Priming of Therapeutic T Cells for Adoptive Immunotherapy

Hong-Ming Hu
B.S., Fudan University, Shanghai, China, 1987
M.S., Academia Sinica, Shanghai, China, 1990

A dissertation presented to the faculty of the
OGI School of Science & Engineering
at Oregon Health & Science University
in partial fulfillment of the
requirements for the degree

Doctor of Philosophy
in
Biochemistry and Molecular Biology

January 2003

The dissertation "Priming and Effector Functions of Therapeutic T Cells for Adoptive Immunotherapy" by Hong-Ming Hu has been examined and approved by the following Examination Committee:

Bernard A. Fox, Advisor
Earle A. Chiles Research Institute
Associate Professor (Joint)
Oregon Graduate Institute/Oregon Health and Science University

Michiko M. Nakano
Research Associate Professor

Peter Zuber
Professor

Andrew D. Weinberg
Associate Professor
Oregon Health and Science University

ACKNOWLEDGMENTS

My greatest debt and appreciation go to Dr. Bernard A. Fox, who brought me to the beautiful state of Oregon and introduced me to the field of Tumor Immunology. It proved to be a wonderful and rewarding journey, not only for my professional life but also for my personal life. His encouragement has allowed me to develop my own research interests and skills and has given me the privilege to enjoy scientific research as a very important part of my life. His determination to help me to develop as an independent scientist has made my childhood dream come true. Without his strong support and advocacy, I would not have the opportunity to head my own research lab.

I would also like to express my deep appreciation to Dr. Walter J. Urba. His leadership and vision have made me firmly believe that I not only can do great science, but I can also help cancer patients by translating scientific discoveries into novel clinical trials at the Earle A. Chiles Research Institute. He has also helped me improve my writing skills and improved the quality of my research papers tremendously.

I thank Dr. Weinberg for being a great source of knowledge in immunology over the past years. I also thank Drs. Michiko Nakano and Peter Zuber for their kindness and willingness to serve on my dissertation committee. It was greatly appreciated.

In addition, I thank Dr. Suyu Shu for his advice and encouragement for many years; he has been the greatest role model for me. I also want to thank the following past and present members of Dr. Fox's lab: Hauke Winter, Yiwei Chu, Jane Yamada, Shawn Jasen, Christian H. Poehlein, Sybren L. Meijer, Annemieke Dols, Tarsem Moudigil, Michael LaCelle, Jun Ma, Dorminik Ludinger, and my first postdoctoral fellow Rui Li. It has been and will continue to be a wonderful experience for me with their support and friendship.

DEDICATION

This thesis is dedicated to my wife,

Zhuwei Cao,

for her support and love.

TABLE OF CONTENTS

ACKNOWLEDGMENTS iii
DEDICATION iv
TABLE OF CONTENTS v
LIST OF TABLES x
LIST OF FIGURES xii
ABSTRACT xiv

CHAPTER 1 INTRODUCTION 1
1.1 The Historical Perspective of Cancer Immunotherapy 1
1.2 Gene Therapy Approaches 4
1.2.1 Gene Transfer to Augment Tumor Immunogenicity 7
1.2.2 Antigen-based Gene Transfer Strategies 9
1.2.3 Allogeneic MHC Gene Transfer 9
1.2.4 Superantigen Gene Transfer 13
1.2.5 Tumor Antigen Gene Transfer 13
1.2.6 Co-stimulatory Signals 15
1.2.7 Adhesion/Accessory Molecules 17
1.3 Immune Deviation as a Mechanism for Immunological Tolerance to Tumor Antigens 19
1.3.1 Definition of Tolerance 19
1.3.2 Type 1 and Type 2 Immune Responses 20
1.3.3 Three-Signals Hypothesis for Priming of Therapeutic T Cells 23
1.4 Summary of Main Chapters of Thesis 30

CHAPTER 2 GENE-MODIFIED TUMOR VACCINE WITH THERAPEUTIC POTENTIAL SHIFTS TUMOR-SPECIFIC T CELL RESPONSE FROM A TYPE 2 TO A TYPE 1 CYTOKINE PROFILE 32
2.1 Introduction 32
2.2 Materials and Methods 34
2.2.1 Mice 34
2.2.2 Tumor Cell Lines 34
2.2.3 Reagents 34
2.2.4 Culture Conditions 35
2.2.5 Immunization and Tumor Challenge 35
2.2.6 Lipofection of Tumor and Vaccination for Adoptive Immunotherapy 35
2.2.7 Separation and Activation of TVDLN 36
2.2.8 Adoptive Immunotherapy 37
2.2.9 Measurement of Cytokines 37
2.2.10 Statistical Analysis 38
2.3 Results 38
2.3.1 D5 Is a Poorly Immunogenic Tumor 38
2.3.2 L-Selectin Expression of TVDLN 40
2.3.3 Reduced L-Selectin Expression Identifies Therapeutic T Cells in LN Draining Gene-Modified Vaccine 43
2.3.4 Cytokine Production by L-Selectin$^{low/-}$ T Cells Following Anti-CD3 and IL-2 43
2.3.5 Cytokine Production by Freshly Isolated TVDLN 48
2.4 Discussion 50

CHAPTER 3 DIVERGENT ROLES FOR CD4^{+} T CELLS IN THE PRIMING AND EFFECTOR/MEMORY PHASES OF ADOPTIVE IMMUNOTHERAPY . 56
3.1 Introduction 56
3.2 Materials and Methods 57
3.2.1 Mice and Tumor Cell Lines 57

3.2.2 Adoptive Immunotherapy 58
3.2.3 Monoclonal Ab-Mediated Depletion of NK Cells or T Cell Subsets 59
3.2.4 Flow Cytometric Analysis 59
3.2.5 Cytotoxicity Assays 60
3.2.6 Survival and Tumor Challenge Experiments 60
3.3 Results and Discussion 61
3.3.1 Increased Number of Dendritic Cells in D5-G6 TVDLN, But Not D5 TVDLN 61
3.3.2 Increased Number of T Cells with Memory Phenotype in D5-G6 TVDLN 61
3.3.3 D5-G6 Primes Therapeutic T Cells in MHC Class II KO Mice . . . 64
3.3.4 $CD4^+$ T Cell-Independent Priming of CTL 65
3.3.5 Regression of Established Pulmonary Metastases Following Adoptive Immunotherapy Is Independent of Host T Cells or NK Cells 69
3.3.6 Long-Term Survival after Adoptive Immunotherapy with $CD8^+$ Effector T Cells 71
3.3.7 Transfer of $CD8^+$ T Cells Prolongs Survival but Does Not Cure MHC II KO Mice 71
3.3.8 Memory Responses in Mice Cured by Adoptive Immunotherapy . . 74
3.3.9 Requirement for $CD4^+$ Helper T Cells in Maintaining the Protective Memory Response 74
3.3.10 Depigmentation in Mice That Survived a Tumor Challenge 76

CHAPTER 4 CD28, TNF RECEPTOR, AND IL-12 ARE CRITICAL FOR CD4-INDEPENDENT CROSS-PRIMING OF THERAPEUTIC ANTITUMOR $CD8^+$ T CELLS 81
4.1 Introduction 81
4.2 Materials and Methods 82
4.2.1 Mice 82

4.2.2 Tumor Cell Lines and mAbs 82
4.2.3 In Vivo Priming and In Vitro Activation 83
4.2.4 Adoptive Immunotherapy 83
4.2.5 Flow Cytometric Analysis 84
4.3 Results 85
4.3.1 Priming of Therapeutic Cells Is Independent of CD40/CD40L Interaction 85
4.3.2 Costimulation by Either CD80 or CD86 Is Critical for Priming Therapeutic T Cells 87
4.3.3 Blocking Costimulatory Molecules Reduces Expression of T Cell Activation Markers 89
4.3.4 IL-12 and TNF Are Required for Priming Therapeutic T Cells in MHC Class II Deficient Mice 89
4.3.5 Costimulation by CD80 and CD86 Is Not Required for T Cell Effector Function 97
4.4 Discussion 97

CHAPTER 5 DEVELOPMENT OF ANTITUMOR IMMUNE RESPONSES IN RECONSTITUTED LYMPHOPENIC HOSTS 104
5.1 Introduction 104
5.2 Materials and Methods 105
5.2.1 Mice, Tumor Cell Lines, and Monoclonal Antibodies 105
5.2.2 Reconstitution, Vaccination and In Vitro Sensitization 106
5.2.3 Adoptive Immunotherapy 106
5.2.4 Intracellular Cytokine Staining and Fluorescence-activated Cell Sorter Analysis 107
5.2.5 Cytotoxicity Assay 107
5.3 Results 107
5.3.1 RLP RAG1 Hosts Developed Strong Tc1 and Th1 Responses . . . 107
5.3.2 Effector T Cells Obtained from Vaccinated RLP RAG1 Mice Are Highly Therapeutic 112

5.3.3 Delayed Vaccination Primes T Cells with Less Therapeutic Activity 114
5.4 Discussion 118

LITERATURE CITED 122
BIOGRAPHICAL SKETCH 148

LIST OF TABLES

1.1 Gene Transfer Options Available to Augment an Immune Response to Tumors 8

1.2 A Three-Signal Model for the Generation for the Design of Therapeutic Antitumor Immunity 27

2.1 Lipofection with an Allogeneic Class I Gene Enables D5 Tumor Cells to Sensitize T Cells with Therapeutic Potential 41

2.2 Vaccination with D5-K^d, but Not Unmodified D5, Sensitizes T Cells in the L-Selectin$^{low/-}$ Populations of TVDLN That Following Adoptive Transfer, Mediate Tumor Regression 44

2.3 Anti-CD3-Activated T Cells from Either Vaccine Contain the Same Distribution of CD4 and CD8 T Cells but Exhibit Opposing Tumor-Specific Cytokine Profiles In Vitro and Antitumor Activity In Vivo 45

3.1 Therapeutic T Cells Can Be Generated from MHC II KO Mice 66

3.2 $CD8^+$ But Not $CD4^+$ Effector T Cells Are Therapeutic 68

3.3 Regression of Established Pulmonary Metastases After Adoptive Transfer Dose Does Not Require Receipt NK, $CD4^+$, or $CD8^+$ T Cells 70

4.1 Anti-CD40L Fails to Block Priming of Therapeutic T Cells 86

4.2 Generation of Therapeutic T Cells from CD40L KO Mice 88

4.3 Anti-CD80 or Anti-CD86 Does Not Inhibit T Cell Priming 90

4.4 Anti-CD80 and Anti-CD86 Inhibits T Cell Priming 91

4.5 IL-12, But Not CD40L, Is Required for T Cell Priming in MHC II KO Mice 94

4.6 Priming of Therapeutic T Cells in MHC II KO Mice 96

4.7 Effect of Blocking CD80 and CD86 after Adoptive Transfer of Effector T Cells 98

5.1 Concomitant Vaccination and Reconstitution in RAG1 Mice Results in Highly Therapeutic T Cells 115
5.2 Delayed Vaccination in RAG1 RLP Mice Results in Less Therapeutic T Cells 117

LIST OF FIGURES

1.1 Basic animal model for tumor vaccination and adoptive immumotherapy with in vitro activated TVDLN T cells 3

1.2 A working hypothesis for tumor vaccines based on gene-modification with allo-MHC molecules 11

1.3 Schematic overview of differences in immune response observed in sentinel nodes draining immunogenic or nonimmunogenic tumors 25

1.4 Clinical trial design for HLA-B7 gene-modified tumor vaccine and adoptive transfer of in vitro activated TVDLN in melanoma and renal cell carcinoma patients 29

2.1 Mice were immunized with 10^7 gamma-irradiated D5 tumor cells and were challenged 14 days later with viable D5 at one of three doses 39

2.2 FACS analysis of L-selectin expression on freshly isolated naive LNC, D5 TVDLN, and D5-K^d TVDLN cells 42

2.3 Anti-CD3 activated and IL-2-expanded effector T cells generated from L-selectin$^{low/-}$ or L-selectin^{high} D5 or D5-K^d TVDLN were assessed for tumor-specific cytokine release at the time cells were adoptively transferred into tumor-bearing hosts 47

2.4 L-selectin$^{low/-}$ cells were isolated from freshly harvested naive or day 8 D5 or D5-K^d TVDLN and activated with anti-CD3 (2c11) for 2 days, and supernatants were harvested 49

3.1 D5-G6 vaccination increased the percentage of dendritic cells in D5-G6 TVDLN 62

3.2 Both CD4 and CD8 T cells are primed in D5-G6 TVDLN 63

3.3 CD4-independent priming of CTL in mice vaccinated with D5-G6 67

3.4 Long-term survival in mice following adoptive immunotherapy 72

3.5 Adoptive immunotherapy fails to cure tumor bearing MHC II KO mice . . 73
3.6 Development of antitumor immunity in mice following adoptive immunotherapy 75
3.7 $CD8^+$ T cells in immune mice play a major role in protection against a secondary tumor challenge 77
3.8 Adoptive transfer of effector T cells from TVDLN of D5-G6 vaccinated mice also induced depigmentation in long-term surviving mice 78
4.1 $CD8^+$ T cell activation in D5-G6-vaccinated mice requires costimulation either by CD80 or CD86, but not CD40L 92
4.2 Schematic of CD4-independent cross-priming of $CD8^+$ effector T cells . . 101
5.1 Expression of MHC class I and class II molecules on melanoma cell lines 109
5.2 Selective expansion of IFN-γ producing and TNF-α producing $CD4^+$ and $CD8^+$ T cells in vaccinated normal and RLP RAG1 mice 110
5.3 TVDLN T cells derived from vaccinated RLP RAG1 mice were highly cytolytic 113
5.4 Concomitant vaccination of reconstituted RAG1 mice generates T cells with greater therapeutic activity than delayed vaccination 116

Abstract

Priming of Therapeutic T Cells for Adoptive Immunotherapy

Hong-Ming Hu

Ph.D., OGI School of Science and Engineering
at Oregon Health & Science University
January 2003

Thesis Advisor: Dr. Bernard A. Fox

One major goal of cancer immunotherapy is to prime tumor-specific T cells that mediate tumor regression. In the past four years, my work has focused on the characterization of T cells that mediate tumor regression using an adoptive transfer model. T cells were derived from tumor vaccine-draining lymph nodes and transferred into mice bearing established tumors after in vitro activation and expansion. We found the following: (1) Therapeutic T cells have a type 1 cytokine profile; (2) non-immunogenic tumors are not ignored by the host's immune system, instead they elicit a non-protective type 2 cytokine response; (3) priming of $CD8^+$ therapeutic T cells can occur independent of $CD4^+$ T cell help when the tumor vaccine produces GM-CSF; (4) $CD4^+$ T cells are critical for long-term survival of tumor-bearing mice after adoptive immunotherapy; (5) mice cured by adoptive immunotherapy also developed autoimmune depigmentation; (6) with a GM-CSF-producing tumor vaccine, CD28, TNF and IL-12 are required for the priming of therapeutic $CD8^+$ T cells in the absence of $CD4^+$ T-cell help; and (7) priming of melanoma-specific T cells by GM-CSF-producing vaccine can be significantly

increased if vaccination is performed in reconstituted lymphopenic hosts. These results can be used to optimize strategies for the development of an effective vaccine for patients with cancer.

CHAPTER 1
INTRODUCTION

1.1 The Historical Prospective of Cancer Immunotherapy

In 1888, Dr. William B. Coley, a surgeon at Memorial Hospital in New York City, performed the first trial of "immunotherapy of cancer" by infecting cancer patients with bacteria and later by vaccination with killed bacteria (Coley's toxins) [Coley, 1991]. Some quite remarkable clinical results were observed in individual patients, but the approach fell out of favor because of toxicity and low response rate. The field of tumor immunology reemerged in the 1940s and 1950s when Gross, Prehn and Main demonstrated that transplantable tumors from inbred animals could be rejected by hosts that had received tumor vaccines prior to tumor transplantation [Schreiber, 1993]. They also discovered that transplantation immunity was primarily mediated by T cells, and that adoptive transfer of T cells from vaccinated animals could mediate regression of established transplanted tumors [Schreiber, 1993]. Later, in the 1980s and 1990s, Dr. Steven A. Rosenberg of the National Cancer Institute, started to translate the adoptive immunotherapy findings from mice into clinical trials for patients with melanoma and renal cell carcinoma. The discovery by Dr. Thierry Boon and colleagues, as well as Dr. Rosenberg and colleagues, that T cells from animals and humans could recognize tumor-specific antigens finally put to rest any doubt that the adaptive arm of the immune system was involved with tumor recognition [Boon et al., 1994; Rosenberg, 1999].

Dr. Suyu Shu started a new immunotherapy approach that combined tumor vaccination with in vitro sensitization of tumor vaccine draining lymph node (TVDLN) T cells, which was applicable to patients with various tumor types [Shu et al., 1989; Yoshizawa et al., 1991a]. Fig. 1.1 shows the flow chart of basic

experiments for performing tumor vaccination, harvesting of TVDLN, in vitro activation with anti-CD3, and expansion in media with low dose IL-2 (60 IU/ml). Subsequently, T cells are adoptively transferred into mice with established lung metastases by intravenous injection of tumor cells 3 days before the T cell transfer. In most experiments, mice were also given i.p. injection of IL-2 for 3 days to support T cell survival and function. The number of lung metastases are enumerated 11–13 days post therapy (Fig. 1.1).

Naive mice (T cell donors) were inoculated with live tumor cells (D5-G6, GM-CSF-producing B16BL6/D5 cells) subcutaneously; 8 days later TVDLN (inguinal lymph nodes) were harvested. Single cell suspensions from TVDLN were activated with anti-CD3 monoclonal antibody (5 μg/ml) at a cell density of 2 million cells/ml complete media (CM) for 2 days. Activated T cells were then expanded with recombinant interleukin-2 containing CM (60 IU/ml) at a cell density of 0.15 million cells/ml for another 3 days. Three days before adoptive transfer of activated T cells, mice were injected with live parent D5 tumor cells (tumor-bearing recipient mice) intravenously. Tumor-bearing mice were then treated with a graded number of adoptively transferred T cells. Recombinant IL-2 (90,000 IU per injection per mouse) was also administrated in mice that received no T cells (IL-2 only treatment control) or T cells twice a day for a total of 3 days. Ten to 13 days after T cell transfer, mice were sacrificed and the number of pulmonary metastases of harvested lung were enumerated. Each treatment group used at least five mice.

The experiments described in this dissertation are based on Dr. Shu's initial observation that adoptive immunotherapy is a powerful approach to augment the immune system against tumors. Over the past four years, we have tried to answer two major questions: (1) What is the requirement for priming tumor-reactive T cells in vivo to be therapeutic upon adoptive transfer, and (2) can the number of tumor-reactive T cells be increased by active immunization? These are some of the questions that were unknown when I started my doctoral research program. To appreciate the answers to these questions additional background information is provided.

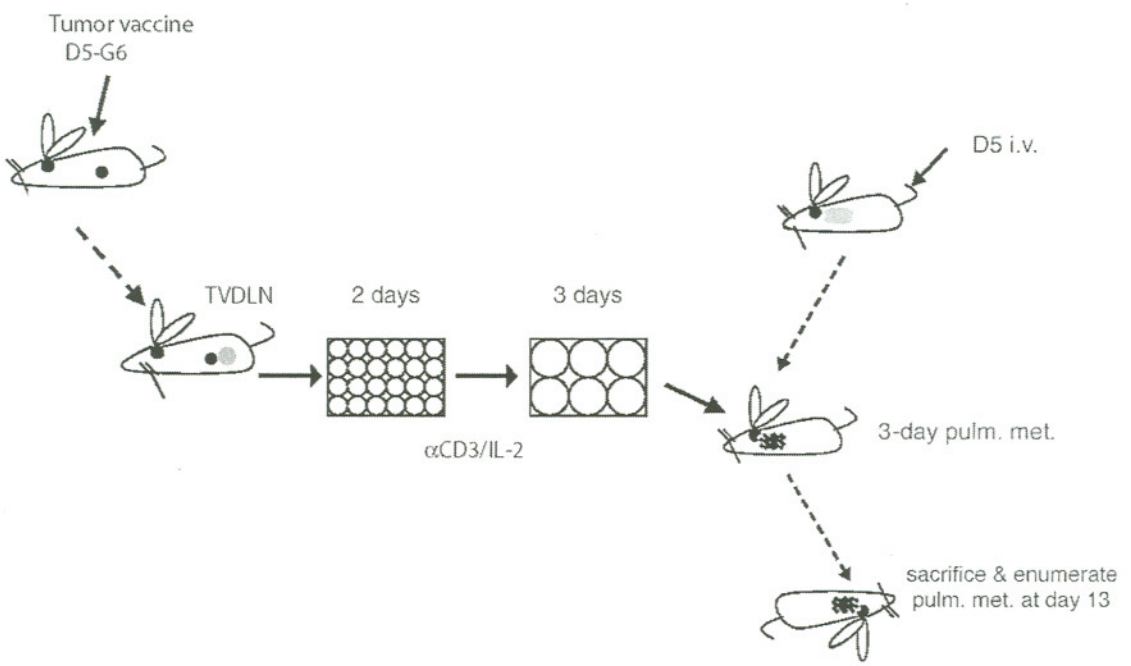


Fig. 1.1 Basic animal model for tumor vaccination and adoptive immunotherapy with in vitro activated tumor vaccine draining lymph node (TVDLN) T cells.

The induction of T cell immune responses to tumor antigens (T cell priming) primarily occurs in TVDLN. The secondary lymphoid tissues are where antigen presenting cells (APC) collect antigens from tumor debris, process them, and present them to naive T cells. Dendritic cells are the key professional APC that play a pivotal role in determining the fate of naive T cells after their initial encounter with antigens. Dendritic cells present peptide antigens to CD4 and $CD8^+$ T cells through association with major histocompatibility complex (MHC) class II or class I molecules, respectively. This process of indirect presentation of tumor antigens via host dendritic cells, rather than tumor cells themselves, is termed "cross-priming". In addition to MHC molecules, dendritic cells also express an array of co-stimulatory molecules and adhesion molecules, as well as produce cytokines. All of these functions determine the fate of the antigen-activated T cells. The combination of signals delivered by these molecules to naive T cells ultimately determines the fate of T cells by turning on or off the programs of cell proliferation, differentiation, survival, and apoptosis. The goal of tumor immunotherapy is to fine-tune the host's immune system so that it initiates and maintains an adequate immune response to tumor-associated antigens. This is particularly true for those responses mediated by tumor-specific memory and effector T cells, because they can ultimately mediate tumor eradication. Understanding the requirements for priming, expansion and skewing of the T cell response to tumor antigens is critical for the design of therapeutic tumor vaccines. But how is it possible to modify the immune response to tumor vaccines? The advent of gene therapy has provided one mechanism to modify the immune response. This has been done by the transfer of genes encoding antigens, co-stimulatory molecules, or adhesion/accessory molecules.

1.2 Gene Therapy Approaches

Tumors grow progressively and kill both animal and human hosts, in part because they are not recognized as foreign by the immune system. This failure of the immune response may be due to a lack of tumor immunogenicity, induction of tolerance, or tumor-induced immunosuppression. The first two possibilities could

result from the absence of a tumor antigen or inadequate antigen presentation in the tumor [Germain, 1993]. The recent description of multiple tumor rejection antigens in melanoma and other tumors, as well as the observations that alterations in intracellular proteins such as p53 and Ras can also act as tumor antigens [Cheever et al., 1993; Yanuck et al., 1993; Stuber et al., 1994], suggests that the former is probably not the major problem. However, deficiencies in the critical antigen presentation pathway could easily result in tumor cells that do not present tumor antigen(s) in a manner necessary to initiate the afferent limb of a cellular immune response. The identical result may also occur if MHC antigens, co-stimulatory or accessory molecule expression on tumor cells, are reduced or eliminated, thereby effectively blocking the process of immune recognition [Weber and Jay, 1988; Garrido et al., 1993, 1997]. Additionally, tumor-associated antigens are frequently among classes of molecules expressed during ontogeny or as molecules unique to specific tissues. Since Burnett and Medawar and colleagues [Silverstein, 1993], this pattern of expression has been accepted as a means of tolerizing the host to self molecules. Thus, tumor-associated antigens are generally thought to possess inherently less potential to illicit a therapeutic anti-tumor immune response. Alternatively, tumors may progress because of a tumor-induced immunosuppressive environment, which interferes with the development of potentially protective immune responses. Recent reports have documented a number of molecular defects in T cells obtained from tumor-bearing hosts that could explain the inability of the immune system to prevent tumor progression [Mizoguchi et al., 1992; Finke et al., 1993; X. Li et al., 1994]. Therein lies the possible explanation for the failure of immune-based strategies to effectively treat cancer. With at least one, and sometimes more, of these scenarios active in any patient, what are the possibilities of successfully altering the natural course of this disease?

Within the last decade, gene transfer technology has evolved so that vectors for a variety of gene products can be designed, delivered, and expressed in tumor cells in vitro or in vivo. This technology provides opportunities to manipulate the host's immune response to the tumor and thereby promote recognition and elimination of the malignancy. While current genetic approaches often focus entirely on

immunological interventions, the introduction of chemo- or radiosensitivity genes may also stimulate an efficient anti-tumor immune response to reduce tumor burden or eliminate metastatic foci. Undoubtedly, certain combinations of these genetic interventions will offer advantages over single intervention strategies. However, the possible combinations are essentially unlimited. Thus, the challenge for the next decade is to logically design and carefully test, in the most appropriate animal models, this next generation of treatment strategies.

This component of my dissertation focuses on one part of the immunological component of these genetic interventions: namely, the potential role for the transfer of genes encoding antigens, co-stimulatory molecules, or adhesion molecules into tumor cells, or other accessory cells, to bolster the immune response to tumors. The role for cytokine gene transfer into tumors or gene transfer into immune effector cells is not discussed; however, a brief review of some possible candidates in each family of specified molecules is included. Their advantages and limitations, and propose combinations that may synergize and offset the immunosuppressed or inadequate anti-tumor immune response is discussed.

The introduction of novel genetic information into eukaryotic cells has been accomplished by chemical, physical, and biological techniques. A number of these gene transfer approaches are readily transferable to clinical settings. These methods include naked DNA, DNA–ligand complexes, DNA–lipid complexes, particle-mediated gene transfer, and viral and bacterial vectors. The chemical/physical gene transfer approaches (naked DNA, DNA–ligand complexes, DNA–lipid complexes, and particle-mediated gene transfer) are relatively inefficient, generally fail to achieve integration of the gene into host chromosomes, and therefore are only expressed transiently and may result in less gene product produced than other vector systems. However, the deficiencies inherent in these approaches may also be viewed as strengths. For instance, the transient expression of a plasmid vector, due to its episomal (nonintegrated) form tends to limit the duration of gene expression. Such limited expression may be advantageous when delivering strong antigens, cytokines or co-stimulatory signals, since extended expression, particularly of certain cytokines may induce significant toxicity. The failure of these plasmids to integrate also

reduces the potential for insertional mutagenesis. The apparent safety of these gene transfer approaches will undoubtedly encourage their use as adjuvant therapy in settings of minimal residual disease, which are more likely to be amenable to immunological interventions. Alternatively, viral vectors offer more efficient delivery and higher levels of gene expression in target tissues. The additional advantage of integration and possible permanent gene expression make this approach necessary for treatment of many genetic disorders. However, the unknown risks associated with these approaches make them less desirable where permanent expression is unnecessary or for vaccination strategies in an adjuvant therapy setting.

A list of strategies with the potential to alter the immune response to tumors is presented in Table 1.1. The following sections divide these approaches into three major categories based on their immunological properties: (1) antigens, (2) co-stimulatory, or (3) adhesion/accessory molecules.

1.2.1 Gene Transfer to Augment Tumor Immunogenicity

Antigen recognition by T cells involves the engagement of the T cell receptor with a small peptide presented by an MHC or human leukocyte antigen (HLA) class I or class II molecule [Hendrick and Eidelman, 1993]. Therefore, immune recognition of tumors by T cells requires that tumors express both an antigen peptide and either an MHC class I or class II molecule. Numerous investigators have demonstrated defects in MHC expression on both human and animal tumors, and proposed a role for this deficit in tumor progression [Weber and Jay, 1988]. A decade ago, Hui and colleagues demonstrated that a tumor, which failed to express detectable levels of MHC class I and grew progressively in syngeneic hosts, was rejected following transfection with a syngeneic MHC class I molecule [Hui et al., 1984]. This observation, since reproduced by multiple investigators, identified one mechanism by which tumors escape recognition by the immune system and strengthened the argument for an immunological approach to cancer therapy [Tanaka et al., 1985, 1986; Wallich et al., 1985; Weber and Jay, 1988].

Table 1.1

Gene Transfer Options Available to Augment an Immune Response to Tumors

(A) Antigens

Alloantigens
Superantigens
Tumor-specific antigens, e.g. mutated Ras, p53
Tumor-associated antigens, e.g. melanoma differentiation antigens, such as gp100.

(B) Co-stimulatory/Accessory Molecules

Receptors	*Ligands*
CD28/CTLA-4	CD80 (B7) and CD86 (B70/B7-2)
OX40	OX40 Ligand
4-1BB	4-1BB Ligand
CD40	CD40 Ligand

(C) Accessory/Adhesion Molecules

T cell receptors	*Ligands/counter-receptors*
CD2(LFA-2)	CD58(LFA-3) and CD59
CD11a/18(LFA-1)	CD54,102,50(ICAM-1,2,3))
CD49d/29(VLA-4)	CD106(VCAM)

1.2.2 Antigen-based Gene Transfer Strategies

What follows is a discussion of possible gene transfer approaches that have the potential to modify tumor immunogenicity by introducing molecules that can elicit either strong or tumor-specific immune responses. These molecules are MHC alloantigen, superantigen, and tumor rejection antigens. The first two have already demonstrated a capacity to indirectly augment or mediate an immune response leading to tumor regression in animal models. The third type raises exciting possibilities for tumor-specific gene transfer approaches that have been made possible by the discovery of tumor-specific or tumor-associated antigens.

1.2.3 Allogeneic MHC Gene Transfer

Tanaka and colleagues found that tumor cells transfected with a syngeneic MHC class I molecule were less tumorigenic in allogeneic animals [Tanaka et al., 1985]. This was the first evidence that transfection of an MHC molecule, allogeneic to the tumor bearing host, could significantly alter tumor progression. While some investigators suggested that allogeneic MHC class I expression alone was insufficient for tumor rejection, others clearly recognized the decrease in tumor genicity following transfection with allogeneic MHC class I genes [Tanaka et al., 1985; Cole et al., 1987; Carlow et al., 1989; Hui et al., 1989; Ostrand-Rosenberg et al., 1990a,b]. Recently, Plautz and colleagues used direct allogeneic MHC class I gene transfer to modify established tumors in mice. The introduction of an allogeneic MHC class I gene into a progressively growing tumor led to the generation of cytotoxic lymphocytes (CTL) reactive to the unmodified tumor and inhibition of tumor growth [Plautz et al., 1993]. Based on these studies, a phase I clinical trial of direct intratumoral allogeneic HLA class I (HLA-B7) gene transfer was initiated in humans with cancer. Five HLA-B7-negative patients were treated without complications and HLA-B7 protein was detected in tumor biopsy tissue from all patients [Nabel et al., 1993]. One patient demonstrated regression of injected nodules following two independent treatments, which was also accompanied by regression of tumor at distant sites. One patient demonstrated an increase in anti-HLA-B7 CTL precursors following gene transfer, demonstrating for the first time that direct gene transfer could

be used in humans to stimulate an immune response. This observation, which was consistent with previous animal studies, provided critical evidence to support the continued development of this immunological approach [Nabel et al., 1992].

We have previously speculated that one advantage of expressing a strongly immunogenic protein on the surface of the tumor is the potential for eliciting an immune response at the tumor site. Generation of an immune response in nature initiates a series of dynamic cellular and humoral events which effectively sensitize a competent host. While the complexity of this response is only partially appreciated, development of the appropriate cytokine cascade is thought to be essential. Thus, the elaboration of appropriate cytokines in response to the newly expressed alloantigen at the tumor site may induce the appropriate inflammatory response and cellular infiltrate, leading to recognition of both the foreign alloantigen and the previously unrecognized tumor-associated antigen(s). Obviously, the success of this approach relies upon recognition of the unique tumor-associated antigen, because recognition of the modified tumor cell alone cannot be expected to be therapeutic against the unmodified tumor at metastatic sites. (A diagram of our working hypothesis, explaining the observations of our experiments, is presented in Fig. 1.2.)

In this proposed model for immune recognition of unmodified tumor cells, T cell recognition of tumor cells is blocked in hosts bearing progressively growing tumors (pathway 1). However, introduction and expression of plasmid DNA (pDNA) encoding an alloantigen (allo-MHC) leads to the generation of CTL against the allo-MHC molecule (pathway 2). The elaboration of the appropriate cytokine milieu and influx of accessory cells leads to the generation of CTL which recognize tumor-specific antigens on both the modified (pathway 3) and unmodified (pathway 4) tumor cells.

Central to this hypothesis is the assumption that it is substantially easier for an antigen to serve as the target for an activated effector T cell than it is for that antigen to stimulate a primary immune response. This assumption was elegantly documented by Restifo and colleagues, when they demonstrated that an apparently non-immunogenic tumor can be recognized by T cells primed against gene modified tumor cells [Restifo et al., 1992].

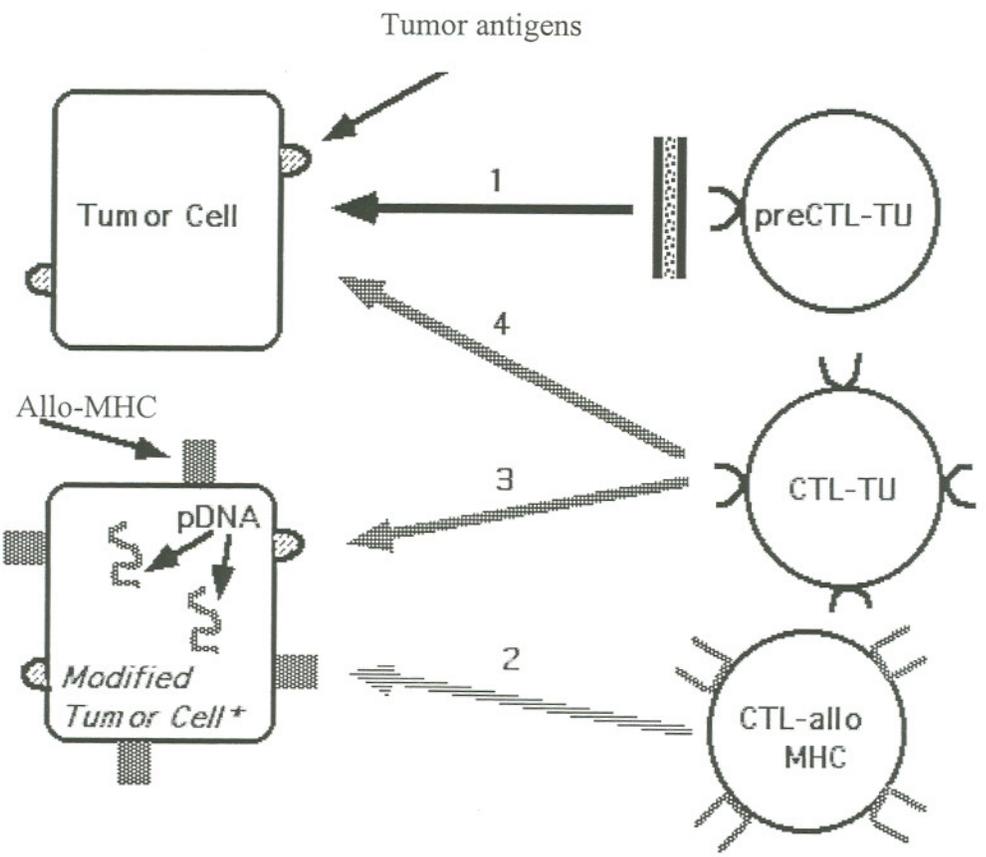


Fig. 1.2 A working hypothesis for tumor vaccines based on gene-modification with allo-MHC molecules.

Unfortunately, some progressively growing tumors may suppress or partially anergize the host's immune response. In one murine model using a poorly immunogenic tumor, direct allogeneic MHC class I gene transfer failed to inhibit tumor growth [Wahl et al., 1995]. However, the allogeneic class I molecule augmented sensitization of immune effector T cells that could be generated by an in vitro activation process from lymph nodes (LN) draining the injected tumors [Wahl et al., 1994]. Allogeneic MHC class I gene transfer was significantly more effective at generating therapeutic T cells in this model than gene transfer with a β-galactosidase control vector [Wahl et al., 1994, 1995].

The transfer of genes encoding syngeneic or allogeneic MHC class II molecules may afford additional opportunities to augment the immunogenicity of tumors. The work of Ostrand-Rosenberg and colleagues offers support for this concept. Their laboratory has documented that transfection of the syngeneic MHC class II gene into tumors that normally do not express this molecule converts the modified tumor cells into an effective vaccine [Ostrand-Rosenberg et al., 1990b]. Approaches such as these may effectively convert the tumor into something similar to antigen processing cell. It is easy to imagine how co-transfection with additional co-stimulatory or accessory molecules may further amplify this approach. Future alloantigen-based therapies may transfer both allogeneic MHC/HLA class I and class II genes into the same tumor vaccine, thereby enabling the activation of both CD4 and CD8 T cells. Theoretically, this would increase the likelihood that the appropriate cytokine cascade would be induced.

An additional advantage of alloantigen-based strategies for the immunotherapy of cancer is that they provide an inherent control to assess the immunological competence of the patient. The capacity of a given patient to mount an immune response to the allogeneic HLA molecule provides a surrogate marker of that patient's immunocompetence. Such assessment of a patient's immune status may be invaluable in evaluating the individual's capacity to mount an anti-tumor immune response.

1.2.4 Superantigen Gene Transfer

Microbial superantigens (SAg) are a family of small peptides that can selectively activate subsets of T cells on the basis of their beta chain of T cell receptor (V_βTCR) expression [Ostrand-Rosenberg et al., 1990b; Herman et al., 1991]. Several investigators have already demonstrated that SAg can be potent inducers of immune responses against tumors. Newell et al. [1991] administered the staphylococcal SAg, superendotoxin type B (SEB), to C3H mice and documented the in vivo activation of the appropriate, complimentary-reactive V_β subsets, while nonreactive V_βTCR subsets were not affected. When mice were administered a dose of SEB SAg at the time of tumor inoculation, there was a significant decrease in the growth of tumor. Although no studies were performed to document the immunologic specificity of this reaction, these results provide a clue to the potential therapeutic effect SAg might have in the manipulation of the host's response to a vaccine or tumor challenge. Subsequently, Shu and colleagues demonstrated that SAg-stimulation of tumor-draining LN cells can induce the differentiation of tumor-specific T cells with therapeutic activity [Shu et al., 1994]. Thus, the direct in vivo use of SAg may stimulate specific and/or non-specific anti-tumor reactivities secondary to the cytokine cascade initiated by SAg activation. Similar to allogeneic MHC class I gene transfer, direct intratumoral SAg gene transfer may have distinct and potent anti-tumor advantages. These potent stimulators of the immune response may offer a new approach to deal with the anergy or suppression long suspected to play a role in the failure of immunotherapy. Furthermore, these agents may offer an adjunctive approach to the more immunologically "tumor-specific" strategies, providing the opportunity to deliver a potent T cell adjuvant along with a tumor-specific gene(s) to the site of interest.

1.2.5 Tumor Antigen Gene Transfer

Another exciting development in immunology over the past decade has been the identification of "common" tumor antigens. Carcinoembryonic antigen (CEA) is one well-characterized antigen associated with colon, breast, gastric, pancreatic, and non-small-cell lung cancer [Thompson et al., 1991]. The CEA gene has been cloned

and incorporated into a vaccinia virus vector. Administration of this recombinant viral vaccine to mice demonstrated anti-tumor effects against a syngeneic tumor expressing human CEA [Kantor et al., 1992]. The preclinical success led investigators to test alternative strategies of generating anti-human CEA immunity without using viral vectors. Conry and colleagues recently demonstrated that direct gene transfer with a plasmid vector encoding CEA generated specific immune reactivity in mice [Conry et al., 1995]. These preclinical observations led to the initiation of a clinical trial of the recombinant vaccinia CEA vaccine in patients with colon cancer.

A number of tumor antigens have recently been described and will likely be the focus of future gene transfer strategies. These include antigens from melanoma, breast, ovarian, cervical, and pancreatic cancers. In each case, T cells recognize specific peptides presented by HLA molecules or as a repeated epitope on a protein core (Muc-1) [Jerome et al., 1991, 1993; van der Bruggen et al., 1991; Ioannides et al., 1993a,b; Slingluff et al., 1993; Storkus et al., 1993; Kawakami et al., 1994b]. Transfection of the genes encoding these peptides into target cells confers susceptibility to lysis by tumor antigen-specific T cells, suggesting a role for those molecules in direct gene transfer vaccination strategies. Currently, these molecules are the focus of a number of clinical trials where patients are vaccinated with defined tumor "peptide" antigens. In the future, a direct gene transfer approach might be used to more efficiently introduce the peptide or protein molecule into host cells in vivo.

Proto-oncogenes can also serve as targets for the immune-mediated destruction of tumor cells. Fenton and colleagues immunized mice to mutated Ras and demonstrated specific protection against a subsequent challenge with tumor cells expressing the appropriate Ras oncogene [Fenton et al., 1993]. Similarly, Carbone and colleagues immunized mice to mutant human p53 protein epitopes and generated CTL that specifically recognized and lysed syngeneic target cells expressing the appropriate mutant p53 [Yanuck et al., 1993]. While both examples used peptides to immunize mice, a similar strategy using plasmid vector constructs can easily be envisioned. These strategies rely on the identification of an appropriate mutated

region of the gene to include in the vector construct, so that the complete proto-oncogene would not be used, and the risk of inducing tumors in normal tissue as a result of gene transfer would be reduced. While these examples represent opportunities to test tumor-specific antigen-based gene transfer strategies, they do not take full advantage of current immunological dogma. For example, they fail to address the apparent requirement for a co-stimulatory molecule to assist the activation of antigen-specific T cells. The following section reviews the role for this class of molecules in anti-tumor gene transfer strategies.

1.2.6 Co-stimulatory Signals

CD28 is surface protein central to the generation of an immune response under most circumstances because of its ability to provide co-stimulation. Co-stimulatory signals provided by CD28 upon recognition of its ligands, CD80 and CD86 (B7, B7-2), leads to increased cytokine production, stabilization of message for IL-2, and maintenance of effector T cell activity [Schwartz, 1992].

Becker and colleagues recently reported that human T cells stimulated by tumor cells in the absence of a co-stimulatory signal were energized and secreted elevated levels of the potentially immunosuppressive cytokine, IL-10 [Becker et al., 1994]. Stimulation of T cells with tumor cells transfected with CD80 did not anergize the T cells and induced the secretion of significantly less IL-10. Furthermore, several animal studies have demonstrated a critical role for CD80 expression by tumor cells in the generation of anti-tumor immune responses [Townsend and Allison, 1993; Y. Li et al., 1994]. Interestingly, Chen and colleagues have subsequently demonstrated that transfection of CD80 into tumor vaccines is only effective when tumors are immunogenic [Chen et al., 1994]. The failure of this approach in poorly immunogenic tumors underlies the importance of our hypothesis that human tumor vaccines may require insertion of genes for additional "antigen components" to generate immune recognition and elaboration of the appropriate cytokine milieu. Nonetheless, results of these and other studies have led investigators to engineer CD80 into tumor cell vaccines. In 1994, a phase I trial of CD80-transfected allogeneic melanoma cells was approved by the Recombinant DNA

Advisory Committee [Sznol, 1994]. The use of an allogeneic tumor vaccine could provide the "antigenic components" necessary to generate an appropriate cytokine milieu at the vaccine site, and the CD80 can provide the co-stimulation signal required to induce a therapeutic immune response. This approach may be similar to allogeneic HLA gene transfer into autologous tumor, with the additional advantage of supplying the CD80 co-stimulatory ligand.

While several groups are investigating the effect of transfecting physiological ligands for CD28 (CD80, CD86) into tumor cell vaccines in an attempt to co-stimulate the anti-tumor immune response, recent evidence suggests an alternative approach may be more efficient. Recently, Nunes and colleagues demonstrated that triggering CD28 with a specific anti-CD28 monoclonal antibody (Mab) stimulated p21ras proteins [Nunes et al., 1994]. This pattern of p21ras activation appeared similar to that observed when the TCR complex was triggered. Interestingly, the CD80 ligand for CD28 did not stimulate the same signaling pathway. This observation suggests that different ligands for CD28 may co-stimulate different intracellular activation pathways more or less efficiently. Therefore, a single approach to co-stimulate CD28 using a physiological ligand may not provide optimal co-stimulation of an anti-tumor immune response. Future experiments may compare the effectiveness of these ligands by generating constructs which anchor an anti-CD28 Mab reagent into the tumor membrane. Tumor cells transfected with CD80, CD86, or membrane-bound anti-CD28 Mab could then be tested in vaccine strategies and the relative effectiveness of each strategy assessed.

Recently, Kubin and colleagues demonstrated that IL-12 synergizes with CD80 co-stimulation of CD28 to increase proliferation and cytokine release of T cells in vitro [Kubin et al., 1994]. Zitvogel et al. [1996b] extended these observations to demonstrate that the anti-tumor advantage confirmed by CD80 transfection into a tumor cell vaccine was further amplified by expression of IL-12 at the vaccine site. These interesting results provide insight into the direction that clinical trials will likely progress.

1.2.7 Adhesion/Accessory Molecules

A number of adhesion/accessory molecules also exert effects that have some co-stimulatory and/or immune amplifying properties that make them likely targets for incorporation into a wide variety of tumor vaccine strategies. While several have properties that could arguably warrant their inclusion on a list of co-stimulatory molecules, none appear as critical to the generation of an immune response as CD28. Therefore, a varied group of molecules were combined under the heading Adhesion/ Accessory Molecules and are discussed below. In fact, there is a wide array of additional accessory molecules that can facilitate T cell recognition and activation [Shevack, 1993]. T cells generally express cell surface molecules that promote antigen recognition by stabilizing the interaction of T cells with the antigen presenting cell (APC). These adhesion molecules give T cells the opportunity to grasp and hold putative targets, providing the TCR an opportunity to determine whether a complimentary ligand exists.

A host of accessory molecules that have the capacity to augment immune responses are listed in Table 1.1. They include CD2, LFA-1 (CD11a/18), CD27, CD30, CD40, CD44, CD49d/29(VLA-4), CD45, CD59, 4-1BB, and OX-40 [Liu et al., 1992; Baum et al., 1994]. Future investigations will undoubtedly focus on the effect of expressing ligands for these molecules in tumor vaccines or in combination with other genes, most notably those for defined tumor antigens, for direct in vivo gene transfer strategies.

One of the most critical accessory molecules on T cells is leukocyte function antigen (LFA-1). Antibodies to LFA-1 can inhibit essentially all activity of immune cells [Shevack, 1993]. This antibody-mediated inhibition of immune function is accomplished by interfering with effector cell adhesion to target cells. These data suggest that the normal interaction of LFA-1 with its ligand(s) intracellular adhesion molecules, ICAM-1, ICAM-2, or ICAM-3, is a critical component of a successful immune response. Galocha and colleagues have demonstrated that the absence of a single ligand for LFA-1, ICAM-1, can reduce or eliminate target lysis by some CTL clones [Galocha et al., 1993]. Since many tumor cell types do not express these ligands effectively, the potential of these tumor cells to act as stimulators or targets of

the immune response may be decreased. This finding suggests that transfer of genes encoding this family of LFA-1 ligands may augment the generation of an anti-tumor immune response or augment the killing by already generated CTL and be useful in the refinement of engineered tumor vaccines.

The same constructs proposed for gene transfer into tumor cells may also be effective if they were expressed in autologous APC, fibroblasts, or muscle cells. These approaches have already been used as an adjunct to tumor vaccine strategies, eliminating the requirement to isolate, culture, and modify the tumor cells themselves [Lotze et al., 1994]. One can also envision the use of autologous APC transduced with appropriate tumor antigen constructs being used to vaccinate patients with malignancies. This approach may be particularly effective given the efficiency of APC function in stimulating immune responses.

Currently, these strategies are predominantly focused on in vitro manipulations of cultured cells. However, future applications are likely to focus on direct in vivo approaches to manipulate these targets. Direct gene transfer strategies that employ simple, safe, plasmid vectors, while being less efficient than their viral counterparts, may be eminently suited for certain vaccination approaches to cancer immunotherapy. This will be particularly true for the development of tumor vaccination in an adjuvant setting, where possible risks of viral gene transfer may be deemed unacceptable given the current state of the art in gene therapy.

Gene therapy provides many opportunities to exploit various immune modulating molecules to enhance host immune response to tumor antigens. Understanding the mechanisms for immunological tolerance to tumor antigens can shed light on which specific gene therapy strategies can lead to optimal antitumor immune responses. Elucidating one potential mechanism for tolerance to tumor antigens has been the major focus of my thesis research project. Gene-modified tumor vaccines have had some success in both preclinical and clinical studies; however, the mechanisms behind various gene therapy approaches are ill-defined. Our own approach has been focused on allo-MHC I gene modification; we have found one mechanism in which the allo-gene-modified tumor vaccine can overcome immune deviation that is induced by tumor cells.

1.3 Immune Deviation as a Mechanism for Immunological Tolerance to Tumor Antigens

The incidence of melanoma, which is the most common malignancy in men and women between the ages of 25 and 30, is increasing faster than that of any other cancer. Melanoma is particularly interesting from an immunological perspective because it contains a wide spectrum of tissue-restricted proteins (e.g., MART-1, MAGE, gp100, tyrosinase, TRP-1, and TRP-2) that serve as targets of effector T cells in vitro [Brichard et al., 1993; Boon et al., 1994; Kawakami et al., 1994a,b]. However, in vivo, adequate spontaneous activation of tumor-specific lymphocytes either does not occur or it results in inefficient tumor protection. Why tumors that are clearly antigenic are so clearly nonimmunogenic has puzzled investigators for years. Since all of these melanoma antigens, and most other tumor antigens described to date, are self-antigens, the role of tolerance in the response to these "antigens" must be considered.

1.3.1 Definition of Tolerance

Burnett and Fenner have defined tolerance rather simply as "non-reactivity against self" [Schwartz, 1993]. If most tumor antigens are indeed self antigens, and if the tumor-bearing host must ensure "non-reactivity against self," then the host seems doomed to succumb to their cancer. This is what happens all too often to our patients. A broader appreciation of tolerance in the tumor-bearing host and how it affects the immune response to tumor antigens is essential to the development of therapeutic cancer vaccines. Our understanding of immunological tolerance has changed dramatically over the past decade. We now recognize a number of measures the host uses to induce tolerance and protect itself from the induction of auto-aggressive T cells. High affinity, auto-reactive T cells may be deleted centrally in the thymus, while other non-deletional pathways are available for induction of tolerance in the periphery [reviewed in Schwartz, 1993]. Functional silencing (or anergy), another mechanism of peripheral tolerance, can occur as a consequence of T cell stimulation by antigen and MHC in the absence of co-stimulation [Linley and

Ledbetter, 1993; Schwartz, 1993; Rocken and Shevach, 1996]. While initially described in vitro, functionally unresponsive T cells have also been identified in several in vivo systems [Arnold et al., 1993; Kyburz et al., 1993; Liblau et al., 1996; Garside and Mowat, 1997; Lanoue et al., 1997]. A recent report presents direct evidence that energized CD4 T cells survive in vivo for months following induction of peripheral tolerance and can recover from the unresponsive state [Pape et al., 1998]. These observations strengthen the role for functional silencing as an important mechanism in peripheral tolerance. However, antigen-specific immune responses can occur in models of tolerance and appear to induce "tolerance" because the response is a non-destructive rather than a destructive immune response. This mechanism has been referred to as immune deviation [Rocken and Shevack, 1996]. These findings are consistent with the following definition of tolerance proposed by Schwartz [1993]: "a physiologic state in which the immune system does not react destructively against self." Thus, the production of inefficient immune responses that are not capable of reacting destructively against self may be responsible for the poor results associated with many immunotherapies.

1.3.2 Type 1 and Type 2 Immune Responses

It is now generally accepted that both T helper (Th) cells and T cytotoxic (Tc) cells can be segregated into two general categories based on their cytokine release patterns [Salgame et al., 1991; Croft et al., 1994; Mosmann and Sad, 1996; L. Li et al., 1997]. A type 1 cell (T1) selectively secretes IFN-γ, TNF-β/LT-α, and IL-2, whereas type 2 cells (T2) secrete IL-4, 5, 6, 9, 10, and 13. They appear to share a common precursor that can differentiate along either pathway. The final pathway is determined by the cytokine milieu in which the T cells are activated and undergo differentiation [Seder and Paul, 1994]. The presence of T1 or T2 cytokines will drive uncommitted T cells to develop a cytokine profile similar to the one to which they are exposed, while at the same time inhibiting the development of cells with the reciprocal phenotype. Thus, IFN-γ selectively expands T1 cells and inhibits proliferation of T2 cells, while IL-4 and IL-10 can selectively inhibit cytokine

secretion by T1 cells [Mosmann and Sad, 1996; Nakamura et al., 1997; Tamada et al., 1997].

This ability to inhibit the maturation of cells producing cytokines of the alternate type may account for the tendency to see a predominant cytokine profile during most immune responses. Therefore, the presence of T2 cytokines during the initial interaction between a T cell and a tumor antigen presented by APC in the draining LN would facilitate the development of a T2 anti-tumor response. This could be caused by secretion of T2 cytokines by tumor cells, by secretion of IL-4 by NK1.1 T cells, or by IL-6 secreted from APC [Mosmann and Sad, 1996; von der Weid et al., 1996; Bendelac et al., 1997; Inoue et al., 1997; Rincon et al., 1997; Tamada et al., 1997]. The secretion of IFN-γ by NK cells or IL-12 by APC would be expected to activate T1 and inhibit T2 cells [Mosmann and Sad, 1996].

Exposure to antigen in the absence of co-stimulation also induces T2 responses [Scharton and Scott, 1993; Bendelac et al., 1997; Constant and Bottomly, 1997]. This in turn blocks the release of IL-12 by dendritic cells, inhibits the production of T1 cells, and allows T2 responses to be established. The dose of antigen used to sensitize T cells also affects the type of cytokine response. High concentrations lead to a predominant T1 response in CD4 cells, whereas low doses of antigen promote differentiation of predominantly T2 cells that produce high levels of IL-4 [reviewed in Hosken et al., 1995 and Constant and Bottomly, 1997]. A T2 profile also may develop as the default response if antigen is presented to T cells in the absence of inflammatory cytokines.

The importance of T1 or T2 responses in the pathogenesis and control of a number of diseases has been extensively described recently [Mosmann and Sad, 1996]. T1 responses protect or cure animals infected by protozoa, bacteria, or fungi and appear to cause the destruction observed in transplant rejection and in the following autoimmune diseases: experimental encephalomyelitis, multiple sclerosis, psoriasis vulgaris, insulin-dependent diabetes mellitus, and rheumatoid and reactive arthritis. In contrast, T2 responses appear to be important in models where helminths are studied and causative for allergic reactions, including atopic asthma and Omenn's syndrome.

The differential effects of T1 and T2 responses in tumor-bearing animals has only been appreciated recently [Aruga et al., 1997b; Tsung et al., 1997]. Aruga et al. [1997b] demonstrated that the efficacy of T cells in an adoptive transfer model could be abrogated by neutralizing Mabs to IFN-γ and GM-CSF, and augmented by treatment with anti-IL-10. In the same MCA 205 tumor model, Tsung et al. [1997] documented that tumors from untreated mice had a rim of $CD3^+$ T cells associated with production of IL-2, 4 and 10 (T2 response), but no IFN-γ, while T cells in tumors from IL-12 treated mice exhibited high levels of IFN-γ production (T1 response) and reduced IL-2, 4, and 10 production. This suggests that the balance between the production of T1 and T2 cytokines is a key determinant of whether or not a therapeutic effect is seen. This concept is further supported by two clinical studies in which T1 responses were associated with tumor regression. Kawakami et al. [1994b] noted higher response rates in patients treated with tumor-infiltrating lymphocytes exhibiting gp100-specific IFN-γ production, and Lowes et al. [1997] demonstrated that mRNA levels for T1 cytokines were significantly higher in spontaneously regressing melanomas compared to progressing lesions. These data support the hypothesis that induction of a T1 response is necessary for tumor regression and that type 2 responses are either ineffective or potentially harmful because of their ability to inhibit T1 responses.

Schwartz's definition of tolerance as it relates to patients with cancer might be better stated as follows: "a physiological state in which the immune system does not react destructively against tumor cells" [Schwartz, 1993]. In my recent publications included in this dissertation I have provided evidence that subcutaneous injection of the "nonimmunogenic" D5 tumor does sensitize tumor-specific T cells in vivo; however, because they produce T2 cytokines, tumor cells are not destroyed. The allo-modified vaccine induces T1 T cells and results in actual destruction of tumor cells in vivo. To test our vaccine in a clinically relevant model, we investigated how the vaccination strategy worked in a mouse bearing disseminated tumor. Systemic D5 tumor blocked the generation of either a tumor-specific T1 or T2 response following vaccination with D5-K^d and cells were not therapeutic. This observation is similar to the functional silencing/anergy seen in other models of peripheral tolerance. We then

showed that our D5-G6 vaccine (GM-CSF secreting) "breaks" tolerance, presumably by providing additional APC and their repertoire of co-stimulatory molecules, and generates cells with therapeutic activity. These observations clearly identify immune deviation as a mechanism of tolerance in naive animals with progressively growing subcutaneous tumors. However, these observations also point to peripheral tolerance playing a role following intravenous inoculation of the same tumor. A better understanding of these two mechanisms of tolerance (immune deviation and peripheral tolerance) that may be operational in cancer could help explain the lack of an effective immune response in patients with a variety of malignancies. Subsequent goals of my dissertation were to define mechanisms that regulate the induction of T1 or T2 tumor-specific immune responses and to develop strategies that promote polarization of a tumor-specific T1 response in vitro and in vivo.

1.3.3 Three-Signals Hypothesis for Priming of Therapeutic T Cells

Development of an immune response against tumor-specific antigens that leads to complete regression of metastatic cancer is the Holy Grail of tumor immunology. Since the LN is the principle site where adaptive, antigen-specific immune responses are initiated, we have considered that analysis of the immune response in sentinel LNs, draining tumor or tumor vaccines may provide clues for the failure the immune system to develop an effective antitumor response.

Before an immune response can be initiated, specialized phagocytes/antigen presenting cells (APC/dendritic cells) need to acquire antigen in the periphery and then migrate to the draining LN to "present" this antigen to naive lymphocytes. Lymphocytes that are continuously recirculating through the LN then have opportunities for rare antigen-specific T cells to encounter antigen-laden APCs. Once antigen-specific T cells encounter their complimentary antigen on APCs and are provided appropriate co-stimulatory signals, they become activated. These activated T cells rapidly expand, acquire effector activity, leave the LN, and search the periphery for their sensitizing antigen. For infectious diseases this process routinely clears infectious agents and provides protection from subsequent exposure to the same

agent. Why a similar process does not occur with cancer cells is not clearly understood.

Thus, as LNs are the site where T cells are educated to antigens, why do tumors that metastasize to LNs fail to sensitize T cells to develop an antitumor immune response? For several decades it was commonly believed that most human tumors lacked tumor or tumor-associated antigens and were not seen by the immune system. With the recent description of normal proteins serving as tumor-associated antigens, the concept of immune tolerance to self-proteins offered an alternative explanation for the failure of tumor vaccine strategies, since it was considered unlikely that an individual could develop a destructive immune response against a normal protein if they had been tolerized to that same protein.

Our recent work has focused on characterizing the immune response in LN draining progressively growing tumors, or tumor vaccines, in mice and humans. We have worked with non-immunogenic murine tumors because they are thought to be more relevant to human disease. Tumors are characterized as non-immunogenic when vaccination uniformly fails to protect the host from a subsequent tumor challenge. This designation suggests that the mouse, like his human counterpart, has not recognized, or is tolerant of the tumor antigens. Our recent studies with three non-immunogenic tumors suggest that this is not true [Boon et al., 1994; Winter et al., 2002]. We have demonstrated that progressively growing subcutaneous tumors sensitize tumor-specific T cells; however, the antigen-reactive T cells are polarized to secrete type 2 (T2) cytokines (e.g., IL-4 and IL-10) and lack therapeutic activity upon adoptive transfer. Conversely, immunogenic tumors induce predominantly type 1 (T1) antitumor responses, exhibiting highly polarized tumor-specific IFN-γ secretion. These results are summarized in the schematic presented in Fig. 1.3.

This cytokine response to nonimmunogenic tumors was identified because we first enriched for the subpopulation of recently activated T cells in the sentinel LN. This was accomplished by isolating LN cells that had downregulated expression of the LN homing receptor, L-selectin (CD62L), a well-established marker for recently activated and memory T cells [van der Bruggen et al., 1991; Brichard et al., 1993; Boon et al., 1994].

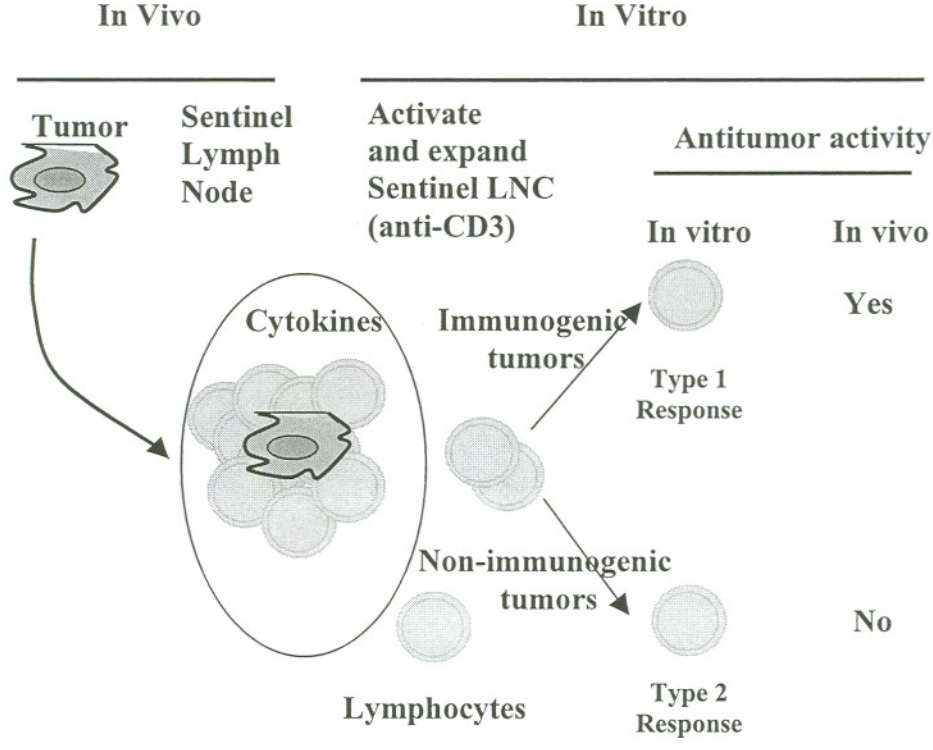


Fig. 1.3 Schematic overview of differences in immune response observed in sentinel nodes draining immunogenic or nonimmunogenic tumors.

These observations suggest that both "nonimmunogenic" and immunogenic tumors are capable of inducing an immune response; however, there is a strong association between the type of tumor-specific response and therapeutic effect. Tumors that elicit T1 T cells can be eradicated, whereas tumors inducing a T2 response continue to grow despite that immune response. The development of a nontherapeutic T2 immune response has been called immune deviation [Kawakami et al., 1994b]. We have developed two novel vaccine strategies that induce therapeutic T cells in a poorly immunogenic tumor model and appear to do so by overcoming this immune deviation, i.e., they skew the T cell response of naive mice from a T2 to a T1 cytokine profile [Coley, 1991; Winter et al., 2002]. In further support of the importance of a T1 response to tumor eradication, we have shown that T cells from LNs draining poorly immunogenic tumors can be shifted from a T2 to a T1 cytokine response by in vitro culture with anti-IL-4 and IL-12 (a potent inducer of IFN-γ), a shift that is accompanied by the acquisition of therapeutic activity in our adoptive transfer model [Winter et al., 2002]. Thus, we have hypothesized that development of a tumor-specific T1 response is critical to the generation of therapeutic T cells. Further, these new results suggest that two signals alone are insufficient to induce a therapeutic response against nonimmunogenic tumors and have led us to adopt a three-signal model for the development of therapeutic antitumor immunity (Table 1.2).

It is already well established that T cell activation requires two signals: one being antigen presented in the context of MHC, and the second being co-stimulation [Linsley and Ledbetter, 1993]. However, progressively growing non-immunogenic tumors induce an immune response in the sentinel LN, a result that presumably requires co-stimulation, but they lack therapeutic activity. Manipulation of the cytokine milieu in which T cells are activated and expanded provides a third signal that can direct the developing immune response. Our results, presented above, suggest that this approach is an effective immunotherapy strategy in naive mice.

Since non-immunogenic tumors prime a T2 immune response, and since a T2 response inhibits development of a T1 response [Schwartz, 1993], we further postulated that immune deviation might explain the inability of vaccines to prime therapeutic T cells in tumor-bearing hosts. The presence of systemic tumor cells in

Table 1.2

A Three-Signal Model for the Generation for the Design of Therapeutic Antitumor Immunity

Signal	Type	Source
one	antigenic	tumor antigens/peptides presented by APC
two	co-stimulatory	CD80, CD86, OX40L, 4-1BBL
three	T1-polarizing	IL-12, IL-18, anti-IL-4, IL-13 and IL-5

the host could lead to the development of a T2 cytokine response, which could subsequently interfere with the induction by vaccination of a potentially therapeutic T1 response. A more thorough understanding of this process has important implications for clinical trials in humans because most vaccines will be administrated to patients with cancer in whom immune deviation may already have occurred.

These observations have encouraged us to initiate a clinical trial which utilizes an autologous tumor vaccine modified by lipid complex-mediated gene transfer to express an allogeneic HLA class I molecule (HLA-B7) (see Fig. 1.4a and 1.4b). Seven to ten days following vaccination (Fig. 1.4c), TVDLN were harvested and T cells isolated (Fig. 1.4d). Following activation and expansion (Fig. 4e), these tumor-sensitized T cells were returned to the patient with a course of systemic IL-2 (Fig. 4f). This trial, which represents a fourth generation of adoptive immunotherapy strategy (following lymphokine-activated killer cells, tumor-infiltrating lymphocytes, and in vitro sensitization of TVDLN), sought to define the toxicity of this approach and characterize the immune response to the gene-modified tumor vaccine (Fig. 4g). Patients were vaccinated with autologous tumor + BCG in one extremity and allogeneic HLA class I gene-modified tumor in the contralateral extremity, providing the opportunity to assess the relative efficacy of each approach.

The type of immune response generated was a key indicator of the success of adoptive immunotherapy in our animal model as described above. A number of reports suggest that there is a strong correlation between the generation of a type 1 response and antitumor activity [Ghosh et al., 1995; Aruga et al., 1997a,b]. Although some groups have shown that a tumor-specific type 2 response can also be therapeutic, the efficacy of type 2 responses is generally less than that observed for a tumor-specific type 1 immune response [Dobrzanski et al., 1999, 2000, 2001]. Therefore, the type of immune response generated may also contribute to the lack of tumor regression in our clinical trial. We observed no difference in tumor-specific IFN-γ secretion between LN lymphocytes draining the gene-modified and unmodified autologous tumor cell vaccines, but LN lymphocytes draining the HLAB7-modified autologous tumor vaccine secreted significantly more IL-5, a type 2 cytokine, when restimulated with autologous tumor. The induction of a type-2 immune response in

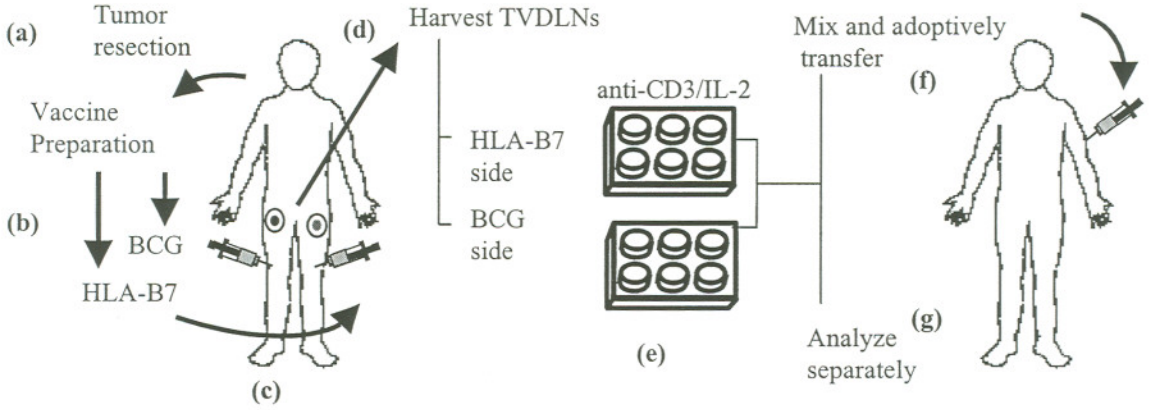


Fig. 1.4 Clinical trial design for HLA-B7 gene-modified tumor vaccine and adoptive transfer of in vitro activated TVDLN in melanoma and renal cell carcinoma patients.

the LN draining the gene-modified vaccine was surprising, because our animal model would have predicted the opposite, namely that vaccination with a tumor modified by lipofection with an allogeneic MHC class I construct, would have elicited a type 1 (IFN-γ) response [Hu et al., 1998]. Why vaccinations with the HLAB7 lipofected autologous tumor induced a tumor-specific IL-5 response is not understood, but it suggests that inflammatory cytokines did not dominate the cytokine milieu in the draining LN. If they had, a more strongly polarized type 1 response would have been expected. The TVDLN were mixed prior to adoptive immunotherapy so the type 2 response elicited in the lymphocytes draining the gene-modified vaccine could have affected those draining the other vaccine. Although it has not been established that a tumor-specific type 2 cytokine response will reduce the efficacy of a type 1 response, one can speculate from other studies that this might be the case. This has led us to suggest a three-signal model for developing a therapeutic antitumor response. The signals include the well-characterized signal 1 (i.e., recognition of antigen by the TCR), signal 2, a co-stimulatory signal provided to the responding T cell, and a third signal that polarizes the development of a strong tumor-specific type 1 cytokine response. Our results from patients are insufficient to confirm the validity of this model. Too few samples were available, no tumor regressions were seen, and we were unable to rigorously examine the response to a well-defined antigen. All of these conditions must be met in future studies before an assessment of the clinical importance of developing a type 1 or 2 cytokine response can be evaluated definitively.

1.4 Summary of Main Chapters of Thesis

Chapters 2–5 analyze the priming of tumor-reactive T cells with tumor vaccines.

Chapter 2, "Gene-modified tumor vaccine with therapeutic potential shifts tumor-specific T cell response from a type 2 to a type 1 cytokine profile," demonstrated that a poorly immunogenic tumor elicited a non-therapeutic type 2 immune response (IL-4 and IL-10) and that a therapeutic vaccine derived from the

same tumor (gene-modified tumor vaccine) skewed the non-therapeutic type 2 immune response to a therapeutic type 1 immune response (IFN-γ). This paper not only uncovered a novel tumor escape mechanism, but also pointed out a new paradigm for vaccine design and a starting point from which to search for mechanisms of T cell-mediated tumor regression.

Chapter 3, "Divergent role for $CD4^+$ T cells in the priming and effector/memory phases of adoptive immunotherapy," demonstrated that $CD8^+$ therapeutic T cells not only cured mice with tumor but also induced an autoimmune reaction that led to depigmentation of the fur. Furthermore, this study demonstrated that the priming of therapeutic $CD8^+$ T cells by a GM-CSF gene-modified tumor vaccine could be independent of $CD4^+$ helper T cells; however, host $CD4^+$ T cells were critical for long-term survival of mice after adoptive immunotherapy and protective immunity against tumor re-challenge.

Chapter 4, "CD28, TNFR, and IL-12 are critical for CD4-independent cross-priming of therapeutic antitumor $CD8^+$ T cells," extended the observations of Chapter 3 to further define the molecules critical for priming therapeutic $CD8^+$ T cells in vivo. Our observation, that priming of antitumor $CD8^+$ T cells depended on co-stimulation by CD28, was not new; however, the demonstration of a requirement for tumor necrosis factor receptor signaling, rather than CD40, was a novel finding.

Chapter 5, "Development of antitumor immune responses in reconstituted lymphopenic hosts," described a novel approach to augment antitumor immune responses by vaccinating hosts during immune reconstitution. During the T cell recovery phase of immune reconstitution of lymphocyte deficient mice, both CD4 and CD8 tumor-reactive T cells can be skewed to significantly higher percentages if the tumor vaccine was given during immune reconstitution. Active immunization in reconstituted lymphopenic cancer patients after chemotherapy or irradiation therapy will be tested in future clinical trials at the Providence Cancer Center.

CHAPTER 2
GENE-MODIFIED TUMOR VACCINE WITH THERAPEUTIC POTENTIAL SHIFTS TUMOR-SPECIFIC T CELL RESPONSE FROM A TYPE 2 TO A TYPE 1 CYTOKINE PROFILE*

2.1 Introduction

Acquired immune responses can be divided broadly into cell-mediated and humoral immunities. The type of immune response generated is a function of the cytokines produced during exposure to antigen (Ag). Production of type 1 cytokines (IL-2, IFN-γ, and TNF-β) leads to cell-mediated immunity, whereas type 2 cytokines (IL-4, -5, -6, -9, -10 and -13) are associated with antibody (Ab) production [reviewed in Mosmann and Sad, 1996]. Initially reported for CD4 T cells, it is now generally accepted that both CD4 and CD8 T cells can be segregated into type 1 and type 2 populations based on their patterns of cytokine release [Salgame et al., 1991; Croft et al., 1994; Mosmann and Sad, 1996; L. Li et al., 1997]. Uncommitted precursors can differentiate along either pathway, with their final phenotype determined by the cytokine milieu at the time they undergo activation and differentiation [Croft et al., 1994; Seder and Paul, 1994]. The presence of IFN-γ and IL-12 tends to promote type 1 cells while inhibiting the development of type 2 cells, whereas IL-4 drives the development of type 2 cells and inhibits type 1 [Mosmann and Sad, 1996; Nakamura et al., 1997].

---

* Originally published in this or similar form in *The Journal of Immunology* and used with permission of The American Association of Immunologists.

Hu, H.-M., Urba, W. J., and Fox, B. A. (1998) Gene-modified tumor vaccine with therapeutic potential shifts tumor-specific T cell response from a type 2 to a type 1 cytokine profile. *J. Immunol.* **161**, 3033-3041.

The biological importance of this dichotomy of T cell responses was first demonstrated in infectious diseases, where the generation of type 1 immune responses conferred protection or produced cure of animals infected with protozoa, bacteria, and fungi [reviewed in Mosmann and Sad, 1996]. Type 1 responses also appear to be responsible for the destructive effects observed in various autoimmune states, e.g., experimental allergic encephalomyelitis, multiple sclerosis, insulin-dependent diabetes mellitus, and rheumatoid arthritis, as well as transplant rejection. In contrast, type 2 responses appear to be curative or protective in models of helminths and have been implicated in allergic reactions, including atopic asthma and Omenn's syndrome.

The differential roles of type 1 and type 2 T cells in tumor-bearing animals have only been described recently. A number of reports suggest that there is a correlation between the generation of a type 1 response and antitumor activity [Kawakami et al., 1994b; Zitvogel et al., 1996a; Aruga et al., 1997b; Lowes et al., 1997; Tsung et al., 1997]. In this study, we show that there is an immune response in local lymph nodes (LN) following vaccination with what has been called a non-immunogenic tumor, B16BL6-D5 (D5). T cells from LN draining tumor vaccine exhibited reduced expression of L-selectin (CD62), a well-described marker of recently activated T cells [Bradley et al., 1991; Mobley and Dailey, 1992; Hou and Doherty, 1993; Andersson et al., 1994; Mobley et al., 1994]. Similar changes are observed in T cells draining a vaccine site containing the immunogenic, gene-modified D5-K^d (D5 transfected with H-2K^d plasmid DNA). This suggests that both tumors had been recognized by T cells. T cells from LN draining each of these vaccines were separated into populations with reduced (L-selectin$^{low/-}$) or normal (L-selectin^{high}) expression of L-selectin, and their antitumor properties were assessed in vivo following in vitro activation with anti-CD3 and IL-2. Adoptive transfer of L-selectin$^{low/-}$ T cells from D5-K^d tumor vaccine draining lymph nodes (TVDLN) mediated significant ($p < 0.05$) regression of pulmonary metastases, while T cells from D5 TVDLN were ineffective. L-selectin^{high} cells failed to mediate tumor regression regardless of the tumor cells used for vaccination. In vitro activated L-selectin$^{low/-}$ T cells draining gene-modified or unmodified D5 vaccines demonstrated tumor-specific cytokine release; however, nontherapeutic D5 TVDLN were highly polarized towards

a type 2 profile (IL-4, IL-10), whereas the therapeutic D5-K^d TVDLN exhibited a type 1 profile (IFN-γ). Polarization of cytokine production was already evident at the time of TVDLN harvest, 8 days after vaccination. These results suggest that the therapeutic failure of some tumor vaccine strategies may result from the generation of an immune response that, while tumor-specific, does not lead to tumor regression.

2.2 Materials and Methods

2.2.1 Mice

Female C57BL/6 (B6) mice were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained in a specific pathogen-free environment. Mice were generally 8 to 12 weeks old when used for experiments. Recognized principles of laboratory animal care were followed [Guide for the Care and Use of Laboratory Animals, National Research Council, 1996], and all animal protocols were approved by The Earle A. Chiles Research Institute animal care and use committee.

2.2.2 Tumor Cell Lines

D5 is a poorly immunogenic subclone of the spontaneously arising B16BL6 melanoma [Blanckaert et al., 1993]. An early passage of the original BL6 tumor was provided by Dr. E. Gorelick and was subcloned by limiting dilution culture in Dr. S. Shu's laboratory. D5 exhibits low to undetectable class I (H-2 D^b and K^b) expression and no class II expression. MPR-4, which is a transformed prostate tumor cell line (provided by Dr. T. C. Thompson, Baylor College of Medicine, Houston, TX) from a C57BL/6 mouse [Thompson et al., 1993], also has very low MHC class I expression.

2.2.3 Reagents

Magnetic beads conjugated with anti-CD62L (L-selectin) were provided by Miltenyi Biotec (Auburn, CA). The 145-2c11 hybridoma (anti-CD3) was a gift of Dr. J. A. Bluestone (University of Chicago, Chicago, IL). Recombinant human IL-2

was provided by the Chiron (Emeryville, CA). DMRIE/DOPE lipid solution was provided by Vical (San Diego, CA).

2.2.4 Culture Conditions

Lymphocytes and tumor cells were cultured in complete medium (CM), which consisted of RPMI 1640 (BioWhittaker, Walkersville, MD) containing 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 μg/ml of gentamicin sulfate. This was further supplemented with 50 mM 2-mercaptoethanol (Aldrich, Milwaukee, WI) and 10% fetal bovine serum (Life Technologies, Grand Island, NY). Tumor cells were harvested two or three times per week by brief trypsinization (BioWhittaker) and maintained in T-75 or T-150 culture flasks.

2.2.5 Immunization and Tumor Challenge

Mice were immunized by subcutaneous (s.c.) inoculation of 10^7 gamma-irradiated (10,000 rad) unmodified D5 tumor cells and were challenged 14 days later in the contralateral flank with viable D5 tumor. Mice were challenged with one of three different doses of tumor cells (10^3, 10^4, or 10^5). A challenge with 10^5 tumor cells induces tumors 100% of the time. Injection of 10^3 or 10^4 tumor cells is below the dose that results in tumors in 100% of animals within 14 days. Naive, age-matched control mice were included for each tumor dose. Results are presented both as the number of animals developing tumor and as the mean tumor size for animals that developed tumors.

2.2.6 Lipofection of Tumor and Vaccination for Adoptive Immunotherapy

An H-2 K^d expression plasmid with the CMV early gene promoter and no selectable marker gene was used for these studies. Plasmid DNA was prepared using Qiagen plasmid purification kits (Santana Clarita, CA). LPS was removed from the preparation by treatment with Triton X-114 as previously described [Conry et al., 1994]. Tumor cells were harvested by trypsinization, washed twice with lactated Ringer's solution (LR) and subsequently resuspended at 2×10^6 cells/ml. Lipofection was performed as follows. Plasmid DNA and lipid complex were

prepared by mixing DNA (2.5 μg plasmid/10^6 tumor cells) and lipid (DMRIE/DOPE, Vical; 10 μg/10^6 tumor cells) in LR. The lipid/DNA complex was incubated for 5 min at room temperature before being gently mixed with D5 in LR to a final concentration of 1 to 2 $\times$ 10^6 cells/ml and incubated at room temperature for 1 h with an orbital shaker rotating at 75 rpm. After incubation, tumor cells were washed twice and resuspended in LR for injection or resuspended in CM and cultured for subsequent determination of *trans*-gene expression. Transfection efficiency, which was determined by flow cytometric analysis of H-2K^d expression, generally ranged from 30 to 70% 24 to 48 h after lipofection. One million unmodified D5 or H-2 K^d gene-lipofected D5 (D5-K^d) tumor cells were injected subcutaneously into both hind flanks of B6 mice.

2.2.7 Separation and Activation of TVDLN

Eight to eleven days following tumor inoculation, the superficial inguinal LN were harvested, and lymphocytes were separated into L-selectin^{high} and L-selectin^{Lo} populations using either panning or CD62L-MACS microbeads (Miltenyi Biotec). For panning, nylon wool-enriched T cells were incubated with anti-L-selectin (American Type Culture Collection, Rockville, MD; catalogue no. HB-132) for 30 min at 4°C and washed twice, and 80 $\times$ 10^6 cells in 4 ml of CM were plated onto T-25 flasks coated with rabbit anti-rat IgG (100 μg/ml; catalogue no. R-9255, Sigma, St. Louis, MO) and incubated for 45 min at 4°C. Nonadherent cells were recovered by vigorous pipetting and subjected to a second panning step on rabbit anti-rat IgG-coated dishes. Nonadherent cells recovered after this step were designated L-selectin$^{low/-}$ cells. Adherent cells from the first and second panning steps were harvested using a cell scraper and pooled (L-selectin^{high}). For magnetic bead separation, lymphocytes were resuspended at 10^8 cells/ml in CM, mixed with 10 μl/ml of CD62L-MicroBeads (497-01, Miltenyi Biotec), and incubated for 20 min at 4°C. Cells were washed once with 50 ml of CM, resuspended at 10^8/ml, and passaged over a VarioMACS magnetic depletion column (type BS 413-04 or CS 413-05, Milteyi Biotec) held by a high energy magnet (VarioMACS separator, Miltenyi Biotec). The column was washed extensively with CM, and nonadherent cells (L-

selectin$^{low/-}$ cells) were collected. The column was then removed from the magnetic field and washed to recover the bound population (L-selectin^{high}) of lymph node cells (LNC). Samples of unseparated, nonadherent and bound cells were stained with either an FITC labeled anti-rat Ig (panning) or an FITC-labeled anti-CD62L (magnetic bead separation) and analyzed on a Coulter flow cytometer for efficiency of separation.

Unfractionated, L-selectin^{high} and L-selectin$^{low/-}$ cells were resuspended at 2 $\times$ 10^6 cells/ml in CM and cultured in 24-well plates with 50 μl of a 1/40 dilution of 2c11 ascites (anti-CD3). This dilution was determined previously to be optimal for T cell activation. After 2 days of activation, the T cells were harvested and expanded in CM containing 60 IU/ml rhIL-2 for 2 or 3 additional days, while culture supernatant was saved for determination of cytokine release. T cells were then harvested, washed twice in HBSS, counted, and used in adoptive transfer and cytokine release assays.

2.2.8 Adoptive Immunotherapy

Experimental pulmonary metastases were established by i.v. inoculation of B6 mice with 1 to 3 $\times$ 10^5 D5 tumor cells. Three days later, when metastases were established, T cells were adoptively transferred i.v. Starting on the day of T-cell infusion, mice received 90,000 IU of IL-2 twice daily for 4 days. Animals were sacrificed by CO_2 narcosis 12 to 15 days following tumor inoculation, the lungs were resected and fixed in Feketes solution, and the number of pulmonary metastases was enumerated in a blinded fashion. Metastases that were too numerous to count accurately were assigned an arbitrary value of 250.

2.2.9 Measurement of Cytokines

After activation and expansion TVDLN were washed and incubated alone or were stimulated with either nothing, D5, MPR-4, or anti-CD3. T cells (2 $\times$ 10^6/well) and tumor cells (2.5 $\times$ 10^5/well) were cultured in 2 ml in 24-well plates. Supernatants were recovered 24 h (IFN-γ, IL-10) and 48 h (IL-4) after simulation and assayed in duplicate by ELISA using commercially available reagents (IFN-γ, PharMingen (San Diego, CA) or Genzyme (Cambridge, MA), IL-4, Genzyme; IL-10,

PharMingen). Supernatants were also recovered from the TVDLN cultures 2 days after activation with anti-CD3 and before addition of IL-2. The concentration of cytokine in the supernatant was determined by regression analysis.

2.2.10 Statistical Analysis

The significance of differences in the number of metastases between experimental groups was determined using the Wilcoxon rank sum test. Two-sided p values of <0.05 were considered significant. Each treatment group consisted of at least five mice, and no animal was excluded from the statistical evaluations. The significance of differences in cytokine secretion was determined using Student's paired t test. Two-sided p values of <0.05 were considered significant.

2.3 Results

2.3.1 D5 Is a Poorly Immunogenic Tumor

D5 has been called a poorly immunogenic/nonimmunogenic tumor because mice cannot be protected from a subsequent tumor challenge by immunization with irradiated tumor. B6 mice immunized with 10^7 gamma-irradiated (10,000 rad) D5 tumor cells were challenged 14 days later with 10^3, 10^4, or 10^5 viable tumor cells. Previous studies determined that 10^5 tumor cells would induce tumors in 100% of the animals. However, mice were also challenged with two lower dosages of D5 to determine whether immunization would afect the host's capacity to reject a dose of tumor cells that were not uniformly tumorigenic and thus provide a more sensitive indication of antitumor immunity. The fraction of mice developing tumors at the three challenge doses and the tumor growth curves are shown in Fig. 2.1. Similar percentages of naive and immunized animals developed tumors at all challenge doses, and inspection of the tumor growth curves revealed that immunization did not slow tumor growth even when mice were challenged with as few as 1000 D5 tumor cells. Thus, immunization does not protect mice from challenge with D5 tumor.

To determine whether inoculation with D5 sensitized potentially therapeutic T cells, we harvested TVDLN, activated them with anti-CD3/IL-2, and then adoptively

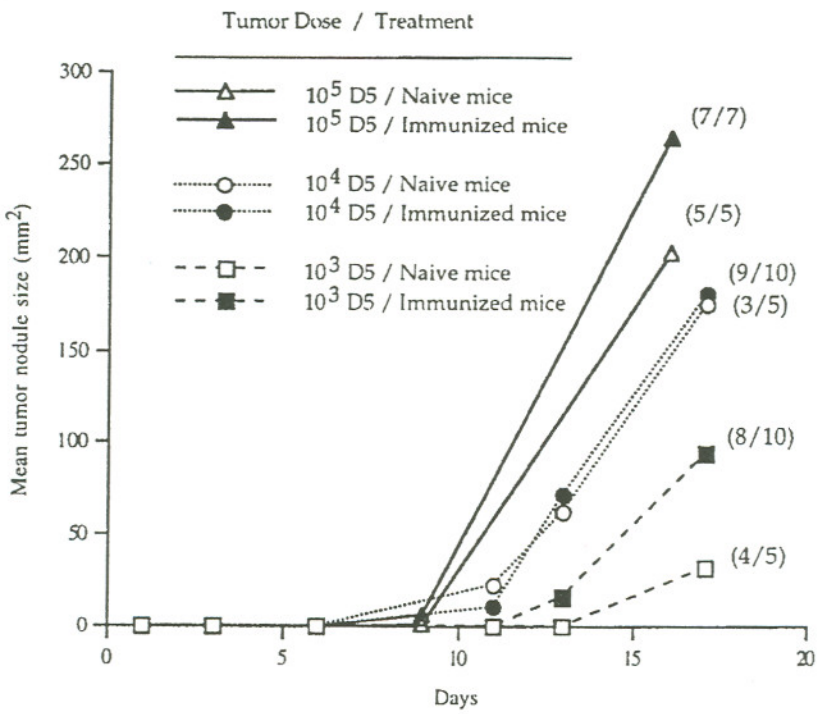


Fig. 2.1 Mice were immunized with 10^7 gamma-irradiated (10,000 rad) D5 tumor cells and were challenged 14 days later with viable D5 at one of three doses (10^3, 10^4, or 10^5). Naive mice were included in each experiment as a control for tumor growth. Mean tumor size for mice that developed tumors is presented. For each group the number of animals that developed tumors is presented, in parentheses, over the total number of animals challenged with tumor (number of mice with tumor/number of mice injected).

transferred them into mice bearing 3-day established pulmonary metastases [Yoshizawa et al., 1991a,b]. In three separate experiments, activated TVDLN from mice vaccinated with D5 failed to exhibit antitumor activity (Table 2.1). In the same experiments, other mice were vaccinated with D5 tumor cells that had been lipofected with an allogeneic MHC class I plasmid vector, H-2 K^d. LN draining this allo-modified vaccine (D5-K^d TVDLN) mediated significant reduction of pulmonary metastases following activation with anti-CD3/IL-2. These results confirm the poorly immunogenic nature of the D5 tumor and demonstrate the capacity of alloAg-modified tumor vaccine to sensitize T cells capable of mediating a therapeutic antitumor response.

2.3.2 L-Selectin Expression of TVDLN

Down-regulation of L-selectin expression is a well-established marker for recently activated T cells and memory T cells [Bradley et al., 1991; Mobley and Dailey, 1992; Hou and Doherty, 1993; Andersson et al., 1994; Mobley et al., 1994]. Recently, Kagamu et al. [1996] demonstrated that tumor-reactive T cells from an LN draining the weakly immunogenic MCA-205 tumor had reduced levels of expression of L-selectin. Furthermore, they showed that all T cells with therapeutic potential were contained in these L-selectin$^{low/-}$ TVDLN. Therefore, we attempted to identify tumor-reactive T cells in LN draining the poorly immunogenic D5 tumor by searching for T cells with reduced expression of L-selectin. Although 10% of lymphocytes in LN from naive mice exhibited reduced L-selectin expression, the L-selectin$^{low/-}$ population doubled to 20% following immunization with D5 tumor (Fig. 2.2A and B). Analysis of L-selectin on D5-K^d TVDLN revealed a similar finding; 25% of LNC exhibited decreased expression (2C). The down-regulation of L-selectin in both D5 and D5-K^d TVDLN implied that T cells were activated in both instances.

Next we investigated whether enrichment for L-selectin$^{low/-}$ cells, presumed to contain the tumor-sensitized T cells, would reveal antitumor activity. D5 or D5-K^d TVDLN harvested 8 to 11 days following tumor inoculation were separated on the basis of L-selectin expression. Fig. 2.2D–F and G–L illustrates the results of typical separations using panning or anti-CD62L magnetic beads to fractionate cells based on

Table 2.1

Lipofection with an Allogeneic Class I Gene Enables D5 Tumor Cells to Sensitize T Cells with Therapeutic Potential

Tumor vaccine[a]	T cells[b]	IL-2[c]	Mean number of pulmonary metastases (SEM)[d]		
			Expt. 1	Expt. 2	Expt. 3
none	none	+	250	>250	124(6)
D5	total LNC	+	250	>250	107(10)
D5-K^d	total LNC	+	114 (27)[e]	24 (10)[e]	10 (4)[e]

[a] Mice were vaccinated in both flanks with a s.c. dose of 10^6 D5 tumor cells.

[b] Single cell suspensions from day 8 to 11 tumor vaccine-draining superficial inguinal lymph nodes were stimulated in vitro at 2×10^6 cells/ml with anti-CD3 (2C11) for 2 days and then expanded for 3 days in CM supplemented with 60 IU/ml IL-2. Cells were harvested and adoptively transferred into animals with established 3-day D5 pulmonary metastases. In experiments 1 and 2 all mice received 3×10^7 T cells. In experiment 3 mice received 3.5×10^7 T cells.

[c] IL-2 was administered i.p. twice daily for 4 consecutive days following adoptive transfer.

[d] Mice were sacrificed 14 days following i.v. inoculation of tumor and the number of pulmonary metastases enumerated in a blinded fashion.

[e] Significantly less than all other groups ($p < 0.05$).

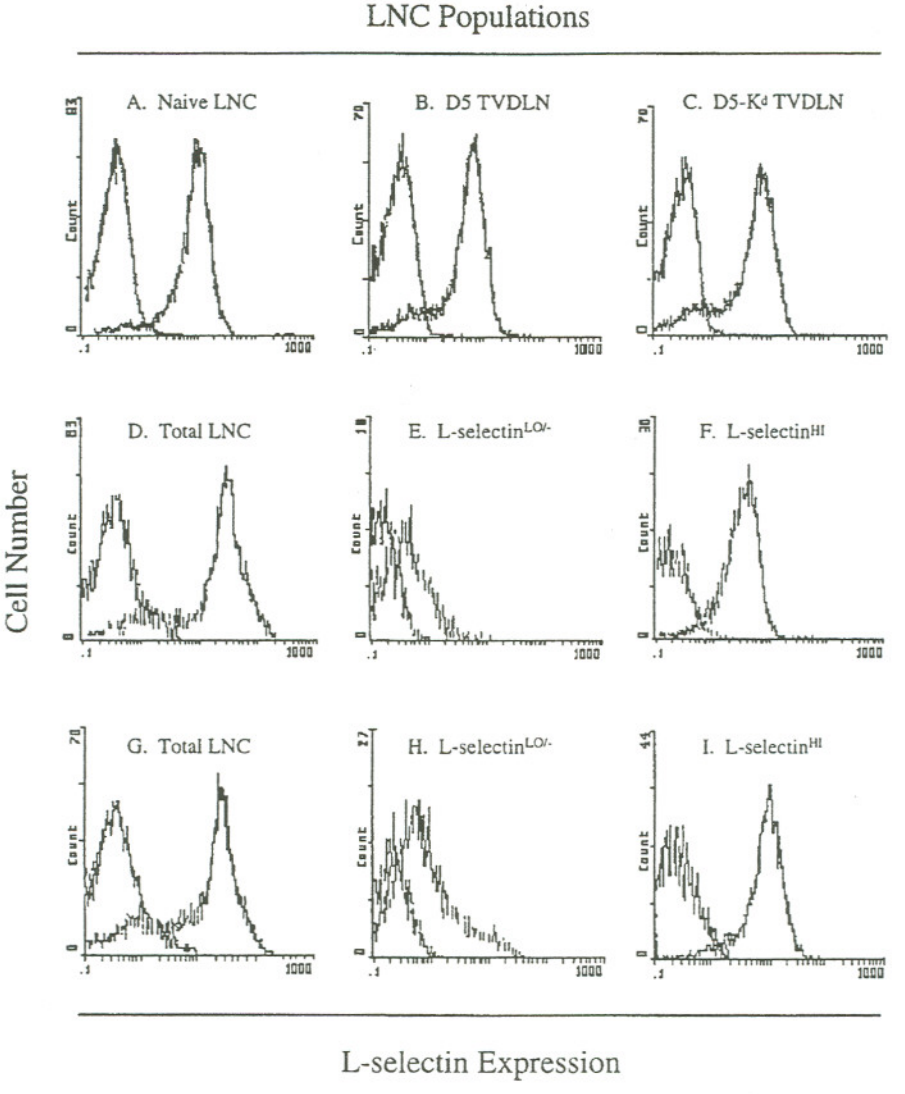


Fig. 2.2 FACS analysis of L-selectin expression on freshly isolated naive LNC (A), D5 TVDLN (B), and D5-K^d TVDLN (C) cells. Lymphocytes from D5 TVDLN were separated into L-selectin$^{low/-}$ or L-selectin^{high} cells by immuno-magnetic beading. FACS analysis of L-selectin expression for a representative experiment is presented for the total unseparated population (D) as well as for the L-selectin$^{low/-}$ (E) and L-selectin^{high} (F) populations. Similar results were obtained for experiments using D5-K^d TVDLN. A representative FACS analysis of L-selectin expression on the total unseparated D5-K^d TVDLN population (G) as well as the L-selectin$^{low/-}$ (H) and L-selectin^{high} (I) populations derived from that preparation are shown.

their L-selectin expression. L-selectin$^{low/-}$ and L-selectin^{high} T cells were then activated with anti-CD3 and expanded in IL-2. As described previously, L-selectin$^{low/-}$ T cells treated with anti-CD3 and IL-2 expanded more than L-selectin^{high} cells [Kagamu et al., 1996; data not shown]. In five experiments the former averaged 7.2 fold expansion compared to 3.7-fold for the L-selectin^{high} cells.

2.3.3 Reduced L-Selectin Expression Identifies Therapeutic T Cells in LN Draining Gene-Modified Vaccine

To measure directly the antitumor activity of in vitro activated L-selectin$^{low/-}$ T cells, they were adoptively transferred into mice bearing 3-day established metastases. Even though D5 TVDLN contained increased numbers of L-selectin$^{low/-}$ cells, presumed to be tumor-reactive, they failed to exhibit therapeutic activity when activated with anti-CD3 and IL-2 and adoptively transferred to tumor-bearing hosts (Table 2.2). Similarly, L-selectin^{high} TVDLN from the same mice also failed to exhibit any therapeutic activity. Thus, enrichment and activation of the T cells presumed to be tumor reactive did not improve efficacy following vaccination with unmodified D5. However, this was not the case when TVDLN from mice vaccinated with alloAg-modified tumor, D5-K^d, were tested. Comparable numbers of L-selectin$^{low/-}$ T cells from D5-K^d TVDLN exhibited significantly greater ($p < 0.05$) therapeutic activity than L-selectin^{high} TVDLN (Table 2.2). Thus, despite the existence of cells with reduced expression of L-selectin in TVDLN from mice immunized with unmodified or alloAg-modified tumor, the only T cells with antitumor potential were found in the LN draining D5-K^d.

2.3.4 Cytokine Production by L-Selectin$^{low/-}$ T Cells Following Anti-CD3 and IL-2

To determine the reason for the differential antitumor effects of the L-selectin$^{low/-}$ cells, which we presumed contained the recently activated tumor-specific cells, we examined the ability of L-selectin$^{low/-}$ T cells from TVDLN of D5 or D5-K^d tumor vaccines to secrete IFN-γ. Tumor-specific IFN-γ secretion has been associated with the therapeutic efficacy of adoptively transferred T cells [Barth et al., 1991; Shu et al., 1994; Zou et al., 1995; Tsung et al., 1997]. Stimulation with anti-CD3

Table 2.2

Vaccination with D5-K^d, but Not Unmodified D5, Sensitizes T Cells in the L-Selectin$^{low/-}$ Populations of TVDLN That Following Adoptive Transfer, Mediate Tumor Regression

Tumor vaccine[a]	T cells[b]	IL-2[c]	Mean number of pulmonary metastases (SEM)[d]		
			Expt. 1	Expt. 2	Expt. 3
none	none	+	165 (5)	250	124(6)
D5	L-selectin^{high}	+	189 (14)	250	107 (10)
D5	L-selectin$^{low/-}$	+	136 (17)	250	110 (6)
none	none	+	250	250	250
D5-K^d	L-selectin^{high}	+	250	219 (14)	250
D5-K^d	L-selectin$^{low/-}$	+	63 (26)[e]	35 (20)[e]	37 (25)[e]

[a] Mice were vaccinated in both flanks with a s.c. dose of 10^6 D5 or D5-K^d tumor cells.

[b] Single cell suspensions from day 8 to 11 tumor vaccine-draining superficial inguinal lymph nodes were stimulated in vitro at 2×10^6 cells/ml with anti-CD3 (2C11) for two days in CM and then expanded for 3 days in CM supplemented with 60 IU/ml IL-2. Cells were harvested and adoptively transferred into animals with established 3-day D5 pulmonary metastases. For experiments where D5 was the tumor vaccine, all mice received 2×10^7 T cells, except in experiment 3, where the L-selectin^{high} group received 4×10^7 T cells. In studies where D5-K^d was used to generate TVDLN, mice in experiment 1 received 10^7 T cells, while mice in experiments 2 and 3 received 2×10^7 T cells.

[c] IL-2 was administered i.p. twice daily for 4 consecutive days following adoptive transfer.

[d] Mice were sacrificed 14 days following i.v. inoculation of tumor and the number of pulmonary metastases enumerated in blinded fashion.

[e] Significantly less than all other groups ($p < 0.05$).

Table 2.3

Anti-CD3-Activated T Cells from Either Vaccine Contain the Same Distribution of CD4 and CD8 T Cells but Exhibit Opposing Tumor-Specific Cytokine Profiles In Vitro and Antitumor Activity In Vivo

Vaccine[a]	LNC[b]	% of cells expressing specified marker[c]				Cytokine profile[d]	Therapeutic activity[e]
		$CD4^+$		$CD8^+$			
		Mean	Range	Mean	Range		
D5	L-selectin$^{low/-}$	13.7	11–15	78.8	75–85	type 2	no
D5-K^d	L-selectin$^{low/-}$	14.6	10–22	79.4	72–90	type 1	yes

[a] Mice were vaccinated bilaterally with a s.c. dose of 10^6 D5 tumor cells or D5 tumor transiently lipofected with an H-2 K^d plasmid. Expression of the H-2 K^d plasmid ranged from 30 to 46% of D5 cells.

[b] Single cell suspensions from day 8 TVDLN were stimulated in vitro at 2×10^6 cells/ml with anti-CD3 (2C11) for 2 days in complete medium (CM) and then expanded for 3 days at a starting concentration of $1–2 \times 10^5$ cells/ml in CM supplemented with 60 IU/ml IL-2. Cells were harvested and characterized for expression of phenotypic markers, cytokine production, and in vivo antitumor activity.

[c] Percentage of $CD4^+$ and $CD8^+$ T cells present in anti-CD3-activated TVDLN was determined by flow cytometry.

[d] Activated TVDLN from either vaccine were characterized for production of cytokine following stimulation with D5 tumor. A type 1 profile was defined as a tumor-specific IFN-γ response with little or no detectable IL-4, while a type 2 profile released IL-4 with little or no release of IFN-γ.

[e] Therapeutic activity was defined as the capacity of adoptively transferred T cells to mediate regression of 3-day established pulmonary metastases of D5 tumor.

induced a similar level of IFN-γ secretion by L-selectin$^{low/-}$ T cells from LN draining either D5 or D5-K^d vaccines. However, only T cells from the D5-K^d TVDLN secreted IFN-γ specifically in response to tumor stimulation (Fig. 2.3A). IFN-γ was not produced when T cells were cultured alone or in the presence of syngeneic but unrelated prostate cancer cells, MPR-4. Thus, tumor-specific release of IFN-γ correlated with the therapeutic activity of these T cells. L-selectin^{high} TVDLN did not secrete IFN-γ in response to stimulation with D5 tumor regardless of the cells used for vaccination (Fig. 2.3D).

We next evaluated IL-4 production by the same T cells. L-selectin$^{low/-}$ T cells from D5 and D5-K^d TVDLN released similar amounts of IL-4 in response to stimulation with anti-CD3 (Fig. 2.3B). However, in contrast to IFN-γ, only T cells from the D5 vaccine secreted IL-4 specifically in response to stimulation with D5 tumor cells. This response was clearly tumor specific, since IL-4 was not secreted by T cells cultured alone or with the unrelated tumor MPR-4. No tumor-specific secretion of IL-4 was observed from L-selectin^{high} T cells from D5 or D5-K^d TVDLN (Fig. 2.3E). To confirm the type 2 cytokine profile of L-selectin$^{low/-}$ D5 TVDLN, we measured IL-10 production (Fig. 2.3C). L-selectin$^{low/-}$ T cells from D5 TVDLN produced more IL-10 following stimulation with D5 than without stimulation or after stimulation with MPR-4 ($p < 0.05$). Interestingly, while L-selectin$^{low/-}$ T cells expanded from both D5 and D5-K^d TVDLN released similar levels of IFN-γ and IL-4 after polyclonal stimulation with anti-CD3, T cells from the D5-K^d TVDLN released less IL-10 than L-selectin$^{low/-}$ T cells from D5 TVDLN ($p < 0.05$). Additional studies have found the anti-CD3-stimulated release of IL-10 to be more variable from experiment to experiment, and current studies are investigating whether this is related to the development of a type 1 cytokine response. Interestingly, L-selectin^{high} T cells from D5 or D5-K^d TVDLN released similar levels of IL-10 following stimulation with anti-CD3 (Fig. 2.3F). Although the cytokine profiles of D5 and D5-K^d TVDLN were markedly different, the phenotypes of the T cells were indistinguishable (Table 2.3).

These results demonstrate that vaccination with the poorly immunogenic D5 tumor does indeed lead to the sensitization of tumor-specific T cells. However, these T cells are polarized toward a type 2 cytokine profile and even after activation with

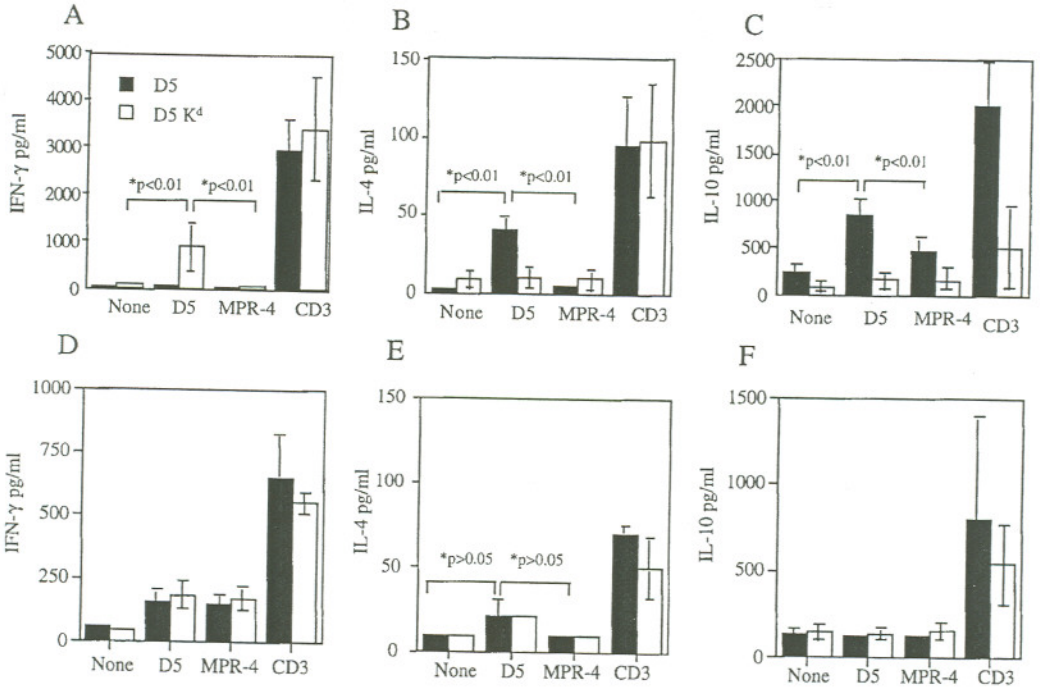


Fig. 2.3 Anti-CD3 activated and IL-2-expanded effector T cells generated from L-selectin$^{low/-}$ (A–C) or L-selectin^{high} (D–F) D5 or D5-K^d TVDLN were assessed for tumor-specific cytokine release at the time cells were adoptively transferred into tumor-bearing hosts. T cells (1×10^6/ml) were cultured alone (none), with anti-xCD3 (α-CD3), with a syngeneic but unrelated prostate (MPR-4) tumor (2×10^5/ml), or with D5 (1×10^5/ml), and supernatants were harvested 18 to 24 h later for IFN-γ determination and 44 to 48 h for IL-4 and IL-10 determinations. Cytokine release into the supernatant was determined by ELISA. Data are presented as the mean of three independent (L-selectin$^{low/-}$ or two (L-selectin^{high}) independent experiments ($\pm$SE). The limit of detection is 10 pg/ml for IFN-γ and IL-4 and 40 pg/ml for IL-10.

anti-CD3 and IL-2 lacked therapeutic efficacy. In striking contrast, vaccination with D5-K^d tumor sensitized T cells with a type 1 cytokine profile that, following anti-CD3 and IL-2 administration, effectively mediated tumor regression.

2.3.5 Cytokine Production by Freshly Isolated TVDLN

Previous studies have demonstrated that the cytokine environment in which T cells are first activated and begin differentiation determines the subsequent cytokine profile of those cells [Croft et al., 1994; Mosmann and Sad, 1996]. Because noncultured TVDLN require additional activation in vitro with tumor cells, microbial superAgs, or Abs to CD3 or the T-cell receptor, in order to develop therapeutic activity, we have concluded that they are not fully differentiated effector T cells [Shu et al., 1989, 1994; Yoshizawa et al., 1991b]. Therefore, to determine whether the cytokines released by these fresh cells during the in vitro activation step might account for the generation of highly polarized effector T cells, we examined which cytokines were released into the culture medium during the in vitro activation step with anti-CD3. L-selectin$^{low/-}$ TVDLN from both D5 and D5-K^d vaccines were activated as described above, and supernatants were recovered at the end of the second day of activation. As a control, LN from naive mice were harvested, and L-selectin$^{low/-}$ cells were isolated and cultured under identical conditions as TVDLN. Analysis of cytokine production by D5 or D5-K^d TVDLN revealed striking differences in the amounts of IFN-γ and IL-4 secreted. Fig. 2.4 depicts the ratios of the concentrations of IFN-γ and IL-4 found in the supernatant after 2 days of culture with anti-CD3, in four experiments. L-selectin$^{low/-}$ T cells from the D5 vaccine exhibited a low ratio of IFN-γ to IL-4, indicating a polarization toward a type 2 response, while the ratio of cells draining the D5-K^d vaccine more closely resembled that of naive LN cells. Thus, these results suggest that the response of L-selectin$^{low/-}$ T cells in D5 TVDLN is already biased toward a type 2 profile by 8 days following vaccination. Furthermore, the apparent excess in IL-4 release during the 2-day culture with anti-CD3 may contribute to the highly polarized tumor-specific cytokine release profile observed after the 5 day in vitro culture. A similar polarization of IL-10 secretion was not observed; L-selectin$^{low/-}$ cells from both D5 and D5-K^d TVDLN

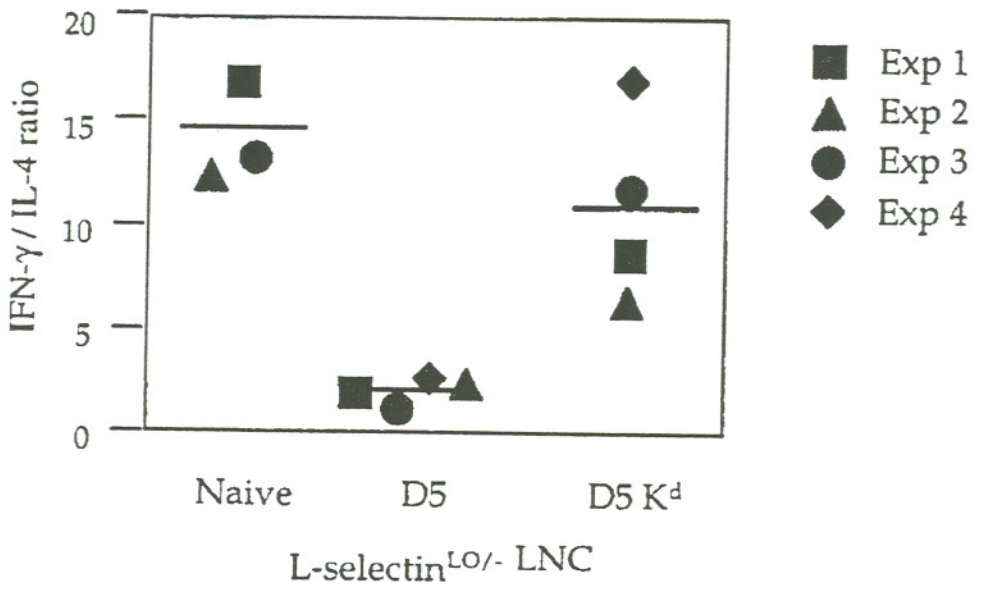


Fig. 2.4 L-selectin$^{low/-}$ cells were isolated from freshly harvested naive or day 8 D5 or D5-K^{d} TVDLN and activated with anti-CD3 (2c11) for 2 days, and supernatants were harvested. The concentrations of IFN-γ and IL-4 in the supernatant were determined by ELISA. The release of IFN-γ and IL-4 (picograms per milliliter) at 48 h was plotted as the ratio of IFN-γ/IL-4 secreted for each population of LNC. Studies with naive L-selectin$^{low/-}$ cells were not run simultaneously with D5 or D5-K^{d} TVDLN.

secreted similar levels of IL-10 following activation with anti-CD3 [data not shown]. This is in agreement with other reports that early expression of IL-4 or IFN-γ, but not of IL-10, directs the subsequent polarization of primary $CD4^+$ T cell cultures [Gollob et al., 1996].

2.4 Discussion

Our inability to immunize mice against the D5 subclone of B16BL6 has led to its being called a poorly immunogenic tumor. Our results suggest otherwise. While D5 is unable to induce a protective immune response, it is clearly an immunogenic tumor. The failure to protect is due to the nature of the immune response, not to its absence. We showed that while vaccination with the unmodified D5 tumor failed to sensitize T cells with therapeutic potential it increased the number of TVDLN with low levels of L-selectin expression, a well-established marker of recently activated or memory T cells. We show that although L-$selectin^{low/-}$ T cells from D5 TVDLN are specifically sensitized to tumor, as evidenced by a D5-specific type 2 cytokine response following in vitro activation with anti-CD3 and IL-2, they are unable to mediate tumor regression upon adoptive transfer. The same tumor, modified by transient lipofection with an allogeneic MHC class I gene (D5-K^d), induces T cells that express a tumor-specific type 1 cytokine profile and induce regression of established tumor. This correlation between cytokine profile and antitumor activity may be particularly important to understanding the elements of tumor regression in vivo and may provide approaches to monitor or predict therapeutic efficacy.

These observation forced us to reevaluate our perspective on the immunogenicity of tumors. The molecular mechanisms for the inherent level of immunogenicity of tumors is poorly understood. There are many explanations for the poorly immunogenic phenotype. Speculation is that poorly immunogenic/ nonimmunogenic tumors fail to exhibit or fail to present tumor-associated Ags. They may lose MHC class I/II molecules, lack accessory and/or costimulatory molecules, or have defects in Ag processing or transport pathways [Restifo and Wang, 1996]. Alternatively, since many of the recently described tumor-associated Ags are normal

proteins with restricted tissue expression (e.g., Melan-A MART-1 and gp100), it is possible that the host may have deleted these self-Ag-reactive T cells during ontogeny and thus be tolerant to these self Ags. Another possibility is that tumor-reactive T cells in the host have been anergized. This may occur because tumor Ags are presented by tumor, or by APC, without the appropriate second signal [Harding et al., 1992; Linsley and Ledbetter, 1993]. All of these explanations assume that there is a failure by the host to develop an antitumor immune response. Recently, immune deviation has been identified as a third mechanism, together with anergy and clonal deletion, capable of inducing tolerance in animals [Rocken and Shevach, 1996; Singh et al., 1996]. Immune deviation results in the inhibition of effector function by generating an opposing immune response against the same target Ag. The results of our study are consistent with the hypothesis that immune deviation is responsible for the poorly immunogenic nature of the D5 tumor.

Our observations support the results in animal models and a clinical trial that demonstrated the efficacy of adoptively transferred T cells is associated with tumor-specific release of IFN-γ or type 1 cytokines [Barth et al., 1991; Kawakami et al., 1994b; Shu et al., 1994]. Others have also observed a correlation between the in situ induction of T cells that exhibit tumor-specific release of type 1 cytokines, particularly IFN-γ, and tumor regression [Zou et al., 1995; Tsung et al., 1997]. Additionally, a recent analysis of spontaneously regressing and progressing melanoma lesions suggested a significant correlation between message for type 1 cytokine and tumor regression, thus further extending the hypothesis that tumor-specific type 1 cytokine profiles are associated with immune destruction of tumor metastases [Lowes et al., 1997]. This paradigm of a therapeutic type 1 response and a nonprotective type 2 response closely resembles that observed in animal models of some parasitic diseases [Mosmann and Sad, 1996].

The immune response to *Leishmania major* is a well-studied model of immunoregulation. C57BL/6 mice, resistant to infection with *L. major*, develop a Th1 response following infection and induce CTL that eliminate the pathogen. BALB/c mice are susceptible to infection with *L. major*, because Th1 effector function is inhibited by Th2 cytokines produced in response to infection [Powrie and

Coffman, 1993; Reiner and Locksley, 1995]. Recently it has been demonstrated that type 1-promoting cytokines such as IL-12 or, to a lesser extent, IFN-γ, have therapeutic action in early infection as well as in the presence of a disease-exacerbating Th2-type response [Afonso et al., 1994; Heinzel et al., 1995; Reiner and Locksley, 1995]. The therapeutic success of IL-12 in several animal tumor models, covering the spectrum of immunogenicity, suggests another comparison between the immune response to this parasitic disease and cancer [Nastala et al., 1994; Tahara et al., 1995; Rodolfo et al., 1996; Tsung et al., 1997]. Although observations made using *L. major* cannot necessarily be extrapolated to tumor immunology, they provide substantial information about the significant and diverse effects polarized cytokine response can mediate in vivo.

A number of studies have identified type 2 T cells infiltrating tumor sites [Schoof et al., 1993; Maeurer et al., 1995]. However, it has not been shown that these type 2 T cells are specifically primed to tumor. Recently, a report by Aruga and his colleagues documented both tumor-specific type 1 (IFN-γ) and type 2 (IL-10) cells in TVDLN draining the weakly immunogenic MCA-207 sarcoma [Aruga et al., 1997b]. However, secretion of tumor-specific type 2 cytokines by TVDLN did not block antitumor activity. The implication is that a strong, predominantly type 1 response by the effector T cells in MCA-207 TVDLN could overcome the possibly weaker, type 2 influence to mediate tumor regression. Similarly, Tsung and colleagues observed that progressively growing tumor nodules were associated with T cells producing both IL-4 and IL-10 without evidence of IFN-γ production [Tsung et al., 1997]. Following treatment with IL-12, IFN-γ expression was observed, and tumors regressed even though IL-4 and IL-10 were still produced at the tumor site. A similar observation has recently been reported in the induction of autoimmune diabetes [Rothe et al., 1994]. These results suggest that it is not a single cytokine that is critical to defining effector activity.

Similarly, it is probably not a single cytokine that is critical in defining the subsequent cytokine profile of the responding T cell, but the ratio of type 1 to type 2 cytokines present during activation/differentiation [Nakamura et al., 1997]. Our own data are consistent with this model. While effector T cells exhibit a highly polarized

cytokine profile, the activation of freshly isolated TVDLN T cells with anti-CD3 induces a cytokine milieu that includes IL-4 and IFN-γ. However, the ratio of IFN-γ to IL-4 is substantially depressed for D5 TVDLN, while the ratio for D5-K^d TVDLN is similar to that observed for naive LNC. These observations raise intriguing questions about how the poorly immunogenic D5 tumor induces this alteration in the ratio of type 1 and type 2 cytokines. The easiest explanation might be that the D5 tumor produces type 2 cytokines that skew the antitumor immune response in that direction. However, we have been unable to identify any secretion of IL-3, IL-4, IL-5, or IL-10 by the D5 tumor, negating a role for established type 2 cytokines in this effect (Fig. 2.3) [data not shown]. Alternatively, a recent report by Rincon and colleagues has suggested that IL-6 may also promote the polarization of type 2 responses by inducing IL-4 release by T cells [Rincon et al., 1997]. Future experiments will evaluate this possibility. Additional possibilities are that generation of an immune response to tumor in the absence of proinflammatory type 1 cytokines allows the induction of a type 2 response by default. In this way, the response to tumor may resemble the immune response to self-Ags in transgenic mice, where development of a Th2 response blocks the development of self-Ag-reactive Th1 cells. A similar pattern exists in the immune response to soluble protein Ags, where type 2 responses predominate. Is the poorly immunogenic tumor, which cannot act as its own APC, serving as a slow release reservoir of soluble Ags and thereby inducing a type 2 antitumor response by default?

If one accepts that the type 2 response is the natural default response to Ag in the absence of proinflammatory cytokines, vaccination with allo-modified D5 may provide additional stimuli that skew the response from the default type 2 pathway to a predominantly type 1 profile. We have previously demonstrated that engineering allo-MHC into tumor vaccines is more therapeutic than lipofection with other genes, and Plautz has identified a requirement for a complete functional allo-molecule in the generation of an antitumor effect [Wahl et al., 1992, 1995; Plautz et al., 1993]. Moreover, since type 1 responses are generally associated with the anti-allo response seen in transplant rejection, it is possible that the expression of allo-MHC at the vaccine site contributes to the observed shift [Bach et al., 1997]. However, it has

been difficult to reproducibly identify the generation of an anti-H-2 K^d immune response in D5-K^d TVDLN [H. M. Hu and B. A. Fox, unpublished observation].

Generation of type 1 responses is also favored when higher concentrations of Ag are present during sensitization/activation, while low concentrations of Ag promote type 2 responses [Constant et al., 1995; Hosken et al., 1995]. Recently, we demonstrated that the process of lipofection leads to increased expression of endogenous MHC class I molecules on tumor cells that normally exhibit low to undetectable levels of class I [Fox et al., 1998]. This effect could increase the concentration of tumor Ags and may promote the development of a type 1 antitumor response. Moreover, the process of lipofecting plasmid DNA into tumor cells inevitably carries with it some low level of endotoxin. While most reports suggest that fairly high levels (100 μg) of endotoxin are required to induce substantial in vivo effects, a recent report by Tough et al. demonstrates that even small doses of LPS (10 ng) administered iv indirectly leads to marked proliferation of $CD8^+$ T cells in the LN and spleen [Tough et al., 1997]. Thus, indirect activation of T cells by contaminating LPS in the vaccine preparation may provide proinflammatory cytokines that could polarize the antitumor response in the direction of a type 1 profile. Additionally, bacterial DNA preparations can also induce IFN-γ secretion indirectly via the stimulation of IL-12 and TNF-α secretion by adherent cells [Halpern et al., 1996]. This effect is sensitive to treatment with DNase, implying a requirement for intact DNA. The induction of this cytokine pattern may explain the general tendency of DNA vaccines to induce predominantly type 1 responses [Pisetsky, 1996]. However, DNA and LPS are unlikely to be the sole inducers of a therapeutic immune response in this model. Evidence from several studies confirm that LPS and DNA, present in preparations of ineffective gene constructs and injected intratumorally or mixed with tumor vaccines, are not sufficient to induce highly therapeutic antitumor responses. Therefore, we propose that these molecules may play an accessory role in promoting the activation and differentiation of T cells skewed towards a type 1 profile.

If these observations in the D5 model can be confirmed in patients with cancer, novel strategies may be applied to shift the nontherapeutic profile toward an

effective profile. Several studies have demonstrated that naive and even memory Th cells with the L-selectin$^{low/-}$ phenotype can be forced to differentiate into polarized memory effector cells of either type 1 or type 2 profiles by activation in the presence of exogenous cytokines. IFN-γ and IL-12 in combination with anti-IL-4 neutralizing mAbs are highly effective at polarizing T cells to type 1 profiles, whereas IL-4 and neutralizing anti-IFN-γ mAb can skew T cell responses toward type 2 profiles. In contrast to these studies, cells from mice chronically infected with *L. major* cannot be forced to change their cytokine profile by manipulation of the cytokine milieu [Mocci and Coffman, 1997]. If patients with cancer do develop polarized tumor-specific type 2 responses it will be important to determine whether they can be modulated towards a type 1 profile. If chronic exposure to tumor has permanently altered the cytokine profile to that of type 2, as observed in *L. major*, it will be critical to determine whether pools of naive T cells remain that can be sensitized and skewed toward a tumor-specific type 1 response.

In summary, the poorly immunogenic/nonimmunogenic D5 tumor for which standard vaccination strategies do not provide protection from a tumor challenge sensitizes T cells in TVDLN that exhibit a tumor-specific type 2 response following in vitro activation with anti-CD3 and IL-2. An allo-modified vaccine of the same tumor induces T cells with a tumor-specific type 1 cytokine response that exhibit therapeutic efficacy.

CHAPTER 3

DIVERGENT ROLES FOR CD4$^+$ T CELLS IN THE PRIMING AND EFFECTOR/MEMORY PHASES OF ADOPTIVE IMMUNOTHERAPY*

3.1 Introduction

CD4$^+$ T cells are required for the generation and maintenance of cytolytic CD8$^+$ T cells [Carbone et al., 1998] and are generally believed to be essential for the generation of both a cellular and humoral antitumor immune response [Bennett et al., 1997; Hung et al., 1998; Ossendorp et al., 1998; Pardol and Topalian, 1998; Toes et al., 1999]. However, the contribution of CD4$^+$ T cells to the development, expression and maintenance of antitumor immunity is still the subject of intense investigation. These investigations generally employ either active-specific or adoptive immunotherapy approaches. Active-specific immunotherapy strategies vaccinate the host to prime and expand therapeutic effector and memory T cells [Pardoll, 1998]. In this model one cannot readily separate the contribution of CD4$^+$ or CD8$^+$ T cells to the priming, expansion, effector, or memory phases of the antitumor immune response. Using active-specific immunotherapy, several studies have demonstrated that CD4$^+$ T cells play a critical role in the development of a therapeutic antitumor immune response [Bour et al., 1998; Mackey et al., 1998a; Pardoll and Topolian, 1998; Toes et al., 1999]. Alternatively, adoptive immunotherapy strategies exploit in vitro conditions to activate and expand primed T cells, generally with IL-2, for infusion to the tumor-bearing host [Shu and Rosenberg, 1985; Rosenberg et al., 1988;

---

* Originally published in *The Journal of Immunology* and used here with permission of the American Association of Immunologists.

Hu, H.-M., Winter, H., Urba, W. J., and Fox, B. A. (2000) Divergent roles for CD4$^+$ T cells in the priming and effector/memory phases of adoptive immunotherapy. *J. Immunol.* **165,** 4246–4253.

Crossland et al., 1991]. Since one animal is vaccinated and used as the source of primed T cells and another animal, with systemic tumor, is the recipient of in vitro expanded effector T cells, the contribution of $CD4^+$ and $CD8^+$ T cells to distinct phases of T cell differentiation can be readily determined. In this system, effector T cells generated from tumor-infiltrating lymphocytes or tumor vaccine draining lymph nodes (TVDLN) of weakly immunogenic tumors are generally $CD8^+$ T cells [Shu and Rosenberg, 1985; Rosenberg et al., 1986]. However, effective elimination of systemic tumor requires the coadministration of either tumor-sensitized $CD4^+$ T cells or systemic IL-2 [Spiess et al., 1987; Yoshizawa et al., 1992]. This suggests that the principal role of $CD4^+$ T cells at the effector stage is to provide IL-2 to support or expand $CD8^+$ effector T cells. However, $CD4^+$ T cell help is required during the in vitro activation and expansion of effector T cells from lymph nodes (LN) draining a non-gene-modified weakly immunogenic tumor vaccine [Yoshizawa et al., 1991a, 1992]. This requirement for $CD4^+$ T cells could not be replaced by addition of IL-2 in the culture medium. Recent studies have demonstrated that tumor cells genetically modified to express certain cytokines were more effective as vaccines [Dranoff et al., 1993; Hock et al., 1993]. In most studies GM-CSF-secreting tumor vaccines were among the most effective vaccine strategies [Dranoff et al., 1993; Arca et al., 1996b; Lipshy et al., 1997]. Since GM-CSF can activate and cause differentiation of dendritic cells [Caux et al., 1992], we examined whether one reason for its effectiveness as a vaccine adjuvant might be its ability to bypass the requirement for $CD4^+$ T cell help. Our studies employed a tumor vaccine genetically engineered to secrete GM-CSF and investigated the requirements for $CD4^+$ T cells during priming, in vitro activation and expansion, therapeutic efficacy following adoptive transfer, and ability to support/maintain a protective antitumor memory response.

3.2 Materials and Methods

3.2.1 Mice and Tumor Cell Lines

Female C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). MHC II gene knockout (KO) mice (C57BL/6 Tac–[KO] Aβ N5) and MHC I

and II double KO mice were purchased from Taconic Farms (Germantown, NY). Recognized principles of laboratory animal care were followed [Guide for the Care and Use of Laboratory Animals, National Research Council, 1996], and all animal protocols were approved by the Earle A. Chiles Research Institute Animal Care and Use Committee. D5 is a poorly immunogenic subclone of the spontaneously arising B16BL6 tumor [Arca et al., 1996a]. D5-G6 is a stable murine GM-CSF-transduced D5 clone, which secretes GM-CSF at 400 ng/ml/10^6 cells/24 h [Arca et al., 1996a]. Both were provided by Dr. Suyu Shu, Cleveland Clinic Foundation (Cleveland, OH). MCA-101 is a chemically induced fibrosarcoma (gift from Dr. Nicholas Restifo, National Cancer Institute, National Institutes of Health) [Restifo et al., 1992]. Tumor cells were cultured in complete medium (CM), which consisted of RPMI 1640 (BioWhittaker, Walkersville, MD) containing 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 μg/ml of gentamicin sulfate. This was further supplemented with 50 μM 2-mercaptoethanol (Aldrich, Milwaukee, WI), and 10% fetal bovine serum (Life Technologies, Grand Island, NY). Tumor cells were harvested two or three times per week by brief trypsinization and maintained in T-75 or T-150 culture flasks.

3.2.2 Adoptive Immunotherapy

Vaccination was performed by injecting 1×10^6 D5-G6 tumor cells, subcutaneously (s.c.) into both the fore and hind flanks of mice. Eight days later, inguinal and axillary LNs (D5-G6 TVDLN) were collected, and single-cell suspensions were prepared and cultured at 2×10^6 cells/ml of CM in 24-well plates with 50 μl of a 1/40 dilution of 2C11 ascites (anti-CD3). This dilution was determined previously to be optimal for T cell activation. After 2 days of activation, the T cells were harvested and subsequently expanded at 0.1×10^6 cells/ml in CM containing 60 IU/ml IL-2 (gift from Dr. Marty Giedlin, Chiron, Emeryville, CA) in Lifecell tissue culture flasks (Nexell Therapeutics, Irvine, CA) for 3 additional days. Effector T cells were then harvested and washed twice in HBSS, and graded numbers of effector T cells were injected i.v. into B6 mice with 3-day experimentally established D5 pulmonary metastases (five mice per group unless indicated

otherwise). Pulmonary metastases were generated by tail vein injection of 0.1–0.2 × 10^6 tumor cells. Starting on the day of T cell infusion, mice received 90,000 IU of IL-2 i.p. once per day for 2 or 4 days. Animals were sacrificed 11–13 days following tumor inoculation by CO_2 narcosis. Lungs were resected and fixed in Feketes solution. The number of pulmonary metastases was counted in a blinded fashion. Metastases that were too numerous to count accurately were assigned a value of 250. In some experiments effector T cells were transferred into mice depleted of T or NK cells either by sublethal irradiation (500 rad) 1 day before T cell transfer or by injection of anti-NK1.1 mAb (PK136). In some experiments T cells were transferred into tumor-bearing MHC II KO mice or MHC I and II double KO mice.

3.2.3 Monoclonal Ab-Mediated Depletion of NK Cells or T Cell Subsets

In vivo depletion of $CD4^+$ and $CD8^+$ T cells was accomplished by treatment with mAbs produced by the GK1.5 (anti-CD4, American Type Culture Collection, Manassas, VA (ATCC); TIB 207), 2.43 (anti-CD8, ATCC; TIB 210), or PK136 (anti-NK1.1, ATCC; HB-191) hybridomas, respectively. Vaccination was performed on day 0, and mice were given 50 μl of ascites (anti-CD4 or CD8) i.p. in 0.5 ml HBSS 1 day before and 2 and 5 days after vaccination. This regimen effectively depleted either $CD4^+$ or $CD8^+$ T cells [data not shown] as determined by flow cytometric analysis with labeled anti-CD4 or CD8 mAb (PharMingen, San Diego, CA). Purified rat Ig (500 μg; I-4131, Sigma, St. Louis, MO) was used as control antibody (Ab). NK cell depletion was achieved by administration of 100 μl PK136 hybridoma ascites i.p. 1 day before T cell transfer.

3.2.4 Flow Cytometric Analysis

Flow cytometery was performed on a FACScan (Becton Dickinson, Mountain View, CA), and data were analyzed with CellQuest software (Becton Dickinson). FITC- or PE-conjugated anti-CD4, CD8, NK1.1, I-Aβ, and CD11c were purchased from Pharmingen. FITC-conjugated OX-40 and Ly6-C Abs were prepared in our laboratory with purified Ly6.2-C Ab (from culture supernatant of hybridoma 143-4-2,

ATCC, CRL-1970) and OX86 Ab (gift from Dr. Andrew W. Weinberg). Purified anti-CD16/CD32 mAb was prepared from culture supernatant of the 2.4G2 hybridoma (ATCC) and used to block nonspecific binding to Fc receptors.

3.2.5 Cytotoxicity Assays

Six-hour ^{51}Cr-release assays were used to detect tumor-specific cytotoxicity of effector T cells. Tumor targets, including D5, D5 treated with IFN-γ, and MCA-101, were labeled with $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA), washed, and incubated with effector T cells as previously described [Fox et al., 1990]. D5 tumor cells were incubated overnight with 5 ng/ml recombinant IFN-γ in order to up-regulate MHC class I expression. Standard 3-day lymphokine-activated killer (LAK) cells, generated by culturing splenocytes with 6000 IU/ml IL-2 for 3 days, were used as a positive control for tumor cell lysis in all experiments.

3.2.6 Survival and Tumor Challenge Experiments

Mice bearing 3-day pulmonary metastases were treated with effector T cells generated as described above, but instead of being sacrificed to count pulmonary metastases, they were observed for overall survival. Mice were sacrificed when they became cachectic or moribund. To determine whether surviving mice were immune, they were rechallenged s.c. with 2×10^4 D5 tumor cells ($10 \times TD_{100}$) 50 or 150 days after adoptive immunotherapy. In some experiments mice were depleted of either CD4 or CD8 subsets by injection of 50 μl of ascites (anti-CD4 or CD8) i.p. in 0.5 ml of HBSS 1 day before and 2 and 5 days after rechallenge with D5 tumor cells. The incidence of tumor formation and tumor growth were assessed every other day. Age-matched naive mice served as controls. The significance of differences in survival time between the two experimental groups was determined by Kaplan-Meier analyses and log rank tests.

3.3 Results and Discussion

3.3.1 Increased Number of Dendritic Cells in D5-G6 TVDLN, But Not D5 TVDLN

It is now well accepted that antitumor immunity is initiated by the acquisition of tumor-associated Ags by antigen presenting cells (APC) and their subsequent presentation to immunocompetent T cells. Dendritic cells are thought to be the key APC responsible for cross-priming the immune response to many different Ags [Albert et al., 1998b; Sigal et al., 1999]. CTL priming by a GM-CSF gene-modified tumor vaccine also occurs via a cross-priming pathway [Huang et al., 1994b, 1996]. To examine whether local secretion of GM-CSF increased the number of dendritic cells available for cross-priming an antitumor immune response in TVDLN, we stained the cells from LN draining either unmodified D5 tumor or GM-CSF-producing D5-G6 tumor with anti-CD11c and anti-MHC II I-Aβ, two markers of dendritic cells. Naive LN and TVDLN of D5-vaccinated mice contained a similar number of dendritic cells (1.4 and 1.6%), whereas TVDLN of D5-G6-vaccinated exchibited a 3-fold increase in the number of double-positive DCs (4.8%) (Fig. 3.1).

3.3.2 Increased Number of T Cells with Memory Phenotype in D5-G6 TVDLN

The activation status of freshly isolated TVDLN T cells 8 days following vaccination was assessed by flow cytometry. Expressions of OX40 and Ly6-C were used as markers for the number of activated CD4 and CD8 T cells, respectively [Weinberg et al., 1994; Walunas et al., 1995]. Vaccination with D5-G6 increased the percentage of $OX40^+CD4^+$ T cells to 11.2% compared with 4.4% for D5 TVDLN or 3.9% for naive LN $CD4^+$ T cells (Fig. 3.2). A similar pattern was seen for Ly6-C^+CD8^+ expression. In naive LNC 28.6% of $CD8^+$ T cells expressed Ly6-C^+. D5-G6 TVDLN had a 16.4% increase (from 28.6 to 45.0%) in Ly6-C^+CD8^+ T cells compared with a 6% increase (from 28.6 to 34.6%) for $CD8^+$ T cells from D5 TVDLN (Fig. 3.2). Thus, there is an increased number of activated $CD4^+$ and $CD8^+$ T cells in D5-G6 TVDLN, consistent with the hypothesis that the increased number of DCs are priming more T cells to tumor-associated Ags.

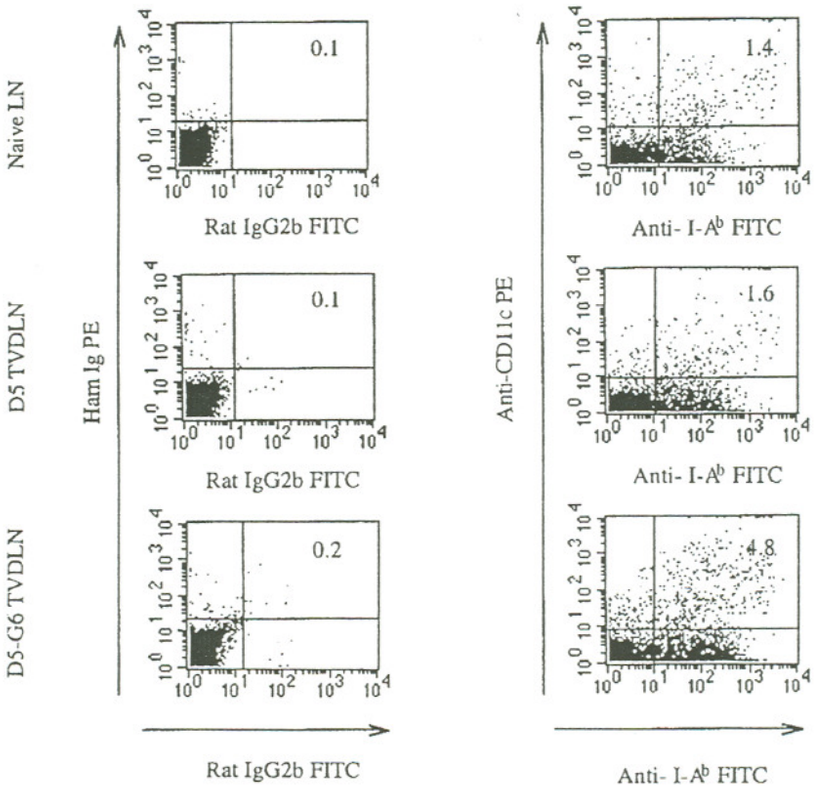


Fig. 3.1 D5-G6 vaccination increased the percentage of dendritic cells in D5-G6 TVDLN. Single-cell suspensions of naive LN, D5, and D5-G5 TVDLN were stained with FITC-labeled control rat IgG2b and PE-labeled control hamster (Ham) IgG, FITC-labeled I-A^b (IgG2a) and PE-labeled CD11c mAb (hamster IgG). Samples were analyzed by CellQuest with FACScan (Becton Dickinson). Ten thousand events were collected and analyzed gated on live cells. The number in the top right quadrant is the percentage of double-positive cells.

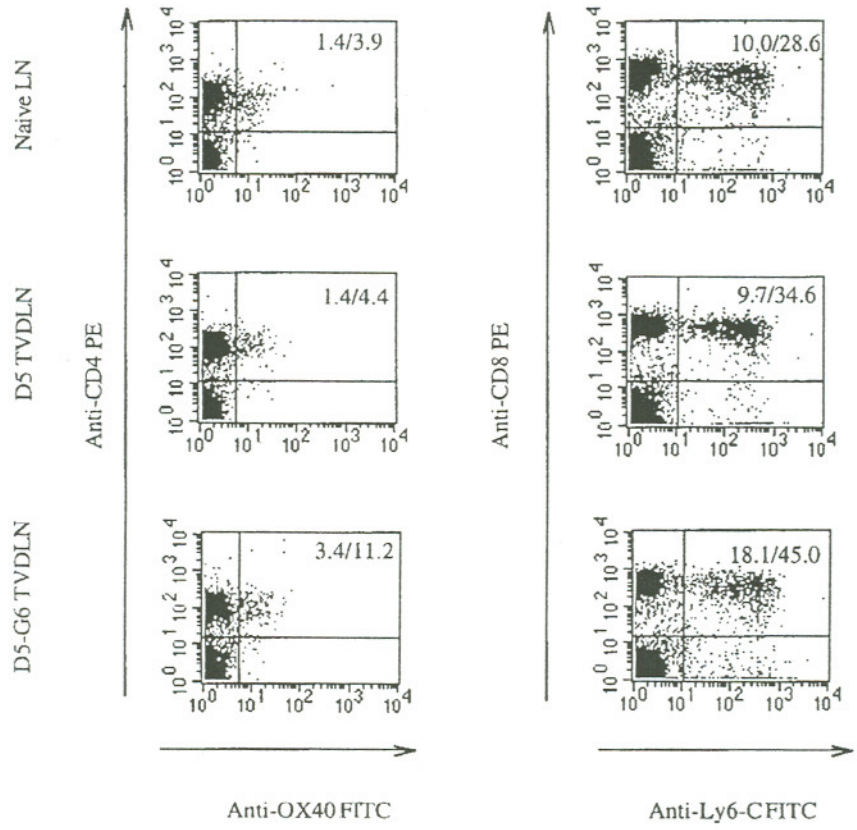


Fig. 3.2 Both CD4 and CD8 T cells are primed in D5-G6 TVDLN. Naive, D5, and D5-G6 TVDLN were stained with either PE-conjugated anti-CD4 Ab and FITC-labeled OX-40 Ab or PE-conjugated anti-CD8 Ab and FITC-labeled anti-Ly6-C Ab. The first number presented in the upper right quadrant is the percentage of double-positive cells in the total population. The second number is the percentage of double-positive CD4 (right three dot plots) or CD8 (left three dot plots) T cells.

3.3.3 D5-G6 Primes Therapeutic T Cells in MHC Class II KO Mice

A major contribution of $CD4^+$ T cells to the cross-priming of $CD8^+$ CTL occurs via the activation of dendritic cells by CD40/CD40L cross-linking [Bennett et al., 1998; Mackey et al, 1998a; Ridge et al., 1998; Schoenberger et al., 1998]. $CD4^+$ T cell help can also be replaced by injecting mice with an anti-CD40 mAb that engages CD40 on dendritic cells, a signal that is normally provided by CD40L on activated $CD4^+$ T cells [Bennett et al., 1998; Schoenberger et al., 1998]. However, some CTL responses to viral Ags develop independently of $CD4^+$ T cell help [Hou et al., 1995]. In this case, direct activation of CTL is mediated by dendritic cells, which, secondary to viral infection, have acquired costimulatory properties in the absence of the cross-linking of CD40 [Ridge et al., 1998]. To address directly the requirement for $CD4^+$ T cell help in the priming of tumor-specific $CD8^+$ T cells, we attempted to generate effector T cells from MHC II KO mice. These mice lack MHC class II-restricted $CD4^+$ T cells and fail to prime CTL responses efficiently [Bennett et al., 1998; Schoenberger et al., 1998]. As shown in Table 3.1, the adoptive transfer of effector T cells generated from MHC II KO mice vaccinated with D5-G6 mediated regression of pulmonary metastases at both high (70×10^6 cells) and low doses (35×10^6 cells) of T cells transferred. This shows that both the priming and subsequent generation of therapeutic $CD8^+$ T cells could be accomplished in the absence of MHC class II-restricted $CD4^+$ Th cells by vaccination with the GM-CSF-secreting tumor, D5-G6. While MHC class II-restricted $CD4^+$ T cells are eliminated in MHC II KO mice, a minor population of $CD4^+$ T cells, many of them restricted by CD1 molecules, have been found in these mice [Cardell et al., 1995]. Recent studies have shown that these CD1-restricted $CD4^+$ T cells can provide help for CTL against *Toxoplasma gondii* or for Ab responses to GPI-linked proteins of *Plasmodium* and *Trypanosoma* [Schofield et al., 1999]. To determine whether this subpopulation of T cells may provide T cell help in the generation of an antitumor immune response in MHC II KO mice, MHC II KO mice were depleted of $CD4^+$ T cells by Ab administration before and after D5-G6 vaccination. Flow cytometric analysis confirmed the depletion (from 4.0 to 0.2%) of this minor population [data not shown]. Effector T cells generated from CD4-depleted mice were highly therapeutic;

complete regression of pulmonary metastases was observed with depletion of minor population of $CD4^+$ T cells (Table 3.1).

3.3.4 $CD4^+$ T Cell-Independent Priming of CTL

To generate both $CD4^+$ and $CD8^+$ effector T cells independently, C57BL/6 wild-type (wt) mice were depleted of $CD4^+$ or $CD8^+$ T cells by Ab administration before and following D5-G6 vaccination. Flow cytometric analysis of TVDLN obtained from anti-CD4 or anti-CD8-treated mice confirmed the depletion of these subsets [data not shown]. Effector T cells generated from nondepleted, CD4-depleted, and CD8-depleted wt mice were then adoptively transferred into mice with established pulmonary metastases. To generate effector T cells from D5-G6 TVDLN, we routinely activate the LN cells with anti-CD3 Ab for 48 h and then expand the activated T cells in CM with low dose IL-2 (60 IU/ml). Previously we have shown that in vitro activated effector T cells generated from D5-G6 vaccinated mice are tumor specific as evidenced by tumor-specific IFN-γ release and tumor-specific cytotoxicity [Winter et al., 1999]. To determine the cytolytic activity of effector T cells generated from CD4-depleted mice, short term ^{51}Cr release assays were performed. Target cells included D5 (very low level of MHC I expression), D5 treated in vitro with recombinant IFN-γ to up-regulate MHC I expression (D5/IFN-γ), and a syngeneic fibrosarcoma, MCA-101. LAK cells were used to prove that targets could be lysed. Both D5 and MCA 101 tumor cells were lysed equally by LAK cells; however, LAK-mediated lysis of IFN-γ-treated D5 was reduced (Fig. 3.3A). We frequently observe this pattern of reduced susceptibility to LAK killing when MHC class I has been upregulated by IFN-γ. The effector T cells generated from TVDLN in D5-G6-vaccinated mice exhibited a low level D5-specific lysis, which became more detectable if D5 cells were pretreated with IFN-γ (Fig. 3.3B). As expected, $CD8^+$ effector T cells (Fig. 3.3C), but not $CD4^+$ effector T cells (Fig. 3.3D), were highly cytolytic.

Effector T cells generated from CD4-depleted mice were highly therapeutic; complete regression of pulmonary metastases was observed at both the high (70×10^6) and low (35×10^6) doses of T cells transferred (Table 3.2). In contrast, effector

Table 3.1

Therapeutic T Cells Can Be Generated from MHC II KO Mice

Donor	Immunotherapy[a]			Mean number of lung metastases		
	Vaccine	T cells $\times 10^6$	IL-2	Expt. 1	Expt. 2	Expt. 3
none			+	250	250	250
MHC II KO	D5-G6	70	+	0[c]	0[c]	0[c]
MHC II KO	D5-G6	35	+	0[c]	0[c]	0[c]
CD4 depleted[b]	D5-G6	70	+	0[c]	0[c]	ND

[a] Eight days after s.c. vaccination of 1×10^6 D5-G6 tumor cells, TVDLNs were harvested from MHC II KO mice, activated with anti-CD3 for 48 h, and then expanded for 3 days in CM with 60 IU/ml IL-2 to generate effector T cells. wt recipient mice were injected with 2×10^5 D5 tumor cells i.v. 3 days before adoptive transfer of effector T cells. IL-2 (90,000 IU in 0.5 ml HBSS) was injected i.p. once per day for 4 days after cell transfer.

[b] CD4 depletion of MHC II KO mice was performed by injection of 50 μl anti-CD4 ascites (GK1.5) 1 day before and 2 and 5 days after vaccination with D5-G6.

[c] $p < 0.05$ compared with group receiving IL-2 alone.

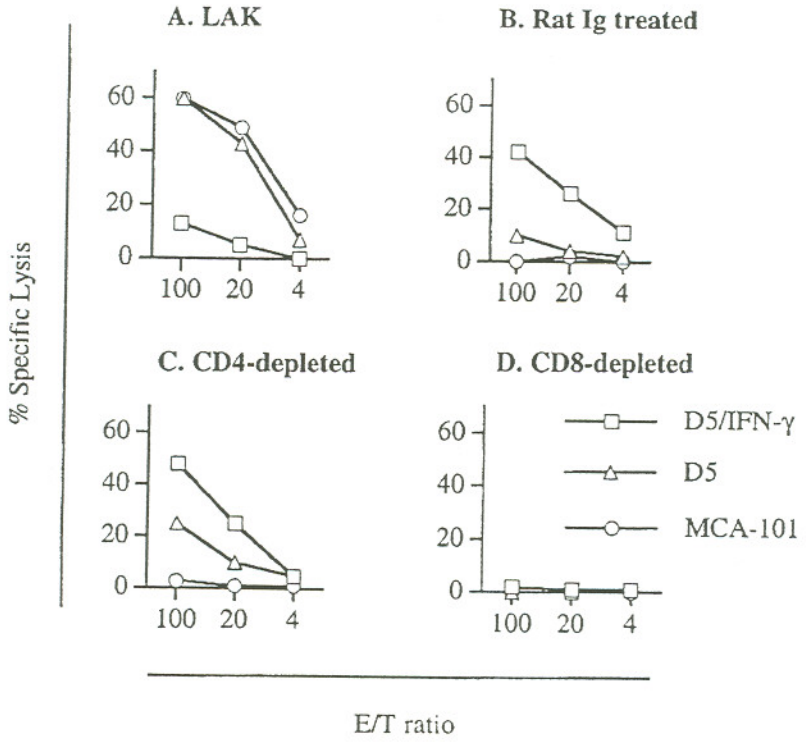


Fig. 3.3 CD4-independent priming of CTL in mice vaccinated with D5-G6. Effector T cells generated from TVDLN of D5-G6 vaccinated, rat IgG-treated, or CD4-depleted mice were tested for cytolytic activity against D5, D5 pretreated with IFN-γ, and MCA-101. Using a 6-h ^{51}Cr release assay, cytolytic activity was determined for LAK (**A**), effector T cells generated from D5-G6 vaccinated rat IgG-treated mice (**B**), effector T cells from D5-G6 vaccinated mice depleted of CD4 (**C**), or CD8 (**D**) cells by mAb treatment. The data represents one of three experiments with similar results.

Table 3.2

$CD8^+$ But Not $CD4^+$ Effector T Cells Are Therapeutic

Donor[a]	Immunotherapy[b]			Mean number of lung metastases (SE)		
	Vaccine	T cells x 10^6	IL-2	Expt. 1	Expt. 2	Expt. 3
none			+	250	250	250
untreated	D5-G6	70	+	0[c]	0[c]	0[c]
untreated	D5-G6	35	+	12 (6)[c]	1 (2)[c]	50 (15)[c]
CD4 depleted	D5-G6	70	+	0[c]	0[c]	0[c]
CD4 depleted	D5-G6	35	+	0[c]	0[c]	0[c]
CD8 depleted	D5-G6	70	+	250	250	250
CD8 depleted	D5-G6	35	+	250	250	250

[a] CD4 and CD8 depletion was done by injection of 50 μl anti-CD4 ascites (GK1.5) and anti-CD8 (2.43) 1 day before and 2 and 5 days after vaccination with D5-G6.

[b] Eight days after s.c. vaccination of 1×10^6 D5-G6 tumor cells, TVDLNs were harvested and activated with anti-CD3 for 48 h and then expanded for 3 days in CM with 60 IU/ml IL-2 to generate effector T cells. Recipient mice were injected with 2×10^5 D5 tumor cells i.v. 3 days before adoptive transfer of effector T cells. IL-2 (90,000 IU in 0.5 ml HBSS) was injected i.p. once per day for 4 days after cell transfer.

[c] $p < 0.05$ compared to group receiving IL-2 or IL-2 plus effector T cells from CD8-depleted D5-G6-vaccinated mice.

T cells from CD8-depleted mice were ineffective at both the high and low doses of T cells transferred (Table 3.2). Consistent with our findings that class II-restricted $CD4^+$ T cells were not required to generate therapeutic T cells in the MHC II KO mice, depletion of $CD4^+$ T cells by mAb treatment did not eliminate the priming of tumor-specific CTL or therapeutic $CD8^+$ effector T cells. This suggests that neither the class II-restricted $CD4^+$ T cells nor the non-MHC II-restricted $CD4^+$ NKT cells are required for efficient priming of $CD8^+$ T cells.

3.3.5 Regression of Established Pulmonary Metastases Following Adoptive Immunotherapy Is Independent of Host T Cells or NK Cells

To ensure that the observed antitumor effects were directly mediated by the adoptively transferred T cells, we had to exclude indirect mechanisms mediated via host T or NK cells. First we transferred effector T cells into tumor-bearing mice ablated of lymphocytes by a sublethal dose of irradiation. While all irradiated control mice treated with IL-2 alone developed >250 pulmonary metastases, complete tumor regression was observed in irradiated mice that received both IL-2 and effector T cells (Table 3.3). Complete eradication of pulmonary metastases was also seen in tumor-bearing mice depleted of NK cells by injection of anti-NK1.1 Ab (PK136) (Table 3.3). Depletion of NK cells by anti-NK1.1 Ab was confirmed by flow cytometry analysis and by elimination of LAK activity as determined by in vitro assays with cells obtained from treated mice [data not shown]. Because irradiation did not completely eliminate all T cells, adoptive immunotherapy was also performed in either CD4-deficient MHC II KO or CD4 and CD8-deficient MHC I and II KO tumor-bearing mice. Again, significant ($p < 0.05$) tumor regression occurred in these mice as well. These results clearly indicate that effector T cells can mediate regression of pulmonary metastases without the participation of host T cells or NK cells.

Table 3.3

Regression of Established Pulmonary Metastases After Adoptive Transfer Dose Does Not Require Receipt NK, $CD4^+$, or $CD8^+$ T Cells

Immunotherapy[a]			Recipient[b]	Mean number of lung metastases (SE)	
Vaccine	T cells	IL-2	Mice	Expt. 1	Expt. 2
none	none	+	wt	250	250
D5-G6	70	+	wt	10 (4)[c]	0
none	none	+	MHC II KO	250	ND
D5-G6	70	+	MHC II KO	9 (7)[c]	ND
none	none	+	MHC I/II KO	146 (36)	ND
D5-G6	70	+	MHC I/II KO	0[c]	ND
none	none	+	Anti-NK 1.1	250	250
D5-G6	70	+	Anti-NK 1.1	7 (4)[c]	1 (1)[c]

[a] Eight days after s.c. vaccination of 1×10^6 D5-G6 tumor cells, TVDLNs were harvested from wt mice, activated with anti-CD3 for 48 h, and expanded for 3 days in CM with 60 IU/ml human recombinant IL-2 to generate effector T cells. Recipient mice were injected with 2×10^5 D5 tumor cells, i.v. 3 days before adoptive transfer of effector T cells. IIL-2 (90,000 IU in 0.5 ml HBSS) was injected i.p. once per day for two days after cell transfer.

[b] MHC II KO and MHC I and MHC II double KO mice were injected with 2×10^5 D5 tumor cells to create tumor-bearing mice deficient in either CD4 or both CD4 and CD8 T cells. Purified anti-NK1.1 mAb (PK136) (500 μg) was injected i.v. into wt tumor-bearing mice 1 day before the adoptive transfer of effector T cells.

[c] $p < 0.05$ compared to mice without T cell transfer.

3.3.6 *Long-Term Survival after Adoptive Immunotherapy with CD8$^+$ Effector T Cells*

To determine whether adoptive immunotherapy with CD8$^+$ effector T cells improved survival, mice bearing 3-day established pulmonary metastases received CD8$^+$ T cells and were observed for survival after adoptive T cell therapy (Fig. 3.4). In experiment 1, 8 of 9 (89%) mice receiving 70 $\times$ 10^6 effector T cells survived for $>$100 days. The one mouse that died did so 21 days after the death of the last control mouse, which received no T cells. In experiment 2, all mice receiving 35 $\times$ 10^6 effector T cells survived $>$100 days, while all control mice that received only IL-2 therapy died before day 25. In the third experiment, 30 of 30 mice that received 35 $\times$ 10^6 effector T cells generated from CD4-depleted mice survived at least 50 days. Thus, effector T cells from D5-G6-vaccinated CD4-depleted mice not only decreased the number of pulmonary metastases, but apparently cured the majority of mice treated.

3.3.7 *Transfer of CD8$^+$ T cells Prolongs Survival but Does Not Cure MHC II KO Mice*

Our results suggest that CD4$^+$ T cells do not play a critical role during either the priming phase or the acute effector phase (as measured by the reduction in pulmonoary metastases) in this adoptive immunotherapy model. To examine whether CD8$^+$ effector T cells can cure mice of systemic tumor without CD4$^+$ T cell help, wt CD8$^+$ T cells were adoptively transferred into MHC II KO mice bearing established pulmonary metastases (Fig. 3.5). Both wt and MHC II KO mice treated with IL-2 alone were dead before day 25. The adoptively transferred CD8$^+$ T cells were highly therapeutic as all wt mice receiving effector T cells survived $>$80 days. However, while MHC II KO mice treated with wt CD8$^+$ T cells survived longer than MHC II KO mice treated with IL-2 alone ($p < 0.05$), they all died before day 70. These MHC II KO mice succumbed to solitary metastases that developed at various anatomic sites (lung, face, hip, and tail). Thus, when only CD8$^+$ effector T cells are adoptively transferred, host CD4$^+$ T cells appear to be critical for either maintaining effector function of CD8$^+$ T cells or for activation of other host effector mechanisms

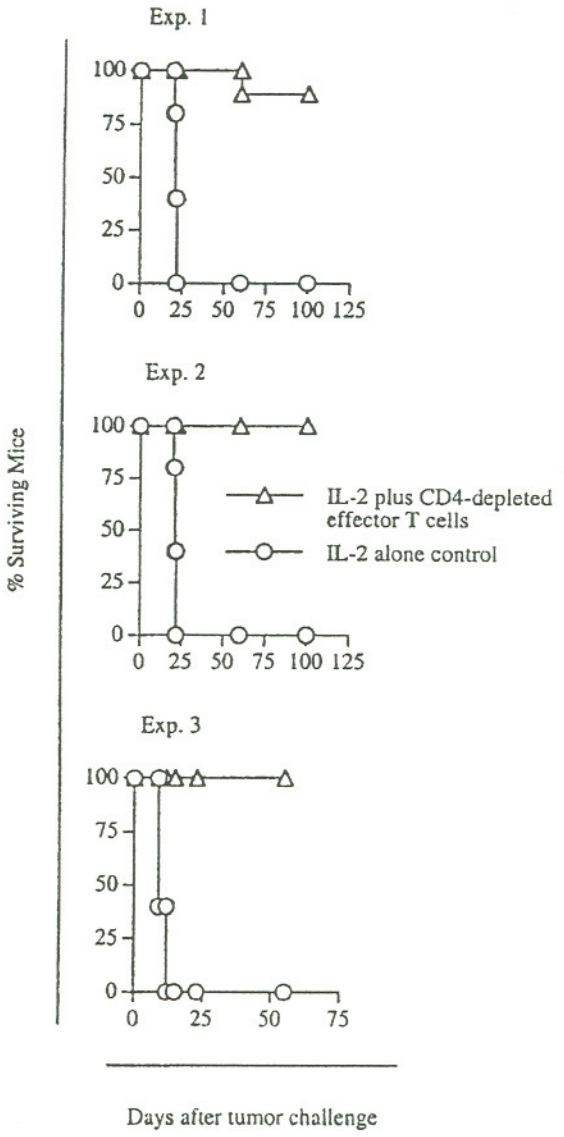


Fig. 3.4 Long-term survival in mice following adoptive immunotherapy. CD4-depleted effector T cells were generated from TVDLN in CD4-depleted wt mice vaccinated with D5-G6. These cells were adoptively transferred into tumor-bearing wt mice created by i.v. injection of 2×10^5 D5 cells 3 days earlier. All mice also received two injection of 90,000 IU of IL-2 i.p. daily. In experiment 1, nine mice were treated with 70×10^6 effector T cells generated from TVDLN of D5-G6-vaccinated CD4-depleted mice and IL-2, and six mice were treated with IL-2 alone (controls). Experiment 2 was the same as experiment 1, except that 35×10^6 T cells were transferred and five mice were used in each group. Experiment 3 was the same as experiment 2, except that 35 mice were used, five of them were used as IL-2 only controls, and 30 mice were treated with CD4-depleted effector T cells.

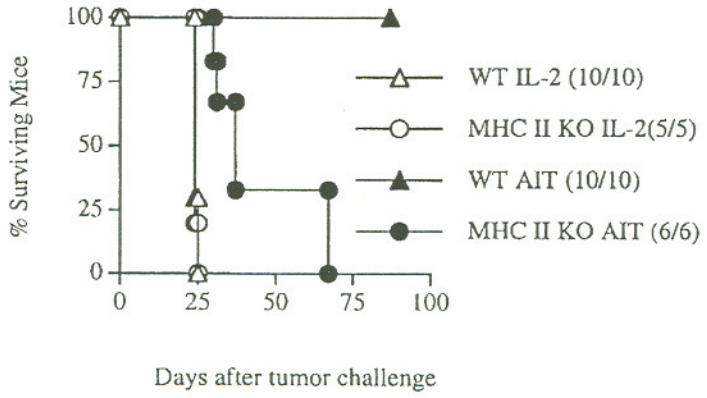


Fig. 3.5 Adoptive immunotherapy fails to cure tumor bearing MHC II KO mice. Adoptive immunotherapy was performed as described in Table 3.1 except that mice were observed for survival. Twenty wt and 11 MHC II KO mice were injected i.v. with D5 tumor cells. Ten wt and six MHC II KO tumor-bearing mice were treated with 70×10^6 effector T cells and IL-2. The remaining mice received IL-2 alone.

that are not required for acute tumor regression in the lung, but are important for complete eradication of residual tumor.

3.3.8 Memory Responses in Mice Cured by Adoptive Immunotherapy

To test whether immunological memory developed in the wt mice cured of systemic tumor by the adoptive transfer of CD4-depleted effector T cells, mice surviving >120 days were subsequently rechallenged with D5 tumor cells. While all naive mice rapidly developed tumor nodules and had to be sacrificed, all eight mice in experiment 1 and three of five mice in experiment 2 were resistant to a subsequent tumor challenge. The two mice in experiement 2 that developed tumor did so much later than the control naive mice that were injected with the same number of tumor cells (Fig. 3.6). Thus, the adoptive transfer of effector T cells from CD4-depleted mice cured the majority of mice, and led to the development of long-term protective immunity in 11 of 13 mice.

3.3.9 Requirement for CD4+ Helper T Cells in Maintaining the Protective Memory Response

Our data clearly show that when vaccination is performed with this GM-CSF-secreting tumor vaccine, neither priming nor the acute effector function of $CD8^+$ T cells requires $CD4^+$ T cell help. However, the requirement for host $CD4^+$ T cells to cure and develop and/or maintenain a protective antitumor $CD8^+$ memory response and the mechanism responsible for this effect need further characterization. Others have reported divergent requirements for $CD4^+$ T cell help depending on the Ag studied and the experimental design. The long-term H-Y-specific CTL memory response is independent of $CD4^+$ T cells [Di Rosa and Matzinger, 1996], although it is generally accepted that $CD4^+$ T cells are important for maintaining a long-term CTL response against viral Ags and for the clearance of chronic viral infections [Stevenson et al., 1998; Zajac et al., 1998]. While our results clearly demonstrated that $CD4^+$ T cells are not critical for priming or effector function immediately following adoptive transfer, we considered it likely that they would be required to maintain a protective antitumor memory response given our results with the MHC II

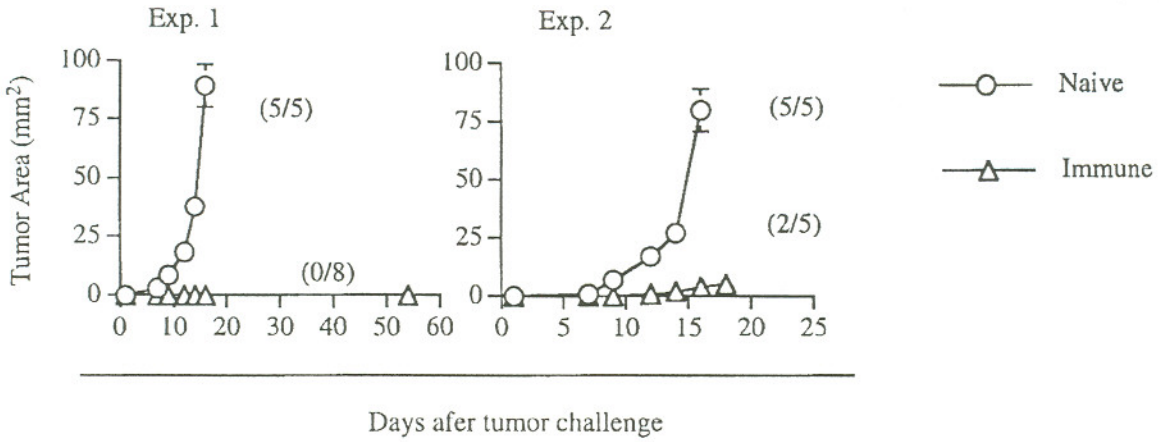


Fig. 3.6 Development of antitumor immunity in mice following adoptive immunotherapy. Surviving mice from were challenged with 2×10^4 D5 tumor cells s.c. about 120 days after adoptive immunotherapy with CD4-depleted effect T cells. The incidence of tumor formation and tumor size were recorded starting 7 days after challenge. The numbers in parentheses indicate the number of mice that developed tumor of the total number of mice challenged.

KO mice. To address the role of $CD4^+$ T cells in maintaining the antitumor memory response, we depleted either $CD4^+$ or $CD8^+$ T cells from mice cured by the adoptive transfer of therapeutic $CD8^+$ effector T cells. These mice had survived either 50 days (short-term memory) or >150 days (long-term memory) and appeared to be tumor free at the time of mAb treatment. These two groups of mice were then challenged with D5 tumor cells and monitored for tumor growth. Mice that had survived 50 days (short-term memory) when they were treated with rat IgG or anti-CD4 were resistant to the tumor challenge, but all mice depleted of $CD8^+$ T cells succumbed to tumor growth ($p < 0.05$) (Fig. 3.7A). Interestingly, at 150 days following adoptive transfer (long-term memory) the majority of CD8-depleted and CD4-depleted mice, but not the control rat Ig-treated mice, developed tumor after the tumor challenge (Fig. 3.7B).

This suggests that $CD8^+$ T cells play the major role in protection against tumor challenge relatively early following adoptive transfer (50 days), but that $CD4^+$ T cells are important in the development and maintenance of long-term memory at later time points (150 days). This does not exclude the probability that $CD4^+$ T cells had played a critical role in maintaining the antitumor $CD8^+$ T cells up until the time they were depleted (at 50 days). Future studies will examine whether the requirement for $CD4^+$ T cells as the time of tumor challenge is a function of there being a sufficient number of $CD8^+$ antitumor T cells available to mediate regression in the absence of T cell help at 50 days, but not at 150 days, following adoptive transfer.

3.3.10 Depigmentation in Mice That Survived a Tumor Challenge

Five of 14 mice that were cured by adoptive immunotherapy with $CD8^+$ effector T cells and survived a secondary challenge of D5 tumor developed various degrees of depigmentation. This started around day 80 after adoptive immunotherapy and ranged from randomly distributed small white patches to a few large spots that continued to progress over time. One mouse with several large areas of depigmentation is shown in Fig. 3.8. A naive untreated mouse is shown for comparison.

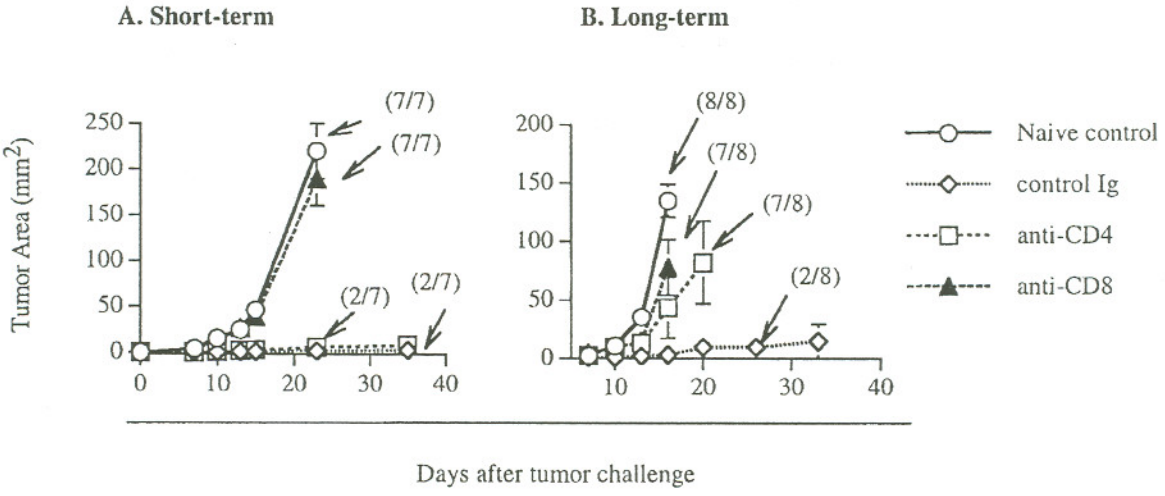


Fig 3.7 $CD8^+$ T cells in immune mice play a major role in protection against a secondary tumor challenge. 50 days (**A**) or >150 days (**B**) after the adoptive transfer of effector T cells generated from D5-G6 TVDLN, mice were treated with control rat IgG, anti-CD4 or anti-CD8 mAb and then challenged with 2×10^4 D5 tumor cells. Age-matched naive mice were included as control. The incidence of tumor formation and tumor size were recorded starting 7 days after challenge. The numbers in parenthesis indicate the number of mice that developed tumor of the total number of mice challenged.



Fig. 3.8 Adoptive transfer of effector T cells from TVDLN of D5-G6 vaccinated mice also induced depigmentation in long-term surviving mice. Fourteen mice treated with therapeutic T cells generated from CD4-depleted D5-G6 vaccinated mice survived >100 days. Five mice developed depigmentation, one of them is shown here with a normal mouse.

Our study demonstrates that a GM-CSF-secreting tumor vaccine is able to prime potent antitumor $CD8^+$ effector T cells without $CD4^+$ T cell help. These effector T cells not only cure the majority of mice with pulmonary metastases, but also induce long-term immunity against tumor challenge. Similar to the memory responses against cytopathic viruses [Stevenson et al., 1998; Belz et al., 2000], the cure and maintenance of long-lasting antitumor immunity generated by adoptive transfer of $CD8^+$ T cells is dependent on $CD4^+$ T cell help. In contrast to viral immunity, the role of Ab responses in long-term tumor immunity is less clear. An Ab response to the melanosome protein, gp75 tyrosinase-related protein (TRP)-1, was shown to be involved in both the regression of B16 melanoma and autoimmune depigmentation [Hara et al., 1995; Takechi et al., 1996].

In the B16 mouse melanoma model, depigmentation can be induced by passive transfer of anti-TRP-1 mAb [Hara et al., 1995] or active immunization with either insect-derived TRP-1 protein [Naftziger et al., 1996], a plasmid DNA containing xenogenic TRP-1 cDNA [Weber et al., 1998], or a recombinant mouse TRP-2 vaccinia virus [Overwijk et al., 1999]. Interestingly, mechanisms required for tumor protection induced by TRP-1 DNA vaccination were different from those required for depigmentation [Weber et al., 1998]. Tumor rejection, but not depigmentation, required $CD4^+$ T cells, $NK1.1^+$ cells, and the Fc receptor γ-chain. Using the same homologous DNA vaccination approach, Bowne et al. were also able to induce tumor immunity by DNA immunization against human TRP-2, but not by immunization with the syngeneic gene [Bowne et al., 1999]. Immunization against human TRP-2 induced both autoantibodies and autoreactive cytotoxic T cells. In this case, both tumor immunity and depigmentation required $CD8^+$ T cells, but not Abs. Interestingly, only depigmentation required perforin; tumor immunity proceeded in the absence of perforin. Thus, immunity induced against two closely related autoantigens, TRP-1 and TRP-2, involved different mechanisms, i.e., Ab versus a $CD8^+$ T cell response [Bowne et al., 1999]. Using vaccinia viruses immunization strageties, Overwijk et al. were able to demonstrate a striking depigmentation and melanocyte destruction in mice vaccinated with murine TRP-1, but not vectors containing other melanocyte differentiation Ags (TRP-2, MART-1, or gp100)

[Overwijk et al., 1999]. These mice also rejected a lethal challenge of B16 melanoma, indicating that the immune response against TRP-1 could destroy both normal and malignant melanocytes. Cytotoxic T lymphocytes (CTL) specific for TRP-1 could not be detected in depigmented mice, but high titers of anti-TRP-1 IgG Abs were present, further supporting the hypothesis that differenc mechanisms (humoral or cellular) can mediate both autoimmunity and antitumor immunity.

To our knowledge, the results presented in this study are the first direct evidence that adoptive transfer of $CD8^+$ T cells alone could induce depigmentation. In agreement with Bowne et al. [1999], we observed that while effector T cells generated from D5-G6-vaccinated wt or perforin knockout mice can cure animals of systemic tumor, only wt effector T cells induce depigmentation [Winter et al., 1999; W. Hauke, H.-M. Hu, and B. A. Fox, manuscript in preparation]. The $CD8^+$ effector T cells used for adoptive immunotherapy were found to recognize the H-2 K^b-restricted dominant TRP-2 peptide [Bloom et al., 1997] and three mouse gp100 peptides [H. M. Hu et al., manuscript in preparation], but not the TRP-1 peptide described by Dyall and colleagues [1998]. Using an ELISA with immobilized proteins from a solubilized melanosome fraction prepared from D5 tumor, we were also able to detect strong anti-melanosome Ab response in long term surviving mice regardless of whether they developed depigmentation [data not shown]. Current studies are investigating the possible contribution of both CD4 Th cells and humoral immunity to the protective antitumor memory response observed in mice cured by adoptive immunotherapy.

Although it remains to be determined whether our observations from the D5 model will extend to other tumor models, elucidation of the mechanism for CD4-independent priming of therapeutic $CD8^+$ T cells will probably yield information critical to the design of therapeutic vaccine and adoptive immunotherapy strategies.

CHAPTER 4
CD28, TNF RECEPTOR, AND IL-12 ARE CRITICAL FOR CD4-INDEPENDENT CROSS-PRIMING OF THERAPEUTIC ANTITUMOR CD8$^+$ T CELLS*

4.1 Introduction

Generation of tumor-specific CD8 T cells via CD4 T cell-dependent cross-priming of tumor Ags by professional antigen presenting cells (APCs), such as dendritic cells or macrophages, is well established [Brossart and Bevan, 1997; Albert et al., 1998b; Fields et al., 1998; Mackey et al., 1998b]. After capturing Ags released from tumor cells, dendritic cells must undergo a maturational process before they are able to prime tumor-specific naive T cells [Brossart and Bevan, 1997; Albert et al., 1998b; Fields et al., 1998; Mackey et al., 1998b]. Dendritic cells maturation occurs following engagement of CD40 on dendritic cells with CD40 ligand (CD40L) on specific CD4 T cells [Bennett et al., 1998; De Saint-Vis et al., 1998; Mackey et al., 1998a; Ridge et al., 1998; Schoenberger et al., 1998; Toes et al., 1998]. Major consequences of this interaction are the upregulation of costimulatory molecules CD80 and CD86 on dendritic cells and the release of inflammatory cytokines, IL-12 and TNF-α.

GM-CSF gene modification of tumor cells has proven to be one of the most effective vaccine adjuvants for priming antitumor T cells in naive mice [Dranoff et al., 1993]. Optimal priming of antitumor CD8$^+$ T cells by a GM-CSF gene-modified

---

* Originally published in this or similar form in the Journal of Immunology and used here with permission of the American Association of Immunologists.

Hu, H.-M., Winter, H., Ma, J., Croft, M., Urba, W. J., and Fox, B. A. (2002) CD28, TNF receptor, and IL-12 are critical for CD4-independent cross-priming of therapeutic antitumor CD8$^+$ T cells. *J. Immunol.* **169**, 4897–4904.

whole cell tumor vaccine requires cross-presentation of tumor Ags by host bone marrow-derived APC, e.g., dendritic cells [Huang et al., 1994a,b, 1996]. However, we have recently shown that the cross-priming of tumor-specific CD8 T cells can occur without $CD4^+$ T cell help. Vaccination of animals depleted of CD4 T cells, as a result of mAb treatment or by targeted mutation of class II, led to the priming of tumor-specific $CD8^+$ T cells, as evidenced by cytotoxicity and IFN-γ release assays [Hu et al., 2000]. Additionally, the tumor-specific $CD8^+$ T cells primed in CD4-deficient animals were highly therapeutic, reducing pulmonary metastases and curing animals with established systemic tumor. These results suggest that molecular interactions other than CD40 and CD40L are involved in the maturation of effective APC when a GM-CSF-secreting tumor is used as a vaccine.

One way a tumor may escape immune destruction is by inducing anergy or tolerance in tumor-specific $CD4^+$ T cells; effectively blocking the priming of therapeutic T cells [Staveley-O'Carroll et al., 1998; Diehl et al., 1999; Sotomayor et al., 1999]. Thus, effective vaccine strategies will need to provide CD4 help or develop CD4-independent approaches to prime therapeutic $CD8^+$ T cells. Our efforts have been focused on the latter, delineating the mechanisms by which CD8 T cells can be primed without CD4 T cell help. To better understand the mechanisms for CD4-independent priming, we examined the role of the important costimulatory molecules, CD40L/CD154, CD80, and CD86, and the inflammatory cytokines IL-12 and TNF during the priming phase of our adoptive immunotherapy model.

4.2 Materials and Methods

4.2.1 Mice

Female C57BL/6J mice and CD40L knockout (KO) mice (B6; 129S-$Tnfsf5t^{m1Imx}$) were purchased from The Jackson Laboratory (Bar Harbor, ME). MHC class II KO mice (C57BL/6Tac-*Abbtm1* N5) were purchased from Taconic (Germantown, NY). Recognized principles of laboratory animal care were followed (Guide for the Care and Use of Laboratory Animals, National Research Council,

1996), and all animal protocols were approved by the Earle A. Chiles Research Institute Animal Care and Use Committee.

4.2.2 Tumor Cell Lines and mAbs

D5 is a poorly immunogenic subclone of the spontaneously arising B16BL6 tumor [Arca et al., 1996a]. D5-G6 is a stable murine GM-CSF-transduced D5 clone, which secretes GM-CSF at 400 ng/ml/10^6 cells/24 h [Huang et al., 1994b]. Both were provided by S. Shu (Cleveland Clinic Foundation, Cleveland, OH). Tumor cells and T cells were cultured in complete medium (CM), which consisted of RPMI 1640 (BioWhittaker, Walkersville, MD) containing 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 μg/ml gentamicin sulfate. This was further supplemented with 50 μM 2-mercaptoethanol (Aldrich, Milwaukee, WI), and 10% fetal bovine serum (Life Technologies, Grand Island, NY). Tumor cells were harvested two-to-three times per week by brief trypsinization and maintained in T-150 or T-225 culture flasks.

Purified rat Ig (500 μg; Sigma-Aldrich I-4131, St Louis, MO) was used as the control Ab. Hamster anti-mouse CD40L (MR1), anti-mouse CD80 (16-10A1) and rat anti-mouse CD86 (GL1) were obtained from American Type Culture Collection (ATCC, Manassas, VA). Ascites fluid was prepared in pristine-primed DBA mice. Hamster anti-mouse CD3 (2c11) and CD28 (PV1) mAbs were purified from culture supernatant by ammonium sulfate precipitation and ion exchange column chromatography. Recombinant soluble TNFR (TNFR-Fc, Enbrel, etanercept) were obtained from Immunex (Seattle, WA). Soluble recombinant receptor activator of NF-κB (RANK) human IgFc fusion protein was a gift from A. Weinberg (Earle A. Chiles Research Institute, Portland, OR) and soluble rLT-βR human IgFc fusion protein was a gift from C. Ware (La Jolla Institute for Allergy and Immunology, San Diego, CA).

4.2.3 In Vivo Priming and In Vitro Activation

In vivo priming of therapeutic T cells was achieved by s.c. injection of D5-G6 tumor cells. Four aliquots of 1×10^6 D5-G6 tumor cells were injected into both the

fore and hind flanks of recipient wild-type (wt), MHC II KO, or CD40L KO mice. When blocking Ab or recombinant fusion proteins were used, they were injected according to the dose and schedule indicated in each table. Eight days after vaccination, enlarged vaccine-draining superficial inguinal and axillary lymph nodes (LN) were collected, and single cell suspensions were prepared by grinding the LN with a pair of sterile glass slides. The resulting cell suspension was washed in HBSS, counted, and activated to generate effector T cells. In some experiments, fresh tumor vaccine-draining LN (TVDLN) were phenotyped, as specified below. Activation was accomplished by culturing TVDLN in CM at 2×10^6 cells/ml in 24-well plates with 5 μg/ml 2c11 Ab (anti-CD3). In some experiments, 10 μg/ml anti-CD28 mAb was added together with the anti-CD3. After 2 days of activation, the T cells were harvested by centrifugation, washed once, and subsequently expanded at 0.1×10^6 cells/ml in CM containing 60 IU/ml IL-2 (kind gift of M. Giedlin, Chiron, Emeryville, CA) in Lifecell tissue culture flasks (Nexell Therapeutics, Irvine, CA) for 3 additional days. Cultured cells were then harvested by centrifugation, washed twice in HBSS, counted, and used in adoptive transfer, cytotoxicity, and cytokine release assays.

4.2.4 Adoptive Immunotherapy

Pulmonary metastases were generated by tail vein injection of 0.2×10^6 tumor cells.

T cells were adoptively transferred i.v. into B6 mice with 3-day experimentally established D5 pulmonary metastases (five mice per group, unless indicated otherwise).

Starting on the day of T cell infusion, mice received 90,000 IU IL-2 i.p. once per day for 2 days. Animals were sacrificed 11–13 days following tumor inoculation by CO_2 narcosis. Lungs were resected and fixed in Fekete's solution. The number of pulmonary metastases was counted in a blinded fashion. Metastases that were too numerous to count accurately were assigned an arbitrary value of 250. In some experiments, effector T cells were transferred into mice injected with 50 μl CD80 and

CD86 mAb ascites diluted in 500 ml HBSS 1 day before and 2 days after T cell transfer.

4.2.5 Flow Cytometric Analysis

Flow cytometry was performed on a B-D FACScan, and data were analyzed with CellQuest software. FITC- or PE-conjugated anti-CD4, CD8, CD11c, CD69, CD80, and CD86 mAb were purchased from BD PharMingen (San Diego, CA). FITC-conjugated anti-mouse OX-40 mAb was prepared in our lab with purified OX86 Ab (gift of A. Weinberg). Purified anti-CD16/CD32 mAb, which was prepared from culture supernatant of the 2.4G2 hybridoma (ATCC), was used to block nonspecific binding to FcR.

4.3 Results

4.3.1 Priming of Therapeutic T Cells Is Independent of CD40/CD40L Interaction

Previously, we documented that priming of therapeutic $CD8^+$ T cells occurred without $CD4^+$ T cell help, if mice were vaccinated with the GM-CSF gene-modified tumor cell line, D5-G6 [Hu et al., 2000]. It is thought that the key contribution of $CD4^+$ T cells is the licensing of APCs that occurs following an interaction between CD40 and CD40L [Toes et al., 1998; Diehl et al., 1999]. This interaction between the $CD4^+$ T cells and APC results in upregulation of costimulatory molecules (CD80 and CD86) and production of inflammatory molecules, IL-12 and TNF-α. Therefore, we sought to determine whether the CD4-independent priming of therapeutic T cells in our model was also CD40L independent. To test this hypothesis, priming with D5-G6 was performed in mice injected with the anti-CD40L blocking Ab, MR-1, using a dose and administration schedule that has been shown to effectively block CD40L function in vivo [Durie et al., 1993]. The results in Table 4.1 clearly show that blocking the interaction between CD40 and CD40L did not inhibit the priming of therapeutic T cells. Complete regression of pulmonary metastases was observed in mice receiving effector T cells generated from either control mice or mice treated with Abs to CD40L (Table 4.1).

Table 4.1

Anti-CD40L Fails to Block Priming of Therapeutic T Cells

Vaccination		Immunotherapy	Pulmonary Metastases Mean (SE)	
Host[a]	Ab[b]	T cells[c]	Expt. 1	Expt. 2
		none	250	250
wt	hamster IgG	35×10^6	0[d]	0[d]
wt	hamster IgG	70×10^6	0[d]	0[d]
wt	anti-CD 154	35×10^6	0[d]	0[d]
wt	anti-CD 154	70×10^6	0[d]	0[d]

[a] Mice were vaccinated with a s.c. dose of 1×10^6 D5G6 tumor cells.

[b] Two days before and 3 and 6 days after vaccination, 50 μl of anti-CD40L (MR-1) Ab (1/10 dilution in HBSS) or control hamster IgG mAb (500 μg) was administered i.p.

[c] Single cell suspensions from day 8 tumor vaccine-draining superficial inguinal LN were stimulated in vitro at 2×10^6 cells/ml with anti-CD3 (2c11) for 2 days in CM, and then expanded for 3 days at a starting concentration of 1.5×10^5 cells/ml in CM supplemented with 10 IU/ml IL-2. A total of 35×10^6 and 70×10^6 cells were adoptively transferred into C57BL/6 mice bearing 3-day D5 pulmonary metastases. A total of 90,000 IU IL-2 was administrated i.p. daily for 3 days after transfer of effector T cells.

[d] Values of $p < 0.01$ compared with IL-2 only treatment group.

Because there was no control to prove the CD40/CD40L interaction was blocked in Ab-treated mice, we sought to ascertain further the contribution of CD40L in the priming of therapeutic T cells by vaccinating CD40L-deficient mice. A critical role for CD40L in both humoral responses and cell-mediated immunity has been documented using these same CD40L knockout mice [Borrow et al., 1996, 1998; Oxenius et al., 1996; Bennett et al., 1998; Grewal and Flavell, 1998; Ridge et al., 1998; Schoenberger et al., 1998; Lefrancois et al., 1999]. TVDLN T cells from both wt and KO mice were activated in vitro by either anti-CD3 alone or anti-CD3 plus anti-CD28, and subsequently expanded in media containing low-dose IL-2. Anti-CD28 Ab was included in the experiment to examine whether in vitro costimulation would effect the generation of therapeutic T cells from wt or CD40L KO mice. Results from Table 4.2 showed that effector T cells generated from both wt and CD40L KO mice were capable of eradicating 3-day established pulmonary metastases (Table 4.2). Although effector T cells generated by stimulation with either anti-CD3 or anti-CD3/CD28 appeared to be equally effective on a per cell basis, stimulation with both anti-CD3 and anti-CD28 doubled the effector T cell yield.

4.3.2 Costimulation by Either CD80 or CD86 Is Critical for Priming Therapeutic T Cells

One major consequence of the interaction between CD40 and CD40L expressed on dendritic cells and CD4 T cells, is the upregulation of CD80 and CD86 expression on the dendritic cell surface. Engagement of CD28 and CD80, or CD86, has been shown to be the critical costimulatory signal for in vivo T cell activation in many different Ag systems. However, there are exceptions. For example, generation of the primary CTL response to lymphocytic choriomeningitis virus (LCMV) has been shown to be CD40L and CD28 independent [Borrow et al., 1996, 1998; Kundig et al., 1996; Oxenius et al., 1996; Andreasen et al., 2000]. To investigate the contribution of CD80 and CD86 to T cell priming in our model, we vaccinated mice in which CD80 and CD86 were blocked alone or in combination with mAbs to mouse CD80 (10-A1) and/or CD86 (GL1). The ability to generate therapeutic T cells was not affected by blocking either CD80 or CD86 alone. Complete tumor regression

Table 4.2

Generation of Therapeutic T Cells from CD40L KO Mice

Vaccination	Immunotherapy		Pulmonary metastases
Host[a]	Stimulation[b]	T cell dose[c]	Mean (SE)
		none	250
wt	CD3	35×10^6	1 (1)[d]
CD40L KO	CD3	35×10^6	14 (6)[d]
wt	CD3/CD28	35×10^6	1 (1)[d]
CD40L KO	CD3/CD28	35×10^6	3 (2)[d]

[a] Wild-type or CD40L KO mice were vaccinated with a s.c. dose of 1×10^6 D5G6 tumor cells.

[b] Single cell suspensions from day 8 tumor vaccine-draining superficial inguinal LN were stimulated in vitro at 2×10^6 cells/ml with anti-CD3 (2c11) or anti-CD3 plus anti-CD28 (PV1) Abs for 2 days in CM, and then expanded for 3 days at a starting concentration of 1.5×10^5 cells/ml in CM supplemented with 10 IU/ml IL-2.

[c] Cells were harvested and adoptively transferred into wt animals with established 3-day D5 pulmonary metastases. A total of 35×10^6 cells were adoptively transferred into C57BL/6 mice bearing 3-day D5 pulmonary metastases. A total of 90,000 IU rIL-2 was administrated i.p. daily for 3 days after transfer of effector T cells.

[d] Values of $p < 0.01$ compared with IL-2-only treatment group.

was observed in mice receiving effector T cells generated from either control mice or mice blocked with anti-CD80 or anti-CD86 in three of three experiments (Table 4.3). However, when mice were vaccinated in the presence of both anti-CD80 and anti-CD86 Abs, therapeutic T cells were not generated by in vitro activation and expansion of these TVDLN (Table 4.4). This strongly suggested that costimulation via CD28, by CD80 or CD86, is critical for the priming of therapeutic T cells, even in a situation that does not require interaction between CD40L and CD40.

4.3.3 Blocking Costimulatory Molecules Reduces Expression of T Cell Activation Markers

If treatment with Abs to both CD80 and CD86 prevented T cell priming in TVDLN, we postulated that the expression of the early T cell activation marker, CD69, would be affected. Therefore, the expression of CD69 was analyzed by flow cytometry. The average percentage of $CD69^+CD8^+$ T cells in naive LNs in three independent experiments was 6.9%. In TVDLNs from D5-G6-vaccinated mice, the average percentage of $CD69^+CD8^+$ T cells increased to 12.3% (Fig. 4.1). This represented a significant increase compared to naive mice ($p < 0.05$). Blocking both CD80 and CD86 resulted in levels of CD69 expression on $CD8^+$ cells that were equal to that expressed by naive lymphocytes. Blocking CD80 or CD86 alone had little effect on CD69 expression in vaccinated mice. Furthermore, treatment with anti-CD40L Ab did not affect the expression of CD69 on T cells. These results are consistent with the notion that CD28, but not CD40 signaling, is critical for T cell priming in our model.

4.3.4 IL-12 and TNF Are Required for Priming Therapeutic T Cells in MHC Class II-Deficient Mice

Previously, we showed that D5-G6 was able to prime therapeutic CD8 effector T cells in MHC II KO mice [Hu et al., 2000]. Ag-loaded MHC II-deficient dendritic cells have been shown to be ineffective vaccines unless preactivated with CD40 ligand [Hermans et al., 1999]. Because CD40L is found on mast cells [Grewal and Flavell, 1998], eosinophils [Gauchat et al., 1995], and platelets [Henn et al., 1998], we

Table 4.3

Anti-CD80 or Anti-CD86 Does Not Inhibit T Cell Priming

Vaccination		Immunotherapy	Mean number of pulmonary metastases (SE)		
Host[a]	Ab[b]	T cells[c]	Expt. 1	Expt. 2	Expt. 3
		none	250	250	250
wt	hamster IgG	35×10^6	0[d]	0[d]	0[d]
wt	hamster IgG	70×10^6	0[d]	0[d]	ND
wt	rat IgG	35×10^6	0[d]	0[d]	0[d]
wt	rat IgG	70×10^6	0[d]	0[d]	ND
wt	anti-CD80	35×10^6	0[d]	0[d]	0[d]
wt	anti-CD80	70×10^6	0[d]	0[d]	0[d]
wt	anti-CD86	35×10^6	0[d]	0[d]	2 (5)[d]
wt	anti-CD86	70×10^6	0[d]	0[d]	0[d]

[a] Mice were vaccinated with a s.c. dose of 1×10^6 D5G6 tumor cells.

[b] Two days before and 3 and 6 days after vaccination, 50 μl of anti-CD80, anti-CD86 (1/10 dilution in HBSS), or control IgG mAb (500 μg) was administered i.p.

[c] Single cell suspensions from day 8 tumor vaccine-draining superficial inguinal LN were stimulated in vitro at 2×10^6 cells/ml with anti-CD3 (2c11) for 2 days in CM, and then expanded for 3 days at a starting concentration of 1.5×10^5 cells/ml in CM supplemented with 10 IU/ml IL-2. Cells were harvested and adoptively transferred into animals with established 3-day D5 pulmonary metastases. A total of 90,000 IU rIL-2 was administrated i.p. daily for 3 days after transfer of effector T cells.

[d] Values of $p < 0.01$ compared with IL-2 only treatment group.

Table 4.4

Anti-CD80 and Anti-CD86 Inhibit T Cell Priming

Vaccination		Immunotherapy	Mean number of pulmonary metastases (SE)			
Host[a]	Ab[b]	T cells[c]	Expt. 1	Expt. 2	Expt. 3	Expt. 4
		none	250	250	250	250
wt	control IgG	35×10^6	13 (18)	0[d]	0[d]	0[d]
wt	control IgG	70×10^6	ND	0[d]	0[d]	0[d]
wt	anti-CD80 and 86	35×10^6	227 (39)	250	250	246 (9)
wt	anti-CD80 and 86	70×10^6	197 (49)	250	ND	250

[a] Mice were vaccinated with a s.c. dose of 1×10^6 D5G6 tumor cells.

[b] Two days before and 3 and 5 days after vaccination, 100 μl of a combination of anti-CD 80 and anti-CD 86 mAb (mouse B7.1 clone 16-10A1 ascites and mouse B7.2 clone GL1 1/10 dilution in HBSS) or 500 μg of control rat IgG plus 500 μg of hamster IgG was administered i.p.

[c] Single cell suspensions from day 8 tumor vaccine-draining superficial inguinal LN were stimulated in vitro at 2×10^6 cells/ml with anti-CD3 (2c11) for 2 days in CM, and then expanded for 3 days at a starting concentration of 1.5×10^5 cells/ml in CM supplemented with 10 IU/ml IL-2. Cells were harvested and adoptively transferred into animals with established 3-day D5 pulmonary metastases. A total of 90,000 IU rIL-2 was administrated i.p. daily for 3 days after transfer of effector T cells.

[d] Values of $p < 0.01$ compared with IL-2-only treatment group.

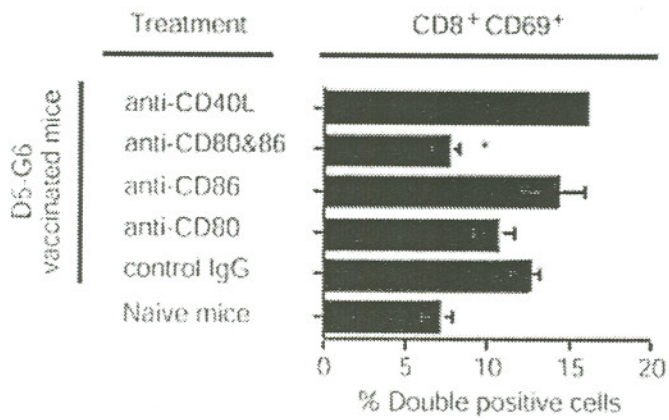


Fig. 4.1 $CD8^+$ T cell activation in D5-G6-vaccinated mice requires costimulation either by CD80 or CD86, but not CD40L. Mice were vaccinated with D5-G6 tumor cells and treated with nothing, anti-CD40L, anti-CD80, anti-CD86 Ab, or a combination of both Abs on the day of vaccination and 3 days later. TVDLN were harvested on day 8, and CD69 expression on CD8 T cells was analyzed by flow cytometry. LN cells (LNC) were stained with FITC-labeled anti-CD8 mAbs and PE-labeled anti-CD69 mAb. Naive LNC were included as a control. Results shown are the mean and SEM of three independent experiments, except data for CD40L were from one experiment.

wanted to rule out the possibility that these cell types could provide CD40 costimulation in MHC class II-deficient mice. Therefore, we performed vaccination and adoptive immunotherapy in MHC class II KO mice that were treated with anti-CD40L blocking Ab. Data presented in Table 4.5 clearly demonstrate that CD40L is not required for the generation of therapeutic T cells in the MHC II KO mice. We also examined the requirement for IL-12, because its production by dendritic cells has been shown to be essential for the generation of antitumor immunity [Zitvogel et al., 1996a; Mackey et al., 1998b]. Treatment of mice with the mAb against the IL-12 p70 heterodimer following vaccination with D5-G6 completely prevented the priming of therapeutic cells (Table 4.5).

Because something other than CD40L must have been inducing costimulatory molecules and triggering IL-12 production by dendritic cells in MHC II KO mice, we sought to determine whether another member of the TNFR superfamily could replace the function of CD40 in our model. We focused on the TNFR superfamily because recent reports have suggested that its members can enhance dentritic cell function as well as T cell expansion and growth [Anderson et al., 1997; Green and Flavell, 1999]. Specifically, the following candidates were examined: TNFR I, lymphotoxin (LT)-βR, and RANK. Both TNFR I and RANK have been implicated in dendritic cell activation [Caux et al., 1992; Anderson et al., 1997; Rieser et al., 1997; Bachmann et al., 1999; Josien et al., 2000], while LT-βR signaling is critical for LN organogenesis and the presence of dendritic cells in the spleen [Futterer et al., 1998; Berger et al., 1999; Endres et al., 1999; Wu et al., 1999].

To determine whether any of these molecules are involved in CD4-independent priming of therapeutic T cells, soluble TNFR I, LT-βR, or RANK human IgFc fusion proteins were used to prevent signaling by TNF-α, LT-α_3, LT-$\alpha_1\beta_2$, LIGHT, and RANK ligand (TNF-related activation induced cytokine). The soluble TNFR I human IgFc protein will block both TNF-α and LT-α_3 signaling, while soluble LT-βR-IgFc blocks both LT-$\alpha_1\beta_2$ and LIGHT signaling [Williams-Abbott et al., 1997; Mauri et al., 1998]. RANK-IgFc is able to neutralize RANK ligand signaling [Anderson et al., 1997]. Although the number of TVDLN cells recovered from MHC II KO mice treated with these blocking agents was not different from the control IgG-treated

Table 4.5

IL-12, But Not CD40L, Is Required for T Cell Priming in MHC II KO Mice

Vaccination		Immunotherapy	Mean number of lung metastases (SE)	
Host[a]	Treatment[b]	T cells[c]	Expt. 1	Expt. 2
		none	250	250
MHC II KO	none	35×10^6	0[d]	0[d]
MHC II KO	hamster Ig	35×10^6	0[d]	0[d]
MHC II KO	CD40L	35×10^6	0[d]	0[d]
MHC II KO	rat Ig	35×10^6	0[d]	0[d]
MHC II KO	IL-12 p70	35×10^6	250	250

[a] C57BL/6 MHC II KO mice were vaccinated with a s.c. dose of 1×10^6 D5G6 tumor cells.

[b] On the day of vaccination, and 3 days later, mice were treated with 50 μl anti-CD40L ascites, anti-IL-12 p70 ascites, 500 μg of control rat Ig, or 500 μg of hamster Ig.

[c] Single cell suspensions from day 8 tumor vaccine-draining superficial inguinal LN were stimulated in vitro at 2×10^6 cells/ml with anti-CD3 (2c11) for 2 days in CM, and then expanded for 3 days at a starting concentration of 1.5×10^5 cells/ml in CM supplemented with 10 IU/ml IL-2. Cells were harvested and adoptively transferred into animals with established 3-day D5 pulmonary metastases. A total of 90,000 IU rIL-2 was administrated i.p. daily for 3 days after transfer of effector T cells.

[d] Values of $p < 0.01$ compared with IL-2-only treatment group.

group [data not shown], a reduction in the percentage of mature dendritic cells (CD11c and CD80 or CD86 positive) was observed in TVDLN from mice treated with soluble TNFR I-IgFc (7.8%) compared to control human IgG (11.04%)-, LT-βR-IgFc (11.08%)-, or RANK-IgFc (11.05%)-treated mice. As a point of reference, mature dendritic cells make up $\sim$1% of the cells from a naive LN.

Unlike treatment with both anti-CD80 and anti-CD86 Abs, the expression of early T cells activation makers (CD69, CD25, or OX40) was not different among control and treated groups [data not shown]. Effector T cells were generated from each group as described, and their therapeutic activity was determined by adoptive transfer into wt mice bearing 3-day established pulmonary metastases. The T cells generated from vaccinated mice that received soluble TNFR I-IgFc fusion protein failed to mediate tumor regression, while T cells generated from vaccinated mice receiving LT-βR or RANK Fc fusion proteins were highly therapeutic (Table 4.6). These data suggest that the TNFR, rather than CD40 signaling, is critical for cross-priming of therapeutic T cells in MHC class II-deficient mice vaccinated with GM-CSF-producing tumor cells. However, it is difficult to appreciate how this happens given only a slight decrease in the number of dendritic cells and the similarities in the T cell activation phenotype of TVDLN from animals treated with TNFR I-IgFc fusion protein.

We hypothesized that treatment with TNFR I-IgFc fusion protein might have interfered with priming of therapeutic $CD8^+$ T cells by blocking production of IL-12 in the TVDLN. To address this, we examined day 8 TVDLN for the presence of mRNA for IL-12 p35 and p40. Although naive LN cells express a very low level of message for p40 and message for p35, day 8 TVDLN exhibited substantial mRNA for both p35 and p40. Surprisingly, we saw no difference in the level of IL-12 mRNA in TVDLN from control mice or animals treated with TNFR I-IgFc fusion protein [data not shown]. Thus, the ability of TNFR I blockade to eliminate the priming of T cells with therapeutic activity cannot be explained by an effect on IL-12.

Table 4.6

Priming of Therapeutic T Cells in MHC II KO Mice

Vaccination		Immunotherapy	Mean number of lung metastases (SE)	
Host[a]	Treatment[b]	T cells[c]	Expt. 1	Expt. 2
		none	250	250
MHC II KO	none	35×10^6	0[d]	0[d]
MHC II KO	human Ig	35×10^6	7 (2)[d]	0[d]
MHC II KO	TNFR-Fc	35×10^6	250	250
MHC II KO	LT-βR-Fc	35×10^6	0[d]	0[d]
MHC II KO	RANK-Fc	35×10^6	0[d]	0[d]

[a] C57BL/6 MHC II KO Mice were vaccinated with a s.c. dose of 1×10^6 D5G6 tumor cells.

[b] On the day of vaccination, and 3 days later, mice were treated with 200 μg of purified human Ig, or fusion proteins i.p.

[c] Single cell suspensions from day 8 tumor vaccine-draining superficial inguinal LN were stimulated in vitro at 2×10^6 cells/ml with anti-CD3 (2c11) for 2 days in CM, and then expanded for 3 days at a starting concentration of 1.5×10^5 cells/ml in CM supplemented with 10 IU/ml IL-2. Cells were harvested and adoptively transferred into animals with established 3-day D5 pulmonary metastases. A total of 90,000 IU rIL-2 was administered i.p. daily for 3 days after transfer of effector T cells.

[d] Values of $p < 0.01$ compared with IL-2 only and TNFR-Fc treatment groups.

4.3.5 Costimulation by CD80 and CD86 Is Not Required for T Cell Effector Function

Recently, we have reported that the adoptive transfer of in vitro activated TVDLN from D5-G6-vaccinated mice can mediate regression of pulmonary metastases directly, without participation of host lymphocytes [Hu et al., 2000]. However, we could not differentiate between the direct cytotoxicity of transferred T cells and the indirect activation of host phagocytes, such as macrophages, as the final effector mechanism responsible for tumor cell killing. Our data indicate that adoptive transfer of $CD8^+$ effector T cells can induce tumor regression in tumor-bearing MHC class I-and class II-deficient mice, thereby implying that direct killing mechanisms are most likely operational in our model. Although we demonstrated that costimulation by either CD80 and CD86 is critical for the priming phase of therapeutic T cells in this model, the role for costimulation during the effector phase of T cell-mediated tumor regression is not known. Where it has been studied, the requirement for costimulation during the effector phase has been reported to vary among systems [Schweitzer and Sharpe, 1998; Chang et al., 1999; Gudmundsdottir et al., 1999; Peterson et al., 1999; Villegas et al., 1999; London et al., 2000; Lumsden et al., 2000; Whitmire and Ahmed, 2000]. To address the contribution of CD80 and CD86 in the effector phase of our adoptive immunotherapy model, therapeutic T cells were transferred into tumor-bearing mice that received either control Ig, or anti-mouse CD80 and anti-mouse CD86 mAbs, before and following T cell transfer. In each case the T cells were highly therapeutic, suggesting that CD80 and CD86 play a minimal role, if any, in the effector phase of our model (Table 4.7).

4.4 Discussion

Our discovery that the priming of therapeutic T cells occurs normally in both CD40L KO mice and mice receiving anti-CD40L blocking mAb demonstrates that priming of $CD8^+$ T cells by D5-G6 tumor cells does not require interaction between CD40 and CD40L. This is consistent with our previous report that $CD4^+$ T cells, which express the CD40L, are not necessary in this model [Hu et al., 2000]. Priming

Table 4.7

Effect of Blocking CD80 and CD86 after Adoptive Transfer of Effector T Cells

Vaccination	Immunotherapy		Mean number of pulmonary metastases (SE)	
Host[a]	T cells[b]	Blocking mAb[c]	Expt.1	Expt. 2
none	none	none	250	250
wt	35×10^6	control IgG	0[d]	0[d]
wt	35×10^6	anti-CD80 and 86	0[d]	0[d]

[a] Mice were vaccinated with a s.c. dose of 1×10^6 D5G6 tumor cells.

[b]Single cell suspensions from day 8 tumor vaccine-draining superficial inguinal LN were stimulated in vitro at 2×10^6 cells/ml with anti-CD3 (2c11) for 2 days in CM, and then expanded for 3 days at a starting concentration of 1.5×10^5 cells/ml in CM supplemented with 10 IU/ml IL-2. Cells were harvested and adoptively transferred into animals with established 3-day D5 pulmonary metastases.

[c] Shortly before adoptive transfer of effector T cells into the tumor-bearing mice, a combination of CD80 and CD86 mAb (mouse B7.1 clone 16-10A1 ascites and mouse B7.2 clone GL1 1/10 dilution in HBSS) or control rat and hamster IgG mAb was administered i.p. The Ab injection was repeated 3 and 6 days after adoptive transfer with the same dose.

[d] Values of $p < 0.01$, compared with IL-2 control group.

of therapeutic T cells was also normal in mice in which costimulation by either CD80 or CD86 was blocked by mAb treatment. However, complete inhibition of priming was observed if CD28 signaling was prevented by administration of mAbs to both CD80 and CD86. This suggests that costimulation via the CD28 pathway is critical for priming of therapeutic T cells, even when a CD40 signal is not required. Furthermore, blocking experiments showed that IL-12 and TNFR signaling are critical for priming therapeutic T cells in CD4-deficient MHC II KO mice, suggesting that TNFR signaling can replace CD40 to activate APCs in our model. In contrast to the priming phase, CD80 and CD86 costimulation is not important during the effector phase of tumor regression, because complete tumor regression occurred in mice receiving T cells in the presence of mAbs to both CD80 and CD86.

The lack of a strong antitumor T cell immune response has been attributed to the lack of expression of CD80 and CD86 on tumor cells. A large number of studies have shown that increased antitumor T cell responses to tumor Ags can be achieved by expressing CD80 or CD86 on tumor cells [Chen et al., 1992; Baskar et al., 1995; Chamberlain et al., 1996]. The efficacy of this approach varies between different tumor models, but the consensus is that protective immune responses can be augmented by exogenous expression of costimulatory molecules only in immunogenic tumor models [Chen et al., 1994]. These findings support a role for tumor cells in direct presentation to T cells. However, most studies show that host professional APC rather than tumor cells are the major presenters of tumor Ags. Therefore, several laboratories have focused on inhibiting CTLA-4 [Hurwitz et al., 1998] or enhancing CD80 and CD86 expression on dendritic cells in order to improve antitumor immunity [Mackey et al., 1998b; Diehl et al., 1999; Sotomayor et al., 1999].

Recently, Lu et al. [2000] have demonstrated that CD4-dependent cross-priming of CTL can occur in a CD40/CD40L-independent fashion. Zhan et al. [2000] recently reported that CD4-independent priming of allogenic tumor-specific CTL is both CD40L and CD28 costimulation dependent. Our data demonstrate that CD4-independent priming of therapeutic $CD8^+$ T cells by a GM-CSF-producing tumor vaccine is CD40L independent, but CD28 dependent. Therefore, cross-priming

of $CD8^+$ effector T cells can be either: (1) dependent on both CD4 Th cells and CD40L; (2) CD4 Th cell dependent, but CD40L independent; (3) CD4 Th independent and CD40L dependent; or (4) independent of both CD4 Th cells and CD40L. In most cases, costimulation via either CD80 or CD86 is critical for cross-priming of $CD8^+$ effector T cells, regardless of whether CD4 Th cells or CD40L are required. However, the Th-independent primary CTL response to LCMV has been found to be both CD40L and CD28 independent [Borrow et al., 1996, 1998; Oxenius et al., 1996; Andreasen et al., 2000]. This observation may be explained by the possibility that a prolonged signal 1 can overcome the requirement for signal 2 [Kundig et al., 1996]. Upon infection with LCMV, the maturation of dendritic cells was found to be CD40 independent while CD8 T cell dependent [Ruedl et al., 1999]. Consistent with the hypotheses that the strength of signal 1 determines the requirement for costimulation and CD4 Th cells, Franco et al. [2000] recently reported that high affinity, but not low affinity MHC class I peptide was able to prime CTLs without CD4 Th cell help. Taken together, there is no generalized requirement for $CD4^+$ T cells and costimulation in CTL cross-priming; the requirement is influenced by multiple factors, including the strength of signal 1 and the cytokine environment.

In this study, we also reported that TNFR and IL-12, but not RANK and LT-βR, are involved in CD4-independent priming of therapeutic CD8 T cells in mice vaccinated with a GM-CSF-transduced tumor vaccine. A similar requirement for TNFR and IL-12 has also been shown for priming therapeutic antitumor T cells by dendritic cells pulsed with tumor peptides [Zitvogel et al., 1996a]. The critical role for TNFR signaling in cross-priming may be explained by either a direct effect on T cells [Joseph et al., 1998; Sepulveda et al., 1999], or indirect effect on dendritic cells, including enhanced induction of IL-12 production [Pape et al., 1997; Vella et al., 1995; Trevejo et al., 2001]. This latter explanation seems less likely given our observation that a similar number of T cells with an activated phenotype, and thus presumably responding to tumor, were recovered from TVDLN of both control and TNFR I-IgFc-treated animals. This argument is further buttressed by the detection of similar levels of IL-12 mRNA in TVDLN of both control and TNFR I-IgFc-treated

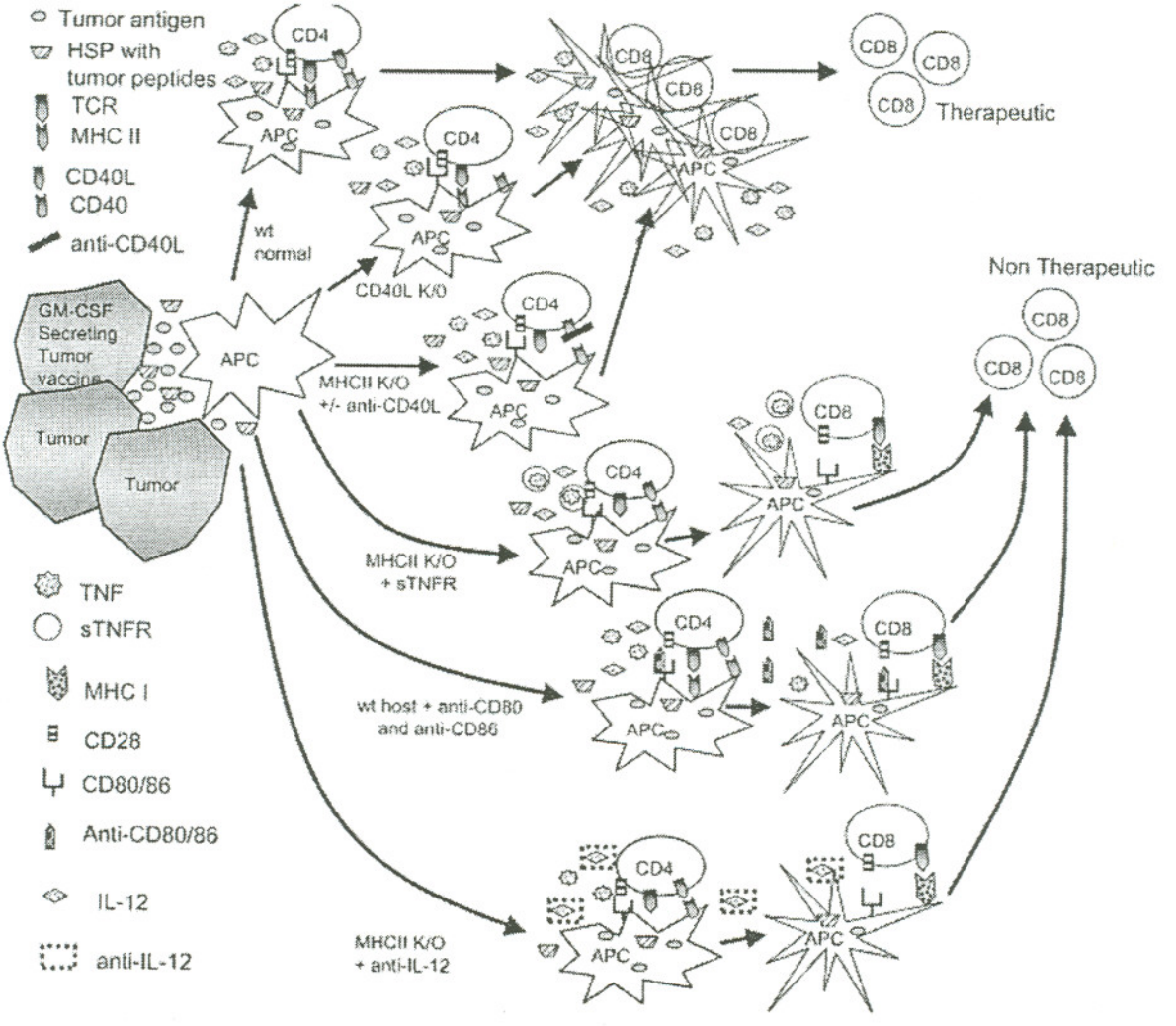


Fig. 4.2 Schematic of CD4-independent cross-priming of $CD8^+$ effector T cells.

animals. Thus, in this model, the critical role for TNF may be a direct effect on $CD8^+$ T cells. Potentially, TNF plays a role in maturing of differentiating these antitumor T cells into fully functional effector cells. Current investigations are trying to identify where in this maturation process TNF plays its critical role.

A more critical question should be how GM-CSF transfection of tumor cells leads to the activation of dendritic cells for the production of IL-12 and TNF. Unfortunately, our studies have not been able to identify which component is responsible for activating the dendritic cells in this model in which high levels of GM-CSF are secreted by the vaccine. In this regard, the following postulation may be feasible. The most likely suspects responsible for activating the dendritic cells in this model are heat shock proteins (hsp). Cross-priming of CTLs by tumor vaccines has been shown to occur through dendritic cell uptake of either apoptotic cells [Albert et al. 1998b; Albert et al., 1998a] or hsp released from necrotic tumor [Udono et al., 1994; Arnold et al., 1995; Cho et al., 2000a; Singh-Jasuja et al., 2000a,b]. Interestingly, the hsp gp96 can mediate dendritic cell maturation in a CD4-independent fashion [Chandawarkar et al., 1999; Cho et al., 2000a], and induce inflammatory cytokines such as IL-12 and TNF-α [Singh-Jasuja et al., 2000a,b]. Further studies will be required to directly investigate whether hsp actually function as an interplayer between GM-CSF transfection and dendritic cell activation. The similarities between the requirements for costimulatory molecules and inflammatory cytokines in our model and that for cross-priming by hsp are so striking that it raises the intriguing possibility that priming by a whole cell vaccine may occur through the hsp representation pathways. In fact, overexpression of hsp70 on B16 melanoma cells not only induced MHC class I expression on the tumor cell surface, it also rendered the B16 melanoma immunogenic [Wells et al., 1997, 1998; Dressel et al., 1999]. Combining this with the capacity of hsp gp96 to induce IL-12 and TNF-α [Singh-Jasuja et al., 2000a,b] provides a possible pathway for CD4-independent cross-priming of tumor-reactive CD8 T cells by dendritic cells [Todryk et al., 1999].

Finally, our studies in animals deficient of MHC class II-restricted T cells or lacking CD40L are likely to be important given the observations that tumor-bearing mice and some humans have significant defects in their CD4 T cell populations.

Therefore, dissecting the critical elements for CD4-independent priming of therapeutic $CD8^+$ T cells may provide a basic paradigm for a therapeutic vaccine strategy for patients with cancer. These findings, summarized in Fig. 4.2, outline the critical signaling pathways that are operational in this mouse model. It is interesting to note that all three appear to exert their effect directly on the effector T cell, and interfering with any one eliminates the generation of therapeutic $CD8^+$ T cells. Clearly, the costimulatory and maturational effects of these molecules play an essential role in antitumor immunity. These findings provide a strong rationale for determining the level of expression of these molecules in TVDLN of patients on clinical vaccine trials. Characterization of these parameters, in addition to monitoring for tumor-specific T cell responses, may provide correlates for objective clinical responses as well as critical insights into possible reasons that vaccines fail. If these characterizations identify defects in CD80/CD86 expression, IL-01 or TNF production, in addition to deficiencies in CD4 T cells, strategies to overcome the defect(s) or supply the appropriate signals will need to be pursued. However, even if CD4-independent cross-priming of therapeutic $CD8^+$ T cells occurs, the effect may not prove curative if CD4 help cannot be induced to maintain the tumor-specific CD8 memory response [Hu et al., 2000].

Overall, our results suggest that in patients in which CD4 responses may be inadequate or deficient, the priming of therapeutic tumor-reactive CD8 T cells can be achieved by augmenting host APC function via local production of GM-CSF. Alternatively, increasing the concentration of peptides chaperoned by hsp may also prime therapeutic $CD8^+$ T cells in the absence of an optimal CD4 response. We are currently engaged in clinical trials of both strategies.

CHAPTER 5
DEVELOPMENT OF ANTITUMOR IMMUNE RESPONSES IN RECONSTITUTED LYMPHOPENIC HOSTS*

5.1 Introduction

Naïve T cells, which normally do not proliferate, or do so at a very slow rate, are driven to proliferate vigorously when they are transferred into lymphopenic hosts (either irradiated or RAG1 knock-out (RAG1) mice) [Surh and Sprent, 2000]. The self-peptides bound by host major histocompatibility complex (MHC) class I and class II molecules not only control T-cell survival [Germain and Stefanova, 1999] but also induce homeostatic proliferation (lymphopenia-driven proliferation) in the periphery of T cell-deficient hosts [Surh and Sprent, 2000]. Homeostatic proliferation occurs in both the CD4 and CD8 T-cell compartments; $CD4^+$ T cells depend on self-MHC II/peptide ligands [Ernst et al., 1999], whereas $CD8^+$ T cells depend on self-MHC I/peptide ligands [Goldrath et al., 2000]. Surprisingly, naïve $CD8^+$ T cells that undergo homeostasis-driven proliferation acquire many characteristics of memory and effector T cells. These characteristics include high levels of expression of CD44 and Ly6C, increased IFN-γ production, and CTL activity [Goldrath and Bevan, 1999; Kieper and Jameson, 1999; Oehen and Brduscha-Riem, 1999; Cho et al., 2000b; Murali-Krishna and Ahmed, 2000]. Recent data also suggested a subset of naïve $CD4^+$T cells acquired memory-like function (secretion of interleukin (IL)-2 and IFN-γ) after expansion in RAG1 mice [Gudmundsdottir and Turka, 2001]. Because

---

* Originally published in this or similar form in *Cancer Research* and used here with permission of the American Association for Cancer Research.

Hu, H.-M., Poehlein, C. H., Urba, W. J., and Fox, B. A. (2002) Development of antitumor immune responses in reconstituted lumphopenic hosts. *Cancer Res.* **62**, 3914-3919.

reconstitution of the T-cell compartment in lymphopenic hosts is regulated by the peptides occupying MHC class I and II molecules at the time of T-cell recovery, there may be an opportunity to skew the T-cell repertoire during T-cell recovery by engaging the available MHC class I and class II molecules with peptides of particular interest. If, as it appears, naïve T cells are more sensitive to activation by weak self-antigens during reconstitution of lymphopenic hosts, there may be a window during which immune tolerance may be broken. If MHC class I and class II molecules presented self peptides, which had been shown to serve as tumor-associated antigens, during a lymphopenic episode, the host may be repopulated with tumor-reactive T cells that could lead to better tumor control. To test this hypothesis, we monitored tumor-specific CD4 and CD8 T cells in tumor vaccine draining lymph nodes (TVDLN) from RAG1 mice vaccinated with melanoma cells following intravenous (i.v.) reconstitution with naïve spleen cells. Adoptive immunotherapy was performed using effector T cells generated from the TVDLNs of reconstituted lymphopenic (RLP) hosts.

5.2 Materials and Methods

5.2.1 Mice, Tumor Cell Lines, and Monoclonal Antibodies

Female C57BL/6J (B6) mice and RAG1 KO mice (B6.129S7-*Rag1*^{tm1Mom} stock number 002216) were purchased from the Jackson Laboratory (Bar Harbor, ME). Recognized principles of laboratory animal care were followed (Guide for the Care and Use of Laboratory Animals, National Research Council, 1996), and all animal protocols were approved by the Earle A. Chiles Research Institute Animal Care and Use Committee. D5 and D5-G6 tumor cells lines have been described previously [Hu et al., 2000]. D5 is a subclone of the B16 melanoma cell line B16BL6. D5-G6, which is a clone produced by transducing D5 with MFG-mGM-CSF retroviral vector, produces ~200 ng/ml/10^6 cells/24 h of GM-CSF. MCA 310 is a chemically induced fibrosarcoma from C57BL/6J mice. B16F10 melanoma is from American Type Culture Collection; B16F10-CIITA.28 is a stable clone that expresses the human MHC class II transactivator, which also exhibits high level I-A^b expression on the cell

surface. CL8-1 is a stable clone of B16 melanoma that was transfected with a plasmid that encodes H-2 K^b. Tumor cells were cultured in complete medium (CM), which consisted of RPMI 1640 (BioWhittaker, Walkersville, MD) containing 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 μg/ml of gentamicin sulfate. This was further supplemented with 50 μM 2-mercaptoethanol (Aldrich, Milwaukee, WI) and 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY). Tumor cells were harvested two to three times per week by brief trypsinization and were maintained in T-150 or T-225 culture flasks. Hamster antimouse CD3 (clone 2c11) and CD28 (clone PV1) mAbs (American Type Culture Collection) were purified from culture supernatant by ammonium sulfate precipitation and ion exchange column chromatography.

5.2.2 Reconstitution, Vaccination and In Vitro Sensitization

RAG1 mice were reconstituted with 20 million naive spleen cells from wild-type B6 mice and then vaccinated with the D5-G6 tumor vaccine as described previously [Hu et al., 2000]. Briefly, four aliquots of 1×10^6 tumor cells each were injected into both the fore and hind flanks of recipient mice. Eight days after vaccination, two enlarged inguinal and two auxillary TVDLNs were collected, and single cell suspensions were prepared by pressing lymph nodes between two sterile glass slides. The TVDLN cells were cultured at 1×10^6 cells/ml of CM in 24-well plates with 5 μg/ml 2c11 antibody (anti-CD3) and 5 μg/ml anti-CD28 mAb. After 2 days of activation, the T cells were harvested and subsequently expanded at 0.1×10^6 cells/ml in CM containing 60 IU/ml IL-2 (Chiron Co., Emeryville, CA) in Lifecell tissue culture flasks (Nexell Therapeutics, Inc., Irvine, CA) for 3 additional days. These in vitro activated and expanded cells are referred to as effector T cells.

5.3.3 Adoptive Immunotherapy

Effector T cells were washed twice in HBSS and injected i.v. into B6 mice in which pulmonary metastases were established 3 days earlier by tail vein injection of 0.2×10^6 D5 tumor cells. Starting on the day of T-cell infusion, mice received 90,000 IU IL-2 i.p. once per day for 3 days. Animals were sacrificed at 13 days

after tumor inoculation by CO_2 narcosis. Lungs were resected and fixed in Fekete's solution. The number of macroscopic pulmonary metastases was counted and metastases that were too numerous to count accurately were assigned a value of 250.

5.2.4 Intracellular Cytokine Staining and Fluorescence-activated Cell Sorter Analysis

For intracellular IFN-γ release assays, four million effector T cells were either not stimulated or stimulated with 2×10^5 D5, MCA 310, F10, F10-CIITA.28 tumor cells, or anti-CD3 antibody in 2 ml of CM in 24-well plates. Brefeldin A was added and the cells were incubated at 37°C for 14–16 h. Cells were harvested and stained with FITC- labeled anti-CD8 antibody, Cy-chrome labeled anti-CD3 antibody, and PE-labeled anti-IFN-γ antibody after fixing and permeablization (Cytofix/Cytoperm kit; PharMingen, San Diego, CA). Fifty thousand gated events based on forward and light scatter were collected and analyzed, all of the analysis was gated on $CD3^+$ cells.

5.2.5 Cytotoxicity Assay

In vitro cytotoxicity of effector T cells was determined as described previously [Hu et al., 2000]. Tumor cells were incubated with 100 mCi of $Na_2{}^{51}CrO_4$ (DuPont NEN, Boston, MA) for 90 min, washed twice, and plated into round-bottomed, 96-well plates with 1×10^4 target cells/well in triplicate. The target cells were incubated with effector T cells at the indicated E:T ratios in a total volume of 200 μl of CM at 37°C in a CO_2 incubator. The supernatant was harvested and counted, and the percentage of specific lysis was calculated as previously described [Hu et al., 2000]. Maximum lysis was determined by incubating target cells with 2% Triton X-100 detergent.

5.3 Results

5.3.1 RLP RAG1 Hosts Developed Strong Tc1 and Th1 Responses

To compare the immune responses in vaccinated normal or RLP hosts, we performed intracellular cytokine staining and flow cytometry on TVDLN lymphocytes

to determine the frequency of tumor-specific T cells. TVDLNs from normal and RLP RAG1 hosts were stimulated with anti-CD3 antibody and anti-CD28 and expanded in IL-2 to generate effector T cells [Hu et al., 2000]. Effector T cells were restimulated in vitro for 14–18 h with D5, MCA 310, F10, F10-CIITA.28, CL8-1, or anti-CD3 antibody in the presence of 5 μM brefeldin A. D5, MCA-310, CL8-1 tumor cells all express class I MHC but do not express MHC class II molecules; F10 and F10-CIITA.28 tumor cells express low and high levels of class II MHC I-A^b molecules, respectively (Fig. 5.1). As expected, the control tumor, MCA 310, failed to stimulate effector T cells from either normal or reconstituted RAG1 hosts. However, the background was significantly higher in RLP RAG1 hosts (Fig. 5.2). In normal hosts, the percentage of TVDLN CD8 T cells that produced IFN-γ ranged from 1.12 to 2.61%, depending on the tumor cells used for stimulation (Fig. 5.2A). The percentage of CD8 T cells stimulated to release IFN-γ in response to the different cell lines is as follows: control MCA-310 (0.88%); D5 (1.83%); F10 (1.12%); F10-CIITA.28 (1.79%); and CL8-1 (2.61%) (Fig. 5.2A). There appears to be a direct correlation between MHC class I expression by the stimulator cells and the percentage of IFN-γ-producing CD8 T cells. The percentage of IFN-γ producing cells at baseline was higher in effector T cells obtained from RLP vaccinated RAG1 mice: 2.24% and 2.5% $CD8^+$ T cells producing IFN-γ without stimulation or stimulated with MCA-310 in RLP hosts, compared to 0.55% with no stimulation and 0.88% with control MCA-310 in normal hosts. There was also a large increase in the percentage of melanoma-specific IFN-γ-secreting CD8 T cells after stimulation of TVDLN effector T cells from RLP RAG1 hosts with different cell lines derived from B16 melanoma: D5 (6.64%); F10 (6.91%); F10-CIITA.28 (10.29%); and CL8-1 (8.16%). Although the "background" stimulation by the control tumor MCA-310 was greater in RAG1 mice compared with normal mice, the increase in melanoma-specific T cells also appeared to be greater in RAG1 mice. Interestingly, although $<1\%$ of CD4 T cells ($CD3^+CD8^-$)from D5-G6 vaccinated normal hosts responded to F10 or F10-CIITA (expressing low and high level of I-A^b molecules, respectively), a much larger proportion of CD4 T cells from vaccinated RLP RAG1 mice were stimulated to

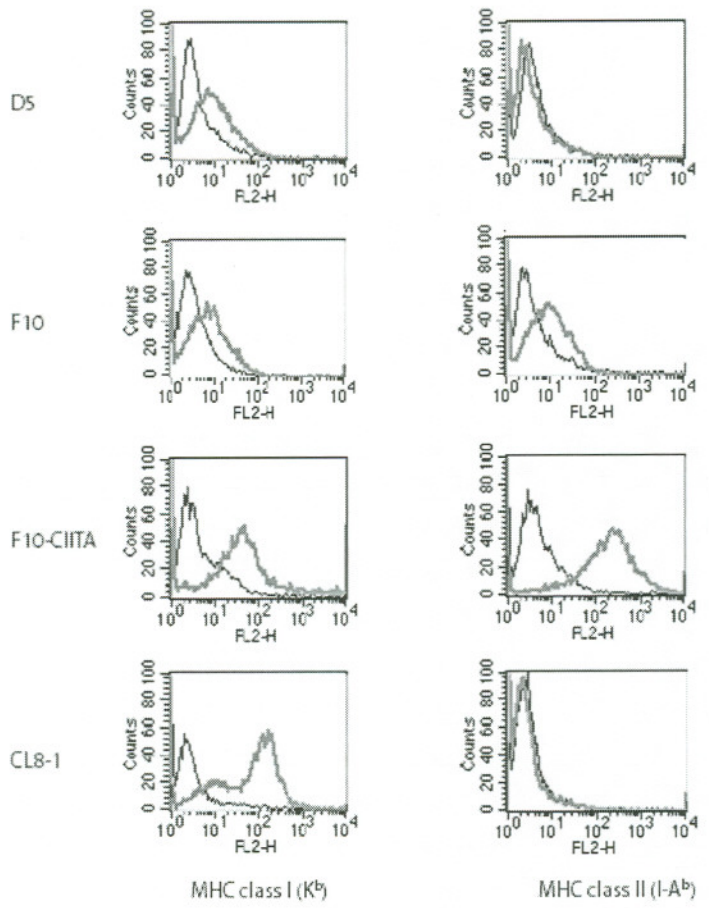


Fig. 5.1 Expression of MHC class I and class II molecules on melanoma cell lines. D5 (B16BL6-D5), F10 (B16BL6-F10), F10-CIITA (clone 28 of F10 cells transfected with human *CIITA* gene), and CL8-1 (clone 8-1 of B16BL6 cells transfected with H-2 K^b gene) are different subclones derived from B16 melanoma. For class I expression, tumor cells were stained with biotin-labeled anti-K^b, biotin-labeled isotype mAbs (PharMingen), and then with phycoerythrin-conjugated strepavidin (Jackson Immune Research Laboratory). For class II expression, tumor cells were directly labeled with phycoerythrin-conjugated anti-I-A^b or isotype mAbs. **Thin line**, background staining by isotype control antibody; **thick line**, staining of the class I or class II antibodies.

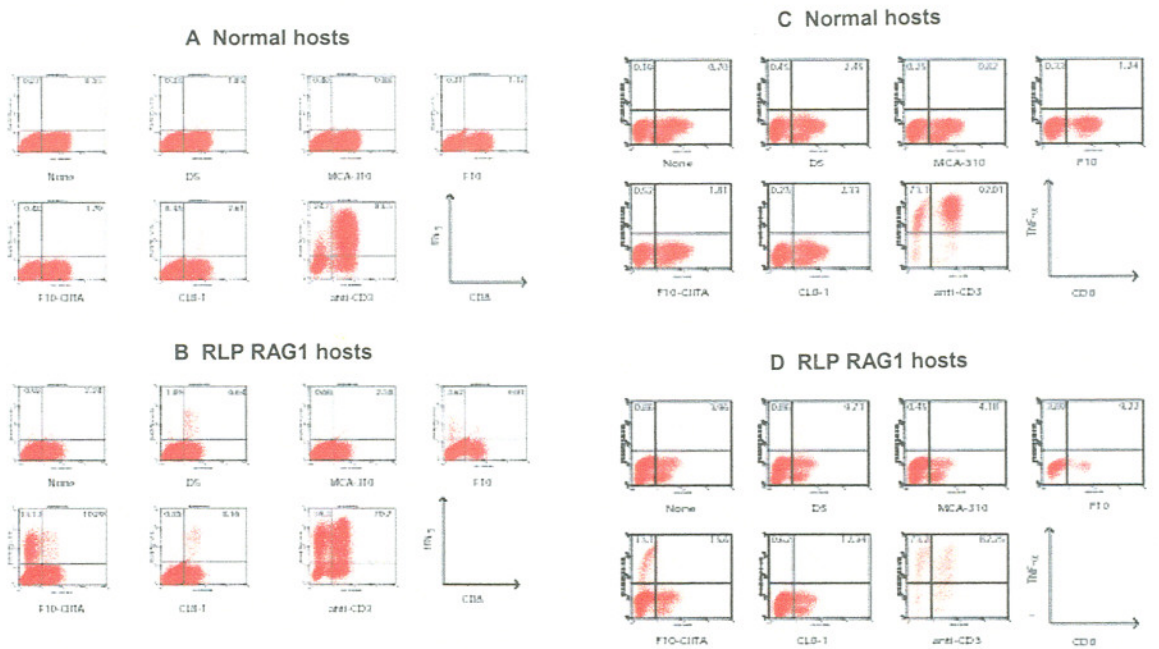
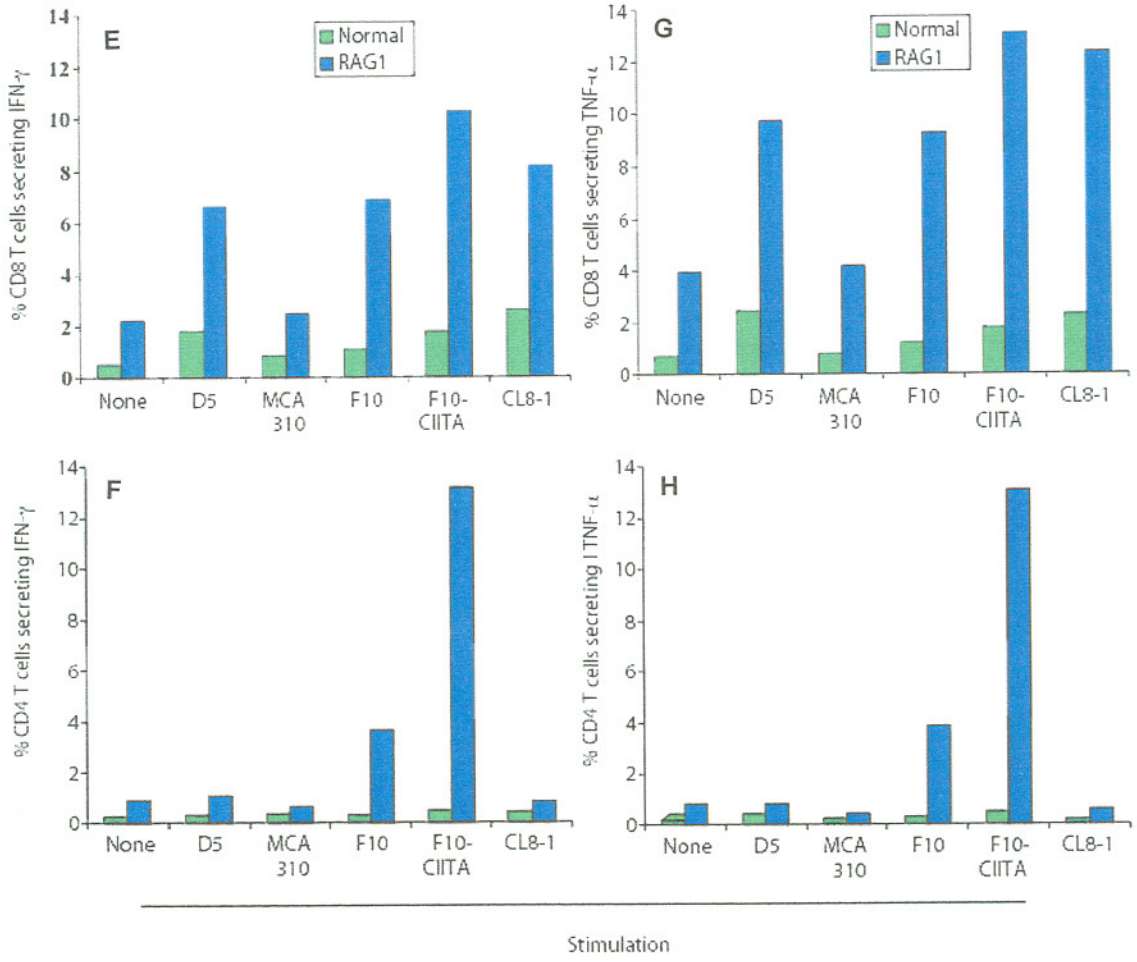


Fig. 5.2 Selective expansion of IFN-γ producing (**A** and **B**) and TNF-α producing (**C** and **D**) $CD4^+$ and $CD8^+$ T cells in vaccinated normal and RLP RAG1 mice. TVDLN effector T cells were derived from D5-G6 vaccinated normal or RLP RAG1 hosts by in vitro stimulation with anti-CD3 and CD28 and expansion in IL-2. T cells were then not stimulated or restimulated in vitro with D5, MCA 310, F10, F10-CIITA.28, CL8-1, or anti-CD3 antibody in the presence of brefeldin A. T cells were harvested and stained with FITC-labeled anti-CD8 antibody and Cy-chrome labeled anti-CD3 antibody. T cells were fixed and permeablized before being stained with either phycoerythrin-labeled isotype control antibodies or anti-IFN-γ or TNF-α antibody. Flow cytometry analysis of labeled cells was performed with a B-D FACScalibur. Fifty thousand "live" cell gated events were collected. All of the dot plots represent $CD3^+$ live cell gated events. The number represent the percentage of IFN-γ^+ in either $CD8^+CD3^+$(top right quarter) or $CD8^-CD3^+$(top left quarter) T cells. **A** and **C** present the intracellular IFN-γ and TNF-α staining results in normal hosts, while **B** and **C** present the results from RLP RAG1 hosts. Similar results were obtained from two other independent experiments. Data are presented as bar graphs for easier comparison (**E–H**, next page).

Fig. 5.2 (continued)



release IFN-γ by F10 (3.62%) and F10-CIITA.28 (13.13%) (Fig. 5.2B). A summary of these data is also presented as a bar graph for easy comparison (Fig. 5.2E and G).

The production of TNF-α by CD4 and CD8 tumor-specific T cells in vaccinated normal and RLP RAG1 hosts was also examined. Results similar to those for IFN-γ production were obtained. CD8 T cells from normal hosts exhibited an increased TNF-α response after stimulation with each of the melanoma cell lines but not the MCA-310 cell line (Fig. 5.2C). Although baseline or nonspecific TNF-α production was higher, the largest increase in melanoma-specific TNF-α-producing CD4 T cells was found in RAG1 RLP compared with normal hosts (Fig. 5.2C and D). A summary of these data is also presented as a bar graph for easy comparison (Fig. 5.2F and H). The significantly higher number of melanoma-specific T cells observed in RLP RAG1 mice supports our hypothesis that antigen-specific T cells can be expanded preferentially in reconstituted lymphopenic hosts if appropriate antigen is presented during immune reconstitution.

To determine whether effector T cells generated from RLP RAG1 mice exhibited tumor-specific cytotoxicity, a 6-h ^{51}Cr-release assay was performed. Although effector T cells from both normal and RLP RAG1 mice failed to kill control tumor MCA 310, effector T cells generated from RLP RAG1 mice, but not normal mice, were highly cytolytic to D5 tumor cells in vitro (Fig. 5.3). These data demonstrate that vaccination during reconstitution of a lymphopenic host can result in a strong type 1 (both Th1 and Tc1) immune response.

5.3.2 Effector T Cells Obtained from Vaccinated RLP RAG1 Mice Are Highly Therapeutic

To determine whether the presence of a higher frequency of Th1 and Tc1 cells in the TVDLNs from RLP RAG1 mice would confer superior therapeutic efficacy in vivo, we adoptively transferred effector T cells into mice bearing established 3-day pulmonary metastases. Each mouse also received 90,000 IU IL-2 by i.p. injection for 3 days after T-cell transfer to support the survival and anti-tumor activity of the T cells. Effector T cells generated from reconstituted RAG1 KO mice exhibited greater potency on a per cell basis compared with effector T cells from normal hosts

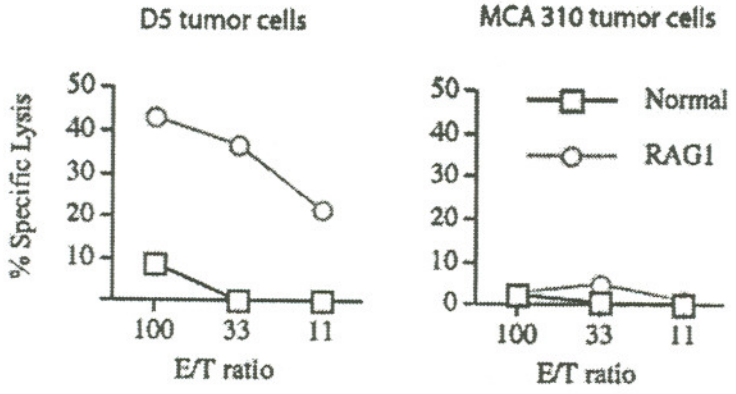


Fig. 5.3 TVDLN T cells derived from vaccinated RLP RAG1 mice were highly cytolytic. D5 and control MCA 310 tumor cells were labeled with ^{51}Cr and then incubated with TVDLN effector T cells derived from either normal or RAG1 hosts for 6 h. Effector T cells derived from RAG1 hosts, but not normal hosts, were highly cytolytic to D5 tumor. Effector T cells from both normal and RAG1 mice failed to kill control MCA 310 tumor cells.

(Table 5.1). Results from two independent experiments demonstrated that 15×10^6 effector T cells generated from reconstituted RAG1 KO mice mediated complete tumor regression (no metastases), whereas the same number of T cells from normal mice failed to mediate complete tumor regression in three independent experiments. In experiment 1, as few as 5×10^6 effector T cells from RAG1 KO mice were able to mediate complete tumor regression, whereas 15×10^6 effector T cells from normal mice failed to eradicate tumor. In experiment 2, 15×10^6 effector T cells from vaccinated RLP RAG1 hosts mediated complete tumor regression, whereas 35×10^6 effector T cells from normal mice were not able to do so. In three of three experiments, 5×10^6 T cells from RLP mice mediated significantly better tumor regression than three times as many, 15×10^6, T cells from normal hosts ($p < 0.05$). Data from these three independent experiments were combined and represented in a graphic format (Fig. 5.4A). These results demonstrated that there is direct correlation between in vivo therapeutic activity and the percentage of melanoma-specific T cells measured by intracellular cytokine staining. This is consistent with our previous observation that $CD8^+$ T cells are the effector T cells that mediate the regression of pulmonary metastases in this animal model [Hu et al., 2000].

5.3.3 Delayed Vaccination Primes T Cells with Less Therapeutic Activity

After transfer into lymphopenic hosts, naïve T cells reach their peak of proliferation between days 5 and 7; their number reaches a plateau at day 20, after which the space is "filled," and the number of T cells in the hosts remains constant [Goldrath et al., 2000]. To determine whether the improvements in vaccination depended on stimulating T cells during homeostatic proliferation or had something to do with the adoptive transfer of naïve T cells, we vaccinated RAG1 mice 1 week after reconstitution with 20×10^6 naïve splenocytes when their proliferation reaches a peak. Normal mice without reconstitution were also vaccinated as the controls. Effector T cells from both groups of mice were generated and adoptively transferred into tumor-bearing mice as described. The results of these experiments are shown in Table 5.2. Vaccination after adoptively transferred naïve T cells have reached their peak of proliferation is less effective than vaccination at the time of T-cell transfer.

Table 5.1

Concomitant Vaccination and Reconstitution in RAG1 Mice Results in Highly Therapeutic T Cells

Group	Immunotherapy[a] at day 3		Mean number of pulmonary metastases[b] at day 13		
	T-cells donor	T cells × 10^6	Expt. 1	Expt. 2	Expt. 3
		none	>250	>250	>250
1	normal	35	0[c]	46 (30)[c]	44 (7)[c]
2	normal	15	19 (29)[c]	189 (49)[c]	>250
3	normal	5	198 (77)	>250	ND
4	RAG1/RLP	35	ND	ND	ND
5	RAG1/RLP	15	0[c,d]	0[c,d]	60 (16)[c,d]
6	RAG1/RLP	5	0[c,e]	78 (22)[c,e]	172 (55)[c,e]

[a] RAG1 KO mice were reconstituted with 20 × 10^6 naïve spleen cells from wt C57BL6 mice. Immediately after reconstitution, both normal and reconstituted RAG1 mice were vaccinated with D5-G6 cells s.c. TVDLN were harvested 8 days later and stimulated with anti-CD3/CD28 for 48 h. T cells were expanded in complete medium with 60 IU/ml IL-2 for 3 days. The indicated number of in vitro activated T cells (effector T cells) were adoptively transferred into tumor-bearing mice. After T-cell transfer, mice received 90,000 IU IL-2 daily for 3 days.

[b] Mice were injected i.v. with 2 × 10^5 D5 tumor cells to establish pulmonary metastases. A total of 5 mice were used for each experimental group. ND, not done.

[c] $P < 0.05$, compared with mice without T-cell transfer.

[d] $P < 0.05$, group 2 compared with group 5.

[e] $P < 0.05$, group 3 compared with group 6.

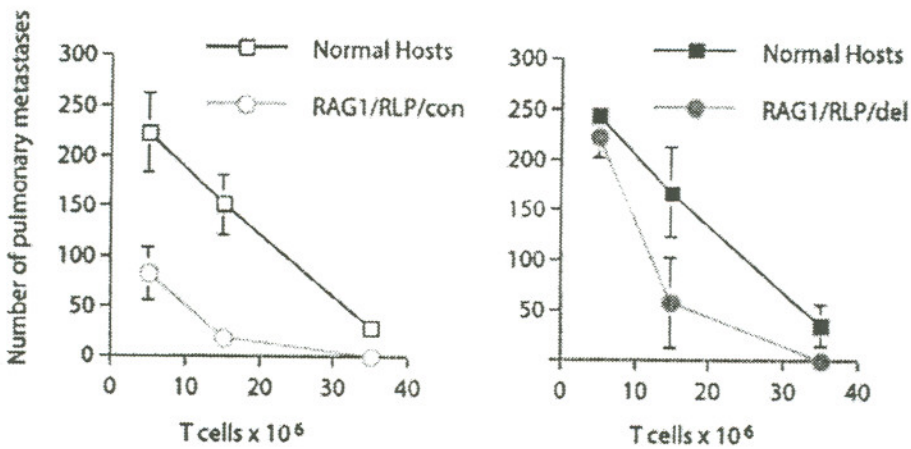


Fig. 5.4 Concomitant vaccination of reconstituted RAG1 mice generates T cells with greater therapeutic activity than delayed vaccination. Normal or RAG1 mice were reconstituted with 20×10^6 naïve spleen cells and vaccinated immediately (A) or delayed for 1 week. TVDLNs were collected 8 days after vaccination and adoptively transfer into mice bearing 3-day D5 lung metastases after activation with anti-CD3 and CD28 mAbs and expanded in IL-2 medium. Five, 15, and 35 million T cells were transferred in each experiment, as indicated in Tables 5.1 and 5.2. The number of lung metastases in mice without T cells transfer is >250. The mean number of lung metastases is from two or three experiments, as indicated in Tables 5.1 and 5.2. **Bars**, SE; for some group of mice the error bar is too small to be seen.

Table 5.2

Delayed Vaccination in RAG1 RLP Mice Results in Less Therapeutic T Cells

Group	Immunotherapy[a] at day 3		Mean number of pulmonary metastases[b] at day 13		
	T cells donor	T cells $\times 10^6$	Expt. 1	Expt. 2	Expt. 3
		none	>250	>250	>250
1	normal	35	0[c]	87 (50)[c]	16 (19)[c]
2	normal	15	113 (66)[c]	ND	221 (24)
3	normal	5	243 (11)	ND	245 (8)
4	RAG1/RLP	35	ND	0[c,d]	1 (1)[c,d]
5	RAG1/RLP	15	38 (33)[c]	36 (27)[c]	96 (88)[c]
6	RAG1/RLP	5	192 (37)[e]	ND	>250[e]

[a] RAG1 KO mice were reconstituted with 20×10^6 naïve spleen cells from wt C57BL6 mice. One week after reconstitution, both normal and reconstituted RAG1 mice were vaccinated with D5-G6 cells s.c. TVDLN were harvested 8 days later and stimulated with anti-CD3/CD28 for 48 h. T cells were expanded in complete medium with 60 IU/ml IL-2 for 3 days. The indicated number of in vitro activated T cells (effector T cells) were adoptively transferred into tumor-bearing mice. After T-cell transfer, mice received 90,000 IU IL-2 daily for 3 days.

[b] Mice were injected i.v. with 2×10^5 D5 tumor cells to establish pulmonary metastases. A total of 5 mice were used for each experimental group. ND, not done.

[c] $P < 0.05$, compared with mice without T-cell transfer.

[d] $P < 0.05$, group 4 compared with group 1.

[e] $P > 0.05$, compared with group 3 and the group without T-cell transfer.

Although concomitant vaccination experiments were not performed at the same time, T cells from RLP RAG1 mice with delayed vaccination were less therapeutic. In contrast to T cells from RLP RAG1 mice with concomitant vaccination, 5 million effector T cells from mice with delayed vaccination were not more effective than 15 million effector T cells from normal hosts and actually were not therapeutic compared with mice without T-cell transfer (Table 5.2 and Fig. 5.4B). These results indicate that vaccination efficacy in RLP mice is influenced by both proliferation rate of naïve T cells and space available for expansion of both antigen-specific and non-specific T cells.

5.4 Discussion

A major goal of current cancer immunotherapy strategies is to increase the immunogenicity of tumor cells. These strategies include mixing tumor cells with bacterial adjuvants, genetically modifying them to produce cytokines or express costimulatory molecules, and combining them as lysates or cell fusions with dendritic cells. In animal models, tumor vaccines have been shown to elicit immune responses that protect animals from tumor growth after subsequent tumor challenge. However, tumor vaccine strategies usually fail to elicit immune responses that are potent enough to eradicate established tumors. The delay in the development of an effective immune response and the overall weakness of the response when it finally develops are possible explanations for the observed lack of efficacy after active immunization of tumor-bearing hosts. Improved immunization strategies are required to produce therapeutic immune responses to tumor vaccines in tumor-bearing hosts. A major impediment continues to be the lack of potent tumor rejection antigens. In melanoma and many other tumors, the potential tumor rejection antigens are weak self-antigens. Because the tumor antigens are self-proteins, high-affinity T cells, which are likely to be the most effective antitumor T cells, will already have undergone negative selection in the thymus and or silencing by other peripheral mechanisms. We know, however, that tumor-bearing hosts are not completely tolerant of these self-antigens, because vaccination with altered peptide ligands or xenogenic proteins (heteroclitic

immunization) can result in a strong immune response to not only the modified antigen but the native antigen as well [Dyall et al., 1998]. For the melanoma antigen gp100, vaccination with modified peptide dramatically enhanced the immune response to the modified peptide but also to native peptides presented by melanoma cells [Lee et al., 1999].

For many cancers, the tumor-associated antigens are unknown, and whole tumor cells are often used as vaccines. One way to enhance an antitumor immune response would be to devise a situation in which tumor-specific T cells are "tuned" to become more sensitive to activation by weak tumor antigens. It has been reported that a naïve T-cell repertoire could be skewed to increase the relative percentage of antigen-specific T cells if antigen was given during immune reconstitution [Machall et al., 1996]. Borrelle et al. [2000] recently demonstrated enhanced efficacy of a GM-CSF gene-modified lymphoma vaccine in lethally irradiated mice reconstituted with T-cell-depleted bone morrow. Our findings extend the report of Borrelle et al. [2000] in several important ways:

(a) Because vaccination is augmented in reconstituted RAG-1 mice that are not irradiated, it documents that the cytokine storm induced by lethal irradiation is not required for the increased antitumor effect.

(b) Because we used spleen cells and vaccinated immediately after reconstitution, it suggests that the augmented antitumor response does not require differentiation of new T cells from bone marrow stem cells. These two findings are likely the most important and clinically relevant observations of our study, since lethal whole body irradiation and bone marrow transplant will likely prove to be roadblocks to widespread clinical application of this vaccine strategy for solid tumors.

(c) In contrast to their use of TCR transgenic T cells and a foreign model tumor antigen, influenza hemaglutinin, to detect an increase in antigen-reactive T cells, we were able to show strong CD4 and CD8 T-cell responses to native antigens presented by tumor cells. These results also infer that vaccination during a time of natural lymphocyte recovery from lymphopenia could increase the magnitude of the immune response to a vaccine. Because lymphocyte recovery occurs regularly in treated cancer patients [Mackall et al., 1994], our data suggest that the development

of transient lymphopenia following chemotherapy or irradiation may be exploited by vaccinating patients during these lymphopenic episodes to boost immunity and possibly induce tumor regression and prevent tumor recurrence.

Currently, little is known about the mechanisms that regulate the homeostasis of naïve T cells in lymphopenic hosts. Two nonexclusive explanations have been proposed to explain this phenomenon. One is the "fill up the space" hypothesis [Tanchot et al., 1997], which states that naive and memory T cells occupy specific survival niches. The induction of lymphopenia empties these niches and allows naïve cells to expand and fill the empty "space." The other regulatory or suppressor cell hypothesis proposes that regulatory cells counter self-reactive T cells and thereby prevent the development of organ-specific autoimmunity [Shevach et al., 2001]. Of course the same process would inhibit the priming of tumor immunity [Sutmuller et al., 2001]. It is possible that regulatory cells were not transferred in sufficient number when RAG-1 mice were reconstituted with normal spleen cells to prevent the expansion and eradication of autoreactive T cells. The spleen cells used for reconstitution in our experiments were unfractionated; therefore, additional transfer experiments with spleen cells depleted of $CD4^+CD25^+$regulatory T cells would be necessary to determine their role in priming therapeutic T cells during reconstitution of a lymphopenic host.

Neither hypothesis directly addresses the factors that drive naïve T cells to proliferate in the absence of exogenous antigenic stimulation. Our results can be explained best by the T-cell activation threshold tuning hypothesis proposed by Grossman and Paul [2000]. Their hypothesis states that the immune system is designed to be moderately self-reactive and that self-reactivity is controlled by activation threshold. It proposes that the immune system responds to episodes of "system perturbation," such as episodes of infection or lymphopenia. In our experimental model, there are two system perturbations, lymphopenia and a tumor vaccine. As a population, T cells respond to lymphopenia by tuning their activation threshold down to a level at which a subgroup of naïve T cells are driven to proliferate, even in the absence of foreign antigens. If tumor antigens are also present, T-cell responses could be focused on these peptides, and tumor specific T

cells may be preferentially driven to proliferate to reach a level sufficient to confer resistance to tumor growth. Repopulation of both CD4 and CD8 niches with a T-cell repertoire skewed in favor of melanoma-reactive T cells following vaccination in reconstituted RAG1 mice would explain our results.

Previously, we demonstrated that priming of therapeutic CD8 effector T cells in normal hosts is CD4 indepdendent and that CD8, but not CD4 effector T cells, mediated tumor regression [Hu et al., 2000]. We observed a large increase in the number of Th1 CD4 effector T cells that appear to be MHC class II restricted and tumor specific in RLP RAG1 mice compared to normal hosts after vaccination with D5-G6. Because a large percentage of CD4 T cells were primed in vaccinated RLP RAG1 mice, we are currently reexamining the role of CD4 T cells during the priming and effector phases of adoptive immunotherapy. Preliminary experiments suggest that the adoptive transfer of either CD4 T cells derived from vaccinated RLP RAG1 mice but not from normal mice are highly therapeutic [C. H. Poehlein and H.-M. Hu, unpublished results]. We also plan to determine whether these CD4 T cells are melanoma-specific and identify the antigens they recognize. Future experiments will determine whether tolerance in melanoma-specific CD4 T cells was broken by vaccination during reconstitution of lymphopenic hosts. Most interestingly, we observed both enhanced tumor protection and the rapid development of vitiligo after tumor challenge with the B16F10 melanoma cell line in reconstituted lymphopenic mice that had been vaccinated with irradiated unmodified B16F10 tumor cells [H.-M. Hu, A poorly immunogenic tumor induces protective antitumor immunity in mice subjected to sublethal irradiation and immune reconstitution, manuscript in preparation]. This supports our hypothesis that vaccination of RLP hosts with whole tumor cell vaccines can break immune tolerance to tumor antigens. In conclusion, our study suggests that active vaccination of T-cell reconstituted lymphopenic hosts can jump start or reboot the immune system in favor of strong immune responses to self-antigens and promote protection against tumor outgrowth. This strategy will be tested in future clinical trials.

LITERATURE CITED

Afonso, L. C., T. M. Scharton, L. Q. Vieira, M. Wysocka, G. Trinchieri, and P. Scott (1994) The adjuvant effect of interleukin-12 in a vaccine against *Leishmania major*. *Science* **263,** 235-237.

Albert, M. L., S. F. A. Pearce, L. M. Francisco, B. Sauter, P. Roy, R. L. Silverstein, and N. Bhardwaj (1998a) Immature dendritic cells phagocytose apoptotic cells via $\alpha V\beta 5$ and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J. Exp. Med.* **188,** 1359-1368.

Albert, M. L., B. Sauter, and N. Bhardwaj (1998b) Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* **392,** 86-89.

Anderson, D. M., E. Maraskovsky, W. L. Billingsley, W. C. Dougall, M. E. Tometsko, E. R. Roux, M. C. Teepe, R. F. DuBose, D. Cosman, and L. Galibert (1997) A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature* **390,** 175-179.

Andersson, E., J. P. Christensen, O. Marker, and A. R. Thomsen (1994) Changes in cell adhesion molecule expression on T cells associated with systemic virus infection. *J. Immunol.* **152,** 1237-1245.

Andreasen, S. O., J. E. Christensen, O. Marker, and A. R. Thomsen (2000) Role of CD40 ligand and CD28 in induction and maintenance of antiviral $CD8^+$ effector T cell responses. *J. Immunol.* **164,** 3689-3697.

Arca, M. J., J. C. Krauss, A. Aruga, M. J. Cameron, S. Shu, and A. E. Chang (1996a) Therapeutic efficacy of T cells derived from lymph nodes draining a poorly immunogenic tumor transduced to secrete granulocyte-macrophage colony-stimulating factor. *Cancer Gene Ther.* **3,** 39-47.

Arca, M. J., J. C. Krauss, S. E. Strome, M. J. Cameron, and A. E. Chang (1996b) Diverse manifestations of tumorigenicity and immunogenicity displayed by the poorly immunogenic B16-BL6 melanoma transduced with cytokine genes. *Cancer Immunol. Immunother.* **42,** 237-245.

Arnold, B., G. Schonrich, and G. J. Hammerling (1993) Multiple levels of peripheral tolerance. *Immunol. Today* **14,** 12-14.

Arnold, D., S. Faath, H. Rammensee, and H. Schild (1995) Cross-priming of minor histocompatibility antigen-specific cytotoxic T cells upon immunization with the heat shock protein gp96. *J. Exp. Med.* **182,** 885-889.

Aruga, A., E. Aruga, M. J. Cameron, and A. E. Chang (1997a) Different cytokine profiles released by $CD4^+$ and $CD8^+$ tumor-draining lymph node cells involved in mediating tumor regression. *J. Leukoc. Biol.* **61,** 507-516.

Aruga, A., E. Aruga, K. Tanigawa, D. K. Bishop, V. K. Sondak, and A. E. Chang (1997b) Type 1 versus type 2 cytokine release by Vβ T cell subpopulations determines in vivo antitumor reactivity: IL-10 mediates a suppressive role. *J. Immunol.* **159,** 664-673.

Awwad, M. and R. J. North (1988) Immunologically mediated regression of a murine lymphoma after treatment with anti-L3T4 antibody. A consequence of removing $L3T4^+$ suppressor T cells from a host generating predominantly Lyt-2^+ T cell-mediated immunity. *J. Exp. Med.* **168,** 2193-2206.

Bach, F. H., C. Ferran, P. Hechenleitner, W. Mark, N. Koyamada, T. Miyatake, H. Winkler, A. Badrichani, D. Candinas, and W. W. Hancock (1997) Accommodation of vascularized xenografts: expression of "protective genes" by donor endothelial cells in a host Th2 cytokine environment. *Nat. Med.* **3,** 196-204.

Bachmann, M. F., B. R. Wong, R. Josien, R. M. Steinman, A. Oxenius, and Y. Choi (1999) TRANCE, a tumor necrosis factor family member critical for CD40 ligand-independent T helper cell activation. *J. Exp. Med.* **189,** 1025-1031.

Barth, R. J., Jr., J. J. Mule, P. J. Spiess, and S. A. Rosenberg (1991) Interferon gamma and tumor necrosis factor have a role in tumor regressions mediated by murine $CD8^+$ tumor-infiltrating lymphocytes. *J. Exp. Med.* **173,** 647-658.

Baskar, S., L. Glimcher, N. Nabavi, R. T. Jones, and S. Ostrand-Rosenberg (1995) Major histocompatibility complex class II+B7-1+ tumor cells are potent vaccines for stimulating tumor rejection in tumor-bearing mice. *J. Exp. Med.* **181,** 619-629.

Baum, P. R., R. B. Gayle, 3rd, F. Ramsdell, S. Srinivasan, R. A. Sorensen, M. L. Watson, M. F. Seldin, E. Baker, G. R. Sutherland, and K. N. Clifford (1994) Molecular characterization of murine and human OX40/OX40 ligand systems: identification of a human OX40 ligand as the HTLV-1-regulated protein gp34. *EMBO J.* **13,** 3992-4001.

Becker, J. C., C. Czerny, and E. B. Brocker (1994) Maintenance of clonal anergy by endogenously produced IL-10. *Int. Immunol.* **6,** 1605-1612.

Belz, G. T., P. G. Stevenson, M. R. Castrucci, J. D. Altman, and P. C. Doherty (2000) Postexposure vaccination massively increases the prevalence of gamma-herpesvirus-specific $CD8^+$ T cells but confers minimal survival advantage on CD4-deficient mice. *Proc. Natl. Acad. Sci. U.S.A.* **97,** 2725-2730.

Bendelac, A., M. N. Rivera, S.-H. Park, and J. H. Roark (1997) Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu. Rev. Immunol.* **15,** 535-562.

Bennett, S. R., F. R. Carbone, F. Karamalis, J. F. Miller, and W. R. Heath (1997) Induction of a $CD8^+$ cytotoxic T lymphocyte response by cross-priming requires cognate $CD4^+$ T cell help. *J. Exp. Med.* **186,** 65-70.

Bennett, S. R., F. R. Carbone, F. Karamalis, R. A. Flavell, J. F. Miller, and W. R. Heath (1998) Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* **393,** 478-480.

Berger, D. P., D. Naniche, M. T. Crowley, P. A. Koni, R. A. Flavell, and M. B. Oldstone (1999) Lymphotoxin-beta-deficient mice show defective antiviral immunity. *Virology* **260,** 136-147.

Blanckaert, V. D., M. E. Schelling, C. A. Elstad, and G. G. Meadows (1993) Differential growth factor production, secretion, and response by high and low metastatic variants of B16BL6 melanoma. *Cancer Res.* **53,** 4075-4081.

Bloom, M. B., D. Perry-Lalley, P. F. Robbins, Y. Li, M. el-Gamil, S. A. Rosenberg, and J. C. Yang (1997) Identification of tyrosinase-related protein 2 as a tumor rejection antigen for the B16 melanoma. *J. Exp. Med.* **185,** 453-459.

Boon, T., J. C. Cerottini, B. Van den Eynde, P. van der Bruggen, and A. Van Pel (1994) Tumor antigens recognized by T lymphocytes. *Annu. Rev. Immunol.* **12,** 337-365.

Borrello, I., E. M. Sotomayor, F. M. Rattis, S. K. Cooke, L. Gu, and H. I. Levitsky (2000) Sustaining the graft-versus-tumor effect through posttransplant immunization with granulocyte-macrophage colony-stimulating factor (GM-CSF)-producing tumor vaccines. *Blood* **95,** 3011-3019.

Borrow, P., A. Tishon, S. Lee, J. Xu, I. S. Grewal, M. B. Oldstone, and R. A. Flavell (1996) CD40L-deficient mice show deficits in antiviral immunity and have an impaired memory $CD8^+$ CTL response. *J. Exp. Med.* **183,** 2129-2142.

Borrow, P., D. F. Tough, D. Eto, A. Tishon, I. S. Grewal, J. Sprent, R. A. Flavell, and M. B. Oldstone (1998) CD40 ligand-mediated interactions are involved in the

generation of memory $CD8^+$ cytotoxic T lymphocytes (CTL) but are not required for the maintenance of CTL memory following virus infection. *J. Virol.* **72,** 7440-7449.

Bour, H., C. Horvath, C. Lurquin, J. C. Cerottini, and H. R. MacDonald (1998) Differential requirement for CD4 help in the development of an antigen-specific $CD8^+$ T cell response depending on the route of immunization. *J. Immunol.* **160,** 5522-5529.

Bowne, W. B., R. Srinivasan, J. D. Wolchok, W. G. Hawkins, N. E. Blachere, R. Dyall, J. J. Lewis, and A. N. Houghton (1999) Coupling and uncoupling of tumor immunity and autoimmunity. *J. Exp. Med.* **190,** 1717-1722.

Bradley, L. M., D. D. Duncan, S. Tonkonogy, and S. L. Swain (1991) Characterization of antigen-specific $CD4^+$ effector T cells in vivo: immunization results in a transient population of MEL-14-, CD45RB-helper cells that secretes interleukin 2 (IL-2), IL-3, IL-4, and interferon gamma. *J. Exp. Med.* **174,** 547-559.

Brichard, V., A. Van Pel, T. Wolfel, C. Wolfel, E. De Plaen, B. Lethe, P. Coulie, and T. Boon (1993) The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med.* **178,** 489-495.

Brossart, P., and M. J. Bevan (1997) Presentation of exogenous protein antigens on major histocompatibility complex class I molecules by dendritic cells: pathway of presentation and regulation by cytokines. *Blood* **90,** 1594-1599.

Carbone, F. R., C. Kurts, S. R. Bennett, J. F. Miller, and W. R. Heath (1998) Cross-presentation: a general mechanism for CTL immunity and tolerance. *Immunol. Today* **19,** 368-373.

Cardell, S., S. Tangri, S. Chan, M. Kronenberg, C. Benoist, and D. Mathis (1995) CD1-restricted $CD4^+$ T cells in major histocompatibility complex class II-deficient mice. *J. Exp. Med.* **182,** 993-1004.

Carlow, D. A., R. S. Kerbel, and B. E. Elliott (1989) Failure of expression of class I major histocompatibility antigens to alter tumor immunogenicity of a spontaneous murine carcinoma. *J. Natl. Cancer Inst.* **81,** 759-767.

Caux, C., C. Dezutter-Dambuyant, D. Schmitt, and J. Banchereau (1992) GM-CSF and TNF-α cooperate in the generation of dendritic Langerhans cells. *Nature* **360,** 258-261.

Chamberlain, R. S., M. W. Carroll, V. Bronte, P. Hwu, S. Warren, J. C. Yang, M. Nishimura, B. Moss, S. A. Rosenberg, and N. P. Restifo (1996) Costimulation

enhances the active immunotherapy effect of recombinant anticancer vaccines. *Cancer Res.* **56,** 2832-2836.

Chandawarkar, R. Y., M. S. Wagh, and P. K. Srivastava (1999) The dual nature of specific immunological activity of tumor-derived gp96 preparations. *J. Exp. Med.* **189,** 1437-1442.

Chang, T. T., C. Jabs, R. A. Sobel, V. K. Kuchroo, and A. H. Sharpe (1999) Studies in B7-deficient mice reveal a critical role for B7 costimulation in both induction and effector phases of experimental autoimmune encephalomyelitis. *J. Exp. Med.* **190,** 733-740.

Cheever, M. A., W. Chen, M. L. Disis, M. Takahashi, and D. J. Peace (1993) T-cell immunity to oncogenic proteins including mutated ras and chimeric bcr-abl. *Ann. N. Y. Acad. Sci.* **690,** 101-112.

Chen, L., S. Ashe, W. A. Brady, I. Hellstrom, K. E. Hellstrom, J. A. Ledbetter, P. McGowan, and P. S. Linsley (1992) Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell* **71,** 1093-1102.

Chen, L., P. McGowan, S. Ashe, J. Johnston, Y. Li, I. Hellstrom, and K. E. Hellstrom (1994) Tumor immunogenicity determines the effect of B7 costimulation on T cell-mediated tumor immunity. *J. Exp. Med.* **179,** 523-532.

Cho, B. K., D. Palliser, E. Guillen, J. Wisniewski, R. A. Young, J. Chen, and H. N. Eisen (2000a) A proposed mechanism for the induction of cytotoxic T lymphocyte production by heat shock fusion proteins. *Immunity* **12,** 263-272.

Cho, B. K., V. P. Rao, O. Ge, H. N. Eisen, and J. Chen (2000b) Homeostasis-stimulated proliferation drives naive T cells to differentiate directly into memory T cells. *J. Exp. Med.* **192,** 549-556.

Cole, G. A., V. K. Clements, E. P. Garcia, and S. Ostrand-Rosenberg (1987) Allogeneic H-2 antigen expression is insufficient for tumor rejection. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 8613-8617.

Coley, W. B. (1991) The treatment of malignant tumors by repeated inoculations of erysipelas. With a report of ten original cases. 1893. *Clin. Orthop.* 3-11.

Conry, R. M., A. F. LoBuglio, F. Loechel, S. E. Moore, L. A. Sumerel, D. L. Barlow, and D. T. Curiel (1994) A carcinoembryonic antigen polynucleotide vaccine has in vivo antitumor activity. *Gene Ther.* **1,** 1-7.

Conry, R. M., A. F. LoBuglio, F. Loechel, S. E. Moore, L. A. Sumerel, D. L. Barlow, and D. T. Curiel (1995) A carcinoembryonic antigen polynucleotide vaccine has in vivo antitumor activity. *Gene Ther.* **2,** 59-65.

Constant, S. L., and K. Bottomly (1997) Induction of Th1 and Th2 $CD4^+$ T cell responses: alternative approaches. *Annu. Rev. Immunol.* **15,** 297-322.

Constant, S., C. Pfeiffer, A. Woodard, T. Pasqualini, and K. Bottomly (1995) Extent of T cell receptor ligation can determine the functional differentiation of naive $CD4^+$ T cells. *J. Exp. Med.* **182,** 1591-1596.

Croft, M., L. Carter, S. L. Swain, and R. W. Dutton (1994) Generation of polarized antigen-specific CD8 effector populations: reciprocal action of interleukin (IL)-4 and IL-12 in promoting type 2 versus type 1 cytokine profiles. *J. Exp. Med.* **180,** 1715-1728.

Crossland, K. D., V. K. Lee, W. Chen, S. R. Riddell, P. D. Greenberg, and M. A. Cheever (1991) T cells from tumor-immune mice nonspecifically expanded in vitro with anti-CD3 plus IL-2 retain specific function in vitro and can eradicate disseminated leukemia in vivo. *J. Immunol.* **146,** 4414-4420.

de Saint-Vis, B., I. Fugier-Vivier, C. Massacrier, C. Gaillard, B. Vanbervliet, S. Ait-Yahia, J. Banchereau, Y. J. Liu, S. Lebecque, and C. Caux (1998) The cytokine profile expressed by human dendritic cells is dependent on cell subtype and mode of activation. *J. Immunol.* **160,** 1666-1676.

Diehl, L., A. T. den Boer, S. P. Schoenberger, E. I. van der Voort, T. N. Schumacher, C. J. Melief, R. Offringa, and R. E. Toes (1999) CD40 activation in vivo overcomes peptide-induced peripheral cytotoxic T-lymphocyte tolerance and augments anti-tumor vaccine efficacy. *Nat. Med.* **5,** 774-779.

Di Rosa, F., and P. Matzinger (1996) Long-lasting CD8 T cell memory in the absence of CD4 T cells or B cells. *J. Exp. Med.* **183,** 2153-2163.

Dobrzanski, M. J., J. B. Reome, and R. W. Dutton (1999) Therapeutic effects of tumor-reactive type 1 and type 2 $CD8^+$ T cell subpopulations in established pulmonary metastases. *J. Immunol.* **162,** 6671-6680.

Dobrzanski, M. J., J. B. Reome, and R. W. Dutton (2000) Type 1 and type 2 $CD8^+$ effector T cell subpopulations promote long-term tumor immunity and protection to progressively growing tumor. *J. Immunol.* **164,** 916-925.

Dobrzanski, M. J., J. B. Reome, and R. W. Dutton (2001) Immunopotentiating role of IFN-γ in early and late stages of type 1 CD8 effector cell-mediated tumor rejection. *Clin. Immunol.* 8, 70-84.

Dranoff, G., E. Jaffee, A. Lazenby, P. Golumbek, H. Levitsky, K. Brose, V. Jackson, H. Hamada, D. Pardoll, and R. C. Mulligan (1993) Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 3539-3543.

Dressel, R., M. Lubbers, L. Walter, W. Herr, and E. Gunther (1999) Enhanced susceptibility to cytotoxic T lymphocytes without increase of MHC class I antigen expression after conditional overexpression of heat shock protein 70 in target cells. *Eur. J. Immunol.* **29,** 3925-3935.

Durie, F. H., R. A. Fava, T. M. Foy, A. Aruffo, J. A. Ledbetter, and R. J. Noelle (1993) Prevention of collagen-induced arthritis with an antibody to gp39, the ligand for CD40. *Science* **261,** 1328-1330.

Dyall, R., W. B. Bowne, L. W. Weber, J. LeMaoult, P. Szabo, Y. Moroi, G. Piskun, J. J. Lewis, A. N. Houghton, and J. Nikolic-Zugic (1998) Heteroclitic immunization induces tumor immunity. *J. Exp. Med.* **188,** 1553-1561.

Endres, R., M. B. Alimzhanov, T. Plitz, A. Futterer, M. H. Kosco-Vilbois, S. A. Nedospasov, K. Rajewsky, and K. Pfeffer (1999) Mature follicular dendritic cell networks depend on expression of lymphotoxin beta receptor by radioresistant stromal cells and of lymphotoxin beta and tumor necrosis factor by B cells. *J. Exp. Med.* **189,** 159-168.

Ernst, B., D. S. Lee, J. M. Chang, J. Sprent, and C. D. Surh (1999) The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery. *Immunity* **11,** 173-181.

Fenton, R. G., D. D. Taub, L. W. Kwak, M. R. Smith, and D. L. Longo (1993) Cytotoxic T-cell response and in vivo protection against tumor cells harboring activated ras proto-oncogenes. *J. Natl. Cancer Inst.* **85,** 1294-1302.

Ferrara, J. L. (1994) Cytokines other than growth factors in bone marrow transplantation. *Curr. Opin. Oncol.* **6,** 127-134.

Fields, R. C., K. Shimizu, and J. J. Mule (1998) Murine dendritic cells pulsed with whole tumor lysates mediate potent antitumor immune responses in vitro and in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 9482-9487.

Finke, J. H., A. H. Zea, J. Stanley, D. L. Longo, H. Mizoguchi, R. R. Tubbs, R. H. Wiltrout, J. J. O'Shea, S. Kudoh, and E. Klein (1993) Loss of T-cell receptor zeta chain and p56lck in T-cells infiltrating human renal cell carcinoma. *Cancer Res.* **53,** 5613-5616.

Fox, B. A., P. J. Spiess, A. Kasid, R. Puri, J. J. Mule, J. S. Weber, and S. A. Rosenberg (1990) In vitro and in vivo antitumor properties of a T-cell clone generated from murine tumor-infiltrating lymphocytes. *J. Biol. Response Mod.* **9,** 499-511.

Franco, A., D. A. Tilly, I. Gramaglia, M. Croft, L. Cipolla, M. Meldal, and H. M. Grey (2000) Epitope affinity for MHC class I determines helper requirement for CTL priming. *Nat. Immunol.* **1,** 145-150.

Futterer, A., K. Mink, A. Luz, M. H. Kosco-Vilbois, and K. Pfeffer (1998) The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. *Immunity* **9,** 59-70.

Galocha, B., D. Lopez, and J. A. Lopez de Castro (1993) Clonal heterogeneity in LFA-3 and ICAM-1 requirement for lysis by alloreactive T lymphocytes. *J. Immunol.* **150,** 1653-1662.

Garrido, F., T. Cabrera, A. Concha, S. Glew, F. Ruiz-Cabello, and P. L. Stern (1993) Natural history of HLA expression during tumour development. *Immunol. Today* **14,** 491-499.

Garrido, F., F. Ruiz-Cabello, T. Cabrera, J. J. Perez-Villar, M. Lopez-Botet, M. Duggan-Keen, and P. L. Stern (1997) Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. *Immunol. Today* **18,** 89-95.

Garside, P., and A. M. Mowat (1997) Mechanisms of oral tolerance. *Crit. Rev. Immunol.* **17,** 119-137.

Gauchat, J. F., S. Henchoz, D. Fattah, G. Mazzei, J.-P. Aubry, T. Jomotte, L. Dash, K. Page, R. Solari, D. Aldebert, M. Capron, C. Dahinden, and J.-Y. Bonnefoy (1995) CD40 ligand is functionally expressed on human eosinophils. *Eur. J. Immunol.* **25,** 863-865.

Gavin, M. A., S. R. Clarke, E. Negrou, A. Gallegos, and A. Rudensky (2002) Homeostasis and anergy of $CD4^+$ $CD25^+$ suppressor T cells in vivo. *Nat. Immunol.* **3,** 33-41.

Germain, R. N. (1993) Antigen processing and presentation. In *Fundamental Immunology*, 3rd ed. (Paul, W. E., ed.), Raven Press, New York, pp. 629-675.

Germain, R. N., and I. Stefanova (1999) The dynamics of T cell receptor signaling: complex orchestration and the key roles of tempo and cooperation. *Annu. Rev. Immunol.* **17,** 467-522.

Ghosh, P., K. L. Komschlies, M. Cippitelli, D. L. Longo, J. Subleski, J. Ye, A. Sica, H. A. Young, R. H. Wiltrout, and A. C. Ochoa (1995) Gradual loss of T-

helper 1 populations in spleen of mice during progressive tumor growth. *J. Natl. Cancer Inst.* **87,** 1478-1483.

Goldrath, A. W., and M. J. Bevan (1999) Low-affinity ligands for the TCR drive proliferation of mature $CD8^+$ T cells in lymphopenic hosts. *Immunity* **11,** 183-190.

Goldrath, A. W., L. Y. Bogatzki, and M. J. Bevan (2000) Naive T cells transiently acquire a memory-like phenotype during homeostasis-driven proliferation. *J. Exp. Med.* **192,** 557-564.

Gollob, K. J., W. O. Dutra, and R. L. Coffman (1996) Early message expression of interleukin-4 and interferon-gamma, but not of interleukin-2 and interleukin-10, reflects later polarization of primary $CD4^+$ T cell cultures. *Eur. J. Immunol.* **26,** 1565-1570.

Green, E. A., and R. A. Flavell (1999) TRANCE-RANK, a new signal pathway involved in lymphocyte development and T cell activation. *J. Exp. Med.* **189,** 1017-1020.

Grewal, I. S., and R. A. Flavell (1998) CD40 and CD154 in cell-mediated immunity. *Annu. Rev. Immunol.* **16,** 111-135.

Grossman, Z., and W. E. Paul (1992) Adaptive cellular interactions in the immune system: the tunable activation threshold and the significance of subthreshold responses. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 10365-10369.

Grossman, Z., and W. E. Paul (2000) Self-tolerance: context dependent tuning of T cell antigen recognition. *Semin. Immunol.* **12,** 197-203; discussion 257-344.

Grossman, Z., and A. Singer (1996) Tuning of activation thresholds explains flexibility in the selection and development of T cells in the thymus. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 14747-14752.

Gudmundsdottir, H., and L. A. Turka (2001) A closer look at homeostatic proliferation of $CD4^+$ T cells: costimulatory requirements and role in memory formation. *J. Immunol.* **167,** 3699-3707.

Gudmundsdottir, H., A. D. Wells, and L. A. Turka (1999) Dynamics and requirements of T cell clonal expansion in vivo at the single-cell level: effector function is linked to proliferative capacity. *J. Immunol.* **162,** 5212-5223.

Halpern, M. D., R. J. Kurlander, and D. S. Pisetsky (1996) Bacterial DNA induces murine interferon-gamma production by stimulation of interleukin-12 and tumor necrosis factor-alpha. *Cell. Immunol.* **167,** 72-78.

Hara, I., Y. Takechi, and A. N. Houghton (1995) Implicating a role for immune recognition of self in tumor rejection: passive immunization against the brown locus protein. *J. Exp. Med.* **182,** 1609-1614.

Harding, F. A., J. G. McArthur, J. A. Gross, D. H. Raulet, and J. P. Allison (1992) CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature* **356,** 607-609.

Heinzel, F. P., R. M. Rerko, F. Ahmed, and E. Pearlman (1995) Endogenous IL-12 is required for control of Th2 cytokine responses capable of exacerbating leishmaniasis in normally resistant mice. *J. Immunol.* **155,** 730-739.

Hedrick, S. M., and F. J. Eidelman (1993) T Lymphocyte antigen receptors. In *Fundamental Immunology*, 3rd ed. (Paul, W. E., ed.), Raven Press, New York, pp. 383-420.

Henn, V., J. R. Slupsky, M. Grafe, I. Anagnostopoulos, R. Forster, G. Muller-Berghaus, and R. A. Kroczek (1998) CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* **391,** 591-594.

Herman, A., J. W. Kappler, P. Marrack, and A. M. Pullen (1991) Superantigens: mechanism of T-cell stimulation and role in immune responses. *Annu. Rev. Immunol.* **9,** 745-772.

Hermans, I. F., D. S. Ritchie, A. Daish, J. Yang, M. R. Kehry, and F. Ronchese (1999) Impaired ability of MHC class II-/- dendritic cells to provide tumor protection is rescued by CD40 ligation. *J. Immunol.* **163,** 77-81.

Hock, H., M. Dorsch, U. Kunzendorf, K. Uberla, Z. Qin, T. Diamantstein, and T. Blankenstein (1993) Vaccinations with tumor cells genetically engineered to produce different cytokines: effectivity not superior to a classical adjuvant. *Cancer Res.* **53,** 714-716.

Hosken, N. A., K. Shibuya, A. W. Heath, K. M. Murphy, and A. O'Garra (1995) The effect of antigen dose on $CD4^+$ T helper cell phenotype development in a T cell receptor-alpha beta-transgenic model. *J. Exp. Med.* **182,** 1579-1584.

Hou, S., and P. C. Doherty (1993) Partitioning of responder $CD8^+$ T cells in lymph node and lung of mice with Sendai virus pneumonia by LECAM-1 and CD45RB phenotype. *J. Immunol.* **150,** 5494-5500.

Hou, S., X. Y. Mo, L. Hyland, and P. C. Doherty (1995) Host response to Sendai virus in mice lacking class II major histocompatibility complex glycoproteins. *J. Virol.* **69,** 1429-1434.

Hu, H. M., W. J. Urba, and B. A. Fox (1998) Gene-modified tumor vaccine with therapeutic potential shifts tumor-specific T cell response from a type 2 to a type 1 cytokine profile. *J. Immunol.* **161,** 3033-3041.

Hu, H. M., H. Winter, W. J. Urba, and B. A. Fox (2000) Divergent roles for $CD4^+$ T cells in the priming and effector/memory phases of adoptive immunotherapy. *J. Immunol.* **165,** 4246-4253.

Huang, A. Y., P. Golumbek, M. Ahmadzadeh, E. Jaffee, D. Pardoll, and H. Levitsky (1994a) Bone marrow-derived cells present MHC class I-restricted tumour antigens in priming of antitumour immune responses. *Ciba Found. Symp.* **187,** 229-240; discussion 240-244.

Huang, A. Y., P. Golumbek, M. Ahmadzadeh, E. Jaffee, D. Pardoll, and H. Levitsky (1994b) Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. *Science* **264,** 961-965.

Huang, A. Y., A. T. Bruce, D. M. Pardoll, and H. I. Levitsky (1996) In vivo cross-priming of MHC class I-restricted antigens requires the TAP transporter. *Immunity* **4,** 349-355.

Hui, K., F. Grosveld, and H. Festenstein (1984) Rejection of transplantable AKR leukaemia cells following MHC DNA-mediated cell transformation. *Nature* **311,** 750-752.

Hui, K. M., T. Sim, T. T. Foo, and A. A. Oei (1989) Tumor rejection mediated by transfection with allogeneic class I histocompatibility gene. *J. Immunol.* **143,** 3835-3843.

Hung, K., R. Hayashi, A. Lafond-Walker, C. Lowenstein, D. Pardoll, and H. Levitsky (1998) The central role of $CD4^+$ T cells in the antitumor immune response. *J. Exp. Med.* **188,** 2357-2368.

Hurwitz, A. A., T. F. Yu, D. R. Leach, and J. P. Allison (1998) CTLA-4 blockade synergizes with tumor-derived granulocyte-macrophage colony-stimulating factor for treatment of an experimental mammary carcinoma. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 10067-10071.

Inoue, M., M. Minami, Y. Fujii, H. Matsuda, R. Shirakura, and T. Kido (1997) Granulocyte colony-stimulating factor and interleukin-6-producing lung cancer cell line, LCAM. *J. Surg. Oncol.* **64,** 347-350.

Ioannides, C. G., B. Fisk, D. Fan, W. E. Biddison, J. T. Wharton, and C. A. O'Brian (1993a) Cytotoxic T cells isolated from ovarian malignant ascites recognize a peptide derived from the HER-2/neu proto-oncogene. *Cell. Immunol.* **151,** 225-234.

Ioannides, C. G., B. Fisk, K. R. Jerome, T. Irimura, J. T. Wharton, and O. J. Finn (1993b) Cytotoxic T cells from ovarian malignant tumors can recognize polymorphic epithelial mucin core peptides. *J. Immunol.* **151,** 3693-3703.

Jerome, K. R., D. L. Barnd, K. M. Bendt, C. M. Boyer, J. Taylor-Papadimitriou, I. F. McKenzie, R. C. Bast, Jr., and O. J. Finn (1991) Cytotoxic T-lymphocytes derived from patients with breast adenocarcinoma recognize an epitope present on the protein core of a mucin molecule preferentially expressed by malignant cells. *Cancer Res.* **51,** 2908-2916.

Jerome, K. R., N. Domenech, and O. J. Finn (1993) Tumor-specific cytotoxic T cell clones from patients with breast and pancreatic adenocarcinoma recognize EBV-immortalized B cells transfected with polymorphic epithelial mucin complementary DNA. *J. Immunol.* **151,** 1654-1662.

Joseph, S. B., K. T. Miner, and M. Croft (1998) Augmentation of naive, Th1 and Th2 effector CD4 responses by IL-6, IL-1 and TNF. *Eur. J. Immunol.* **28,** 277-289.

Josien, R., H. L. Li, E. Ingulli, S. Sarma, B. R. Wong, M. Vologodskaia, R. M. Steinman, and Y. Choi (2000) TRANCE, a tumor necrosis factor family member, enhances the longevity and adjuvant properties of dendritic cells in vivo. *J. Exp. Med.* **191,** 495-502.

Kagamu, H., J. E. Touhalisky, G. E. Plautz, J. C. Krauss, and S. Shu (1996) Isolation based on L-selectin expression of immune effector T cells derived from tumor-draining lymph nodes. *Cancer Res.* **56,** 4338-4342.

Kantor, J., K. Irvine, S. Abrams, H. Kaufman, J. DiPietro, and J. Schlom (1992) Antitumor activity and immune responses induced by a recombinant carcinoembryonic antigen-vaccinia virus vaccine. *J. Natl. Cancer Inst.* **84,** 1084-1091.

Kawakami, Y., S. Eliyahu, C. H. Delgado, P. F. Robbins, L. Rivoltini, S. L. Topalian, T. Miki, and S. A. Rosenberg (1994a) Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 3515-3519.

Kawakami, Y., S. Eliyahu, C. H. Delgado, P. F. Robbins, K. Sakaguchi, E. Appella, J. R. Yannelli, G. J. Adema, T. Miki, and S. A. Rosenberg (1994b) Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in vivo tumor rejection. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 6458-6462.

Kieper, W. C., and S. C. Jameson (1999) Homeostatic expansion and phenotypic conversion of naive T cells in response to self peptide/MHC ligands. *Proc. Natl. Acad. Sci. U.S.A.* **9,** 13306-13311.

Kubin, M., M. Kamoun, and G. Trinchieri (1994) Interleukin 12 synergizes with B7/CD28 interaction in inducing efficient proliferation and cytokine production of human T cells. *J. Exp. Med.* **180,** 211-222.

Kundig, T. M., A. Shahinian, K. Kawai, H. W. Mittrucker, E. Sebzda, M. F. Bachmann, T. W. Mak, and P. S. Ohashi (1996) Duration of TCR stimulation determines costimulatory requirement of T cells. *Immunity* **5,** 41-52.

Kyburz, D., P. Aichele, D. E. Speiser, H. Hengartner, R. M. Zinkernagel, and H. Pircher (1993) T cell immunity after a viral infection versus T cell tolerance induced by soluble viral peptides. *Eur. J. Immunol.* **23,** 1956-1962.

La Gruta, N. L., I. R. Driel, and P. A. Gleeson (2000) Peripheral T cell expansion in lymphopenic mice results in a restricted T cell repertoire. *Eur. J. Immunol.* **30,** 3380-3386.

Lanoue, A., C. Bona, H. von Boehmer, and A. Sarukhan (1997) Conditions that induce tolerance in mature $CD4^+$ T cells. *J. Exp. Med.* **185,** 405-414.

Lee, K. H., E. Wang, M. B. Nielsen, J. Wunderlich, S. Migueles, M. Connors, S. M. Steinberg, S. A. Rosenberg, and F. M. Marincola (1999) Increased vaccine-specific T cell frequency after peptide-based vaccination correlates with increased susceptibility to in vitro stimulation but does not lead to tumor regression. *J. Immunol.* **163,** 6292-6300.

Lefrancois, L., S. Olson, and D. Masopust (1999) A critical role for CD40-CD40 ligand interactions in amplification of the mucosal CD8 T cell response. *J. Exp. Med.* **190,** 1275-1284.

Li, L., S. Sad, D. Kagi, and T. R. Mosmann (1997) CD8Tc1 and Tc2 cells secrete distinct cytokine patterns in vitro and in vivo but induce similar inflammatory reactions. *J. Immunol.* **158,** 4152-4161.

Li, X., J. Liu, J. K. Park, T. A. Hamilton, P. Rayman, E. Klein, M. Edinger, R. Tubbs, R. Bukowski, and J. Finke (1994) T cells from renal cell carcinoma patients exhibit an abnormal pattern of kappa B-specific DNA-binding activity: a preliminary report. *Cancer Res.* **54,** 5424-5429.

Li, Y., P. McGowan, I. Hellstrom, K. E. Hellstrom, and L. Chen (1994) Costimulation of tumor-reactive $CD4^+$ and $CD8^+$ T lymphocytes by B7, a natural ligand for CD28, can be used to treat established mouse melanoma. *J. Immunol.* **153,** 421-428.

Liblau, R. S., R. Tisch, K. Shokat, X. Yang, N. Dumont, C. C. Goodnow, and H. O. McDevitt (1996) Intravenous injection of soluble antigen induces thymic and peripheral T cell apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 3031-3036.

Linsley, P. S., and J. A. Ledbetter (1993) The role of the CD28 receptor during T cell responses to antigen. *Annu. Rev. Immunol.* **11,** 191-212.

Lipshy, K. A., P. J. Kostuchenko, G. G. Hamad, C. E. Bland, S. K. Barrett, and H. D. Bear (1997) Sensitizing T-lymphocytes for adoptive immunotherapy by vaccination with wild-type or cytokine gene-transduced melanoma. *Ann. Surg. Oncol.* **4,** 334-341.

Liu, Y., B. Jones, A. Aruffo, K. M. Sullivan, P. S. Linsley, and C. A. Janeway, Jr. (1992) Heat-stable antigen is a costimulatory molecule for CD4 T cell growth. *J. Exp. Med.* **175,** 437-445.

London, C. A., M. P. Lodge, and A. K. Abbas (2000) Functional responses and costimulator dependence of memory $CD4^+$ T cells. *J. Immunol.* **164,** 265-272.

Lotze, M. T., J. T. Rubin, S. Carty, H. Edington, P. Ferson, R. Landreneau, B. Pippin, M. Posner, D. Rosenfelder, C. Watson, T. Carlos, J. Kirkwood, B. Lembersky, T. Logan, M. Rosenstein, M. E. Rybak, T. Whiteside, E. Elder, R. C. Moen, W. Jacob, Y. Chen, R. L. Pinkus, and J. Bryant (1994) Gene therapy of cancer: a pilot study of IL-4-gene-modified fibroblasts admixed with autologous tumor to elicit an immune response. *Hum. Gene Ther.* **5,** 41-55.

Lowes, M. A., G. A. Bishop, K. Crotty, R. S. Barnetson, and G. M. Halliday (1997) T helper 1 cytokine mRNA is increased in spontaneously regressing primary melanomas. *J. Invest. Dermatol.* **108,** 914-919.

Lu, Z., L. Yuan, X. Zhou, E. Sotomayor, H. I. Levitsky, and D. M. Pardoll (2000) CD40-independent pathways of T cell help for priming of $CD8^+$ cytotoxic T lymphocytes. *J. Exp. Med.* **191,** 541-550.

Lumsden, J. M., J. M. Roberts, N. L. Harris, R. J. Peach, and F. Ronchese (2000) Differential requirement for CD80 and CD80/CD86-dependent costimulation in the lung immune response to an influenza virus infection. *J. Immunol.* **164,** 79-85.

Mackall, C. L., T. A. Fleisher, M. R. Brown, I. T. Magrath, A. T. Shad, M. E. Horowitz, L. H. Wexler, M. A. Adde, L. L. McClure, and R. E. Gress (1994) Lymphocyte depletion during treatment with intensive chemotherapy for cancer. *Blood* **84,** 2221-2228.

Mackall, C. L., C. V. Bare, L. A. Granger, S. O. Sharrow, J. A. Titus, and R. E. Gress (1996) Thymic-independent T cell regeneration occurs via antigen-driven

expansion of peripheral T cells resulting in a repertoire that is limited in diversity and prone to skewing. *J. Immunol.* **156,** 4609-4616.

Mackey, M. F., R. J. Barth, Jr., and R. J. Noelle (1998a) The role of CD40/CD154 interactions in the priming, differentiation, and effector function of helper and cytotoxic T cells. *J. Leukoc. Biol.* **63,** 418-428.

Mackey, M. F., J. R. Gunn, C. Maliszewsky, H. Kikutani, R. J. Noelle, and R. J. Barth, Jr. (1998b) Dendritic cells require maturation via CD40 to generate protective antitumor immunity. *J. Immunol.* **161,** 2094-2098.

Maeurer, M. J., D. M. Martin, C. Castelli, E. Elder, G. Leder, W. J. Storkus, and M. T. Lotze (1995) Host immune response in renal cell cancer: interleukin-4 (IL-4) and IL-10 mRNA are frequently detected in freshly collected tumor-infiltrating lymphocytes. *Cancer Immunol. Immunother.* **41,** 111-121.

Mauri, D. N., R. Ebner, R. I. Montgomery, K. D. Kochel, T. C. Cheung, G. L. Yu, S. Ruben, M. Murphy, R. J. Eisenberg, G. H. Cohen, P. G. Spear, and C. F. Ware (1998) LIGHT, a new member of the TNF superfamily, and lymphotoxin alpha are ligands for herpesvirus entry mediator. *Immunity* **8,** 21-30.

Meijer, S. L., A. Dols, W. J. Urba, H.-M. Hu, J. W. Smith II, J. Vetto, W. Wood, T. Doran, Y. Chu, P. Sayaharuban, W. G. Alvord, and B. A. Fox (2002) Adoptive cellular therapy with tumor vaccine draining lymph node lymphocytes after vaccination with HLA-B7/β2-microgloblulin gene-modified autologous tumor cells. *J. Immunother.* **25,** 359–372.

Mizoguchi, H., J. J. O'Shea, D. L. Longo, C. M. Loeffler, D. W. McVicar, and A. C. Ochoa (1992) Alterations in signal transduction molecules in T lymphocytes from tumor-bearing mice. *Science* **258,** 1795-1798.

Mobley, J. L., and M. O. Dailey (1992) Regulation of adhesion molecule expression by CD8 T cells in vivo. I. Differential regulation of gp90MEL-14 (LECAM-1), Pgp-1, LFA-1, and VLA-4 alpha during the differentiation of cytotoxic T lymphocytes induced by allografts. *J. Immunol.* **148,** 2348-2356.

Mobley, J. L., S. M. Rigby, and M. O. Dailey (1994) Regulation of adhesion molecule expression by CD8 T cells in vivo. II. Expression of L-selectin (CD62L) by memory cytolytic T cells responding to minor histocompatibility antigens. *J. Immunol.* **153,** 5443-5452.

Mocci, S., and R. A. Coffman (1997) The mechanism of in vitro T helper cell type 1 to T helper cell type 2 switching in highly polarized *Leishmania major*-specific T cell populations. *J. Immunol.* **158,** 1559-1564.

Mosmann, T. R., and S. Sad (1996) The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol. Today* **17,** 138-146.

Murali-Krishna, K., and R. Ahmed (2000) Cutting edge: naive T cells masquerading as memory cells. *J. Immunol.* **165,** 1733-1737.

Nabel, E. G., G. Plautz, and G. J. Nabel (1992) Transduction of a foreign histocompatibility gene into the arterial wall induces vasculitis. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 5157-5161.

Nabel, G. J., E. G. Nabel, Z. Y. Yang, B. A. Fox, G. E. Plautz, X. Gao, L. Huang, S. Shu, D. Gordon, and A. E. Chang (1993) Direct gene transfer with DNA-liposome complexes in melanoma: expression, biologic activity, and lack of toxicity in humans. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 11307-1311.

Naftzger, C., Y. Takechi, H. Kohda, I. Hara, S. Vijayasaradhi, and A. N. Houghton (1996) Immune response to a differentiation antigen induced by altered antigen: a study of tumor rejection and autoimmunity. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 14809-14814.

Nakamura, T., R. K. Lee, S. Y. Nam, E. R. Podack, K. Bottomly, and R. A. Flavell (1997) Roles of IL-4 and IFN-gamma in stabilizing the T helper cell type 1 and 2 phenotype. *J. Immunol.* **158,** 2648-2653.

Nastala, C. L., H. D. Edington, T. G. McKinney, H. Tahara, M. A. Nalesnik, M. J. Brunda, M. K. Gately, S. F. Wolf, R. D. Schreiber, and W. J. Storkus (1994) Recombinant IL-12 administration induces tumor regression in association with IFN-gamma production. *J. Immunol.* **153,** 1697-1706.

Newell, K. A., J. D. Ellenhorn, D. S. Bruce, and J. A. Bluestone (1991) In vivo T-cell activation by staphylococcal enterotoxin B prevents outgrowth of a malignant tumor. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 1074-1078.

Nunes, J. A., Y. Collette, A. Truneh, D. Olive, and D. A. Cantrell (1994) The role of p21ras in CD28 signal transduction: triggering of CD28 with antibodies, but not the ligand B7-1, activates p21ras. *J. Exp. Med.* **180,** 1067-1076.

Oehen, S. and K. Brduscha-Riem (1999) Naive cytotoxic T lymphocytes spontaneously acquire effector function in lymphocytopenic recipients: a pitfall for T cell memory studies? *Eur. J. Immunol.* **29,** 608-614.

Ossendorp, F., E. Mengede, M. Camps, R. Filius, and C. J. Melief (1998) Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. *J. Exp. Med.* **187,** 693-702.

Ostrand-Rosenberg, S., G. A. Cole, M. I. Nishimura, and V. K. Clements (1990a) Transfection and expression of syngeneic H-2 genes does not reduce malignancy of H-2 negative teratocarcinoma cells in the autologous host. *Cell Immunol.* **128,** 152-164.

Ostrand-Rosenberg, S., A. Thakur, and V. Clements (1990b) Rejection of mouse sarcoma cells after transfection of MHC class II genes. *J. Immunol.* **144,** 4068-4071.

Overwijk, W. W., D. S. Lee, D. R. Surman, K. R. Irvine, C. E. Touloukian, C. C. Chan, M. W. Carroll, B. Moss, S. A. Rosenberg, and N. P. Restifo (1999) Vaccination with a recombinant vaccinia virus encoding a "self" antigen induces autoimmune vitiligo and tumor cell destruction in mice: requirement for $CD4^+$ T lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 2982-2987.

Oxenius, A., K. A. Campbell, C. R. Maliszewski, T. Kishimoto, H. Kikutani, H. Hengartner, R. M. Zinkernagel, and M. F. Bachmann (1996) CD40-CD40 ligand interactions are critical in T-B cooperation but not for other anti-viral $CD4^+$ T cell functions. *J. Exp. Med.* **183,** 2209-2218.

Pape, K. A., A. Khoruts, A. Mondino, and M. K. Jenkins (1997) Inflammatory cytokines enhance the in vivo clonal expansion and differentiation of antigen-activated $CD4^+$ T cells. *J. Immunol.* **159,** 591-598.

Pape, K. A., R. Merica, A. Mondino, A. Khoruts, and M. K. Jenkins (1998) Direct evidence that functionally impaired $CD4^+$ T cells persist in vivo following induction of peripheral tolerance. *J. Immunol.* **160,** 4719-4729.

Pardoll, D. M. (1998) Cancer vaccines. *Nat. Med.* **4,** 525-531.

Pardoll, D. M., and S. L. Topalian (1998) The role of $CD4^+$ T cell responses in antitumor immunity. *Curr. Opin. Immunol.* **10,** 588-594.

Peterson, K. E., G. C. Sharp, H. Tang, and H. Braley-Mullen (1999) B7.2 has opposing roles during the activation versus effector stages of experimental autoimmune thyroiditis. *J. Immunol.* **162,** 1859-1867.

Pisetsky, D. S. (1996) Immune activation by bacterial DNA: a new genetic code. *Immunity* **5,** 303-310.

Plautz, G. E., Z. Y. Yang, B. Y. Wu, X. Gao, L. Huang, and G. J. Nabel (1993) Immunotherapy of malignancy by in vivo gene transfer into tumors. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 4645-4649.

Powrie, F., and R. L. Coffman (1993) Cytokine regulation of T-cell function: potential for therapeutic intervention. *Immunol. Today* **14,** 270-274.

Prlic, M., B. R. Blazar, A. Khoruts, T. Zell, and S. C. Jameson (2001) Homeostatic expansion occurs independently of costimulatory signals. *J Immunol.* **167,** 5664-5668.

Reiner, S. L., and R. M. Locksley (1995) The regulation of immunity to *Leishmania major. Annu. Rev. Immunol.* **13,** 151-177.

Restifo, N. P., P. J. Spiess, S. E. Karp, J. J. Mule, and S. A. Rosenberg (1992) A nonimmunogenic sarcoma transduced with the cDNA for interferon gamma elicits $CD8^+$ T cells against the wild-type tumor: correlation with antigen presentation capability. *J. Exp. Med.* **175,** 1423-1431.

Restifo, N. P., and M. Wang (1996) Antigen processing and presentation. In *Tumor Immunology: Immunology and Cancer Vaccines* (Dalgleish, A. G., and M. J. Browning, eds.), Cambridge University Press, New York, pp. 39-68.

Ridge, J. P., F. Di Rosa, and P. Matzinger (1998) A conditioned dendritic cell can be a temporal bridge between a $CD4^+$ T-helper and a T-killer cell. *Nature* **393,** 474-478.

Rieser, C., G. Bock, H. Klocker, G. Bartsch, and M. Thurnher (1997) Prostaglandin E2 and tumor necrosis factor alpha cooperate to activate human dendritic cells: synergistic activation of interleukin 12 production. *J. Exp. Med.* **186,** 1603-1608.

Rincon, M., J. Anguita, T. Nakamura, E. Fikrig, and R. A. Flavell (1997) Interleukin (IL)-6 directs the differentiation of IL-4-producing $CD4^+$ T cells. *J. Exp. Med.* **185,** 461-469.

Rocha, B., N. Dautigny, and P. Pereira (1989) Peripheral T lymphocytes: expansion potential and homeostatic regulation of pool sizes and CD4/CD8 ratios in vivo. *Eur. J. Immunol.* **19,** 905-911.

Rocken, M., and E. M. Shevach (1996) Immune deviation—the third dimension of nondeletional T cell tolerance. *Immunol. Rev.* **149,** 175-194.

Rodolfo, M., C. Zilocchi, C. Melani, B. Cappetti, I. Arioli, G. Parmiani, and M.P. Colombo (1996) Immunotherapy of experimental metastases by vaccination with interleukin gene-transduced adenocarcinoma cells sharing tumor-associated antigens. Comparison between IL-12 and IL-2 gene-transduced tumor cell vaccines. *J. Immunol.* **157,** 5536-5542.

Rosenberg, S. A. (1999) A new era for cancer immunotherapy based on the genes that encode cancer antigens. *Immunity* **10,** 281-287.

Rosenberg, S. A., P. Spiess, and R. Lafreniere (1986) A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes. *Science* **233,** 1318-1321.

Rosenberg, S. A., B. S. Packard, P. M. Aebersold, D. Solomon, S. L. Topalian, S. T. Toy, P. Simon, M. T. Lotze, J. C. Yang, C. A. Seipp, C. Simpson, C. Carter, S. Rock, D. Schwartzentruber, J. P. Wei, and D. E. White (1988) Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. *N. Engl. J. Med.* **319,** 1676-1680.

Rothe, H., A. Faust, U. Schade, R. Kleemann, G. Bosse, T. Hibino, S. Martin, and H. Kolb (1994) Cyclophosphamide treatment of female non-obese diabetic mice causes enhanced expression of inducible nitric oxide synthase and interferon-gamma, but not of interleukin-4. *Diabetologia* **37,** 1154-1158.

Ruedl, C., M. Kopf, and M. F. Bachmann (1999) $CD8^+$ T cells mediate CD40-independent maturation of dendritic cells in vivo. *J. Exp. Med.* **189,** 1875-1884.

Salgame, P., J. S. Abrams, C. Clayberger, H. Goldstein, J. Convit, R. L. Modlin, and B. R. Bloom (1991) Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. *Science* **254,** 279-282.

Scharton, T., and P. Scott (1993) Natural killer cells are a source of interferon gamma that drives differentiation of $CD4^+$ T cell subsets and induces early resistance to *Leishmania major* in mice. *J. Exp. Med.* **178,** 567-577.

Schoenberger, S. P., R. E. Toes, E. I. van der Voort, R. Offringa, and C. J. Melief (1998) T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* **393,** 480-483.

Schofield, L., M. J. McConville, D. Hansen, A. S. Campbell, B. Fraser-Reid, M. J. Grusby, and S. D. Tachado (1999) CD1d-restricted immunoglobulin G formation to GPI-anchored antigens mediated by NKT cells. *Science* **283,** 225-229.

Schoof, D. D., Y. Terashima, G. E. Peoples, P. S. Goedegebuure, J. V. Andrews, J. P. Richie, and T. J. Eberlein (1993) $CD4^+$ T cell clones isolated from human renal cell carcinoma possess the functional characteristics of Th2 helper cells. *Cell. Immunol.* **150,** 114-123.

Schreiber, H. (1993) Tumor immunology. In *Fundamental Immunology*, 3rd ed. (Paul, W. E., ed.), Raven Press, New York, pp. 1143-1178.

Schwartz, R. H. (1992) Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. *Cell* **71,** 1065-1068.

Schwartz, R. H. (1993) Immunological tolerance. In *Fundamental Immunology*, 3rd ed. (Paul, W. E., ed.), Raven Press, New York, pp. 677-731.

Schweitzer, A. N., and A. H. Sharpe (1998) Studies using antigen-presenting cells lacking expression of both B7-1 (CD80) and B7-2 (CD86) show distinct requirements for B7 molecules during priming versus restimulation of Th2 but not Th1 cytokine production. *J. Immunol.* **161,** 2762-2771.

Seder, R. A., and W. E. Paul (1994) Acquisition of lymphokine-producing phenotype by $CD4^+$ T cells. *Annu. Rev. Immunol.* **12,** 635-673.

Sepulveda, H., A. Cerwenka, T. Morgan, and R. W. Dutton (1999) CD28, IL-2-independent costimulatory pathways for CD8 T lymphocyte activation. *J. Immunol.* **163,** 1133-1142.

Shevach, E. M. (1993) Accessory molecules. In *Fundamental Immunology*, 3rd ed. (Paul, W. E., ed.), Raven Press, New York, pp. 531-575.

Shevach, E. M., R. S. McHugh, A. M. Thornton, C. Piccirillo, K. Natarajan, and D. H. Margulies (2001) Control of autoimmunity by regulatory T cells. *Adv. Exp. Med. Biol.* **490,** 21-32.

Shu, S., and S. A. Rosenberg (1985) Adoptive immunotherapy of a newly induced sarcoma: immunologic characteristics of effector cells. *J. Immunol.* **135,** 2895-2903.

Shu, S. Y., T. Chou, and K. Sakai (1989) Lymphocytes generated by in vivo priming and in vitro sensitization demonstrate therapeutic efficacy against a murine tumor that lacks apparent immunogenicity. *J. Immunol.* **143,** 740-748.

Shu, S., R. A. Krinock, T. Matsumura, J. J. Sussman, B. A. Fox, A. E. Chang, and D. S. Terman (1994) Stimulation of tumor-draining lymph node cells with superantigenic staphylococcal toxins leads to the generation of tumor-specific effector T cells. *J. Immunol.* **152,** 1277-1288.

Sigal, L. J., S. Crotty, R. Andino, and K. L. Rock (1999) Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen. *Nature* **398,** 77-80.

Silverstein, A. M. (1993) The history of immunology. In *Fundamental Immunology*, 3rd ed. (Paul, W. E., ed.), Raven Press, New York, pp. 21-41.

Singh, R. R., B. H. Hahn, and E. E. Sercarz (1996) Neonatal peptide exposure can prime T cells and, upon subsequent immunization, induce their immune deviation: implications for antibody vs. T cell-mediated autoimmunity. *J. Exp. Med.* **183,** 1613-1621.

Singh-Jasuja, H., H. U. Scherer, N. Hilf, D. Arnold-Schild, H. G. Rammensee, R. E. Toes, and H. Schild (2000a) The heat shock protein gp96 induces maturation of dendritic cells and down-regulation of its receptor. *Eur. J. Immunol.* **30,** 2211-2215.

Singh-Jasuja, H., R. E. Toes, P. Spee, C. Munz, N. Hilf, S. P. Schoenberger, P. Ricciardi-Castagnoli, J. Neefjes, H. G. Rammensee, D. Arnold-Schild, and H. Schild (2000b) Cross-presentation of glycoprotein 96-associated antigens on major histocompatibility complex class I molecules requires receptor-mediated endocytosis. *J. Exp. Med.* **191,** 1965-1974.

Slingluff, C. L., Jr., A. L. Cox, R. A. Henderson, D. F. Hunt, and V. H. Engelhard (1993) Recognition of human melanoma cells by HLA-A2.1-restricted cytotoxic T lymphocytes is mediated by at least six shared peptide epitopes. *J. Immunol.* **150,** 2955-2963.

Sotomayor, E. M., I. Borrello, E. Tubb, F. M. Rattis, H. Bien, Z. Lu, S. Fein, S. Schoenberger, and H. I. Levitsky (1999) Conversion of tumor-specific $CD4^+$ T-cell tolerance to T-cell priming through in vivo ligation of CD40. *Nat. Med.* **5,** 780-787.

Spiess, P. J., J. C. Yang, and S. A. Rosenberg (1987) In vivo antitumor activity of tumor-infiltrating lymphocytes expanded in recombinant interleukin-2. *J. Natl. Cancer Inst.* **79,** 1067-1075.

Staveley-O'Carroll, K., E. Sotomayor, J. Montgomery, I. Borrello, L. Hwang, S. Fein, D. Pardoll, and H. Levitsky (1998) Induction of antigen-specific T cell anergy: an early event in the course of tumor progression. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 1178-1183.

Stevenson, P. G., G. T. Belz, J. D. Altman, and P. C. Doherty (1998) Virus-specific $CD8^+$ T cell numbers are maintained during gamma-herpesvirus reactivation in CD4-deficient mice. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 15565-15570.

Storkus, W. J., H. J. Zeh, 3rd, M. J. Maeurer, R. D. Salter, and M. T. Lotze (1993) Identification of human melanoma peptides recognized by class I restricted tumor infiltrating T lymphocytes. *J. Immunol.* **151,** 3719-3727.

Stuber, G., G. H. Leder, W. T. Storkus, M. T. Lotze, S. Modrow, L. Szekely, H. Wolf, E. Klein, K. Karre, and G. Klein (1994) Identification of wild-type and mutant p53 peptides binding to HLA-A2 assessed by a peptide loading-deficient cell line assay and a novel major histocompatibility complex class I peptide binding assay. *Eur. J. Immunol.* **24,** 765-768.

Surh, C. D., and J. Sprent (2000) Homeostatic T cell proliferation: how far can T cells be activated to self-ligands? *J. Exp. Med.* **192,** F9-F14.

Sutmuller, R. P., van Duivenvoorde, L. M., van Elsas, A., Schumacher, T. N., Wildenberg, M. E., Allison, J. P., Toes, R. E., Offringa, R., and Melief, C. J. (2001) Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of $CD25^+$ regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. *J. Exp. Med.* **194,** 823-832.

Sznol, M. (1994) Regulatory issues: addition to Appendix D of NIH Guidelines regarding human gene transfer protocol. *Hum. Gene Ther.* **9,** 1182-1183.

Tahara, H., L. Zitvogel, W. J. Storkus, H. J. Zeh, Jr., T. G. McKinney, R. D. Schreiber, U. Gubler, P. D. Robbins, and M. T. Lotze (1995) Effective eradication of established murine tumors with IL-12 gene therapy using a polycistronic retroviral vector. *J. Immunol.* **154,** 6466-6474.

Takechi, Y., I. Hara, C. Naftzger, Y. Xu, and A. N. Houghton (1996) A melanosomal membrane protein is a cell surface target for melanoma therapy. *Clin. Cancer Res.* **2,** 1837-1842.

Tamada, K., M. Harada, K. Abe, T. Li, H. Tada, Y. Onoe, and K. Nomoto (1997) Immunosuppressive activity of cloned natural killer (NK1.1+) T cells established from murine tumor-infiltrating lymphocytes. *J. Immunol.* **158,** 4846-4854.

Tanaka, K., K. J. Isselbacher, G. Khoury, and G. Jay (1985) Reversal of oncogenesis by the expression of a major histocompatibility complex class I gene. *Science* **228,** 26-30.

Tanaka, K., H. Hayashi, C. Hamada, G. Khoury, and G. Jay (1986) Expression of major histocompatibility complex class I antigens as a strategy for the potentiation of immune recognition of tumor cells. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 8723-8727.

Tanchot, C., M. M. Rosado, F. Agenes, A. A. Freitas, and B. Rocha (1997) Lymphocyte homeostasis. *Semin. Immunol.* **9,** 331-337.

Tanchot, C., H. V. Fernandes, and B. Rocha (2000) The organization of mature T-cell pools. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **355,** 323-328.

Thompson, J. A., F. Grunert, and W. Zimmermann (1991) Carcinoembryonic antigen gene family: molecular biology and clinical perspectives. *J. Clin. Lab. Anal.* **5,** 344-366.

Thompson, T. C., L. D. Truong, T. L. Timme, D. Kadmon, B. K. McCune, K. C. Flanders, P. T. Scardino, and S. H. Park (1993) Transgenic models for the study of prostate cancer. *Cancer* **71,** 1165-1171.

Todryk, S., A. A. Melcher, N. Hardwick, E. Linardakis, A. Bateman, M. P. Colombo, A. Stoppacciaro, and R. G. Vile (1999) Heat shock protein 70 induced during tumor cell killing induces Th1 cytokines and targets immature dendritic cell precursors to enhance antigen uptake. *J. Immunol.* **163,** 1398-1408.

Toes, R. E. M., S. P. Schoenberger, E. I. H. van der Voort, R. Offringa, and C. J. M. Melief (1998) CD40-CD40Ligand interactions and their role in cytotoxic T lymphocyte priming and anti-tumor immunity. *Semin. Immunol.* **10,** 443-448.

Toes, R. E., F. Ossendorp, R. Offringa, and C. J. Melief (1999) CD4 T cells and their role in antitumor immune responses. *J. Exp. Med.* **189,** 753-756.

Tough, D. F., S. Sun, and J. Sprent (1997) T cell stimulation in vivo by lipopolysaccharide (LPS). *J. Exp. Med.* **185,** 2089-2094.

Townsend, S. E., and J. P. Allison (1993) Tumor rejection after direct costimulation of CD8+ T cells by B7-transfected melanoma cells. *Science* **259,** 368-370.

Tsung, K., J. B. Meko, G. R. Peplinski, Y. L. Tsung, and J. A. Norton (1997) IL-12 induces T helper 1-directed antitumor response. *J. Immunol.* **158,** 3359-3365.

Udono, H., D. L. Levey, and P. K. Srivastava (1994) Cellular requirements for tumor-specific immunity elicited by heat shock proteins: tumor rejection antigen gp96 primes CD8+ T cells in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 3077-3081.

van der Bruggen, P., C. Traversari, P. Chomez, C. Lurquin, E. De Plaen, B. Van den Eynde, A. Knuth, and T. Boon (1991) A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* **254,** 1643-1647.

Vella, A. T., J. E. McCormack, P. S. Linsley, J. W. Kappler, and P. Marrack (1995) Lipopolysaccharide interferes with the induction of peripheral T cell death. *Immunity* **2,** 261-270.

Villegas, E. N., M. M. Elloso, G. Reichmann, R. Peach, and C. A. Hunter (1999) Role of CD28 in the generation of effector and memory responses required for resistance to *Toxoplasma gondii*. *J. Immunol.* **163,** 3344-3353.

von der Weid, T., A. M. Beebe, D. C. Roopenian, and R. L. Coffman (1996) Early production of IL-4 and induction of Th2 responses in the lymph node originate from an MHC class I-independent $CD4^{+}NK1.1$- T cell population. *J. Immunol.* **157,** 4421-4427.

Wahl, W. L., G. E. Plautz, B. A. Fox, G. J. Nabel, S. Shu, and A. E. Chang (1992) Generation of therapeutic T lymphocytes after in vivo transfection of a tumor with a

gene encoding allogeneic class I major histocompatability complex antigen. *Surg. Forum* **43,** 476-478.

Wahl, W. L., J. J. Sussman, S. Shu, and A. E. Chang (1994) Adoptive immunotherapy of murine intracerebral tumors with anti-CD3/interleukin-2-activated tumor-draining lymph node cells. *J. Immunother. Emphasis Tumor Immunol.* **15,** 242-250.

Wahl, W. L., S. E. Strome, G. J. Nabel, G. E. Plautz, M. J. Cameron, H. San, B. A. Fox, S. Shu, and A. E. Chang (1995) Generation of therapeutic T-lymphocytes after in vivo tumor transfection with an allogeneic class I major histocompatibility complex gene. *J. Immunother. Emphasis Tumor Immunol.* **17,** 1-11.

Wallich, R., N. Bulbuc, G. J. Hammerling, S. Katzav, S. Segal, and M. Feldman (1985) Abrogation of metastatic properties of tumour cells by de novo expression of H-2K antigens following H-2 gene transfection. *Nature* **315,** 301-305.

Walunas, T. L., D. S. Bruce, L. Dustin, D. Y. Loh, and J. A. Bluestone (1995) Ly-6C is a marker of memory $CD8^+$ T cells. *J. Immunol.* **155,** 1873-1883.

Weber, J. S., and G. Jay (1988) MHC class I gene expression by tumors: immunotherapeutic implications. *Curr. Top. Microbiol. Immunol.* **137,** 140-147.

Weber, L. W., W. B. Bowne, J. D. Wolchok, R. Srinivasan, J. Qin, Y. Moroi, R. Clynes, P. Song, J. J. Lewis, and A. N. Houghton (1998) Tumor immunity and autoimmunity induced by immunization with homologous DNA. *J. Clin. Invest.* **102,** 1258-1264.

Weinberg, A. D., J. J. Wallin, R. E. Jones, T. J. Sullivan, D. N. Bourdette, A. A. Vandenbark, and H. Offner (1994) Target organ-specific up-regulation of the MRC OX-40 marker and selective production of Th1 lymphokine mRNA by encephalitogenic T helper cells isolated from the spinal cord of rats with experimental autoimmune encephalomyelitis. *J. Immunol.* **152,** 4712-4721.

Wells, A. D., S. K. Rai, M. S. Salvato, H. Band, and M. Malkovsky (1997) Restoration of MHC class I surface expression and endogenous antigen presentation by a molecular chaperone. *Scand. J. Immunol.* **45,** 605-612.

Wells, A. D., S. K. Rai, M. S. Salvato, H. Band, and M. Malkovsky (1998) Hsp72-mediated augmentation of MHC class I surface expression and endogenous antigen presentation. *Int. Immunol.* **10,** 609-617.

Whitmire, J. K., and R. Ahmed (2000) Costimulation in antiviral immunity: differential requirements for $CD4^+$ and $CD8^+$ T cell responses. *Curr. Opin. Immunol.* **12,** 448-455.

Williams-Abbott, L., B. N. Walter, T. C. Cheung, C. R. Goh, A. G. Porter, and C. F. Ware (1997) The lymphotoxin-α (LTα) subunit is essential for the assembly, but not for the receptor specificity, of the membrane-anchored LTα1β2 heterotrimeric ligand. *J. Biol. Chem.* **272,** 19451-19456.

Winter, H., H. M. Hu, W. J. Urba, and B. A. Fox (1999) Tumor regression after adoptive transfer of effector T cells is independent of perforin or Fas ligand (APO-1L/CD95L). *J. Immunol.* **163,** 4462-4472.

Winter, H., H. M. Hu, K. McClain, W. J. Urba, and B. A. Fox (2001) Immunotherapy of melanoma: a dichotomy in the requirement for IFN-gamma in vaccine-induced antitumor immunity versus adoptive immunotherapy. *J. Immunol.* **166,** 7370-7380.

Winter, H., H.-M. Hu, J. Bashy, D. Lashley, B. Lowe, E. Huntziker, J. Yamada, C. H. Poehlein, G. Alvord, W. J. Urba, and B. A. Fox (2002) Tumor-induced polarization of tumor vaccine-draining lymph node T cells to a type 1 cytokine profile predicts inherent strong immunogenicity of the tumor and correlates with therapeutic efficacy in adoptive transfer studies. *Immunology*, in press.

Wu, Q., Y. Wang, J. Wang, E. O. Hedgeman, J. L. Browning, and Y. X. Fu (1999) The requirement of membrane lymphotoxin for the presence of dendritic cells in lymphoid tissues. *J. Exp. Med.* **190,** 629-638.

Yanuck, M., D. P. Carbone, C. D. Pendleton, T. Tsukui, S. F. Winter, J. D. Minna, and J. A. Berzofsky (1993) A mutant p53 tumor suppressor protein is a target for peptide-induced $CD8^+$ cytotoxic T-cells. *Cancer Res.* **53,** 3257-3261.

Yoshizawa, H., A. E. Chang, and S. Shu (1991a) Specific adoptive immunotherapy mediated by tumor-draining lymph node cells sequentially activated with anti-CD3 and IL-2. *J. Immunol.* **147,** 729-737.

Yoshizawa, H., K. Sakai, A. E. Chang, and S. Y. Shu (1991b) Activation by anti-CD3 of tumor-draining lymph node cells for specific adoptive immunotherapy. *Cell Immunol.* **134,** 473-479.

Yoshizawa, H., A. E. Chang, and S. Y. Shu (1992) Cellular interactions in effector cell generation and tumor regression mediated by anti-CD3/interleukin 2-activated tumor-draining lymph node cells. *Cancer Res.* **52,** 1129-1136.

Zajac, A. J., J. N. Blattman, K. Murali-Krishna, D. J. Sourdive, M. Suresh, J. D. Altman, and R. Ahmed (1998) Viral immune evasion due to persistence of activated T cells without effector function. *J. Exp. Med.* **188,** 2205-2213.

Zhan, Y., A. J. Corbett, J. L. Brady, R. M. Sutherland, and A. M. Lew (2000) CD4 help-independent induction of cytotoxic CD8 cells to allogeneic P815 tumor cells is absolutely dependent on costimulation. *J. Immunol.* **165,** 3612-3619.

Zitvogel, L., J. I. Mayordomo, T. Tjandrawan, A. B. DeLeo, M. R. Clarke, M. T. Lotze, and W. J. Storkus (1996a) Therapy of murine tumors with tumor peptide-pulsed dendritic cells: dependence on T cells, B7 costimulation, and T helper cell 1-associated cytokines. *J. Exp. Med.* **183,** 87-97.

Zitvogel, L., P. D. Robbins, W. J. Storkus, M. R. Clarke, M. J. Maeurer, R. L. Campbell, C. G. Davis, H. Tahara, R. D. Schreiber, and M. T. Lotze (1996b) Interleukin-12 and B7.1 co-stimulation cooperate in the induction of effective antitumor immunity and therapy of established tumors. *Eur. J. Immunol.* **26,** 1335-1341.

Zou, J. P., N. Yamamoto, T. Fujii, H. Takenaka, M. Kobayashi, S. H. Herrmann, S. F. Wolf, H. Fujiwara, and T. Hamaoka (1995) Systemic administration of rIL-12 induces complete tumor regression and protective immunity: response is correlated with a striking reversal of suppressed IFN-γ production by anti-tumor T cells. *Int. Immunol.* **7,** 1135-1145.

BIOGRAPHICAL SKETCH

Hong-Ming Hu was born in Yong Kang, Zhejiang Providence of China. Between 1983-1987, he studied Biochemistry at Fudan University, Shanghai, China, and received his B.S. degree. After graduation, he continued his training at the Shanghai Institute of Biochemistry, Academia Sinica, and received his M.S. degree in Molecular Biology in 1990. In 1991, he moved to the United States and worked as Research Associate in the laboratory of Dr. V. M. Dixit, Department of Pathology, University of Michigan. In 1994, he moved to the Earle A. Chiles Research Institute, Portland, Oregon, with Dr. Bernard A. Fox. He started his graduate studies in Tumor Immunology in 1998 with Dr. Fox in the Department of Biochemistry and Molecular Biology, OGI School of Science and Engineering, Oregon Health & Science University (formerly the Oregon Graduate Institute of Sceince and Technology). In 2001, he become a citizen of the United State of America. He became the Chief of Laboratory of Cancer Immunobiology at Earle A. Chiles Research Institute in 2002.

Publications:

1. Opipari, A. W., Jr., **Hu, H.-M.**, Yabkowitz, R., and Dixit, V. M. (1992) The A20 zinc finger protein protects cells from tumor necrosis factor cytotoxicity. *J. Biol. Chem.* **267,** 12424–12427.

2. Laherty, C. D.*, **Hu, H.-M.***, Opipari, A. W., Wang, F., and Dixit, V. M. (1992) The Epstein-Barr virus LMP1 gene product induces A20 zinc finger protein expression by activating nuclear factor kappa B. *J. Biol. Chem.* **267,** 24157–24160. [*Equal contributors]

3. **Hu, H.-M.**, O'Rourke, K., Boguski, M. S., and Dixit, V. M. (1994) A novel RING finger protein interacts with the cytoplasmic domain of CD40. *J. Biol. Chem.* **269,** 30069–30072.

4. Zhang, Y., **Hu, H.-M.**, Cai, R., Feng, Y.-M. et al. (1996) Secretory expression of a single-chain insulin presuror in yeast and its conversion into human insulin. *Sci. China C, Life Sci.* **39,** 225–233.

5. **Hu, H.-M.** and Fox, B. A. (1996) Transfer of genes encoding antigens, co-stimulatory molecules, or adhesion/accessory molecules as a strategy to augment the anti-tumor immune response. In *Gene Therapy of Cancer* (Brenner, M. K. and Moen, R. C., Eds.), Marcel Dekker, New York, pp. 121–138.

6. **Hu, H.-M.**, Urba, W. J., and Fox, B. A. (1998) Gene-modified tumor vaccine with therapeutic potential shifts tumor-specific T cell response from a type 2 to a type 1 cytokine profile. *J. Immunol.* **161,** 3033–3041.

7. Fox, B. A., Drury, M., **Hu, H.-M.**, Cao, Z., Huntzicker, E. G., Qie, W., and Urba, W. J. (1998) Lipofection indirectly increases expression of endogenous major histocompatibility complex class I molecules on tumor cells. *Cancer Gene Ther.* **5,** 307–312.

8. Chu, Y., **Hu, H.-M.**, Winter, H., Wood., W. J., Doran, T, Lashley, D., Bashey, J., Schuster, J., Wood, J., Lowe, B. A., Vetto, J. T., Weinberg, A. D., Puri, R., Smith, J. W., 2nd, Urba, W. J., and Fox, B. A. (1999) Examining the immune response in sentinel lymph nodes of mice and men. *Eur. J. Nucl. Med.* **26,** S50–S53.

9. Winter, H., **Hu, H.-M.**, Urba, W. J., and Fox, B. A. (1999) Tumor regression after adoptive transfer of effector T cells is independent of perforin or Fas ligand (APO-1L/CD95L). *J. Immunol.* **163,** 4462–4472.

10. **Hu, H.-M.**, Winter, H., Urba, W. J., and Fox, B. A. (2000) Divergent roles for $CD4^+$ T cells in the priming and effector/memory phases of adoptive immunotherapy. *J. Immunol.* **165,** 4246–4253.

11. Winter, H., **Hu, H.-M.**, McClain, K., Urba, W. J., and Fox, B. A. (2001) Immunotherapy of melanoma: a dichotomy in the requirement for IFN-gamma in vaccine-induced antitumor immunity versus adoptive immunotherapy. *J. Immunol.* **166,** 7370–7380.

12. Meijer, S. L., Dols, A., **Hu, H.-M.**, Jensen, S., Poehlein, C. H., Chu, Y., Winter, H., Yamada, J., Moudgil, T., Wood, W. J., Doran, T., Justice, L., Fisher, B., Wisner, P., Wood, J., Vetto, J. T. Mehrotra, R., Rosenheim, S., Weinberg, A. D., Bright, R., Walker, E., Puri, R., Smith J. W. 2nd, Urba, J. W., and Fox, B. A. (2001) Immunological and molecular analysis of the sentinel lymph node: a potential approach to predict outcome, tailor therapy,

and optimize parameters for tumor vaccine development. *J. Clin. Pharmacol.* **Suppl.** 81S–94S.

13. **Hu, H.-M.**, Poehlein, C. H., Urba, W. J., and Fox, B. A. (2002) Development of antitumor immune responses in reconstituted lymphopenic hosts. *Cancer Res.* **62,** 3914–3919.

14. Meijer, S. L., Dols, A., Urba, W. J., **Hu, H.-M.**, Smith, J. W. II, Vetto, J., Wood, W., Doran, T., Chu, Y., Sayaharuban, P., Alvord, W. G., and Fox, B. A. (2002) Adoptive cellular therapy with tumor vaccine draining lymph node lymphocytes following vaccination with HLA-B7/β2-microgloblulin gene-modified autologous tumor cells. *J. Immunother.* **25,** 359–372.

15. **Hu, H.-M.**, Winter, H., Ma, J., Croft, M., Urba, W. J., and Fox, B. A. (2002) CD28, TNF receptor, and IL-12 are critical for CD4-independent cross-priming of therapeutic antitumor $CD8^+$ T cells. *J. Immunol.* **169,** 4897–4904.

16. Poehlein, C. H., Winter, H., Jensen, S., Yamada, J., Urba, W. J., **Hu, H.-M.**, and Fox, B. A. (2003) Mechanisms of T cell-mediated tumor regression. *Mod. Aspects Immunol.*, in press.

17. Winter, H.*, **Hu, H.-M.***, Bashy, J., Lashley, D., Lowe, B., Huntziker, E., Yamada, J., Poehlein, C. H., Alvord, G., Urba, W. J., and Fox, B. A. (2003) Tumor-induced polarization of tumor vaccine-draining lymph node T cells to a type 1 cytokine profile predicts inherent strong immunogenicity of the tumor and correlates with therapeutic efficacy in adoptive transfer studies. *Immunology*, in press. [* Equal contributors]

18. Poehlein, C. H., **Hu, H.-M.**, Yamada, J., Urba, W. J., and Fox, B. A. (2003) Tumor necrosis factor plays an essential role in tumor regression after adoptive transfer of perforin/IFN-γ double knockout effector T cells. *J. Immunol.*, in press.

19. Ma, J., Urba, W. J., Si, L., Wang, Y., Fox, B. A., and **Hu, H.-M.** (2002) Enhanced protective anti-tumor immunity in mice subjected to sublethal irradiation and immune reconstitution. Submitted.

20. **Hu, H.-M.**, Dols, A., Meijer, S. L., Walker, E., Urba, W. J., and Fox, B. A. (2002) Immunological monitoring of peptide vaccination in melanoma patients using soluble peptide/HLA-A2 dimer complexes. Submitted.