses have made the technique highly popular for monitoring immunotherapy trials. Similar to all assays, ongoing validation is

helpful in reducing variability and increasing the sensitivity and specificity of the assay.

Flow Cytometry

Multiparametric characterization of cytokine production on a single-cell basis with high throughput of samples is an attractive feature of flow cytometric analysis. The technique allows simultaneous use of four or more antibodies and is thus particularly useful for the small subsets of lymphocytes modulated by immunotherapy. Cytokines are particularly good candidates for multiplexed analysis, although numerous other functional molecules can be used. Through intricate networks and complex feedback mechanisms, cytokines modulate each other as well as a multitude of cellular events and play a critical role in activation of immune responses after cancer vaccination. Historically, the quantity and breadth of tumor antigen-specific $CD8^+$ T cell responses have not always correlated with clinical outcome. Thus, it is critical to develop immune monitoring assays that can assess the quality of the tumor antigen-specific $CD8^+$ T cell response by measuring multiple $CD8^+$ T-cell-specific functions simultaneously.

Flow cytometry requires monoclonal antibodies against specific molecules of interest, such as cytokines or other functional proteins. The assay can be used to stain cell surface markers, which can be used to identify subpopulations of cells, for example, $CD4^+$ versus $CD8^+$ T cells. The assay can also be used to define intracellular molecular targets, such as IFNγ or CTLA4, by permeabilizing the cells. The technique is powerful and requires few cells but cannot define individual T cell clones. It can, however, be combined with tetramer analysis to identify individual clones, or with ELISpot to provide another measure of cytokine-producing cell frequency. As refinements in technology have been developed, this technique has been applied to a variety of tissue samples with excellent results. As in other assays, validation and standardization is critical before these assays can be used in clinical immune monitoring. Newer flow-cytometry-based approaches have allowed for the simultaneous analysis of five or more cytokines as well as the expression of memory-defining markers on tumor antigen-specific T cells. This analysis finely dissects the immune response and enables identifications of functional subsets of cells not previously known. Multiparameter flow cytometry standardization is important since the tumor-specific $CD8^+$ T cell response in vivo would be defined by many criteria such as homing, expansion, and effector functions of individual cells [36]. A good example of the utility of multiparameter analysis can be found in a trial monitoring quantitative and qualitative immune response in PBMC of 41 bladder cancer patients [37]. The study revealed that percentages of cells expressing CD3, CD4, and CD8, or CD56 were found to be significantly lower in patients than healthy controls. The mean percentage of cells expressing CD4 was significantly lower in patients showing recurrence as compared to patients without recurrent disease. The percentages of $CD4^+$ T cells producing IFNγ, IL-2, and TNFα were significantly reduced, while the mean levels of IL-4, IL-6, and IL-10 in patient T cells were significantly higher as compared to healthy controls. Patients with bladder cancer

appear to develop a T_H2-type response, which is associated with disease progression. The lymphocyte evaluation along with cytokine measurement thus provides a sensitive and valuable tool for evaluating the function of cell-mediated immunity in these patients and can also find application in therapeutic monitoring of bladder cancer patients as new targets for immunotherapy are discovered [37].

Similar to other methods, flow cytometry analysis has its own advantages and disadvantages. The technique can be used to evaluate other molecular players in the immune response, such as perforin, granyzmes, and costimulatory molecules. Cells with similar surface phenotype but different cytokine production profiles, and cells exhibiting different functional characteristics can be differentiated using this technique [38]. Caution must be observed, however, for cross-reactivity of multiple cytokine antibodies, and interpretation may be difficult at times because of changes in the phenotype of individual cells undergoing differentiation. The assay allows a functional readout without a restriction for MHC haplotype as in the tetramer assay, and the response can be read for a broad set of T cells simultaneously in a relatively short period of time. Like other assays that measure functional features, the variation between assays is always a major concern, making proper standardization imperative.

Quantitative Real-Time RT-PCR

The polymerase chain reaction (PCR) revolutionized the field of molecular biology and allowed for accurate detection of gene transcripts from minute volumes of genetic material. The technique is based on an amplification process that occurs when small probes complementary to known DNA sequences within a gene are amplified during DNA replication in vitro. The technique can be used to directly quantitate the level of gene transcripts within a sample through a technique called *quantitative real-time PCR* (qRT-PCR). This method uses a fluorescent dye attached to the specific gene probe, which will be diluted with each round of replication. Thus, the degree of fluorescence dilution will correlate with the number of gene transcripts present in the samples. In contrast to the cellular immune assays discussed previously, the major advantage of qRT-PCR is in the accurate measurement of relevant gene expression levels within target tissues, such as T cell populations or the tumor microenvironment [39,40]. The assay has been most useful in evaluating the tumor microenvironment, where there are quantitative or qualitative differences in immune modulating molecules and cells, for example, the balance between effector and regulatory T cell accumulation in tumors. The qRT-PCR assay may also be more reliable since a few studies have suggested that cellular immune assays may not be reliable for cells located within established tumors [41–43].

Quantitative real-time PCR is a powerful and rapid method to quantify gene transcripts, and it measures the buildup of fluorescence with each PCR cycle and generates quantitative fluorescence data at the earliest phases of the PCR cycle when replication fidelity is highest [44,45]. Guadagni and others demonstrated that CEA mRNA expression measured by qRT-PCR in the blood of carcinoma patients was strongly correlated with disease status [46]. The potential use of qRT-PCR

for immune monitoring came from an early study using clone-specific primers to determine T cell composition and frequency. The technique was used to determine T cell clonal expansion, contraction, and deletion following adoptive transfer [47]. The major advantages of qRT-PCR are the high sensitivity, ability to quantify transcripts, and reproducibility of the assay. The procedure can detect as few as five molecules with excellent dynamic range and in a linear manner over several orders of magnitude. The disadvantages include the cost of instruments and reagents, the inability to currently employ high-throughput systems, and the inherent danger of false positives due to RNA carryover or contamination. The availability of RNA is also required and various techniques for RNA amplification from small samples have been proposed to identify gene expression even from minute samples [39,48,49].

Microarray Analysis

Microarray technology is based on the availability of an expressed sequences tag (EST) database of the human genome and allows collection of extensive biological data. These data can be used to enhance our understanding of physiological processes and disease states by profiling tissue and single-cell populations at the RNA and protein levels. The technique is particularly useful for comparative analysis of genomewide mRNA expression patterns. In cancer research, high-throughput evaluation of genetic or protein profiles in specific tissues can be used to identify unique signatures in tumor compared to normal tissue, or in primary compared to metastatic tissue. Microarray analysis has generated significant information on tumor-associated and tumor-specific biomarkers for cancer diagnosis and as targets for therapeutic drug development [50–52]. Currently, a variety of array protocols and software are available for analysis. Originally, cDNA-based microarrays employed the most popular and widely used form of cDNA robotically printed onto glass slides and subsequently hybridized to fluorescently labeled probes to compare cellular expression patterns from different samples. Another leading technology relies on the use of oligonucleotides and has the advantages of using synthetic DNA from a large number targets rather than a limited number of cDNAs.

Using a functional microarray analysis, $CD8^+$ T cells from 10 melanoma patients after gp100 peptide vaccination were found to have a diverse set of patient- and antigen-specific cytokine expression profiles, indicating differences in clinical responses [53]. For example, melanoma patients with increased secretion of IFNγ and TNFα in response to gp100-based vaccination remained free of disease recurrence, whereas patients who showed discordant secretion of IFNγ and TNFα experienced recurrence sooner [53]. Similarly, genes involved in patient survival were identified through a 17,500-cDNA microarray of 43 tumor tissues from 38 patients with stage III/IV melanoma [54]. Expression data analysis revealed that patients with increased expression of genes related to innate and acquired immunity lived longer than did patients without such expression profiles, confirming the interplay between immunologic mechanisms and the natural history

of melanoma. Future subset analyses of the specific genes may provide important clues to how the immune system interacts with melanoma to prevent disease or respond to immunotherapy used in treatment of established disease. Evaluation of protein levels can be used to complement data obtained from microarray analysis and may provide additional information that cannot be obtained from focusing on expression patterns at the RNA level. This may relate to a poor correlation between mRNA and protein levels or to posttranslational modifications resulting in multiple protein isoforms [55].

Proteomics Analysis

While PCR and microarray data provide information on gene and gene expression status, it is clear that gene expression does not always correlate with actual protein expression. The direct analysis of proteins within cells or tissue samples is referred to as *proteomics analysis*. This technique can provide data on all proteins in a particular tissue sample, and has been used for a variety of diagnostic and monitoring purposes. The use of proteomics for immune monitoring remains theoretical but merits mention because of the type of information that can be provided, and it represents an improvement from standard gene techniques that may be misleading regarding the in vivo environment. Much of the potential for proteomics has focused on identification of biomarkers in serum samples.

Proteomics may offer a novel approach to immunologic monitoring that complements other immunologic assays. In principle, quantitative protein expression profiling is a crucial part of proteomics and requires methodology that is able to efficiently provide accurate and reproducible differential expression values for proteins in two or more biological samples. By studying the T cell activation status and levels of protein expression, it may be possible to quantitate the magnitude of immunologic responses. Proteomics could also provide a tool for obtaining valuable information regarding underlying regulatory mechanisms and pathways as elegantly demonstrated in a study of pancreatic cancer [56]. The proteomic analysis indicated that many of the differentially expressed proteins between cancer and normal healthy pancreatic tissues are involved in protein-driven interactions between ductal epithelium and extracellular matrix remodeling to promote tumor growth, migration, angiogenesis, invasion, metastasis, and immunologic escape. These findings will no doubt facilitate identification of the candidate biomarkers for early responses to therapeutic interventions. Major disadvantages of this technique include the labor- and resource-intensive costs, the lack of quantitative data, low sensitivity, and a high rate of false-positive results. The assay requires a lengthy time for sample preparation, protein quantification and identification, and extensive bioinformatics analysis, which result in long assay times. Most importantly, it has been difficult to establish automation or to perform a full proteomics analysis in large-scale clinical trials. Nonetheless, future advances in high-throughput technology and bioinformatics processing mechanisms will likely improve the use of proteomics as a tool for immune monitoring.

T Cell Proliferation Assay

Although more sophisticated techniques, such as ELISpot and flow cytometry, are typically used for monitoring immune responses, standard T cell proliferation and cytotoxicity assays can often be used to help validate the more complex assays. The T cell proliferation assay is based on the ability of T cells to undergo proliferation on antigen exposure (usually in the presence of APC), and the proliferation can be measured by standard ^{3}H- thymidine incorporation during DNA synthesis. The assay is relatively easy to perform but can be a lengthy procedure that requires radioactive material. The proliferative status is often reported as a stimulation index (SI), which is calculated as the ratio between cell proliferation in the presence versus absence of antigenic stimuli. The SI has proved useful in measuring immune responses to a variety of vaccines [57]. SI values, however, do not adequately reflect the proliferative capacity of low-frequency responder T cells, and repeated in vitro nonspecific stimulation can influence the outcome. In fact, functional T cell anergy in vivo may be reversed during in vitro stimulation, and thus these assays may not reflect the in vivo status of patients [58]. The utility of this assay has nonetheless shown promise in the clinical setting. For example, in a trial using a p53 cancer vaccine in small cell lung cancer patients, clinical responses to subsequent chemotherapy were closely associated with an increased proliferation of p53-specific T cells [59]. In order to circumvent the use of radioactive material and to better define T cell responses, T cell proliferation has been measured by labeling the cells with the succinimidyl ester of carboxyfluorsecein diacetate (CFSE). As the CFSE-labeled cells proliferate in response to antigenic stimulation, the dye is diluted out in the dividing cells. The dilution of CFSE can be used to determine the number of cell divisions and calculate a proliferative index. This method also allows for better characterization of proliferating T cells as the cells are analyzed by flow cytometry to assess proliferation [60,61].

T Cell Cytotoxicity Assay

The potential cytotoxic activity of $CD8^{+}$ T cells can be directly measured by lysis of target cells expressing the cognate antigen recognized by the T cells. MHC-expressing cells pulsed with specific peptides or intact MHC-restricted tumor cells have been used as targets in T cell cytotoxicity assays. Traditionally, target cells are loaded with ^{51}Cr prior to incubation with different ratios with effector cells. The cytotoxicity is determined by the amount of radioactive ^{51}Cr released from the target cells after in vitro culture for several hours. The percent specific lysis is calculated by dividing the total release by maximum release with spontaneous release subtracted from both. This in vitro–specific cell lysis is considered to directly reflect the potency of cytotoxic effector cell killing in vivo. For this reason, many studies of cancer vaccines designed to induce $CD8^{+}$ T effector cells have attempted to demonstrate specific target cell killing in vitro using this assay. This has often been complicated in clinical trials because of difficulty in obtaining autologous tumor cells, the lack of defined antigenic peptides, the low

sensitivity and high background of the assay, and the need for large numbers of T cells. Another drawback to this assay is the lack of information at the single-cell level, which is possible with many of the other more sophisticated T cell assays. In addition, the frequent necessity for T cell in vitro stimulation (IVS) prior to cytotoxicity assay can also distort the true magnitude of effector T cell cytotoxicity in vivo. Alternatively, analysis of the molecules released during cell death, such as lactate dehydrogenase (LDH), is replacing the traditional ^{51}Cr release assay. In addition, it is most likely that flow cytometric analysis at the single-cell level of antigen-specific cell lysis will replace the bulky ^{51}Cr release assay in the future.

Advances in In Vivo Imaging

Direct in vivo imaging of the intact immune system is a rapidly advancing field and could provide real-time identification of various immune components responding to vaccines or immunotherapy. While the application of direct imaging in patients is just beginning, intriguing data from murine studies have supported this as a powerful approach for following in vivo immune responses. Currently available technologies to study tumors in murine models include bioluminescent imaging (BLI), magnetic resonance imaging (MRI), computed tomography (CT), and positron emission topography (PET). The BLI method enables tracking of the cellular flow patterns by following the images produced by luminescent or fluorescent molecules that have been integrated into the genomic DNA of the cells of interest. The most common reporter genes in use today are the green fluorescent protein (GFP) and luciferase (Luc) genes [62,63]. The use of BLI to monitor cells in vivo is quickly being applied to the characterization and treatment of various diseases, including cancer. In one study, human prostate cancer cells were transfected with a Luc gene and injected into SCID mice [89]. Careful analysis of tumor cells after transplantation demonstrated increased fluorescence over 14 days as the tumor grew, indicating the ability for tumor cells to stably express luciferase and use the technique for in vivo monitoring of tumor growth [64]. BLI has also been shown to be effective in monitoring the response to treatment of intraperitoneal tumors [65]. The human cervical carcinoma line HeLa transfected with Luc was injected into the peritoneal cavity of SCID mice. BLI was able to document a detailed regression of tumor in the mice after adoptive transfer of cytokine-induced killer (CIK) T cells [65]. Hu and coworkers elegantly demonstrated the trafficking of preactivated T cells labeled with superparamagnetic iron oxide nanoparticles in tumors that were undergoing immune rejection using MRI [66]. In another study, differentially labeled $CD4^+CD25^+$ Tregs and T_H cells could be tracked in the same lymph node using two-photon laser scanning microscopy. This study found that Tregs limited T_H cell access to the lymph node and decreased contact time with DCs, thereby preventing T_H cell activation [67].

Whole-body imaging can also be helpful in monitoring the immune response to immunotherapy. Thorne and colleagues were able to demonstrate that Luc-expressing CIK T cells infected with vaccinia virus were effective in eradicating ovarian tumors by releasing the virus only after infiltrating the tumor

[68]. This labeling technique of CIK cells further illustrates the potential for tracking immune cells in vivo. MRI, CT, and PET all offer more accurate three-dimensional structural imaging of tumors than BLI provided T cell clones can be appropriately labeled for detection without affecting their functional capability. The use of MRI, CT, and PET are quite expensive and require significant image collection time, which may limit their development. In contrast, BLI offers the advantage of being relatively inexpensive with short image collection times. The current application of BLI, however, is complicated by poor resolution profiles and difficulties in orienting tumor signals in a two-dimensional plane. The eventual application of in vivo imaging is a promising area, and further refinements in optics and labeling technology will likely see an expansion of this technique for real-time immune monitoring of cancer patients.

17.3 ASSAY VALIDATION

All immunologic monitoring assays should include extensive quality control and assay validation studies before use in cancer immunotherapy trials. There should be clear definition of a positive immunologic response to immunotherapeutic treatment taking into account assay imprecision. Preanalytical variability should be minimized by following standardized sample collection and processing and strict adherence to the standard operating procedures (SOP) of optimized assays. The inherent assay variability should be determined by performing assays in replicate samples (intraassay) and also by analyzing replicate samples from the same blood at different timepoints (interassay). Currently, there are joint efforts by laboratories across academics and industry to develop and optimize in vitro assays monitoring T cell function, including standardized protocols for use of all reagents, controls, and standards. The assays have to be developed for the maximum optimization of specificity and reproducibility. It is acknowledged that the best means of validating assays will be achieved through large multicenter cancer vaccine clinical trials [6]. This will allow for assay comparison among the participating laboratories in order to achieve universal standards with acceptable interlaboratory variations.

17.4 CONCLUSIONS

Each assay reviewed herein has unique advantages and disadvantages, and the selection of appropriate immune assays for monitoring clinical trials is not a trivial issue. The evaluation of cancer vaccines and immunotherapy regimens will require standardization of the selected assays and statistical analysis plans in place before an assay can be used for immune monitoring. The standardization of current monitoring assays is of high scientific priority for cancer vaccine evaluation. There are three key aspects for assay standardization and validation:

1. A set of robust assays needs to be established that are standardized and validated in both academic and industry laboratories. This requires some degree of cooperation among experts to select and develop particular assays.
2. Assays selected for patient monitoring require constant evaluation and characterization with attention to the use of standardized reagents (such as peptides, tetramers, control sera, virus panels), and these reagents need to be made available for common use with quality control measures.
3. Development of novel assays of potential value should be an ongoing effort and subject to review as they undergo translation from preclinical to clinical settings. Obviously, the ideal assay would correlate with clinical responses, and continued use of immune monitoring techniques as part of well designed clinical trials will help provide data to better define the potential effectiveness of individual assays. Assay selection may also depend on the type of vaccine or immunotherapeutic intervention.

The development of reliable, sensitive, accurate, and specific in vitro or in vivo immune monitoring assays that can successfully quantify and characterize immune responses in patients has been a major goal for tumor immunologists. Such assays would be beneficial for predicting vaccine efficacy and would also provide a method for furthering our understanding of antitumor immune mechanisms. Current data and prevailing opinion support the use of multiple functional assays, such as ELISpot and CFC in combination with other quantitative assays, like tetramer staining, for immune monitoring considering that immune responses to cancer vaccines involve a complex array of cellular and soluble factors [69,70]. Development and application of quantitative molecular approaches to measure the properties of single cells and complex tissue samples is also possible using molecular profiling and bioinformatic networking programs. The direct imaging of cells and molecular components of the immune response in vivo is in the early stages of development but offers an opportunity to directly visualize the immune response in patients. These new techniques should prove to be helpful in providing more relevant immune monitoring methods for predicting clinical responses to immunotherapy and will no doubt improve our understanding of the complex interactions between tumors and the host immune system.

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18

IMMUNE EVALUATION OF CANCER VACCINES

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18.1 INTRODUCTION

Antitumor vaccines are delivered to cancer patients with the goal of reversing the state of immune unresponsiveness to the tumor and inducing robust tumor-specific reactivity. This strategy is based on the principle that the host's immune system is involved in cancer development, progression, and elimination [1,2]. It has been documented that immune surveillance plays a role in protection from cancer development, and immune elimination of tumor cells involves a combination of innate and adaptive responses [3]. At the same time, the immune system may promote tumor survival by immune selection of tumor variants resistant to immune-mediated intervention [4]. In addition, tumors develop various mechanisms for evasion from the host immune system [5]. Thus, complex interactions between the host immune system and the tumor evolve and become established during cancer progression. Antitumor vaccines are designed to perturb these interactions and shift the balance in favor of the host. The most successful vaccines should be able to induce robust antitumor cellular and humoral immunity, culminating in tumor rejection and

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establishment of long-term immunologic memory. To determine how successful a vaccine is in achieving these goals, it is necessary to serially evaluate changes in tumor-specific responses before, during, and following vaccine administration.

Immune monitoring has become an essential component of all clinical trials testing vaccines and their antitumor efficacy. However, immune monitoring of patients with cancer represents a challenge for several reasons. First, the presence of cancer is associated with a degree of immune suppression, which may be quite considerable, if the tumor burden is large. The resulting immunologic imbalance may be difficult to measure and quantify, because responses to nontumor antigen and mitogens are normal, and only tumor-specific responses are compromised. This could reflect the existence of tolerance to self, as the majority of tumor-associated antigens are "self" [6]. It makes sense that patients who are to be enrolled in clinical protocols testing a therapeutic cancer vaccine should be evaluated for the integrity of their immune response. In patients with cancer, delayed-type hypersensitivity (DTH) responses to recall antigens are intact, except in advanced disease, and those to tumor epitopes are absent. Confounding effects of age, previous therapies, even a cancer type, which may or may not be strongly immunosuppressive, all complicate evaluation of the immune status in individual patients, so that consistent results across patient groups are a rare finding. Further difficulty arises from the fact that no established universal criteria are available for evaluation of tumor-specific immunity in candidates for cancer vaccines. Thus, the question of which patient is immunologically eligible for a vaccine remains unanswered. It would seem reasonable to assume that patients whose immune system is compromised by tumor presence are not the optimal candidates for antitumor vaccines. A general consensus has evolved, largely on the basis of experience, that vaccination after surgery or other primary therapy, in the setting of minimal residual disease (MRD) is likely to be more effective than that administered in the presence of tumor. However, no test currently exists that predicts a response to the vaccine. This creates a problem with selection of patients and selection of methods for monitoring. It further emphasizes the need for defining the criteria that would help in establishing guidelines for immune monitoring and evaluations of cancer vaccines.

18.2 TOOLS NECESSARY FOR SUPPORT OF VACCINATION THERAPIES

To be able to initiate and conduct vaccine-based therapeutic trials, a specialized laboratory, equipped and prepared to support vaccine production and postvaccine monitoring, is necessary. Services required of such a laboratory are extensive and varied (Table 18.1). The process of experimental vaccine introduction to the clinic begins with extensive preclinical evaluations. The laboratory responsible for vaccine production plays a critical role at this stage. In the case of investigator-initiated clinical trials, preclinical studies that lead to vaccine development are often done in experimental animals. The process of translating the concept and methods used in animal vaccination studies to the clinical arena may require extensive adaptation. In general, this involves reproducing vaccine generation under cGMP conditions

TABLE 18.1. Laboratory Services Necessary for Support of Cancer Vaccine Trials

Preclinical services
Adapt and translate research findings to humans
Establish vaccine composition and release criteria
Establish vaccine production process (for cellular vaccines)
Upscale vaccine production to meet clinical requirements
Vaccine preparation (cGMP)
Vaccine formulation and delivery to the clinic
Process human tissues to be used as an antigen source
Process leukapheresis products
Produce cellular vaccines
Test for safety and quality
Vaccine monitoring (GLP)
Prevaccine testing: HLA typing, DTH tests
Serial specimen collection, login, processing
Cryopreservation and banking
Assays on "batched" serial specimens
Result interpretation
QA/QC program to ensure reliability and meet regulatory requirements

with human tissues or cells and limited testing of the immunogenic potential of the vaccine in ex vivo experiments with human cells. Not all cancer vaccines require this type of preclinical investment, but cellular vaccines, such as dendritic cell (DC)-based vaccines, cannot be initiated without prior establishment and validation of the production–release process. As these steps are necessary for filing of an investigator-initiated drug (IND) application with FDA, all cellular vaccines undergo the preclinical development in a cGMP facility, which will be ultimately responsible for their production in a clinical trial. At this stage, the vaccine composition and its final characteristics (e.g., cell viability, purity, and potency) and the release criteria, which must include absence of microorganisms (vaccine sterility) and endotoxin levels (<5 endotoxin units (EU)/kg body weight/dose), are defined.

Vaccine production for therapy is a regulated process that should be performed in a cGMP facility. Even when noncellular vaccines are used, their formulation, including mixing of cGMP-grade peptides with an adjuvant and placing the vaccine in a syringe, requires a cGMP facility. Further, vaccines made with naturally occurring antigens, such as tumor-associated antigens (TAAs) present in apoptotic tumor cells, tumor cell lysates, or tumor cells fused with DC, require that tissue processing or tumor cell dissociation take place in a cGMP facility and be accompanied by sterility and endotoxin testing of all materials prior to their acceptance as vaccine components. An additional requirement is that the production facility be equipped to handle human tissues and large volumes of blood (i.e., leukapheresis products) operating under universal safety precautions established for blood and tissue products. In this context, all processed human specimens are considered to be potentially infectious and are handled as such. The vaccine production

takes place in a cleanroom, and it proceeds strictly according to standard operating procedure (SOP). The final product is tested as per specifications listed in the SOP, and it is released for therapeutic use only if the release criteria, which define safety and quality, are met.

Following the vaccine administration, it is advisable to establish its immunologic effects. To this end, serial monitoring of vaccine-specific or tumor-specific immune responses prior to, during, and after vaccine administration is performed. Monitoring of vaccine-induced immune responses is an essential component of all clinical trials testing antitumor vaccines and their efficacy. However, such monitoring is challenging and requires services of a laboratory skilled in performing serial large-scale testing and cognizant of a need for a high degree of precision that is an essential requirement for assay interpretation. Monitoring begins prior to vaccination, and is concerned not only with establishing an immunologic baseline but also with screening for the determination of subjects' eligibility, such as HLA typing or delayed-type hypersensitivity (DTH) reactivity. The former is needed when an immunizing epitope(s) is HLA-restricted, and the latter is often used as evidence that the patient can mount an immune response upon challenge with a recall antigen.

Specimens collected from subjects prior to, at defined intervals during, and at the end of vaccine-based therapy are delivered to the laboratory. The specimens usually consist of peripheral blood collected into heparinized tubes, but may include tumor or other tissues and body fluids (e.g., pleural or peritoneal fluids, ascites), as well as specially collected interstitial fluids from sites of cannulation [7]. The specimens for immune monitoring are harvested at intervals specified in the clinical protocol and arrive at the laboratory within 24 h of harvest. This requires an overnight delivery of specimens originating at a distant location. They are bar-coded and processed immediately on arrival. The separated cells are either cryopreserved at -80°C in 2-mL vials and banked or are immediately tested in assays that cannot be performed with cryopreserved/thawed cells. The monitoring laboratory is cognizant of assays that have to be performed on fresh as opposed to cryopreserved/thawed cells, and will be prepared to handle the specimens accordingly.

Changes occurring in the immune cell phenotype or function in response to therapy may be difficult to detect, unless sensitive and reliable monitoring assays are available. To decrease interassay variability, immune monitoring is generally performed with "batched" specimens, representing the entire collection of samples obtained from one subject throughout therapy. This type of design mandates that all collected specimens be cryopreserved under controlled conditions, thawed with a minimal loss of viability, and tested in the same assay. It also requires that the monitoring laboratory be equipped to perform cryopreservation and to bank and maintain samples on a large scale for prolonged periods of time. Assay "reliability" in this context depends on selection of those immune markers or functions and those procedures that are least affected by cryopreservation/thawing, which has to be a priori ascertained by the monitoring laboratory. This is done through comparisons of fresh and frozen specimens tested in the same assay. Experience shows that the

correctly performed process of freezing–thawing of immune or other cells is by far the most crucial determinant for preserving their true functional potential.

Interpretation of immune monitoring assays calls for special expertise that combines skills of a clinical immunologist with that of experienced biostatistician. The clinical immunologist is likely to be knowledgeable not only about the assays performed but also about the mechanisms presumably responsible for the observed immune activity. The statistician has to be familiar with the monitoring assays as well as hypotheses being tested and the rationale for the clinical trial design. Together with a clinician responsible for the trial design and execution, this team of collaborating investigators should be well poised to examine and analyze the data with a goal of establishing potential correlations between immune responses to the vaccine and clinical endpoints.

Finally, to ensure that the monitoring laboratory delivers reliable results, a QA/QC program that meets the cGTP standards is required. The program consists of numerous components, assembled to ensure that a laboratory is operated in compliance with the guidelines recommended by FDA. The QA/QC program supervised by a knowledgeable employee, who is independent of the laboratory director/supervisor and does not participate in the monitoring process, will almost certainly improve the quality of testing.

18.3 OBJECTIVES OF THERAPEUTIC CANCER VACCINES

Anticancer vaccines are designed to induce generation and activation of immune effector cells able to interact with tumor cells and eliminate them. In addition, anticancer vaccines should aim at establishing tumor-specific longlasting immunologic memory to prevent tumor recurrence or its metastasis. Still another desirable effect would be to induce alterations in the tumor microenvironment to enhance infiltrations with immune cells and impede tumor growth [8]. Once generated or activated in response to the vaccine, immune cells need to be sustained and protected from functional impairments or death, which is their fate in patients with cancer [9]. These objectives may not be easily achievable in view of immune suppression often associated with cancer [5,9]. While tumor-reactive antibodies and T cells specific for tumor epitopes are detectable in many patients with cancer [10,11], immune responses directed at the tumor are weak and, obviously, not effective in most cases. This is because the majority of tumor-associated antigens are known to be “self” [6], and the state of tolerance or the “lack of danger” results in a dampened response [12]. The presence in increased numbers of regulatory T cells (Treg) in the tumor milieu and in the periphery of patients with cancer further contributes to downregulation of antitumor responses [13]. In addition, tumor is known to produce factors and induce signals that inhibit antitumor responses by a variety of ingenious mechanisms leading to tumor escape (reviewed in Ref. 14). Therefore, an effective antitumor vaccine, which is expected to boost and sustain immunity to TAA, has to overcome a considerable array of obstacles to achieve its objectives. It is for these reasons that most of therapeutic cancer vaccines

delivered to cancer patients to date have not resulted in clinical responses, although in a number of instances, an enhancement of immune responsiveness against TAA was demonstrable [15,16]. One possibility is that patients with cancer can selectively respond to certain antigens but not others, in which case a selection of vaccinating epitopes becomes important. This possibility was considered by a team of Japanese investigators who advocate the use of "personalized peptide vaccination" [17]. Prevaccination blood specimens (cells and plasma) were collected from cancer patients and examined ex vivo for the presence of cellular or humoral responses to 25 HLA-restricted peptides derived from TAA. Only the reactive peptides (maximum of four) were selected for in vivo administration as components of the vaccine [17]. This type of vaccine, also containing Montanide as adjuvant, was shown to result in clinical responses in patients with malignant glioblastoma and to correlate with vaccine-induced immune responses [17]. In patients with cancer, the development of vaccine-induced immunity is uncertain, and thus, the selection of immunogens, use of adjuvants, and serial immune monitoring may all be necessary to realize the objectives of immunotherapy. Also, serial immune monitoring is the only way to link the vaccine-induced changes in immunity to clinical endpoints. Evaluation of immune responses to the vaccine as well as the tumor is, therefore, an integral and important part of a cancer vaccination program.

18.4 EVALUATION OF IMMUNE COMPETENCE PRIOR TO VACCINATION

Patients recruited to clinical vaccination protocols should be evaluated for the integrity of their immune system. For example, an immune screen before entry on the protocol could be performed. Such a screen could perhaps be used to define the eligibility criteria for the protocol, and could provide investigators with valuable insights about potential effectiveness of vaccination therapies in individual patients. However, the problem arises as to which immune assays to select for this immune screen and how to interpret the generated data in the context of a subsequent immunologic response to the vaccine. This is a dilemma, because no single immune marker associated with cancer progression has emerged so far. Consequently, a broad immune screen may not be relevant, as patients with cancer are immunologically incompetent with respect to their tumor but may have normal responses to mitogens or nontumor bacterial or viral antigens. A DTH response to the autologous tumor, when available, and to recall antigens and vaccine components, could be performed, and the serum could be analyzed for IgG and IgM antibodies (Abs) to the vaccine, such as described above for "personalized vaccinations" [17]. But so far, it has not been possible to gauge the degree of cancer-related incompetence any further, and it remains unclear whether the above-referenced prescreen is predictive of immune responses to the vaccine or how it correlates to clinical endpoints. More recent advances in immunology and biotechnology provide a basis for implementation of more sophisticated methods to both prescreening and monitoring of patients with cancer, as described below.

There are good reasons for concern about the immune status of patients with cancer. In view of well-documented immune dysfunction, especially evident in patients with advanced disease, vaccinations would not be expected to succeed. Even in the setting of minimal residual disease (MRD), the immune system may not be sufficiently recovered to effectively respond to a vaccine. The problem is related to the tumor escape from immune control, and the presence of multiple and insidious tumor-induced mechanisms enabling the escape [5,9]. These mechanisms appear to target T cells and persist even after the tumor is removed or treated [18]. For example, many of the effector T cells recovered from tumor sites, lymph nodes, or peripheral blood of cancer patients have signaling defects and are in the process of early apoptosis (e.g., bind Annexin V), making them "unfit" T cells [19,20]. An imbalance in circulating lymphocyte subsets and a persistently altered lymphocyte homeostasis are hallmarks of cancer, which do not readily normalize following oncologic therapies [21].

In view of these findings, the fate of immune cells, and especially of $CD8^+$ effector T cells, in patients with cancer remains of great concern [20]. The loss of these $CD8^+$ effector T cells through apoptosis does not bode well for results of immune therapies and provides a rationale for more careful evaluation of lymphocyte imbalance before vaccination. Clearly, a delivery of vaccines to patients who have compromised lymphocyte homeostasis and whose antitumor effector cells are dying does not make much sense. Therefore, a careful selection of patients with a minimal immune imbalance and of screening assays able to detect this imbalance *before* vaccination seems to be a reasonable strategy to adopt, although some would argue against such selection.

18.5 SELECTION OF ASSAYS FOR MONITORING OF CANCER VACCINES

Immune monitoring of vaccination protocols requires assays that can accurately measure vaccine-induced changes in the frequency and function of antitumor effector cells. These assays have to be adaptable to serial monitoring with a minimal loss of accuracy and to a high-volume testing. In all cases, it is important to select an assay that can accurately measure therapy-induced changes in an immune response relative to that measured at baseline.

Technical advances and new insights into immunologic mechanisms have led to the recent development of many new types of immune assays. Today, it is possible to choose between phenotypic and functional, specific versus nonspecific, direct versus indirect, or single cell versus bulk assays (reviewed in Ref. 22). Current emphasis has been on targeted assays able to assess specific activation pathways, even individual signaling molecules, identify subsets of cells or individual cells engaged in a response to a specific stimulus, or define levels of expression in components of the antigen processing pathway in a cell [23]. In general, tumor antigen-specific assays that can now be reliably and precisely performed are replacing older and less informative nonspecific tests. Multiplex assays able to define an immunologic profile, such as the Th1–Th2 cytokine profile, are

rapidly replacing assays able to measure only one analyte. A better understanding of immune mechanisms and hypothesis-driven monitoring are being combined in establishing immune measures as biomarkers of tumor progression or patient survival. An overall aim is to be able to reliably use immune measures as surrogate endpoints of clinical responses to therapy.

It is, therefore, not surprising that selection of a "right" assay for evaluation of responses to the vaccine is an important decision. An assay that accurately reflects vaccine-induced changes in the phenotype or function of immune cells may require adaptation to serial monitoring necessary for evaluation of cryopreserved "batched" specimens. Such specimen batching is routine and is done in order to test all serial samples collected from a patient in the course of therapy in the same assay. This practice avoids interassay variability and increases reliability. However, it imposes on the laboratory a need to compare the assay performance with fresh versus cryopreserved/thawed cells prior to its acceptance for monitoring. Certain assays, notably those that measure cytotoxicity, cannot be reliably performed with cryopreserved/thawed mononuclear cells [24]. Assays that must be performed on freshly harvested specimens require documentation of interassay variability, so that vaccine-induced changes in an immune measure can be distinguished from assay-related daily variability. Assays that are reliable when performed with cryopreserved/thawed specimens are the best candidates for serial monitoring of cancer vaccines.

Immunologic monitoring of cancer vaccine protocols has long ago progressed beyond ^{51}Cr-release bulk-type assays. Single-cell assays that detect the frequency of epitope-specific effector T lymphocytes have been widely used for evaluation of immune responses to cancer vaccines. Among these, cytokine flow cytometry (CFC), ELISpot, and tetramer binding measure the frequency of epitope-specific effector T lymphocytes in the mononuclear cell specimen [25–27]. All three assays are based on T-cell receptor (TCR) recognition of cognate peptides presented by MHC class I or class II molecules on the surface of antigen-presenting cells (APCs) to the responder T cells. However, no consensus exists as to which of these three assays should be selected to best monitor vaccination results. The common perception is that these assays are equivalent, that is, provide the same results. This is not correct, however. A comparison of the assays in monitoring frequencies of melanoma peptide-specific $CD8^+$ T cells in the peripheral circulation of subjects with metastatic melanoma who had received multiepitope DC-based vaccines was conducted in the author's laboratory [28]. This study estimated concordance between the three assays using a 3×3 scatterplot matrix design constructed for each of the four peptides tested in all three assays before and after vaccination therapy was completed. The three single-cell assays were not found to be concordant in measuring the frequency of immune effector cells in the peripheral blood of vaccinated subjects. The results for tetramer staining were consistently higher than those obtained with the ELISpot or CFC assay [28].

The ELISpot assay measures production of cytokines (most commonly either IFNγ or IL-5) by individual T cells in the plated population with a theoretical detection sensitivity of 1 in 100,000 cells [29]. CFC identifies single responding T cells

(1 in 50,000) with expression of a cytokine in the Golgi zone. Tetramer binding detects peptide-specific T cells expressing the relevant TCR with a theoretical detection sensitivity of 1 in 10,000 cells. The assays not only have different sensitivities of detection but also differ in specificity. ELISpot and CFC are antibody-based and, by definition, are highly specific. In contrast, tetramers, which are complexes of peptides sitting in grooves of four MHC molecules held together by a streptavidin–biotin scaffold [30], bind to T lymphocytes expressing the relevant TCR with variable affinity. Tetramers might easily dissociate or nonspecifically bind to B cells, monocytes, or apoptotic cells [31,32]. Thus, tetramer specificity needs to be carefully controlled. Further, T cells that bind tetramers may not be functional, as TCR signaling could be compromised, as often happens in cancer [33,34]. This reduces the tetramer-binding assay to a phenotypic category, because it detects T cells that bind tetramers but are not always functional [33]. CFC measures cytokine expression in a cell but not its secretion (although it is commonly assumed that the expressed cytokine would be secreted). Cell permeablization, necessary for intercellular staining of a cytokine in the Golgi zone, might introduce problems with immunodetection in CFC assays. ELISpot is based on a principle similar to that of CFC, but it measures cytokine *secretion* from individual stimulated responder cells that are plated as a monolayer of individual cells on a nitrocellulose membrane to allow for adequate spot display. Only ELISpot measures function of individual responder cells by identifying those that produce and secrete the measured cytokine. ELISpot also does not require cell permeabilization or the use of a flow cytometer for cytokine detection [26]. Because it is always preferable to measure function rather than phenotype, ELISpot would be an assay of choice. However, CFC and tetramer binding are flow cytometry-based assays and thus allow for surface labeling of responder cells and their identification. It is possible to select $CD8^+$ or $CD4^+$ T cell subsets on antibody-charged columns prior to ELISpot [35], and two-color ELISpot now available offers a possibility of identifying T cells simultaneously producing two cytokines [36]. In addition, supernatants from ELISpot wells can be collected and tested for cytokine levels in multiplex assays. On the other hand, tetramer binding can be combined both with surface staining to determine cellular phenotype and with intracytoplasmic staining for the detection of cytokine production [33]. While most informative, especially in situations when some of tetramer-binding cells do not express cytokines, this technology is time-consuming and labor-intensive: clearly, not the best choice for serial monitoring. The recommended solution would be to monitor responses by ELISpot or CFC (but not both), depending on considerations such as sample numbers, time, labor, cost, and access to a flow cytometer, and to use tetramer binding as a confirmatory assay in situations where it is important to demonstrate a functional deficiency of tetramer-binding T cells. ELISpot performed under strictly controlled, preferably good laboratory practice (GLP) conditions, is most likely to provide accurate estimates of the frequency of functionally competent effector T cells in batched, serial samples obtained from subjects enrolled in clinical studies. Compared to CFC and tetramer binding, the cost of ELISpot is reasonable enough to permit its use in a high-volume testing. However, the ELISpot assay is not easy

TABLE 18.2. Limitations of Immune Monitoring for Detection of Antigen-Specific T Cells after Administration of Cancer Vaccines

Single-cell assays measuring the frequency of antigen-specific T cells vary in sensitivity; sensitivity may be too low
Antigen-specific T cells localize to tumor sites or tumor-draining lymph nodes; not detectable in the peripheral blood
Paucity of well-characterized tumor antigens that could serve as targets of immune intervention; vaccine not immunogenic
Immune suppression in advanced disease; effector cells dysfunctional or undergoing apoptosis
Technical requirements are rigorous; assays not qualified for rare-event analysis of multiple clinical samples

to standardize, and responder–stimulator interactions might result in unacceptably high background spot counts, in which case the assay becomes uninterpretable.

Single-cell, antigen-specific assays are increasingly being used for monitoring of anticancer vaccines. However, many factors limit their usefulness, and it is necessary to be aware of these limitations (see Table 18.2). For example, antigen-specific T cells may be present with a low frequency in the peripheral circulation because of their localization to the tumor. Thus, even when rare-event analysis is performed using blood, antigen-specific effector cells may not be detected. Alternatively, the frequency of antigen-specific T cells might be low because of activation-induced cell death (AICD) or tumor-driven apoptosis of vaccine-induced effector cells [20]. Detection of antigen-specific T cells requires technically rigorous analysis that should be qualified for analysis of clinical samples.

To date no in vivo measures of immune response to the vaccine are available, except for DTH skin tests, which are not commonly used. They represent a simple and efficient strategy that could be useful not only for assessing the general status of recall immunity but also for determining the likelihood of mounting a response to the vaccine. It has been reported that a good qualitative relationship exists between DTH responses and ex vivo T cell responses to peptides used for vaccination in patients with cancer [37]. Most important, a conversion from a negative to positive skin test to the vaccine is an excellent sign of vaccine-induced immunity, and it has been reported to correlate with clinical responses in patients with melanoma. For these reasons, consideration should be given to a more frequent use of DTH tests measuring responses to vaccine components and administered prior to and after vaccination as an indicator for initiating and designing immunologic monitoring.

As discussed previously [38], a positive DTH skin test to the vaccine after its administration provides a strong rationale for immune monitoring, using peripheral blood or tumor-draining lymph nodes, if the latter are available. In all instances, vaccine-specific responses should be measured, although responses to PMA/ ionomycin, anti-CD3 antibody or mitogens measured in parallel could be used as a general guide for immune responsiveness of an individual patient. There are two

reasonable initial approaches: (1) measure antibody responses to the vaccine, and especially look for a shift from IgM to IgG responses in serial serum specimens; and (2) measure proliferative responses of lymphocytes (or cytokine production) to the vaccine in bulk cultures. Both can be considered first-line assays. It is essential that prevaccine and postvaccine specimens be tested together in the same assay. If both assays yield negative results, but a DTH skin test is positive, a good reason exists for further ex vivo testing because the bulk assays might not be sensitive enough to detect antigen (peptide)-specific responses. In this case, a decision is required of whether to proceed with ELISpot, CFC, or tetramer assay(s) *without* in vitro sensitization (IVS), although results may be negative if the precursor frequency is low, or to resort to single-cell assays performed after IVS. This latter strategy allows for in vitro expansion of antigen (peptide)-specific T cells before the assay, but it is time-consuming, labor-intensive, and costly. If both "screening" assays give positive results, it might be reasonable to proceed to single-cell assays *without* IVS for determining the frequency of antigen (peptide)-responsive T cells. A lack of systemic in vivo response to antigens (peptides) in the vaccine is strongly suggestive that ex vivo assays will be negative as well and that the patient is unlikely to respond immunologically to the vaccine. Nevertheless, immune monitoring with the most sensitive methods is necessary to formally document the absence of cellular immunologic responses. In such circumstances, the availability of a standardized, robust single-cell assay measuring function in addition to the cellular phenotype is of particular importance.

18.6 THE QA/QC PROGRAM AND ASSAY QUALITY

As indicated above, immune monitoring of patients enrolled in cancer vaccine trials should be performed in laboratories with established quality assurance (QA) and quality control (QC) programs that meet current standards. The QC of immunologic assays, especially cellular assays, is difficult, and that of serial immunologic measures represents a special challenge. To meet the challenge, a rigorously operated QA/QC program is the requirement for the monitoring laboratory. The program generally consists of several components, including a manual of standard operating procedures (SOP), policy manual, personnel training, instrument maintenance, reagent and temperature control, secure database, adverse-event review, quality review, and proficiency testing. Currently, no model QC program exists for immunologic monitoring, and the laboratories are encouraged to follow the self-imposed guidelines and confirm compliance by inspections offered by professional groups such as the College of American Pathologists (CAP). No proficiency testing programs are available for most immunologic assays, except those designed for flow cytometry or hematology (i.e., leukocyte count and differential). Participation in these programs is highly recommended. Largely, however, the monitoring laboratories are required to establish their own QA/QC program to ensure that acceptable data are generated in compliance with the CAP standard. In cases where this is not possible, services of a reference immune monitoring lab can be sought. Advantages

TABLE 18.3. Advantages of a Central Laboratory Operated as a GLP Facility

QA/QC program in place, ensuring the quality and reliability of monitoring
State-of-the-art technologies in use
Scaleup and high-throughput assays available
Assay development and standardization
Decreased cost of monitoring due to the large assay volume
Result interpretation by a clinical immunologist in conjunction with statisticians aware of immune-based analyses
Banking of samples that are accompanied by clinical outcome data for future research

of a central laboratory operated as a GLP facility are listed in Table 18.3. Such facilities exist in large medical centers or may be associated with NIH-supported cooperative groups.

Some of the QA/QC issues critical for vaccine monitoring are discussed below. Arrival of serial specimens in the monitoring laboratory has to be recorded either manually or electronically or, preferably, by both means. Blood samples for immunologic monitoring need to be routinely collected in the morning to avoid diurnal variability. Tissue samples and body fluids are processed differently than blood, and the laboratory has to be prepared to handle these specimens as they arrive. Specimens must reach the laboratory within 24 h of collection to be processed. A history of each sample is maintained, and problems are documented in writing and reviewed. As the decision to use fresh or cryopreserved cells for an assay will have been made prior to a clinical protocol initiation on the basis of preliminary comparative studies with fresh versus cryopreserved cells, the specimens are directed accordingly. Cryopreservation, using a rate control freezing device, is a routine but extremely important step in monitoring as is banking of cryopreserved specimens in liquid N_2 vapors. Both are performed under stringent controls because subsequent testing depends on the quality of cells that are stored frozen and thawed for testing. Similarly, aliquoted body fluids that are banked and batched for future testing must be protected from temperature fluctuations or inadvertent equipment failure. Hence, a temperature control program has to be in effect around the clock. An automated temperature control system is advisable. These activities are components of the QA/QC program that is necessary for reliable immune monitoring.

Assays routinely used for monitoring have to be standardized. The standardization data are obtained by repeatedly performing the assay with cells or body fluids obtained from healthy donors under previously established optimized invariant conditions to establish the mean, median, and 80% normal range and coefficient of variation. Intraassay variability is also determined, as batching of specimens in one assay is a usual operating procedure. A set of appropriate controls is selected, and these depend on the type of specimens. For example, in assays performed with fresh PBMC, which are collected at various timepoints, interassay variability is of concern. With freshly harvested cells, it is always advisable to include

fresh control cells obtained from a healthy volunteer. A cryopreserved lot of vialed PBMC obtained from a normal donor can be prepared, its range of reactivity can be determined, and thawed cells can be used in the assay to monitor day-to-day variability.

As an example, ELISpot assays established in the author's laboratory are performed according to the SOP requiring not only that appropriate positive and negative controls be included but that they also contain an assay control of PBMC obtained from a single donor and be cryopreserved in small aliquots [29,39]. These cells are thawed and tested after stimulation with PMA and ionomycin or OKT3 every time the ELISpot assay is performed. The CV for the assay calculated from 50 daily assays is 16%. The approach for determining inter- and intraassay variation of the ELISpot assay is outlined by Bennouna and colleagues [39] and various problems that often limit the usefulness of ELISpot vaccine monitoring as well as solutions are covered by Whiteside [26].

Batching of serial samples obtained from one patient in the same assay may dispense with concerns of interassay variability; however, even with this monitoring strategy in place, it is necessary to control for day-to-day differences to ensure that the assay performs equally well for all patients on the protocol whose batched specimens are likely to be tested on different days. The data obtained from control samples and evaluated in parallel with each patient sample can help to ensure the results validity. Whenever available, universal standards (such as cytokine standards available from NIH or WHO) should be regularly included in the monitoring assays.

Assay *standardization* should not be confused with assay *validation*. The former is a requisite part of the QC program; the latter is a formal evaluation of an assay performed as defined in the Bioanalytical Method Validation issued by FDA in May 2001 (21 CFR part 58) and qualifying it for use in phase III clinical trials as an immunologic endpoint. None of the assays currently available for monitoring of cancer vaccines have been validated. Validation consists of a series of experiments designed to evaluate accuracy, precision, selectivity, sensitivity, reproducibility, and stability characteristics of the method. While validation criteria are defined for chemical assays, immunologic assays do not always fit into these criteria. Nevertheless, because no separate guidelines exist for immunologic assays, those developed for chemical assays are generally followed. Validation of immunologic assays is expensive and faces a number of problems, especially with cellular assays, which typically have interassay CVs exceeding 20%. Further, no referenced standards for cellular assays are currently available. In contrast, validation of immune assays measuring soluble products, such as immunoglobulins or cytokines, can follow methods defined for the development of chemical assays.

18.7 SUMMARY AND CONCLUSIONS

Therapeutic cancer vaccines are expected to induce and sustain tumor-specific immune responses. Therefore, monitoring of immune responses to the vaccine

as well as the tumor is an important part of biotherapy. A broad range of antibody-based and cellular assays have been developed to measure phenotypic and functional attributes of tumor-reactive lymphocytes, regulatory T cells, natural killer (NK) cells, and other inflammatory cells contributing to or interfering with antitumor responses induced by the vaccine. Among them, single-cell assays: ELISpot, cytokine flow cytometry (CFC), and multimer binding, which can measure the frequency in the population of epitope-specific T cells, have become the core of a monitoring repertoire. Selection of assay(s) for monitoring of responses to a vaccine is an important decision requiring an understanding of the assay limitations. The sensitivity, specificity, and precision, including the coefficient of variation, of the assay(s) selected for monitoring should be established a priori. Its satisfactory performance with cryopreserved/thawed versus fresh specimens has to be confirmed. The challenges inherent to immune monitoring of serially acquired specimens are best met by an experienced reference laboratory operating under good laboratory practice (GLP) guidelines.

Immune monitoring of cancer vaccines is a costly and demanding but necessary task [40,41]. Its obvious purpose is to assess whether the patient reacts positively to the vaccine. Today, many ex vivo assays are available for use as monitoring tools (reviewed in Ref. 22). Among them, single-cell assays that determine the frequency of epitope-specific T cells in the peripheral circulation or body fluids have gained popularity and are being widely used. In general, emphasis has been on tumor antigen-specific humoral and cellular responses, although more recently, measurements of autoimmune responses in cancer patients receiving vaccination therapies have been reported to be useful in differentiating clinical responders from nonresponders [42]. Assays aiming at the detection of antigen-specific T cells have limitations and may require rare-event analyses. On the other hand, antibody detection is relatively straightforward and should be a routine part of immune monitoring. Functional assays represent a special challenge, as cryopreservation and specimen batching often interfere with cellular functions. Assay standardization and the selection of robust, reliable monitoring methods used in the setting of an established QA/QC program are key to successful evaluations of cancer vaccines.

Immediate future directions in cancer vaccine evaluations are likely to include targeted immunologic assays measuring regulatory T cells, transcription factors, antigen-processing machinery, and apoptosis. Multiplex formats and arrays will replace individual assays to allow for defining of immune profiles. As our understanding of molecular mechanisms responsible for immune responsiveness or nonresponsiveness to vaccines increases, monitoring is likely to become hypothesis-driven. Immune biomarkers will be identified that may become surrogate endpoints of disease progression and response to vaccination therapies. Finally, it is expected that criteria for validation of immune (cellular) assays will be established, thus enabling reliable evaluation of antigen-specific responses to cancer vaccines.

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