ANALYZING T CELL RESPONSES

Analyzing T Cell Responses

How to Analyze Cellular Immune Responses against Tumor Associated Antigens

Edited by

DIRK NAGORSEN
Charité University Medicine Berlin, Berlin, Germany

and

F.M. MARINCOLA
National Institutes of Health, Bethesda, Maryland, U.S.A.

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LIST OF CONTRIBUTORS

Victor Appay
Pluridisciplinary Oncology Center (CePO), Hôpital Orthopédique, Lausanne, Switzerland

Anne Marie Asemissen
Hematology, Oncology, and Transfusion Medicine, Charité-Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany

Mario Assenmacher
Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

Gideon Berke
Department of Immunology, Weizmann Institute of Science, Rehovot, Israel

Michael Campoli
Roswell Park Cancer Institute and Department of Immunology, School of Medicine, State University of New York at Buffalo, Buffalo, NY, USA

William R. Clark
University of California Los Angeles, Los Angeles, CA, USA

Elissa K. Deenick
Walter and Eliza Hall Institute for Medical Research, Melbourne, Australia

Soldano Ferrone
Roswell Park Cancer Institute and Department of Immunology, School of Medicine, State University of New York at Buffalo, Buffalo, NY, USA

Amanda V. Gett
Walter and Eliza Hall Institute for Medical Research, Melbourne, Australia

Tim F. Greten
Department of Gastroenterology, Hepatology and Endocrinology, Medizinische Hochschule Hannover, Germany

Jhaguaral Hasbold
Walter and Eliza Hall Institute for Medical Research, Melbourne, Australia

Edwin D. Hawkins
Walter and Eliza Hall Institute for Medical Research, Melbourne, Australia

Philip D. Hodgkin
Walter and Eliza Hall Institute for Medical Research, Melbourne, Australia

Mirja Hommel
Walter and Eliza Hall Institute for Medical Research, Melbourne, Australia

Steven Jacobson
Neuroimmunology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD, USA

Udai S. Kammula
Surgery Branch, National Cancer Institute, Bethesda, MD, USA

Ulrich Keilholz
Hematology, Oncology, and Transfusion Medicine, Charité – Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany

Firouzeh Korangy
Department of Gastroenterology, Hepatology and Endocrinology, Medizinische Hochschule Hannover, Germany

Peter P. Lee
Department of Medicine, Division of Hematology, Stanford University School of Medicine, Stanford, CA, USA

Anne Letsch
Hematology, Oncology, and Transfusion Medicine, Charité – Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany

Anatoli Malyguine
Laboratory of Cell-Mediated Immunity, Clinical Services Program, SAIC-Frederick, Inc., National Cancer Institute at Frederick, Frederick, MD, USA

Francesco M. Marincola
Immunogenetics Section, Department of Transfusion Medicine, Clinical Center, NIH, Bethesda, MD, USA

Markus J. Maeurer
Department of Medical Microbiology, University of Mainz, Mainz, Germany

Dirk Nagorsen
Hematology, Oncology, and Transfusion Medicine, Charité – Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany

Paul F. Robbins
Surgery Branch, National Cancer Institute, National Institutes of Health, Bethesda. MD, USA

Carmen Scheibenbogen
Hematology, Oncology, and Transfusion Medicine, Charité – Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany

Alexander Schmittel
Hematology, Oncology, and Transfusion Medicine, Charité – Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany

Pamela J. Skinner
University of Minnesota Department of Veterinary and Biomedical Sciences, St. Paul, USA

Eckhard Thiel
Hematology, Oncology, and Transfusion Medicine, Charité – Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany

Utano Tomaru
Division of Pathophysiological Science, Department of Pathology/Pathophysiology, Hokkaido Univ. Graduate School of Medicine, Sapporo, Japan

Hilary F. Todd
Walter and Eliza Hall Institute for Medical Research, Melbourne, Australia

Ena Wang
Immunogenetics Section, Department of Transfusion Medicine, Clinical Center, National Institutes of Health, Bethesda, MD, USA

Theresa L. Whiteside
University of Pittsburgh Cancer Institute and Departments of Pathology, Immunology and Otolaryngology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Yoshihisa Yamano
Third Department of Internal Medicine, Faculty of Medicine, Kagoshima University, Japan

Chapter 1

MONITORING ANTIGEN-SPECIFIC T CELL RESPONSES

Dirk Nagorsen, Francesco M. Marincola
Med. Klinik III, Hämatologie, Onkologie und Transfusionsmedizin, Charité – Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany(DN); Immunogenetics Section, Department of Transfusion Medicine, Clinical Center, NIH, Bethesda, MD, USA (F.M.M.)

Abstract: Tumor immunology has made rapid progress since the discovery of tumor-associated antigens. Antigen-specific T cell responses can now be reliably induced by vaccination. Some preliminary studies suggest that induction of a specific T cell response correlates with the clinical response. Several methods have been successfully utilized for monitoring TAA-specific T cells in tumor patients. The ELISPOT assay, cytokine flow cytometry, and tetramers have emerged as standard first-line T cell assays. Further assays complement the analysis for specific scientific questions. T cell analysis is not yet a defined surrogate marker for clinical efficacy. However, it indicates the immunological potency of a vaccine. A detailed T cell analysis can shed light on the T cell phenotype and function and may thus promote the development of clinically more effective vaccines.

Key words: cancer, vaccine, monitoring, CD8

Tumor treatment basically relies on surgery, radiotherapy, and chemotherapy. Hormone therapy and passive antibody transfer are established additional options for a few malignant diseases. Despite great success in some areas, cancer treatment obviously remains a major challenge. Some clinical situations cannot be adequately addressed. These include, in particular, the prevention of recurrence after a complete response and the management of therapy-resistant carcinomas.

Fighting tumors with the host's own immune system is an idea that has intrigued researchers for many decades. It was, to our knowledge, as early as the mid-nineteenth century that the first scientific report was published on tumor shrinkage that was probably mediated by an immune response to

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therapeutic infection with erysipelas (1). Later Coley (2) developed his famous toxin from gram-positive *Streptococcus pyogenes* and gram-negative *Serratia marcescens* and was relatively successful in treating sarcoma patients (3). These and other attempts to stimulate the immune system against tumors were made by a handful of physicians and surgeons on the basis of observations in only a few patients. The scientific foundation for immunotherapy of cancer in humans was not laid until many years later. Particularly the discovery of tumor-associated antigens in the early nineteen-nineties (4, 5, also see Chapter 2) turned tumor immunology into a major field of research. Animal studies as well as observations in humans support the theory of immune surveillance against malignant cells (reviewed in 6, 7). Findings obtained by these investigations include an increased prevalence of certain tumors following immunosuppression and a correlation between intralesional T cells and an improved clinical outcome for various solid tumors (6), including malignant melanoma (8), colorectal cancer (9), esophageal carcinoma (10), and ovarian cancer (11). However, these studies do not indicate whether infiltrating T cells are directed against tumor-associated antigens.

Spontaneous T cell responses directed against specific TAA have been detected in peripheral blood or bone marrow of tumor patients (reviewed in 12) with various histotypes, including melanoma (13, 14), colorectal cancer (15), AML (16), breast cancer (17), and neuroblastoma (18). Findings in a few selected cases suggest a favorable clinical course in tumor patients with peripheral natural TAA-directed T cells (19, 20). However, in a recent study, again with a limited number of patients, we did not find a significant survival difference between colorectal cancer patients with and without TAA-directed T cell responses (21). There is not yet enough data to draw firm generalized conclusions about the clinical impact of spontaneous T cell responses against TAA.

Also, it is still unclear how spontaneous TAA-directed T cell responses influence the efficiency of vaccination therapies in cancer patients. Some investigators think that a detectable peripheral (precursor) T cell response might be necessary for an effective vaccination (22, 23), while others suggest that T cells with such responses might have lost their anti-tumor effectiveness (24). With improved vaccination schedules, TAA-directed T cell responses can be reliably induced using various vaccination approaches (reviewed in 25). Several reports have described a correlation between a vaccination-induced TAA-directed T cell response and a clinical response (26, 27, 28, 29). Preliminary data also suggest a possibly favorable clinical effect of vaccine-induced T cells in adjuvant vaccination therapy (30, 31, 32, 33). However, some studies have also described the coexistence of vaccine-induced TAA-specific T cells and TAA-bearing tumors (34). This paradox is

not fully understood. Tumor escape mechanisms and T cell dysfunctions are discussed as basic reasons (see chapter 3; 35).

Besides active vaccination, a further experimental treatment option using TAA-specific T cells is the autologous administration of isolated tumor-infiltrating TAA-specific cells after *in vitro* expansion and activation in patients with certain malignant diseases, e.g. melanoma (36). Some clinical success has been achieved in selected patients using specific T cell transfer, particularly after nonmyeloablative chemotherapy (37). Monitoring the presence and localization of adoptively transferred T cells is crucial for evaluating and improving these adoptive-cell-transfer therapies and of course also for gaining insights into the interaction between tumors and the immune system.

The role of T cells in host defense against tumors is not yet fully understood and thus requires a more comprehensive investigation. Several methods have been successfully utilized for monitoring TAA-specific T cells in tumor patients. The ELISPOT assay, cytokine flow cytometry, and tetramers have emerged as standard first-line T cell assays (38). Further methods such as qRT-PCR, proliferation assays, dimers, TCR analysis, cytotoxicity assays, or GFP-HLA complement the analysis for specific scientific questions (see Chapters 5, 6, and 13 through 16). First studies have also been performed on microarray analyses of enriched TAA-specific T cells (see Chapter 17, (39)). Each method has its own specific advantages and disadvantages. One major requirement for a first-line up-to-date monitoring technique is single-cell analysis. This can be done by ELISPOT, flow cytometric cytokine assays and tetramer staining. While functional cytokine assays (ELISPOT, intracellular cytokine flow cytometry) shed light on cytokine production in response to peptide in single cells, tetramers indicate specific binding between a single T cell and a target HLA/peptide complex irrespective of the functional state of the specific cell. Thus, even anergic or areactive specific T cells can be detected by tetramers.

Antibody-based TCR analyses can also be performed on a single-cell level. However, while antibody-based analysis can only detect 70% of the αβ TCR repertoire, molecular analysis has the advantage of full coverage. Unfortunately, molecular methods cannot distinguish between T cell subsets. Their analysis requires previous separation by techniques such as tetramer staining or cytokine capture assay with subsequent cell sorting or separation by magnetic microbeads. This emphasizes the importance of combining different methods. A further example of successful combinations is the application of tetramer staining together with CD107 as a marker of degranulation (as described in Chapter 11). Most *ex vivo* functional assays are based on the measurement of epitope-induced cytokine production. Two further important functions of active T cells are their ability to proliferate

and their cytotoxic potential (Chapters 5 and 6). This book describes all relevant established techniques in separate chapters. In addition, we have included two techniques whose role has not yet been definitely determined: in situ MHC tetramer staining and peptide-HLA-GFP complexes. Though little data is available on these methods, they are very promising, and we expect them to provide important new information on tumor-infiltrating TAA-specific T cells and HLA-TCR interaction.

Scientists from other fields of immunology, especially virologists and rheumatologists, have a major impact on the development of better methods for T cell analysis. In particular, epitopes derived from CMV, EBV, influenza, and HIV are common targets for frequent "autologous" specific T cell responses. Moreover, our impression is that virus-specific T cells have a higher affinity to their targets. HPV vaccination, clinically successful in preventing cervical cancer (40, reviewed in 41), is an excellent example for overlapping questions and challenges in viral and tumor immunology.

There is a trend toward more and more detailed analysis of T cell responses. Especially for virus-specific T cell responses, several subsets of T cells have been described and related to specific functions and stages of development. In particular, CD45RA, CCR7, CD27, and CD28 have proven helpful for determining functional T cell subsets (42, 43, 44). Furthermore, analysis of cytokine expression within cell subsets, such as IL-4, IL-10, TGFβ, or IL-2, helps in assigning functional properties to T cells. The determination of further chemokine receptors on T cells is also of interest because, in combination with the local chemokine milieu, they are indicative of the homing ability of T cells (45).

All these latter analyses can be done by cytokine flow cytometric methods and tetramer staining. In contrast, ELISPOT assays, microarrays, molecular TCR analyses, and qRT-PCR do not allow further determination of T cell subsets unless preceded by a usually laborious cell separation. Before applying analysis methods, some investigators enhance *ex vivo* immunity by using protocols such as culturing T cells for 1-2 weeks with peptide and cytokines (IL-2, IL-7). This stimulation step is useful for analyzing extremely rare T cells and has its role in sensitive semiquantitative analyses. However, phenotypic, functional, and quantitative changes in T cells during *in vitro* stimulation obviously limit the information obtained after *in vitro* stimulation (46). Therefore, when reporting on an assay, it is important to differentiate between *ex vivo* T cell analysis and T cell analysis after *in vitro* stimulation.

There are basically three therapeutic situations in which TAA-specific T cells appear in cancer patients: spontaneously without prior immunotherapy, after active immunization, and after adoptive T cell transfer. Although some clinical success has been achieved, the actual role of antigen-specific T cells

in tumor patients remains to be determined, and the therapeutic niche of T-cell-targeted therapy has yet to be defined. However, first results are promising and strongly support the further development of immunotherapeutic approaches aimed at the induction or modification of T cell responses. Reliable and detailed immune monitoring of T cell responses is essential for further development of cancer vaccination and adoptive T cell transfer studies. Suitable utilization and further improvement of T cell analysis also serve other fields of immunology, including virology and rheumatology.

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Chapter 2

TUMOR ASSOCIATED ANTIGENS

Paul F. Robbins
Surgery Branch, National Cancer Institute, National Institutes of Health,Bethesda, MD, USA

Abstract: The results of an extensive series of studies that were first carried out in the early 1990s have revealed that human tumor reactive T cells recognize a diverse array of antigens. These include antigens expressed in normal tissues, mutated gene products as well as novel epitopes encoded within alternative open reading frames, intronic sequences, as well as the products that result from protein splicing. This antigenic diversity provides opportunities as well as challenges for the development of more effective immunotherapies for the treatment of patients with cancer.

Key words: antigen, epitope, tumor association

1. INTRODUCTION

Studies initiated in the early 1980s demonstrated that CD8+ cytotoxic T cells (CTL) that recognized tumor cell in an MHC restricted manner could be generated by incubation of tumor infiltrating lymphocytes (TIL) with high doses of IL-2, as well as by the *in vitro* sensitization of peripheral blood mononuclear cells (PBMC) from cancer patients with autologous or HLA matched allogeneic tumor cells in mixed lymphocyte tumor cultures (MLTC). These studies lead to the identification of the first antigen recognized by human tumor reactive T cells, MAGE-1, in 1991 (1). Since that time, human tumor reactive T cells have been found to recognize peptides that are processed from the products of more than 50 distinct genes. These include antigens that are derived from normal gene products that are expressed in a tissue specific manner, products that are limited in their expression to tissues such as the testis that do not appear to represent

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immunologic targets, products that are over-expressed in tumors and mutated gene products. Several tumor reactive HLA class II restricted T cells have also been identified, many of which recognize epitopes from proteins that have also been shown to be recognized by HLA class I restricted T cells.

Several mechanisms influence the repertoire of T cells that is available to recognize antigens expressed on tumor cells. Tumor antigens recognized by TIL as well as by T cells generated by direct tumor stimulation have been shown in many cases to results from novel gene processing as well as antigen processing mechanisms that include the translation of RNA transcripts that contain unspliced introns (2), translation from alternative open reading frames (3, 4) and protein splicing (5, 6). The universe of potential antigens thus appears to be significantly larger than that which is based upon known or predicted translated gene products. At the same time, antigen processing mechanisms appear to result in the presentation of only a small proportion of the pool of peptides that are capable of binding to an individual HLA allele, and self-tolerance mechanisms may further limit the available repertoire of T cell specificities, hampering studies aimed at identifying immunogenic candidate T cell epitopes. Nevertheless, antigen identification studies carried out with tumor reactive T cells have provided a large array of targets that can be used for the development of cancer immuotherapies.

2. ANTIGEN IDENTIFICATION

A variety of methods have been employed to identify tumor antigen epitopes. Tumor antigens recognized by HLA class I restricted T cells have primarily been identified using expression cloning methods developed in the early 1980s that relied on the used of highly transfectable cell lines to introduce genes from tumor cell cDNA libraries. For this approach, the appropriate HLA class I restriction element involved with presentation of the T cell epitope must first be identified either by blocking T cell antigen recognition using antibodies that are specifically reactive with particular HLA alleles, or by assaying a series of tumor cells lines that either do or do not share particular HLA gene products with autologous tumor cells for their ability to stimulate the tumor reactive T cells. The appropriate HLA class I restriction element, once identified, is then used to transfected a target cell line such as the monkey kidney cell line COS or the human embryonic kidney cell line 293. Target cells are then transfected with pools of cDNAs generated from tumor cell mRNA, and transfectants assayed for their ability

to stimulate cytokine release from either tumor reactive T cell lines or T cell clones.

Additional studies have demonstrated that cell lines such as 293 could process and present antigens recognized by HLA class II restricted T cells. The HLA class II gene complex consists of an HLA-DR locus that contains from one to three functional genes that vary according to the haplotypes, along with the HLA-DP and DQ loci. Individual HLA-DR molecules consist of a highly polymorphic DRβ gene product that is associated with a nearly invariant DRα chain, while the HLA-DP and DQ products consist of polymorphic α and β chains. Transfected 293 cells that express HLA class II molecules as well as additional molecules associated with class II processing such as invariant chain and HLA-DM could efficiently process antigens recognized by HLA class II restricted T cells and, as discussed further below, has been used to identify unknown tumor antigens (7, 8). Alternatively, target cells generated by transfection of 293 cells with constructs encoding the appropriate HLA class II gene products as well as the HLA class II transactivator (CIITA) gene, which up-regulates the expression of multiple gene products associated with class II processing (9), have also been used to identify genes that encode antigens recognized by HLA class II restricted T cells (10, 11).

Biochemical approaches have also been utilized to identify antigens recognized by tumor reactive T cells (12, 13). Pools of peptides that were eluted from MHC class I molecules were fractionated using reversed phase HPLC columns and tested for their ability to sensitize target cells for T cells recognition. Additional fractionation steps were then carried out using a variety of columns or buffers, and peptides associated with T cell recognition were identified on the basis of their elution profile. The sequence of candidate peptides was then determined using tandem mass spectrometry, and synthetic peptides corresponding to these sequences were then generated and tested for their ability to be recognized by specific T cells. Antigen presenting cells such as dendritic cells (DC) and B cells can efficiently process whole proteins through an exogenous pathway that leads to presentation of HLA class II restricted T cell epitopes. An antigen recognized by HLA class II restricted T cells by fractionating proteins isolated from tumor cells using a variety of biochemical techniques (14).

The screening of tumor cell cDNA expression libraries with patient sera, designated SEREX (serological analysis of gene expression), has resulted in the identification of a large number of antigens, many of which appear to represent widely expressed auto-antigens that may not be recognized by tumor reactive T cells. Nevertheless, a small subset of the antigens identified using the SEREX technique have been found to be recognized by HLA class I and class II restricted T cells (15, 16).

Tumor antigens have also been identified through a process termed reverse immunology, whereby candidate epitopes are first identified from a protein that is over-expressed in a particular tumor type though the use of HLA binding motifs. The motifs represent consensus sequences that have been derived by direct measurement of MHC-peptide binding affinities (17), as well as by identifying common residues that appear at particular positions in known T cell epitopes and peptides that have been eluted from individual MHC molecules (18). The majority of HLA class I peptide epitopes consist of between 8 and 10 amino acids, although longer peptides have been shown to bind to certain HLA class I alleles. Amino acids that predominate at 2 or 3 positions, designated the anchor amino acids, directly interact with pockets in the MHC class I molecule and therefore strongly influence peptide binding affinity, although other secondary anchor positions that have a less important influence on binding have been identified. Peptides that bind to the HLA-A2 class I gene, which is expressed by approximately 50% of Caucasians and represents the most common allele expressed in this population, generally possess an aliphatic residue such as L or M at position 2 (P2) and a V or L residue at the carboxy terminus of the peptide (P9 or P10). The amino and carboxyl termini of HLA class II binding peptides are less well defined than class I binding peptides, as the class II peptide binding pocket has a more open structure than the class I binding pocket. Nevertheless, HLA class II binding peptides generally contain a core region of approximately 10 amino acids that contains two or three anchor positions. A general correlation has been noted between the peptide affinity and the ability to generate specific T cells (19); however, many of the peptides derived from normal, non-mutated gene products that are recognized by tumor reactive T cells appear to bind to HLA class I gene products with relatively low affinity. Presumably, the expression of certain antigens in normal adult tissues can lead to the deletion of T cells that recognize epitopes that bind to HLA molecules with high affinity.

3. ANTIGENS RECOGNIZED BY HLA CLASS I RESTRICTED, TUMOR REACTIVE T CELLS - DIFFERENTIATION ANTIGENS

The antigens recognized by human tumor reactive T cells can be categorized according to their expression profiles in normal tissues and tumors. The differentiation antigens are limited in their expression in the adult primarily to one tissue type and are dominated by the melanocyte differentiation antigens (MDA), which represent gene products that are expressed in melanomas as well as normal skin melanocytes and retinal

tissue. Epitopes have been identified from several MDA that play a role in the synthesis of melanin, including gp100, tyrosinase, TRP-1 and TRP-2, as well as proteins of unknown function such as MART-1 (Table 1).

Table 1. HLA Class I restricted shared tumor specific antigens.

GENE	HLA Allele	Epitope	Reference
BAGE-1:2-10	Cw16	ARAVFLAL	100
DAM-6:271-279	A2	FLWGPRAYA	101
GAGE-1/2/8:9-16	Cw6	YRPRPRRY	102
GAGE-3/4/5/6/7:10-18	A29	YYWPRPRRY	45
GnT-V(intron)	A2	VLPDVFIRCV	103
LAGE-1:157-165	A2	SLLMWITQC	42
LAGE-1:AORF*	A2	MLMAQEALAFL	104
LAGE-1:AORF	A31	LAAQERRVPR	43
MAGE-1:161-169	A1	EADPTGHSY	105
MAGE-1:96-104	A3	SLFRAVITK	106
MAGE-1:222-231	A68	EVYDGREHSA	106
MAGE-1:135-143	A24	NYKHCFPEI	107
MAGE-1:127-136	B37	REPVTKAEML	108
MAGE-1:258-266	B53	DPARYEFLW	106
MAGE-1:62-70	Cw2	SAFPTTINF	106
MAGE-1:230-238	Cw3	SAYGEPRKL	106
MAGE-1:230-238	Cw16	SAYGEPRKL	109
MAGE-2:112-120	A2	KMVELVHFL	110
MAGE-2:157-166	A2	YLQLVFGIEV	110
MAGE-2:156-164	A24	EYLQLVFGI	111
MAGE-2:127-136	B37	REPVTKAEML	108
MAGE-3:168-176	A1	EVDPIGHLY	39
MAGE-3:271-279	A2	FLWFPRALV	40
MAGE-3:112-120	A2	KVAELVHFL	49
MAGE-3:195-203	A24	IMPKAGLLI	112
MAGE-3:97-105	A24	TFPDLESEF	113
MAGE-3:167-176	B18	MEVDPIGHLY	114
MAGE-3:168-176	B35	EVDPIGHLY	115
MAGE-3:127-136	B37	REPVTKAEML	108
MAGE-3:114-122	B40	AELVHFLLL	94
MAGE-3:167-176	B44	MEVDPIGHLY	116
MAGE-3:143-151	B52	WQYFFPVIF	115
MAGE-4:169-177	A1	EVDPASNTY	117
MAGE-4:230-239	A2	GVYDGREHTV	118
MAGE-4:156-163	B37	SESLKMIF	119
MAGE-6:290-298	A34	MVKISFFPR	120
MAGE-6:127-136	B37	REPVTKAEML	108
MAGE-10:254-262	A2	GLYDGMEHL	121
MAGE-10:290-298	B53	DPARYEFLW	106
MAGE-12:271-279	A2	FLWGPRALV	40
MAGE-12:170-178	Cw7	VRIGHLYIL	122
NY-ESO-1:157-165	A2	SLLMWITQC	42
NY-ESO-1:AORF	A2	MLMAQEALAFL	104

GENE	HLA Allele/Epitope		Reference
NY-ESO-1:53-62	A31	ASGPGGGAPR	43
NY-ESO-1:AORF	A31	LAAQERRVPR	43
NY-ESO-1:94-102	B51	MPFATPMEA	123
NY-ESO-1:92-100	Cw3	LAMPFATPM	124
NY-ESO-1:80-88	Cw6	ARGPESRLL	124
SSX-2:41-49	A2	KSEKIFYV	16
TRP-2:intron	A68	EVISCKLIKR	46

The MART-1 antigen, also designated Melan-A, was independently cloned by 2 groups by the expression screening of melanoma cDNA libraries (20, 21). MART-1 represents an immunodominant antigen and is recognized by the majority of melanoma reactive TIL derived from HLA-A2 patients (22). Following the screening of 23 candidate MART-1 peptides that conformed to the HLA-A2 binding motif, a single nonapeptide, AAGIGILTV(MART-1:27-35), as well as two overlapping decapeptides, EAAGIGILTV (MART-1:26-35) and AAGIGILTVI (MART-1:28-36) were recognized by a MART-1 reactive T cell clone as well as three MART-1 reactive TIL. The MART-1:27-35 and MART-1:26-35 peptides both contain an alanine residue at the P2 primary anchor position which appears to be responsible for the relatively low affinity binding affinity of these peptides. Modifications of MART-1:27-35 peptide that resulted in enhanced binding to HLA-A2 generally generated peptides that are recognized less well than the unmodified peptide, whereas substitution of leucine for the alanine residue at the presumed P2 primary anchor position in the MART-1:26-35 peptide (ELAGAGILTV) resulted in the generation of a peptide with enhanced binding to HLA-A2, designated MART-1:26-35(2L) (23) Tumor reactive T cells were more readily generated when the modified MART-1:26-35(2L) peptide was used than when the un-modified peptide was used to carry out the *in vitro* sensitization of PBMC.

Soluble chimeric molecules that contain MHC class I or class II gene products bound to an appropriate peptide and that provide a direct measurement of the percentage of cells reactive with a particular epitope, termed MHC tetramers (24) have been used to characterize immune responses to MART-1 as well as additional tumor antigens. Low levels of cells reactive with the MART-1:26-35(2L) tetramer were detected in six out of ten normal donors and seven out of ten melanoma patients (25). Studies of the phenotype of tetramer positive cells indicated that those cells from normal donors were exclusively of the naïve phenotype, whereas in three out of the ten melanoma patients, cells expressed a more mature phenotype and expressed low levels of CD45RA and CD28, providing evidence that tumor cell stimulation may have lead to activation of the MART-1 reactive T cells.

Additional MART-1 epitopes recognized in the context of HLA-B35 (26) and B45 (27) encompass the MART-1 HLA-A2 epitope, suggesting that this region of the molecule may possess characteristics that lead to the efficient processing.

The gp100 glycoprotein, which was originally identified as an enzyme involved with melanin synthesis, was subsequently found to represent an antigen recognized by HLA-A2 restricted, melanoma reactive T cells (28). In one study, 13 out of 30 HLA-A2 restricted TIL were found to recognize gp100, and three peptides, KTWGQYWQV (gp100:154-162), ITDQVPFSV (gp100:209-217), and YLEPGPVTA (gp100:280-288) were recognized in the context of HLA-A2 by seven, six and six out of the thirteen gp100 reactive TIL, respectively (22). The gp100:280-288 peptide was also identified in an independent study in which peptides that have been eluted from the surface of melanoma cells were fractionated and used to sensitize target cells for T cell recognition (13). In this study, HLA-A2 expressing target cells pulsed with the gp100:280-288 peptide were recognized by five out of five melanoma reactive, HLA-A2 restricted T cell lines generated by in vitro stimulation with tumor cells.

The observation that the dominant gp100 epitopes contained non-optimal anchor residues lead to attempts to enhance the antigenicity as well as the immunogenicity of these peptides by substituting consensus anchor residues at these positions. The gp100:209-217 peptide contained a threonine at position two and possessed a relatively low HLA-A2 binding affinity of 172 nM; however, substitution of a leucine, methionine or isoleucine at the second position resulted in the generation of a peptide that had a significantly higher HLA-A2 binding affinity as well as an enhanced capacity to stimulate peptide and tumor reactive T cells *in vitro* (29). The results of in vitro sensitization studies carried out with PBMC from melanoma patients demonstrated that a variant containing a substitution of methionine for the threonine at position 2 of the native peptide, gp100:209-217(2M), was more effective at stimulating tumor reactive T cells than the parental peptide. Similarly, the gp100:280-288 peptide possessed a binding affinity of only 455 nM, and a peptide containing a substitution of valine for the alanine at position nine possessed a 10-fold higher HLA-A2 binding affinity than the parental peptide and was more effective at stimulating peptide and tumor reactive T cells than the parental peptide (29).

Several additional melanosomal proteins have been shown to represent targets of tumor reactive T cells. Results presented in an initial report indicated that melanoma reactive T cells recognized two HLA-A2 restricted epitopes of tyrosinase, the enzyme that catalyzes the first step in melanin synthesis from the amino acid tyrosine (30). One of the tyrosinase epitopes, MLLAVLYCL(tyr:1-9), was derived from the first 9 amino acids of the

tyrosinase signal peptide. The second epitope, YMNGTMSQV (tyr:369-377), was subsequently shown to contain a post-translational modification resulting from a substitution of aspartic acid for the asparagine residue at position 3 (31). The modified tyr:369-377 peptide was also recognized by CD4+ T cells (32), and antibody blocking studies demonstrated that the CD4 and CD8 co-receptors did not contribute to the recognition of this epitope, indicating that the CD4+ T cells may recognize this epitope with a relatively high avidity. A third HLA-A2 restricted epitope derived from the tyrosinase signal peptide, CLLWSFQTSA (tyr:8-17), was identified using a candidate epitope approach (33). Additional MDA that include TRP-1 (3) TRP-2 (34, 35) and AIM-1 have been identified as the targets of tumor reactive T cells, and tumor reactive T cells were generated by stimulating PBMC from melanoma patients with candidate epitopes from the OA1 (36) and P. polypeptide (37) proteins.

4. TUMOR SPECIFIC ANTIGENS

This category of tumor antigens, which represents non-mutated gene products that are not processed and presented on normal adult tissues, is predominantly comprised of members of the cancer/testis (C/T) family of genes (Table 2). The expression of C/T gene products is limited in adult tissues to the normal testis, which lacks expression of HLA class I and class II molecules. The prototype for this family, MAGE-1, represents the first antigen to be identified as a target of human tumor reactive T cells, and was cloned by transfecting an autologous tumor cell line that appeared to have lost antigen expression with a cosmid library containing genomic DNA from antigen positive tumor cells (1). The cancer/testis genes have been clustered into 10 families, 6 of which have been mapped to the X chromosome (38), and subsequent studies have lead to the identification of T cell epitopes expressed on multiple products derived from these gene families. The MAGE gene family includes 17 members, but additional families appear to contain only a small number of sequences, as the NY-ESO-1 families contains only 2 genes very closely related genes, NY-ESO-1 and LAGE-1. Members of these families are expressed at frequencies of up to 60% in melanoma, breast, lung, bladder and prostate cancers. Two epitopes recognized in the context of HLA-A1 have been identified from homologous regions of the MAGE-A1 and MAGE-A3 antigens (39). A reverse immunology approach was also used to identify an HLA-A2 restricted epitope of MAGE-A3 (40). This epitope may not generally be processed endogenously at levels sufficient to lead to T cell recognition, however, as

Table 2. HLA class I restricted differentiation antigens.

Gene	HLA Allele	/Epitope	Reference
AIM-1:41-50	A2	AMFGREFCYA	125
CEA:605-613	A2	YLSGANLNL	126
CEA:691-699	A2	IMIGVLVGV	49
CEA:61-69	A3	HLFGYSWYK	127
gp100:154-162	A2	KTWGYWQV	128
gp100:209-217	A2	ITDQVPFSV	128
gp100:280-288	A2	YLEPGPVTA	13
gp100:457-466	A2	LLDFTATLRL	128
gp100:476-485	A2	VLYRYGFSV	128
gp100:177,178-186	A2	(A)MLGTHTMEV	129
gp100:619-627	A2	RLMKQDFSV	130
gp100:639-647	A2	RLPRIFCSC	130
gp100:614-622	A3	LIYRRRLMK	130
gp100:17-25	A3	ALLAVGATK	131
gp100:intron	A24	VYFFLPDHL	81
gp100:182-191	A68	HTMEVTVYHR	132
gp100:71-78	Cw8	SNDGPTLI	35
mammaglobin-A:23-31	A3	PLLENVISK	133
MART-1:26,27-35,36	A2	(E)AAGIGILTV(A)	134
MART-1:32-40	A2	ILVILGVL	135
MART-1:24-33,34	B45	AEEAAGIGIL(T)	27
MC1R:244-252	A2	TILLGIFFL	136
MC1R:283-291	A2	FLALIICNA	136
OA1:194:202	A24	LYSACFWWL	36
PSA:165-174	A2	FLPKKLQCV	137
PSA:178-187	A2	VISNDVCAQV	137
P. polypeptide:427-435	A2	IMLCLIAAV	37
RAGE-1:11-20	B7	SPSSNRIRNT	138
SOX-10:331-340	A2	AWISKPPGV	139
TRP-2:180-188	A2	SYVYDFFVWL	140
TRP-2:360-368	A2	TLDSQVMSL	141
TRP-2:455-463	A2	YAIDLPVSV	125
TRP-2:197-205	A31,A33	LLGPGRPYR	142
TRP-1:AORF	A31	MSLQRQFLR	3
TRP-2:387-395	Cw8	ANDPIFVVL	35
TRP-2:403-411	A2	ATTNILEHV	139
TRP-2:288-296	A2	SLDDYNHLV	143
TRP-2:455-463	A2	YAIDLPVSV	125
tyrosinase:243-251	A1	KCDICTDEY	144
tyrosinase:146-156	A1	SSDYVIPIGTY	130
tyrosinase:1-9	A2	MLLAVLYCL	30
tyrosinase:369-377	A2	YMDGTMSQV	30
tyrosinase:8-17	A2	CLLWSFQTSA	33
tyrosinase:206-214	A24	AFLPWHRLF	145
tyrosinase:312-320	B35	LPSSADVEF	146
tyrosinase:192-200	B44	SEIWRDIDF	147

results presented in a second report indicated that T cells generated with this peptide failed to recognize untreated tumor cells (41).

The C/T antigen NY-ESO-1 was initially identified when serum from a patient with esophageal squamous cell carcinoma was used to carry out SEREX analysis (15). Subsequent studies have resulted in the identification of HLA class I restricted epitopes of NY-ESO-1 that are recognized in the context of HLA-A2 (42) and HLA-A31 (43). Antibodies directed against tumor antigens recognized by T cells have generally only been detected in a very low percentage of cancer patients; however, over 50% of patients bearing NY-ESO expressing tumors possess significant titers of anti-NY-ESO-1 antibodies (44). High titers of anti-NY-ESO-1 antibodies are correlated with the presence of bulky disease or multiple tumor metastases, and tumor regression in these patients was associated with a reduction of anti-NY-ESO-1 antibody titers, indicating that the presence of a substantial tumor mass is involved with maintaining anti-NY-ESO-1 antibody responses.

A relatively small number of antigens have been identified that are encoded by transcripts whose expression appears to be strictly limited to tumor cells. A T cell epitope was derived from a GnT-V gene transcript that is expressed only in tumor cells and that was initiated from the intron of a normal gene (45). A partially spliced transcript derived from the TRP-2 gene that appears to be expressed in melanoma cells but not in normal melanocytes also encodes an epitope recognized by tumor specific T cells (46).

5. ANTIGENS OVER-EXPRESSED IN TUMORS

Tumor reactive T cells have been shown in some cases to recognize proteins that are over-expressed in tumor cells relative to normal cells (Table 3). Several of the antigens in this category were initially identified by stimulating PBMC with candidate epitopes, although contradictory results have been obtained when investigators have evaluated the ability of T cells generated with certain peptides to recognize un-manipulated tumor cells. Initial studies indicated that candidate epitopes from the HER-2/neu protein, which is over-expressed in breast and ovarian carcinomas, resulted in the generation of tumor reactive T cells (47-49). In a subsequent clinical study, however, HLA-A2 positive patients with breast, ovarian, or colorectal cancer were immunized with the HER-2/neu:369-377 peptide in an attempt to raise the precursor frequency of T cells reactive with this antigen and hopefully also the frequency of tumor reactive T cells (50). Highly avid peptide reactive T cells could be readily generated from immunized patients;

however, the peptide reactive T cells failed to recognize either tumor cells expressing natural levels of HER-2/neu or HLA-A2 positive target cells that were transfected with a construct encoding HER-2/neu.

Table 3. HLA class I restricted antigens over-expressed in tumor cells.

GENE	HLA ALLELE/EPITOPE		REFERENCE
AIM-2:AORF	A1	RSDSGQQARY	90
AFP:542-550	A2	GVALQTMKQ	148
BING-4:AORF	A2	CQWGRLWQL	149
CPSF:250-258	A2	KVHPVIWSL	150
CPSF:1360-1369	A2	LMLQNALTTM	150
FGF-5:172-176/217-220 A3	A3	NTYASPRFK	54
G250:24-262	A2	HLSTAFARV	151
HER-2/neu:369-377	A2	KIFGSLAFL	47
HER-2/neu:5-13	A2	ALCRWGLLL	49
HER-2/neu:435-443	A2	ILHNGAYSL	49
HER-2/neu:952-961	A2	YMIMVKCMI	48
HER2/neu:665-673	A2	VVLGVVGFI	48
HER2/neu:689-697	A2	RLLQETELV	48
HER2/neu:654-662	A2	IISAVVGIL	152
HER-2/neu:754-762	A3	VLRENTSPK	127
iCE:AORF	B7	SPRWWPTCL	153
M-CSF:AORF	B35	LPAVVGLSPGEQEY	154
MUC-1:950-958	A2	STAPPAHGV	155
MUC-1:12-20	A2	LLLLTVLTV	155
p53:264-272	A2	LLGRNSFEV	156
p53:5-73	A2	RMPEAAPPV	157
p53:125-134	A24	TYSPALNKMF	158
p53:99-107	B46	SQKTYQGSY	159
PRAME:100-108	A2	VLDGLDVLL	92
PRAME:142-151	A2	SLYSFPEPEA	92
PRAME:300-309	A2	ALYVDSLFFL	92
PRAME:425-433	A2	SLLQHLIGL	92
PRAME:301-309	A24	LYVDSLFL	
PSMA:178-186	A24	NYARTEDFF	160
RU2AS:antisense	B7	LPRWPPPQL	161
survivin:95-104	A2	ELTLGEFLKL	162
hTERT:540-548	A2	ILAKFLHWL	91
hTERT:865-873	A2	RLVDDFLLV	163

Candidate epitopes identified from proteins that are over-expressed in many tumor cell types have also been utilized in attempts to generate widely applicable tumor antigen vaccines. Initial findings indicated that tumor reactive T cells could be generated following *in vitro* sensitization of PBMC with the telomerase:540-548 peptide (51). Results presented in an additional study, however, indicated that T cells generated using this peptide failed to

recognize target cells expressing the full length telomerase protein (52). Furthermore, results presented in this report indicated that this epitope could not be generated following incubation of purified proteasomes with longer precursors that encompassed this peptide, in contrast to the majority of naturally processed T cell epitopes (53).

The FGF-5 (5), iCE (54), and PRAME (55) antigens were cloned using PBMC that were sensitized *in vitro* with tumor cell lines, and thus contain epitopes that are naturally processed and presented on the cell surface. While expression of the FGF-5 and iCE gene products is not strictly limited to tumor cells, the level of expression observed in normal cells appears to be insufficient to allow T cell recognition of these antigens. The antigen PRAME antigen was expressed in a variety of tumor types but was also expressed in a variety of normal adult tissues that included testis, endometrium, ovary and adrenal tissues (55). The PRAME reactive T cells expressed high levels of the NK inhibitory receptor p58.2 that recognizes the HLA-Cw7 class I molecule, which was expressed on normal cells but not tumor cells from this patient. Thus, expression of p58.2 appeared to be at least partially responsible for inhibiting the response of PRAME reactive T cells to normal cells.

6. TUMOR ANTIGENS DERIVED FROM MUTATED GENE PRODUCTS

Tumor reactive T cells have been shown to recognize a variety of mutated gene products (Table 4). In general an individual mutation represents a relatively rare event that occurs in only one or a very small percentage of tumors, making identification of the HLA restriction element utilized for T cell recognition more difficult. Several mutations that are expressed in more than one tumor cell, however, have been identified. Expression screening carried out using a T cell clone that recognized a head and neck carcinoma resulted in the isolation of a caspase-8 (CASP-8) gene product containing a point mutation in the stop codon that resulted in the generation of an extension of the normal open reading frame (ORF) (56). The CASP-8 T cell epitope was encoded by sequences that spanned mutation, and amino acids 5 to 9 of the peptide FPSDSWCYF were encoded within the extended ORF. The screening of 150 additional tumor samples failed to result in the identification of additional tumor that expressed this mutation, indicating that this represented a relatively rare event. A mutated CDK4 gene product was cloned using tumor reactive CD8+ T cells, and 2 out of the 28 tumor cell lines that were analyzed expressed a product with the same mutation (57). Screening of an autologous tumor cDNA library

with an HLA-A24 restricted T cell clone resulted in the identification of a mutated β-catenin gene product (58). The analysis of β-catenin gene products expressed by multiple melanoma cell lines indicated that the identical mutation was observed in 4 of the 46 samples that were analyzed (59) and (Robbins, P., unpublished data). Use of an HLA-A11 restricted T cell to screen an autologous cDNA library resulted in the isolation of a transcript of the CKDN2A tumor suppressor gene locus containing a single nucleotide deletion, and the T cell epitope was derived from the frame-shifted region of this transcript (60). Two out of 18 additional melanomas that were screened expressed frame-shifted transcripts resulting from nucleotide deletions that encoded the T cell epitope.

Table 4. HLA class I restricted mutated antigens.

GENE	HLA ALLELE	EPITOPE	REFERENCE
α-actinin-4:118-127	A2	FIASNGVKLV	164
β-catenin:29-37	A24	SYLDSFIHF	58
BCR-ABL:926-934	A2	SSKALQRPV	66
BCR-ABL:922-930	B8	GFKQSSKAL	66
Caspase-8:476-484	B35	FPSDSWCYF	56
CDK-4:23-32	A2	ACDPHSGHFV	57
ELF2M:581-589	A68	ETVSEQSNV	12
ETV6-AML1:334-342	A2	RIAECILGM	165
HSP70-2M:286-295	A2	SLFEGIDIYT	166
	HLA-A*0201[b]		167
	HLA-A*1101[b]		60
KIAA0205:262-270	B44	AEPINIQTV	168
K-ras:7-15	B35	VVVGAVGVG	63
MUM-1:30-38	B44	EEKLIVVLF	2
MUM-2:123-133	B44	SELFRSGLDY	169
MUM-2:126-134	Cw6	FRSGLDSYV	169
MUM-3:322-330	A68	EAFIQPITR	170
N-ras:55-64	A1	ILDTAGREEY	64
Myosin-m:911-919	A3	KINKNPKYK	171
OS-9:438-466	B44	KELEGILLL	172
p14ARF:AORF[c]	A11	AVCPWTWLR	60
p16INK4a:AORF[c]	A11	AVCPWTWLR	60
MART-2:341-351	A1	FLEGNEVGKTY	173

a. The residue or residues that arise as a result of mutations are underlined.

b. Tumor specific T cells directly recognize the mutated class I HLA molecules.

c. Nucleotide deletions in the CDKN2a locus resulting in translation of the +1 and +2 open reading frames of the p14:ARF and p16INK4a gene products, respectively, result in generation of the T cell epitope.