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From Naturally Occurring Tumor Immunity to Supernatural T Cells: Isolation and Characterization of a Murine T Cell Receptor Specific for Human Breast and Ovarian Tumor Antigen Cdr2

Bianca D. Santomaso

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Isolation and Characterization of a Murine T Cell Receptor Specific
for Human Breast and Ovarian Tumor Antigen Cdr2

A thesis presented to the faculty of
The Rockefeller University
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

by

Bianca D. Santomaso

June 2007

From Naturally Occurring Tumor Immunity to Supernatural T cells:
Isolation and Characterization of a Murine T Cell Receptor Specific for Human
Breast and Ovarian Tumor Antigen Cdr2

Bianca D. Santomaso
The Rockefeller University 2007

Patients with paraneoplastic cerebellar degeneration (PCD), a form of neuronal autoimmunity, have a co-occurring natural immune response against a protein called cdr2 in their breast and ovarian carcinomas, and thus provide an innovative starting point for understanding how to harness the immune system to fight cancer. We previously demonstrated cdr2-specific cytotoxic T lymphocytes (CTL) in the peripheral blood of HLA-A2.1+ PCD patients, suggesting that CTLs mediate tumor immunity in these patients. Cdr2 is expressed by a large proportion of breast and ovarian tumors from individuals who do not develop neurological disease, suggesting that immune responses to this antigen may develop independently of autoimmune responses. Here we explore establishing cdr2 as a target for breast and ovarian cancer immunotherapy by identifying naturally processed A2.1-restricted epitopes of cdr2. Immunization of A2.1 transgenic mice with recombinant adenovirus encoding human full length cdr2 led to the identification of two naturally processed A2.1-restricted human cdr2 peptides: cdr2(289-297) and cdr2(290-298). Mouse-derived A2.1-restricted cdr2(289-297)-specific CTLs were able to target cells expressing endogenous human cdr2, but also cross-reacted with endogenous mouse cdr2, resulting in partial tolerance to this epitope. In contrast,

mouse-derived A2.1-restricted cdr2(290-298)-specific CTL were capable of recognizing tumor cells expressing endogenous human cdr2, but were unable to recognize mouse cdr2 due to nonhomology of the human and mouse cdr2(290-298) epitopes. cdr2(290-298)-specific CTL clones were isolated, and their TCR gene cloned. Transfer of the mouse-derived TCR into human CD8⁺ T cells turned them into efficient cdr2-specific CTLs. We have detected CD8⁺ T cells specific for both cdr2(289-297) and cdr2(290-298) in peripheral blood from A2.1⁺ PCD patients by tetramer staining. This correlates the presence of T cells specific to these epitopes with PCD and effective anti-gynecologic tumor immunity, and suggests that these are bona fide tumor-associated CTL epitopes. We conclude that gene transfer of TCR specific for cdr2(290-298) could provide the basis for potent breast and ovarian cancer immunotherapies, while cdr2(289-297)-specific T cells, able to target both mouse and human cdr2, offer a platform for generating a humanized animal model to investigate whether cdr2-TCR gene transfer is possible without inducing neuronal autoimmunity.

*For Adam, my lifetime collaborator,
& Owen Eugene, our greatest experiment*

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CHAPTER I - INTRODUCTION

Summary

Patients with paraneoplastic cerebellar degeneration (PCD), a form of neuronal autoimmunity, have a co-occurring natural immune response against a protein called cdr2 in their breast and ovarian carcinomas, and thus provide an innovative starting point for understanding how to harness the immune system to fight cancer. We previously demonstrated cdr2-specific cytotoxic T lymphocytes (CTL) in the peripheral blood of HLA-A2.1+ PCD patients, suggesting that CTL mediate tumor immunity in these patients. Cdr2 is expressed by a large proportion of breast and ovarian tumors from individuals who do not develop neurological disease, suggesting that immune responses to this antigen may develop independently of autoimmune responses. Here we explore establishing cdr2 as a target for breast and ovarian cancer immunotherapy by identifying naturally processed A2.1-restricted epitopes of cdr2. In the following chapters, we will discuss the process of identifying these epitopes and then discuss our isolation of a molecular reagent for generating T cells specific to them. Finally, we discuss the implications of this research for generating human immunotherapies and a humanized animal model to test their safety. Before doing that, the rest of this chapter will introduce tumor associated antigens and adoptive cell transfer (ACT) therapy, a promising strategy for generating immunity to them in the face of a state of functional tolerance in most tumor bearing hosts. This will set the stage for an introduction to the paraneoplastic neurologic degenerations (PNDs) in general and PCD in specific,

which we will view not from the perspective of the clinically-evident neuronal autoimmunity, but rather from the invisible tumor immunity that we aim to harness in the service of developing gynecologic immunotherapies.

Challenges Posed by The Immunosurveillance Hypothesis

In the five decades since Thomas and Burnet postulated that “small accumulations of tumor cells may...provoke an effective immunological reaction with regression of the tumor”^{1,2}, suppression of tumor cells by the human immune system has become a widely accepted, if not widely observed, phenomenon^{3,4}. Tumor rejection antigens meeting the important criteria of expression in a large proportion of tumors and ability to be recognized by CD8+ cytotoxic T lymphocytes (CTL) have been identified, and induction of CTL specific for these tumor antigens, through either vaccination or adoptive T cell transfer protocols, constitute a major strategy for treating cancer patients⁵. However, few of these antigens are associated with naturally robust immune responses and spontaneous regressions. Furthermore, efforts to target tumor associated antigens (TAA) with antigen specific CTL have yielded limited clinical responses^{6,7}.

Tumor Associated Antigens in Mice

Evidence for immune control of tumors initially came from studies in mice. The observation that immunodeficiency is associated with increased tumor incidence^{8,9}, and the classic studies demonstrating immune rejection of

chemically- and irradiation-induced syngeneic tumors^{10,11}, led to the understanding that CD8+ CTL play a particularly crucial role in antitumor immunity. The antigens targeted by CTL in the murine mutagenized tumor models were newly induced “altered self” antigen epitopes that were unique to the tumor and thus robustly recognized by the immune system^{10,12}.

Tumor Associated Antigens in Humans

Unlike the tumor rejection antigens targeted in immunogenic mouse tumors, most human tumor associated antigens are self antigens. Thus, they are expressed by normal tissues in addition to tumor tissues. TAA are traditionally classified into five groups based on their origin, structure, and tissue expression. Several of the earliest identified TAA were melanoma-melanocyte differentiation antigens¹³⁻¹⁵. These antigens, which include MART-1/MelanA, gp100, and tyrosinase, are expressed exclusively by cells of the melanocyte lineage. They are considered to be shared antigens because they are expressed by the vast majority of melanomas tested^{13,14,16,15,17}. A second group of antigens called cancer/testis antigens, which include the melanoma-associated antigen-1 (MAGE-1)¹⁸ and NY-ESO-1¹⁹, are expressed by normal testis and a variety of human tumors including melanoma, breast, colon, lung, bladder, ovarian, neuroblastoma, head and neck, and prostate cancers^{3,20,21}. Rather than being expressed by all tumors of a particular type, these antigens are seen in only a fraction of any tumor type^{18,19,22-24}. A third category includes antigens derived from viral proteins that are found on tumors that are induced by viral infection of human cells^{3,20}. This category

includes antigens such as EBNA-3 on Epstein Barr virus-induced lymphomas and the E6 and E7 proteins on human papilloma virus-induced cervical cancers²⁵⁻²⁷. The fourth group of antigens is created by mutations and include β -catenin and CDK4-kinase mutations. Finally, there are antigens that are ubiquitously expressed normal tissue at low level and overexpressed in tumors. These include proteins such as p53²⁸, telomerase²⁹, and survivin³⁰ which have been implicated in tumor growth or progression and are thought to be good antigens to target because they are likely to be indispensable to the tumor cell.

CD8+ CTLs recognize these tumor associated antigens as short peptides, generally 8 to 10 amino acids long, presented on the surface of MHC class I molecules³¹. These peptides, often referred to as CTL epitopes, are generated in the cytosol of cells after proteolytic processing of endogenous antigens by the proteasome, or in some cases, are generated from exogenous tumor fragments collected by dendritic cells (DCs) in a process called cross-presentation³². While most of the TAA mentioned above were identified by spontaneous CTL or antibody responses generated toward these antigens in tumor bearing hosts, few of these antigens are associated with clinically effective immune responses or documented tumor regressions⁴.

Paraneoplastic neurologic degenerations (PNDs)

Paraneoplastic neurologic disorders (PNDs) are remote effects of systemic malignancies that are not attributable to metastasis^{33,34}. Although they are rare

conditions, estimated to affect only 0.1-1.0% of cancer patients³³, they are probably the best known examples of naturally occurring tumor immunity in humans^{35,36} and as such provide an innovative (albeit often overlooked) source of bone fide tumor rejection antigens. In PND patients, their tumors, most commonly breast, ovarian, or lung tumors are thought to trigger an immune response that successfully suppresses the cancer³⁴. Tumor suppression in PND is significant. Patients with PND-associated tumors have limited disease and an improved prognosis relative to patients with histologically identical tumors unassociated with PND³⁷. Among patients with a PND associated with gynecologic tumors called paraneoplastic cerebellar degeneration or PCD, two-thirds present neurologic symptoms before the diagnosis of cancer and nearly 90% have limited oncologic disease when diagnosed. In contrast, only 50-60% of unselected breast cancer patients and 25% of ovarian cancer patients present with limited stage disease³⁸. Similar findings of limited stage disease characterize patients with the Hu syndrome and small cell lung cancer and there are several reports of spontaneous regressions of tumors associated with paraneoplastic disease^{39,40}. In addition, low titer PND antibodies and tumor immunity have been found in patients with small cell lung cancer (SCLCa) who do not have PND⁴¹. The tumor immunity in PND patients is often so effective that it would go unnoticed were it not for a second event--neuronal autoimmunity--that co-occurs in these patients and brings them to clinical attention.

As alluded to above, the immune response provides the link between the tumor regression and the neuronal degeneration. This critical insight was first offered by Jerome Posner, who demonstrated the presence of high titer antibodies in the serum and cerebral spinal fluid (CSF) of patients with PND^{42,43}. This discovery led to the realization that neuron-specific antigens were being aberrantly expressed by a systemic tumor, which presumably initiated the immune activation that resulted in the tumor immunity and subsequent neuronal autoimmunity. These antibodies were used to clone cDNAs for several target PND tumor-brain (or so-called “onconeural”³⁴) antigens including the cdr2, the antigen associated with PCD, which is the focus of this thesis.

While high titer circulating antibodies remain a diagnostic criteria, and serve to establish a link between the tumor immunity and the autoimmune neuronal degeneration in PNDs, the role of antibodies in the pathogenesis of these disorders remains elusive. Specifically, passive transfer of patient sera into mice fails to induce neuropathology even when it is delivered by intrathecal injection⁴⁴. Mice immunized with PND antigens develop high titers of anti-cdr2 antibody with no detectable neurologic abnormalities⁴⁵. In humans, treatments that reduce the titer of PND antibodies, such as plasmapheresis or intravenous immune globulin, have not been shown to affect the clinical course of disease⁴⁶ suggesting that antibodies may not be sufficient to cause disease. Moreover, PND antigens are intracellular proteins, suggesting that antibodies may not be sufficient to cause the disease.

Paraneoplastic Cerebellar Degeneration

Paraneoplastic Cerebellar Degeneration (PCD), one of the best characterized of the paraneoplastic neurologic disorders, is a disorder that most often complicates breast and ovarian tumors. As is the case for most of the PNDs, the neurologic symptoms, in this case pancerebellar symptoms, often precede the detection of an underlying gynecologic malignancy. Since other PNDs can have a similar clinical presentation of cerebellar symptoms, the diagnosis of PCD is usually confirmed by the presence of a high titer polyclonal antibody, termed “Yo” by Posner and Furneaux⁴⁷, found both in the serum and the CSF. As is the case with all of the PNDs, the antibody selectively reacts with both the regions of the nervous system affected by the disorder and the tumor tissue. PCD antiserum recognizes (and was used to clone) three immunoreactive antigens, cdr1-3^{48,49,50}, but PCD tumors express only the cdr2 antigen⁵¹.

Cdr2 tumor antigen-specific cytotoxic T cells in patients with PCD

Recent studies from our lab involving patients with paraneoplastic cerebellar degeneration (PCD) have presented the first evidence that humans with naturally occurring tumor immunity harbor tumor antigen-specific CTLs. The target antigen in PCD is cdr2, a Purkinje cell-specific protein aberrantly expressed by breast and ovarian tumor cells^{51,52}. While the presence of a cdr2-specific antibody is a feature of PCD, Albert and co-workers demonstrated the presence of circulating cdr2-specific CTL in the blood of these patients⁴⁶. Specifically, CTL restricted to peptides derived from the cdr2 antigen were found

to be present in 5/5 HLA-A2.1 + PCD patients. Moreover, a cytotoxic T cell response was detected directly from the blood (without prior *in vitro* restimulation) of patients in the acute phase of disease. This study provided the first suggestion that CTLs are critical mediators of the natural tumor immunity in these patients.

Along with this critical observation, Albert and co-workers answered an important unanswered question in the field of immunology: Despite evidence of these activated tumor-specific CTLs, no physiologic mechanism had been defined to account for such a phenomenon. Specifically, it was unknown how the tissue restricted PND antigens, which are all intracellular proteins, might be handled by professional antigen presenting cells (APCs) for the generation of MHC I/peptide complexes, a pre-requisite for their detection by CD8+ T cells of the immune system. Instances in which the PND immune responses of individual patients correlated with chemotherapy, and consideration of the work of Antony Rosen and Michael Bevan, which demonstrated that autoimmune lupus antigens are packaged into apoptotic bodies of irradiated keratinocytes⁵³ and the phenomenon of “cross-priming”⁵⁴, respectively, led Darnell³⁴ and Albert to consider the possibility that apoptotic material from dying tumor cells might provide a means for the transfer of antigen to antigen presenting cells (APCs). This hypothesis was documented experimentally⁴⁶. It was demonstrated that dendritic cells (DCs) cross-presenting cdr2-expressing apoptotic HeLa cells, which endogenously express cdr2, are capable of activating cdr2-specific T cells

in patients with PCD but not normal controls. This work helped to define an exogenous (or 'indirect') pathway for the generation of MHC I/peptide complexes and the activation of killer T cells directed towards tumor antigens, which led our lab to propose the following model for the initiation of tumor-specific immunity through an apoptosis-dependent exogenous pathway: 1) Immature DCs in the periphery phagocytose apoptotic tumor cells, 2) The DC processes antigen derived from the engulfed tumor cell for the generation of MHC I and MHC II/peptide complexes and migrates to the draining lymph organ, 3) The DC engages tumor-specific CD8+ and CD4+ T cells for the induction of potent anti-tumor immunity.

A question raised by these findings is why PCD patients are able to reject their tumors while others succumb to breast and ovarian cancer. A surprising discovery is that the PND antigens are expressed in the tumors from the general population of cancer patients. Up to 60% of ovarian tumors and 25% of breast tumors present in neurologically normal cancer patients express the cdr2 antigen⁵⁵. Therefore, while tumor antigen expression is necessary, it is clearly not a sufficient trigger for tumor immunity. Tumors actually employ a variety of counter strategies to evade the immune system, including the secretion of immunosuppressive cytokines such as IL-10 and transforming growth factor β (TGF β)^{56,57}, the generation of regulatory T cells (Tregs), down regulation of MHC molecules, sequestration or destruction of APCs, and tolerization of antigen specific T cells resulting from cross presentation of tumor antigens by APCs in

the absence of CD4 help⁵⁸. We are particularly interested in this last example of immune evasion as it may represent a subversion of the antigen cross-presentation pathway active in PND patients. It is possible that different classes of immune responses are present in cancer patients harboring cdr2-expressing tumors—ranging from those without tumor immune responses who may have active tolerization (by inactivation or deletion) of cdr2-specific T cells, to those who have tumor immunity and demonstrate productive cross-priming of CD4+ and CD8+ responses (a clinically invisible subset of patients), to those who have tumor immunity and PND, having somehow broken immune tolerance to neuronal antigens expressed in the brain. Understanding what factors account for the dramatic difference in the tumor immune response of the PCD patient versus the neurologically normal cancer patient will likely provide insights that will be broadly applicable to the generation of effective immunity to other tumor antigens.

Adoptive T cell transfer (ACT) Therapy

Generating immunity to TAA

A major hurdle for tumor immunotherapy, other than the identification of tumor rejection antigens to target, is the generation of tumor antigen-specific T cells in tumor bearing hosts. These T cells need to be functional, exhibit high avidity^{59,60}, and their numbers must reach levels above the threshold required to mediate regression of established tumors^{61,62}. Generation of memory cells to prevent tumor recurrence is also desirable. However, because most TAA are nonmutated

self antigens, the TAA-specific T cell repertoire is generally of small size and of low avidity. As a matter of self preservation, central or peripheral tolerance deletes most autoreactive high affinity T cells during development or renders them anergic^{63,64}. Antigen expression by tumor tissue may also have a direct negative impact on T cell function⁶⁵. The results of these mechanisms have been seen in both preclinical studies and clinical trials where a lack of T cells with the required reactivity is a major factor limiting T-cell-based immunotherapy. For example, murine studies have demonstrated that tumor specific T cell responses can be readily induced by vaccination when targeting a foreign tumor associated antigen. However, when the same tumor-associated antigen is considered “self” by the available T cell repertoire, the reactivity to these antigens is highly reduced⁶⁶. In human cancer patients, circulating CD8+ self/tumor antigen-specific T cells can be sometimes be found, as in a study looking at patients with metastatic melanoma⁶⁷, but these cells are usually selectively unresponsive to tumor. Similarly, attempts at the immunotherapy of patients with metastatic melanoma by direct immunization with TAA have elicited both humoral and cellular responses but tumor regression has only rarely been observed⁶.

Ex vivo manipulation and expansion of tumor antigen-specific T cells represents one strategy to overcome tolerizing mechanisms and the effects of an immunosuppressive tumor microenvironment to augment antitumor T cell responses^{68,69}. In a transgenic mouse model where a tumor associated viral antigen is expressed in normal tissues during development, the T cell response to

the target antigen is anergic and the tumor cells are not rejected; however, T cell function can be recovered after appropriate ex vivo manipulation⁷⁰. Similarly, B16 melanoma cells, which express dominant T cell antigens which are shared by normal melanocytes, are not rejected unless one of several methods for breaking immune tolerance to a self-protein is used, such as ex vivo manipulation of T cells and co-administration with high-dose IL-2 and antigen specific vaccination⁷¹.

Proof of Principle for Adoptive T cell transfer (ACT) therapy in humans

Along these lines, replacement of an ineffective endogenous T cell compartment through the combination of allogeneic stem cell transplantation (SCT) and donor lymphocyte infusion (DLI) is an effective treatment for patients with hematologic malignancies such as chronic myelogenous leukemia. It is capable of inducing durable complete remissions in 60-75% of patients⁷²⁻⁷⁴. Notably, the antileukemic effect of this therapy is dependent on the recognition of minor histocompatibility antigens of the recipient as non-self by the infused donor lymphocytes, and the development of T cell responses against these antigens is predictive of remission⁷⁵. One drawback of this therapy is that the introduced nonself-reactive lymphocytes are not tissue-specific in their reactivity against minor histocompatibility antigens; therefore they can target non-malignant cell types and cause graft versus host disease (GvHD). Allo-SCT and DLI may also fail due to tumor cells not expressing the immunodominant minor histocompatibility antigens. Nevertheless, it and a similar approach involving the use of Epstein-Barr virus (EBV)-reactive T cells to treat patients with post-transplant

lymphoproliferative diseases^{76,77}, have established proof of principle for adoptive T cell therapy of human malignancies.

ACT for the treatment of solid malignancies

The adoptive transfer of EBV antigen-specific T cells to treat EBV-induced malignancies has yielded significant objective responses in patients and has demonstrated the feasibility of targeting tumor associated antigens by adoptive immunotherapy. However, most solid tumors pose more formidable immunologic as well as physical barriers than do EBV-associated tumors since T cells specific for non-viral self antigens tend to be less frequent and of lower avidity. Still, adoptive cell transfer (ACT) of autologous tumor-reactive T cells into the solid tumor-bearing host appears to hold some promise. In the case of melanoma, ACT is capable of mediating some tumor regression^{71,78,79}. These effects are even more pronounced in the absence of host lymphocytes^{68,80}. The promise of this approach was highlighted by the recent demonstration that about 50% of standard-treatment-refractory metastatic melanoma patients experienced tumor regression when they were reconstituted with activated autologous tumor infiltrating lymphocytes after chemotherapy-induced lymphodepletion^{68,81}.

One large hurdle for ACT is its reliance on the successful isolation and expansion tumor reactive lymphocytes (usually tumor infiltrating lymphocytes or TIL^{82 83}) pre-existing in the patient. Even for melanoma, where ACT has seen the greatest success, there are many patients for whom T cells of the appropriate anti-tumor

specificity and avidity cannot be isolated, even after immunization⁸⁴. The hurdles are even greater for tumors such as breast and ovarian carcinoma where reactive TIL can be more difficult to isolate.

Generating tumor reactive T cell populations by TCR gene transfer

Regardless of whether the absence of therapeutically useful autologous T cells represents a failure of the tumor to stimulate the generation of a tumor-specific T cell response (ignorance) or the active suppression of an immune response (tolerance or deletion), shortcomings in the T cell repertoire can be overcome by combining adoptive T cell transfer with the genetic modification of T cells with high avidity TCR genes specific for tumor associated antigens^{85,86}. Both *in vitro*⁸⁷⁻⁸⁹ and *in vivo*⁹⁰⁻⁹², TCR gene transfer has been demonstrated to be sufficient to redirect T cell specificity to the antigen of choice. In a recent clinical application of this approach, HLA-A2.1-restricted TCR specific for the melanocyte antigen MART-1 was isolated from a patient who experienced tumor remission following adoptive T cell therapy for melanoma^{68,93} and was used to treat HLA-A2.1-positive patients who share this disease⁷. While only 2 out of the 15 patients in this first clinical study of TCR gene transfer demonstrated a clinical response, this strategy still holds therapeutic promise. Potentially therapeutic TCRs can be isolated from the allogeneic T cell repertoire^{94,95} or from HLA-A2.1 transgenic mice^{96,97}, when the autologous repertoire is tolerant⁹⁸.

Specific Aims

There are two big hurdles in tumor immunology. One is to find tumor-associated antigens to target. A corollary to this is identifying naturally processed MHC class I and class II-restricted epitopes for CD8+ CTL and CD4 Th cell recognition, respectively. The second hurdle relates to the fact that most TAA are self antigens and thus, for tumor antigen-targeted immunotherapy to be effective, there is often a need to overcome T cell tolerance resulting from either deletion or anergy. In the remaining chapters, we will discuss our identification of two new tumor associated antigen epitopes for breast and ovarian carcinoma antigen cdr2 and our findings on the ability of TCR gene transfer to generate class I-restricted cdr2-specific CTL. Finally, we will place these findings in a larger context and discuss how our findings may lead to new immunotherapies as well as a humanized animal model to test their safety.

CHAPTER II. MATERIALS AND METHODS

HLA-A2.1 binding peptide prediction

Human cdr2 protein sequence was submitted to analysis by computerized HLA-binding prediction based on the freely available online databases, SYFPEITHI (<http://syfpeithi.bmi-heidelberg.com>)⁹⁹, and the BIMAS “HLA-peptide binding prediction” site:

(http://bimas.dcrt.nih.gov/molbio/hla_bind)¹⁰⁰. Both programs provide peptide sequences that are likely to be presented by the selected HLA molecules along with a ranking or score. SYFPEITHI provides scores based on the presence of MHC class I anchor motifs. BIMAS supplies a ranking according to the estimated half-life of the peptide/MHC complex. The top 2% of peptides from each analysis were chosen for further analysis.

iTopia™ peptide screen

The peptide library for iTopia™ screening was purchased from Jerini Peptide Technologies (Berlin, Germany). All screening assays were performed according to the manufacturer’s instructions using the provided reagents (Beckman Coulter Immunomics, San Jose, CA). Data were analyzed with the iTopia™ software using Prism (GraphPad, San Diego, CA).

Peptides

All other synthetic peptides were ordered from Invitrogen (Carlsbad, CA) or American Peptide Company (Sunnyvale, CA) and were determined to be greater

than 90% pure. The sequences of the peptides used in this study are as follows: human cdr2 (289-297) SLLEEMFLT, murine cdr2 (289-297) SLLEEMFLA, human cdr2 (290-298) LLEEMFLTV, human cdr2 (289-298) SLLEEMFLTV, human cdr2 (273-281) KLVPSLYV, and FluM1 (58-66) GILGFVFTL¹⁰¹.

Cell lines and transfectants

EL4-A2/K^b (EA2K^b) (provided by Dr. Alan Houghton, Sloan-Kettering Institute, New York, NY) is a transfectant of the murine thymoma EL4 (H-2^b haplotype) that expresses A2/K^b. T2 is a lymphoblastoid cell line deficient in TAP function, whose HLA proteins can easily be loaded with exogenous peptides¹⁰². Other human cell lines used were the Adenovirus (Ad)-5 transformed cell line HEK293 (CRC 1573; ATCC; Rockville, MD), the cervical carcinoma cell line HeLa (CCL-2; ATTC), the breast carcinoma cell line SKBR2 (HTB-30; ATTC), the breast carcinoma cell line MCF7, and the ovarian carcinoma cell line COV413 (both provided by Dr. Victor Engelhard, University of Virginia, Charlottesville, VA). Other murine cell lines used were the melanoma line B16-F10 (provided by Dr. Alan Houghton, Sloan-Kettering Institute, New York, NY), and B16.AAD, an AAD stable transfectant of B16-F1 (provided by Dr. Victor Engelhard). The AAD plasmid (provided by Dr. Victor Engelhard) is a hybrid MHC class I molecule that contains the α 1 and α 2 domains from HLA-A2.1 and the α 3 domain of the H-2D^d molecule, and has been described previously¹⁰³. The stable AAD-expressing cell lines HeLa.AAD and MCF7.AAD were generated by transfecting HeLa and MCF7 cells, respectively, with the AAD plasmid using Fugene6

(Roche, Indianapolis, IN) followed by selection with G418 (Gibco). The HeLa.A2.1 stable transfectant was generated by transfecting HeLa cells by the same method with pA2.1 plasmid (a gift from Dr. Paul Robbins NIH/NCI, Bethesda, MD).

Culture Media

The T2 and COV413 cell lines were maintained in “R10 medium”, i.e., RPMI 1640 medium (Mediatech, Herndon, VA) supplemented with 10% (v/v) FBS (Hyclone, Logan, UT), 2mM Glutamax (Gibco), sodim pyruvate, non-essential amino acids, 15 mM Hepes buffer, 50 μ M β -mercaptoethanol, and gentimycin. All other cell lines mentioned above were maintained in “D10 medium”, i.e., DMEM high glucose (Mediatech) containing all of the same supplements as R-10 except for β -mercaptoethanol. Stable transfectants were maintained in the complete medium of the parental cell type supplemented with G418 (Gibco Life Technologies, Grand Island, NY). Cells were washed out of G418 containing media for the 48 hours prior to use in coculture assays.

Mice

The A2.1 transgenic HHD2 mice (referred to as HHD in this text) were provided by Dr. Francios Lemonnier (Pasteur Institute). They are derived from a strain deficient for mouse β 2-microglobulin and H-2D^b molecules and transgenic for a chimeric MHC class I molecule, HLA-A0201/D^b, linked to human β 2-microglobulin. AAD mice, which express the α 1 and α 2 domains from the HLA-

A2.1 molecule, and the $\alpha 3$ domain from the murine H-2D^d molecule¹⁰⁴, and AAA mice, which have fully human A2.1 molecules¹⁰⁵, were obtained from Jackson Laboratories and bred in our colony at LARC. (See Newberg et al.¹⁰⁴ for description of hybrid class I genes). AAD transgenic mice were monitored for expression of HLA-A2.1 on peripheral blood cells by flow cytometry using the BB7.2 antibody. Mice were maintained in specific pathogen-free facilities at the Rockefeller University and all protocols were approved by the Institutional Animal Care and Use Committee at the Rockefeller University.

Recombinant Adenovirus

E1/E3-deleted recombinant adenovirus encoding full-length human cdr2 (Ad-hcdr2), full-length mouse cdr2 (Ad-mcdr2), or full-length influenza matrix protein (Ad-FluM1), were constructed using the AdEasy vector system. Briefly, genes were inserted into the pShuttle-CMV vector multiple cloning site--or no transgene in the case of Ad-GFP--and the resulting plasmid was coelectroporated into BJ5183 E. coli bacteria along with the pAdEasy-1 vector to allow for recombination in bacteria as previously described by He et al.¹⁰⁶. The resulting plasmid was then used to transfect HEK 293 cells. After incubation for 7 days, the cells were frozen and thawed to release the virus; the crude viral lysate was used for further purification following expansion on HEK 293 cells. Adenovirus was purified either by CsCl₂ density centrifugation according to published procedures¹⁰⁷, or using an Adenopure kit (Puresyn) according to the manufacturer's instructions, titrated as pfu per ml and frozen at -80°C until use.

T2 binding assays

T2 cells resuspended in R-10 media were seeded in 96-well U-bottom plates at a density of 2×10^5 /ml with β 2-microglobulin plus peptide at the indicated concentrations and incubated overnight at RT. After incubation, brefeldin A was added to a final concentration of 10 μ g/ml, followed by incubation at 37 °C for 3 h. The cells were washed and stained with mouse anti-human HLA-A2 antibody (clone: BB7.2; Pharmingen), followed by goat anti-mouse PE. Duplicate samples were run on a FACScaliber flow cytometer and the data were analyzed using Flow-Jo software (Treestar). Binding activity of each peptide was calculated by a fluorescence ratio (mean fluorescence of T2 cells loaded with peptide / mean fluorescence of T2 cells without peptide).

Western blot analysis

Tissue culture cells were washed twice with PBS followed by lysis in Passive Lysis buffer (Promega) for 15 min according to the manufacturer's instructions. Membranes were pelleted by centrifugation and protein concentration was determined by Bradford assay. 10 μ g of each total cell protein was separated by 10% SDS-PAGE and transferred to PVDF membrane (Millipore, Bedford, MA). Membranes were blocked for one hour at room temperature in 10% non-fat milk, incubated with PCD patient antiserum overnight at 4°C. After washing with PBS + 0.1% Tween, membranes were incubated with rabbit anti-human-HRP (Jackson) at a dilution of 1:10000 at room temperature for 1 h.

After washing, bound protein was detected by chemiluminescence (NEN) on Biomax MR film (Kodak).

Generation of peptide-specific HLA-A2.1 restricted CTL

8-10 wk-old female mice were immunized intradermally in the flank with 10^9 pfu of recombinant adenovirus. As an adjuvant, 400 ng of pertussis toxin (Sigma) was administered intraperitoneally at 0 and 48 h. 12-18 d after immunization, spleens were removed and RBCs were lysed with ACK buffer (Biofluids). Cells were plated at 3.5×10^6 cells/well in a 24 well plate in R-10 media with 0.5 μ M free peptide. After 7 to 10 d of culture, and every 9-14 d thereafter, IFN- γ production was assessed by ELISPOT and cells were restimulated by incubating 10^5 CTL with 3.5×10^6 peptide-pulsed irradiated (3000 rad) RBC-depleted splenocyte stimulators in 24 well plates in R-10 media supplemented with 10 IU /ml IL-2 (Chiron).

Preparation of primary murine kidney epithelial cell cultures

Primary kidney cell cultures were made by mashing mouse kidneys with the back of a syringe, pipetting until a single cell suspension was obtained, and passing the suspension over a 70 μ M cell strainer. After washing, cells were cultured in D10 medium in 10cm tissue culture dishes (Falcon). Cells were fed by replacing medium on days 4 and 7. Between d7 and d9, 30 U/ml recombinant mouse IFN γ (R&D Systems) was added to the cells to increase surface MHC class I expression. 24 h later, 10^7 pfu of purified adenovirus was added to each plate.

The next day, cells were washed three times with PBS and harvested with trypsin EDTA for use in the ELISPOT assay.

Cytotoxicity Assay

Target cells were labeled with 0.1 mCi ^{51}Cr /10⁶ cells for 2 h. The target cells were washed and counted and 5 x 10³ target cells/well were mixed with CTL at several E:T ratios in a standard 4 h cytotoxicity assay using 96-well round-bottom plates. Tumor cells were pretreated with 100 U/ml IFN- γ (R&D Systems) for 48 h. Percent-specific lysis was calculated as follows: (experimental cpm - spontaneous cpm)/(maximum cpm - spontaneous cpm) x 100.

Cloning of murine cdr2-specific, HLA-A2.1-restricted TCR- α and TCR- β cDNA

Total RNA was extracted with the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions from 2 x 10⁵ CD8-purified (MACS, Miltenyi Biotech) clone 11 and clone 12 T cells. One microgram of total RNA was used to clone the TCR cDNAs by a RACE method (GeneRacer kit; Invitrogen Life Technologies, Carlsbad, CA). Before synthesizing the single strand cDNA, the RNA was dephosphorylated, decapped, and ligated with an RNA oligonucleotide according to the instruction manual of the 5'-RACE GeneRacer kit. Superscript III RT and the GeneRacer Oligo(dT) were used for reverse-transcribing the RNA oligonucleotide-ligated mRNA to single strand cDNAs. 5'-RACE was performed using the 5' Generacer primer and 3' primer of gene-

specific primer TCR-CaRev (5'-ACTGGACCACAGCCTCAGCGTCAT-3'); TCR-Cb1Rev (5'-TGAATTCTTTCTTTTGACCATAGCCAT-3'); or TCR-Cb2Rev (5'-GGAATTTTTTTTCTTGACCATGGCCAT-3') as 3' primers for murine TCR α -, β 1-, or β 2-chain, respectively. The PCR products were cloned into pCR[®]4-TOPO vector and then transformed into One Shot TOP10 competent Escherichia coli (Invitrogen Life Technologies). For each of the two CTL clones, plasmid DNAs were prepared from 16 individual clones from TCR α -chain cDNA, and 16 clones from TCR β -chain cDNA. Full-length insert of all 64 plasmids was confirmed by sequencing in both directions. Two independent 5'RACE PCR reactions were performed for each T cell clone.

Preparation of *in vitro* transcribed mRNA

mRNA encoding GFP from pEGFP-N1 (Clontech) and mRNAs encoding TCR α and β chains were prepared from PCR products made using gene specific primer pairs containing the T7 RNA polymerase promoter sequence (Figure 1). mMACHINE High Yield Capped RNA transcription Kit (Ambion Inc. Austin, TX) was utilized to generate *in vitro* transcribed (IVT) RNA according to the manufacturer's instructions. The IVT RNA was purified using an RNeasy Mini Kit (Qiagen) and purified RNA was resuspended in RNase free water at 1-3 mg/ml.

Forward: T7 Priming; Kozak consensus sequence, gene
Reverse: Poly A tail (T)₆₄; reverse complement of stop codon; gene

EGFP (from pEGFP-N1 Clontech plasmid) Genebank U55762:

Forward
5'TAATACGACTCACTATAGGGAGAGCCACCATGGTGAGCAAGG
GCGAGG-3'

Reverse
5'TTT
TTTTTTTTTTTTTTTTTTTAACTTGTACAGCTCGTCC-3'

cdr2-290 TCR α (V14D-1*01):

Forward
5'TAATACGACTCACTATAGGGAGAGCCACCATGGACAAGATTC
TGACAGC-3'

Reverse
5'TTT
TTTTTTTTTTTTTTTTTTTAACTGGACCACAGCCTCAGCGTC-3'

cdr2-290 TCR β 2 (V12-1*01)

Forward
5'TAATACGACTCACTATAGGGAGAGCCACCATGTCTAACACTG
TCCTCGC-3'

Reverse
5'TTT
TTTTTTTTTTTTTTTTTTTAAAGGAATTTTTTTTCTTGACCATGGCC-3'

Figure 1. Sequences of the gene specific primer pairs used to generate templates for cdr2 TCR α , cdr2 TCR β , and GFP for IVT RNA

Electroporation of *in vitro* transcribed RNA

PBLs were collected by leukopheresis, and lymphocytes were separated by centrifugation on a Ficoll-Hypaque (Pharmacia) cushion, washed in RPMI1640, and then cryopreserved in 10% human serum albumin/10% DMSO/ RPMI until use. For the stimulation of the PBL cells, 1 vial of 10^8 PBMC was thawed into 100 ml (for a cell concentration of 1×10^6 /ml) of complete Stemline medium (CSM), i.e. Stemline T cell Expansion Medium (Sigma; St Louis, MO) supplemented with 5% (v/v) FBS (Hyclone), Glutamax (Invitrogen), and gentamycin. PBL were stimulated with IL-2 (300 IU/ml, Chiron Corp; Emeryville, CA) plus 50 ng/ml OKT3 for 3 to 4 days. Following the stimulation, the cells were enriched for CD8+ T cells by MACS separation (Miltenyi Biotec). Populations were greater than 98% pure after this step (data not shown). The purified CD8+ T cells were subsequently cultured in CSM with IL-2 (300 IU/mL) and no OKT3 for 10-17 days before electroporation. Cells were adjusted with CSM to maintain at a concentration of 10^6 cells/ml during this expansion period. The stimulated CD8+ T cells were subjected to electroporation following resuspension in OPTI-MEM (Invitrogen) medium at a final concentration of 25×10^6 cells/ml. Cells and cuvettes were pre-chilled by putting them on ice for > 5min. For electroporation, 0.2 ml of the cells were mixed with 2 μ g/ 1×10^6 T cells of IVT RNA and electroporated in a 2 mm gap cuvette (Harvard Apparatus BTX, Holliston, MA), using an ECM830 Electro Square Wave Porator (Harvard Apparatus BTX, Holliston, MA) at 500 V/500 μ s. Immediately after electroporation, the cells were

transferred to fresh CSM without IL-2 and incubated at 37°C until use 6-18 h later.

Electroporated T cell cytokine release assays

RNA-electroporated CD4⁺ or CD8⁺ T cells were tested for specificity in cytokine release assays. For these assays, 1×10^5 responder cells and 1×10^5 stimulator cells (peptide-pulsed T2 or Ad-transduced KECs) in were incubated for 18-20 h in a 0.2 ml culture volume in individual wells of 96-well plates. Cytokine secretion was measured in culture supernatants diluted so as to be in the linear range of the assay using an IFN- γ ELISA kit (Endogen, Cambridge, MA) according to the manufacturer's recommendations.

IFN- γ ELISPOTs murine and human

Enzyme-linked immunospot (ELISPOT) assay was used to quantify antigen-specific IFN- γ -producing effector cells. Briefly, nitrocellulose-bottomed 96-well plates (MultiScreen HA, Millipore; Bedford, MA) were coated overnight at 4°C with anti-IFN- γ mAb (clone AN18 at 5 μ g/ml for mouse, clone 1-DIK at 10 μ g/ml for human; both from Mabtech, Stockholm, Sweden). Wells were washed three times with PBS and blocked for 2 h with R-10 culture medium at 37°C. For direct *ex vivo* mouse ELISPOTs, CD8⁺ T cells were isolated by positive selection from spleens using MACS purification (Miltenyi Biotec) and 2×10^5 CD8⁺ T cells were cocultured with 5×10^4 stimulator cells. For ELISPOTs with CTL lines or clones, 1×10^4 CD8⁺ T cells, purified by negative selection using a mouse CD8 isolation kit

(Miltenyi Biotec), were cocultured with 5×10^4 stimulator cells. For human ELISPOTs with RNA electroporated human PBL, 1×10^5 CD8⁺ T cells were cocultured with 5×10^4 irradiated stimulator cells. After incubation for 18 h at 37°C, plates were washed 6 times with PBS + 0.05% Tween-20. Biotinylated IFN- γ mAb (clone R4-6A2 for mouse, Pharmingen; clone 7-B6-1 for human, Mabtech), the conjugate (avidin-peroxidase complex; Vectastain avidin-biotin complex method Elite Kit; Vector Laboratories, Burlingame, CA) and AEC substrate (Sigma) were then used for spot development according to the manufacturer's instructions. All conditions were performed in duplicate or triplicate wells (as indicated). Colored spots represent IFN- γ -releasing cells are reported as spot-forming-cells per 10^6 cells. The ELISPOT plate evaluation was performed in a blinded fashion by an independent evaluation service (Zellnet Consulting; Fort Lee, NJ) using an automated ELISPOT reader (Carl Zeiss; Thornwood, NY) with KS Elispot 4.8 software.

FACs analysis

All surface antibodies were purchased from Beckton Dickinson (San Jose, CA). Cell surface expression of murine TCRV β , murine CD8, murine CD4, human CD8, and human CD107a were measured using FITC or PE-conjugated antibodies according to the manufacturer's instructions. Cell surface expression of the native A2.1 and the chimeric A2.1 molecules (A2/K^b, AAD, or HHD) on human and murine tumor cell lines was measured using FITC-conjugated A2.1 (clone BB7.2; BD Pharmingen).

Tetramer staining.

PE-labeled cdr2(290-298)/HLA-A2.1, cdr2(289-297)/HLA-A2.1, FluM1(58-66)/HLA-A2.1, PSMA(4-12)/HLA.A2.1 and negative tetramer/HLA-A2.1 were purchased from Immunomics (iTag MHC Tetramer, Beckman Coulter, Fullerton, CA, USA) and used according to the manufacturer's recommendations. Where indicated, cdr2(289-297)HLA-A2.1 and cdr2(355-363)HLA-A2.1 tetrameric PE conjugates from the NIH tetramer facility were used. Human cells were stained in a FACS buffer made of PBS, 1%(v/v) FBS (Hyclone), and 1% (v/v) PHS. Where indicated, human FACS buffer was supplemented with 0.02% NaN₃. For tetramer staining of human PBMC or electroporated CD8⁺ T cells, 1.0 × 10⁶ cells were incubated with 1:20 dilution of tetramer for 20 minutes at room temperature. Antibody to CD8 was then added for an additional 10 minutes. For tetramer staining of murine CTL, cells were used at least 7 days after restimulation. CD8⁺ T cells were purified by negative selection (MACs; Miltenyi Biotec) and incubated for 20 minutes on ice in Fc block (BD Pharmingen) in a FACS buffer made of PBS, 5% (v/v) FBS (Hyclone), 5% (v/v) normal goat serum, and 1% (v/v) PHS. For tetramer staining 2 × 10⁵ - 1.0 × 10⁶ murine cells were incubated with tetramer for 20 minutes in FACS buffer at room temperature. Where indicated, antibody to CD8 was then added to the cells during the final 10 minutes. All human and murine cell samples were washed and analyzed immediately. Cells were gated on the CD8⁺ population. Data was collected on a FACScaliber and analyzed using Flowjo software (Treestar).

CD107a assay

The cell surface mobilization of CD107a was determined as a measure of degranulation and functional reactivity. 10^5 tumor cells were placed into one well of a 24-well plate along with 10^5 TCR-electoporated or mock-electoporated CD8⁺ T cells in a total volume of 1 mL. 20 μ L of FITC-conjugated CD107a antibody and 1 μ l of GolgiStop (Beckton Dickenson, San Jose, CA) were added to the well according to the manufacturer's instructions. The coculture was incubated for 4 h at 37°C followed by washing and flow cytometry.

CHAPTER III. IDENTIFICATION OF HLA-A2.1 RESTRICTED NATURAL CTL EPITOPES OF HUMAN CDR2

Introduction

While some effective therapies have been described, many human cancers remain resistant to the classical therapeutic modalities of surgery, chemotherapy, and radiation therapy. Understanding how the immune system participates, or fails to participate, in the recognition and effective destruction of tumors may offer a novel approach to treatment. This has been an area of intense research as it has some very attractive features such as 1) the ability to generate immunity that is specific to tumor antigen, thus minimizing destruction of normal tissue (e.g. bone marrow toxicity observed in chemotherapy), 2) the ability to generate a response with prolonged clinical efficacy through the generation of immunological memory, and 3) potential applicability to the whole spectrum of human cancers. Still, there has been difficulty realizing this potential, as the potency of current immunotherapeutic strategies appears limited.

One constraint is the need to identify bone fide tumor rejection antigens and CTL epitopes against which immunotherapies can be directed. In particular, there is a great ongoing effort to identify immunogenic targets for solid tumors other than melanoma. While identification of many TAAs has relied upon the immune system of the tumor-bearing host, we have approached this endeavor from a unique angle, the study of a rare cohort of patients who have clinically evident natural immunity to their tumors.

The paraneoplastic neurologic disorders (PNDs) provide examples of naturally occurring tumor immunity and as such provide an innovative starting point for understanding how to harness the immune system in order to fight cancer. For example, patients with paraneoplastic cerebellar degeneration (PCD) develop a robust antitumor immune response directed against their breast and ovarian carcinomas^{108,109}. Tumor immunity in PCD is clinically evident. Patients with PND-associated tumors have limited disease and an improved prognosis relative to patients with histologically identical tumors unassociated with PND.

The immune system is thought to initiate PCD when *cdr2*, a protein whose expression is normally restricted to cerebellar Purkinje neurons, becomes aberrantly expressed and recognized as a foreign antigen when abnormally made in breast or ovarian tumors. Although *cdr2* was first identified using autoimmune antisera from PCD patients, cloning of the antigen revealed that it was an intracellular cytoplasmic protein, and we subsequently demonstrated *cdr2*-specific cytotoxic T lymphocytes (CTL) in the peripheral blood of 5/5 (HLA-A2.1+) PCD patients⁴⁶, suggesting that CTLs are critical components of the tumor immunity in these patients. This observation marked the first description of expanded populations of killer T cells in humans with known tumor immunity. Interestingly, *cdr2* is expressed by a large proportion of breast (25%) and ovarian (60%) tumors from individuals who do not develop neurological disease⁵⁵. Taken together with observations of immune responses made in other PNDs^{41,110}, *cdr2* expression, and perhaps immune response to it, may develop

independently of autoimmune responses. This also suggests that a large population of breast and ovarian cancer patients could benefit from cdr2-directed immunotherapy.

Specific Aims of this Chapter

To explore the possibility of establishing cdr2 as a target for immunotherapy of breast and ovarian cancer, we first set out to identify naturally processed HLA-A2.1-restricted epitopes of cdr2 by screening peptides covering the entire human cdr2 protein for HLA-A2.1 binding. To determine which of these epitopes were naturally processed, we used HLA class I transgenic mice, which have been extensively used to evaluate the immunogenicity and natural processing of candidate human tumor associated antigens (TAA)^{28,111,112}. Immunization of HLA-A2.1-transgenic mice with an adenoviral vector encoding human full length cdr2 (Ad-hcdr2) led to the identification of two naturally processed HLA-A2.1-restricted human cdr2 peptides, cdr2(289-297) and cdr2(290-298). Mouse-derived A2.1-restricted CTLs specific for cdr2(289-297) targeted cells expressing endogenous human and mouse cdr2. Because these CTL cross-react with endogenous mouse cdr2, we observed what appears to be partial tolerance to cdr2(289-297) which precluded the isolation of a high avidity cdr2(289-297)-specific CTL line.

In contrast, A2.1 transgenic mouse-derived CTL specific for cdr2(290-298) were capable of recognizing and killing a number of tumor cell lines expressing

endogenous human cdr2, but were unable to recognize mouse cdr2. Due to amino acid differences between human and mouse cdr2 in the 290-298 epitope, a high percentage of CTL isolated from immunized mice were able to recognize human cdr2(290-298) in a CD8-independent manner.

To correlate these findings with those in human PCD patients, we examined the peripheral blood of two A2.1+ PCD patients for the presence of cdr2(289-297) and cdr2(290-298)-specific CD8+ T cells. We detected cells specific for both epitopes by tetramer staining, indicating that these two newly defined CTL epitopes are bone-fide tumor antigen targets associated with the effective anti-tumor immune response seen in PCD.

Results

Prediction of potential human cdr2 peptides that bind to HLA-A2.1

To identify potential HLA-A2.1-restricted epitopes of human cdr2, we used two publicly available prediction algorithms of peptide/MHC interactions, the Bioinformatics and Molecular Analysis Section of the National Institutes of Health (BIMAS) and the University of Tübingen (SYFPEITHI). According to SYFPEITHI, the top 2% of predicted peptides should contain the naturally presented epitopes in 80% of predictions (www.syfpeithi.de). Therefore, as a preliminary screen for A2.1-restricted epitopes of cdr2, we synthesized the top 2% (10) of 446 possible human cdr2 nonamer peptides predicted by both

algorithms (Table 1), and examined their ability to stabilize the expression of HLA-A2.1 on T2 cells, a gauge of HLA-A2.1 binding ability. Of all the peptides tested, cdr2(289-297) SLLEEMFLT, a human cdr2 peptide previously found to be recognized by peripheral blood CTLs of A2.1+ PCD patients⁴⁶, was best able to stabilize A2.1 expression on T2 cells in a stabilization assay (Figure 2). Two other peptides that demonstrated efficient binding to HLA-A2.1 were the mouse homologue of cdr2(289-297) SLLEEMFLA and cdr2(290-298) LLEEMFLTV. The latter is similar to a cdr2 decapeptide (SLLEEMFLTV) that was also shown to be targeted by A2.1+ PCD patient peripheral blood CTLs (M. Albert, unpublished observation).

Predicted by SYFPEITHI ^a			Predicted by BIMAS ^b		
Position	Sequence ^c	Score	Position	Sequence ^d	Score
114	SLTETIECL	27	289	SLLEEMFLT	1508.045
128	HLQSQVEEL	26	273	<u>KLVPDSL</u> LYV	1338.876
290	LLEEMFLTV	26	260	MLQSEHPFV	650.311
37	TLLDRNTEL	25	37	<u>TLLDRNTEL</u>	324.068
65	YLTQVVELL	25	424	ALFKEIFSC	245.211
75	<u>QMNEQHAKV</u>	25	75	<u>QMNEQHAKV</u>	205.951
111	KILSLTETI	24	65	YLTQVVELL	110.747
121	CLQTNIDHL	24	114	<u>SLTETIECL</u>	98.709
273	<u>KLVPDSL</u> LYV	24	290	<u>LLEEMFLTV</u>	58.949
355	ALKVKYEEL	24	203	TMLQAQLSL	57.085

^aWebsite found at www.syfpeithi.de

^bWebsite found at bimas.dcrtnih.gov/molbio/hla_bind

^cMain anchors according to SYFPEITHI are in bold, secondary anchors are underlined

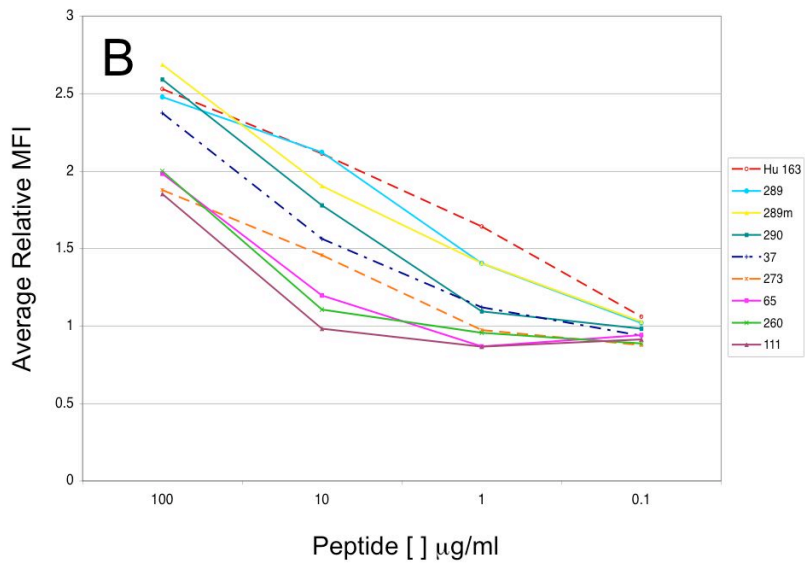
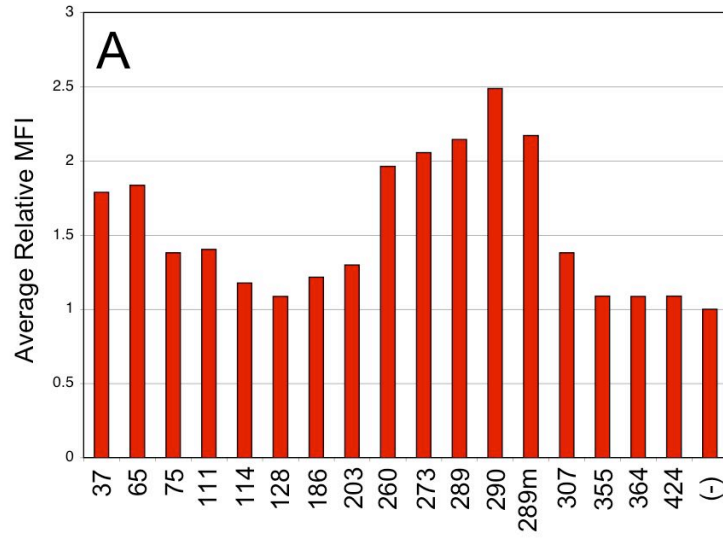
^dPeptides also identified by SYFPEITHI are underlined

Table 1. Predicted human cdr2 A2.1 binding nonamers

The entire protein sequence of human cdr2 was scanned using the epitope prediction algorithms (SYFPEITHI, <http://syfpeithi.bmi-heidelberg.com>; BIMAS, [http://bimas.dcrtnih.gov/molbio/hla bind](http://bimas.dcrtnih.gov/molbio/hla_bind)) to identify nonamer peptides predicted to bind to HLA-A2.1. Listing of the top 2% of peptides.

Figure 2. Binding of human cdr2-derived peptides to HLA-A2.1.

To assess peptide binding, 17 peptides with high scores by both algorithms were synthesized to at least 90% purity and pulsed with β 2-microglobulin onto TAP-deficient T2 cells. Stabilization of HLA-A2.1 on control unpulsed and peptide pulsed T2 cells using (A) 50 μ g peptide, or (B) the indicated peptide concentrations of the top binding peptides in (A), was determined by flow cytometry using mAb BB7.2. Results are expressed as average relative MFI, which was calculated as the mean fluorescence in the presence of peptide over the mean fluorescence for unpulsed T2 cells. Values are the average of triplicate conditions.



Comprehensive screen to identify human cdr2 peptides that bind to HLA-A2.1

To verify the findings above, we undertook a more comprehensive screening approach and assayed a complete library of 446 overlapping human cdr2 nonamers for binding to HLA-A2.1 plate bound monomers. This was part of a larger effort aimed at identifying human cdr2 HLA binding peptides for the 8 most common human alleles (data not shown). After an initial screen of the complete library, peptides binding at a level of greater than 30% of the positive control were characterized further by affinity for HLA-A2.1 over a range of peptide concentrations (10^{-4} M to 10^{-9} M), and by an off-rate assay to determine the relative stability of the MHC/peptide complex formed. Peptides were ranked by a net score that integrated the peptide binding score, the ED_{50} , and the half-life for the MHC/peptide complex for each peptide to plate bound HLA-A2 molecules (iTopiaTM iScore; Table 2). The three A2.1-restricted human cdr2 peptides previously found to be recognized by peripheral blood CTLs of A2.1+ PCD patients^{46,113} (i.e., cdr2(289-297) SLLEEMFLT, cdr2(273-281) KLVPDSLYV, and cdr2(355-363) ALKVLYEEL) were among the top 38 scoring peptides with net scores greater than 0.1 (on a scale of 0 to 1; Table 1). Additionally, the best binder to HLA-A2.1 in this screen was cdr2(290-298) LLEEMFLTV. Therefore, the comprehensive screen of all 446 possible cdr2 nonamers confirmed our T2 binding assay results of a small panel of predicted peptides.

Table 2. Binding of human cdr2-derived peptides to HLA-A2.1

Rank	Position	Sequence	ED50	$\tau_{1/2}$	iScore
1	290–298 ^d	LLEEMFL TV	6.00E-07	5.9	0.815
2	203–211	TMLQAQLSL	8.00E-07	9.8	0.811
3	289–297 ^b	SLLEEMFL T	6.00E-06	13.9	0.803
4	341–349	RGISLLHEV	1.00E-06	7	0.752
5	65–73	YLTKQVELL	2.00E-06	8.1	0.645
6	260–268	MLQ SE HPFV	2.00E-06	5.4	0.584
7	424–432	ALFKEIFSC	3.00E-05	18.7	0.546
8	217–225	VT MEEEY GL	2.00E-06	6.5	0.528
9	114–122	SLTETIECL	4.00E-06	9.3	0.518
10	111–119	KILSLTETI	7.00E-06	10.5	0.512
11	273–281 ^a	KLVPDSLYV	3.00E-06	6.1	0.499
12	384–392	AAAKDLTGV	3.00E-06	6.5	0.469
13	246–254	RALELEAV	1.00E-06	2.7	0.352
14	128–136	HLQSQVEEL	1.00E-05	7.7	0.323
15	168–176	RQHFVYDHFV	6.00E-06	3.7	0.304
16	391–399	GVNAOSE PV	1.00E-05	6.4	0.292
17	372–380	DSL SHKAVQ	9.00E-06	6.3	0.262
18	171–179	FVYDHVFAE	1.00E-05	5.5	0.282
19	405–413	LASVNPE PV	4.00E-06	1.9	0.244
20	313–221	TILSSLAGS	2.00E-06	1.1	0.238
21	204–212	MLQAQLSLE	5.00E-06	5.1	0.235
22	345–353	LLHEVDTOY	7.00E-06	3.5	0.204
23	197–205	HLK KTV TML	1.00E-05	2.5	0.199
24	164–172	LYDLRQHFV	5.00E-07	0.4	0.199
25	400–408	ASGWEL ASV	1.00E-06	0.5	0.187
26	120–128	ECLQTNIDH	3.00E-05	5.4	0.178
27	121–129	CLQTNIDHL	2.00E-05	2.4	0.176
28	107–113	ASQQKILSL	8.00E-06	3.4	0.174
29	125–133	NIDHLQSQV	6.00E-06	1.3	0.166
30	29–37	QLAAELGKT	2.00E-05	1.4	0.15
31	274–282	LVPDSLYVP	1.00E-05	2.4	0.154
32	82–90	KVYEQLDVT	8.00E-06	1.3	0.139
33	201–209	TVT MLQAQL	3.00E-06	0.6	0.132
34	237–245	LGAT GAYRA	1.00E-05	1.2	0.13
35	373–381	SL SHKAVQT	4.00E-06	0.8	0.129
36	225–233	LVL KENSEL	7.00E-06	0.8	0.119
37	355–363 ^c	ALKV KYEEL	7.00E-06	0.8	0.107
38	176–184	VFAEKITSL	5.00E-06	0.4	0.106

Table 2. Binding of human cdr2-derived nonamers to HLA-A2.1

^acdr2-1, ^bcdr2-2, ^ccdr2-7 (nomenclature from Albert et al.⁴⁶). The concentration of peptide required to fold 50% of the plate bound monomer (ED₅₀) and the amount of time in hours required for 50% of the formed complexes to decay (T_{1/2}) is listed for peptides with a relative binding of >30% of the control for HLA.A2.1. Residues underlined in bold are nonhomologous to the mouse sequence.

In cases of both nonself^{114,115} and self antigens¹¹⁶⁻¹¹⁸, it has been suggested that epitope immunogenicity is correlated with MHC class I binding affinity¹¹². Therefore we chose to first evaluate cdr2(289-297), the peptide with the highest affinity for A2.1 in both of our screens that demonstrated an associated CTL response in A2.1 PCD patients, for natural processing and presentation on HLA-A2.1.

Impaired cdr2(289-297)-specific responses in A2.1 transgenic mice.

Prior studies have demonstrated the utility of HLA class I transgenic mice for evaluating immunogenicity and natural processing of candidate human tumor associated antigens (TAA)^{28,111,112}. To investigate cdr2(289-297) as a candidate tumor antigen, we generated a CTL line specific for human cdr2(289-297) by immunizing A2.1 transgenic HHD mice with replication-defective adenovirus expressing full-length human cdr2 (Ad-hcdr2). Because HHD mice harbor human HLA-A2.1 molecules on an H-2D^b/mouse β 2-microglobulin double-knockout background, all CTL generated in this mouse strain will be restricted to the A2.1 molecule¹¹⁹. (See Figure 3 for comparison of A2.1 transgenic mouse strains used in this study.) In initial experiments, one of two HHD mice immunized with Ad-hcdr2 had spleen CD8⁺ T cells which were responsive to cdr2(289-297) peptide-pulsed EA2/K^b cells (EL4 cells transfected with the chimeric A2.1 molecule A2/K^b) by IFN- γ ELISPOT assay, but had no reactivity to EA2/K^b cells pulsed with an irrelevant A2.1 peptide (Figure 4). As a positive control, both mice were immunized in parallel with adenovirus expressing full-

length influenza matrix protein (Ad-FluMP), and both developed spleen CD8⁺ T cells specific for the immunodominant FluM1(58-66) epitope pulsed on EA2/K^b cells (Figure 4).

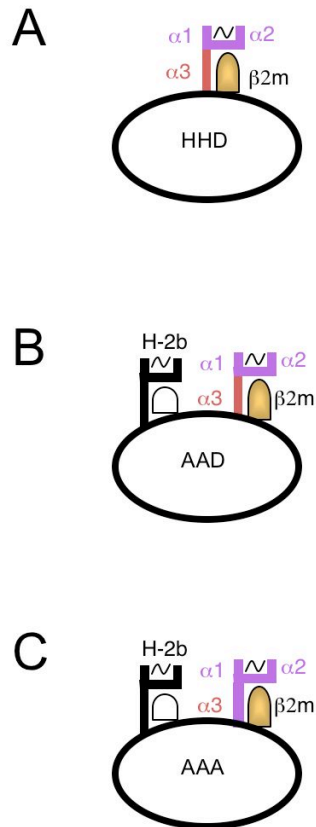


Figure 3. Schematic of the MHC class I molecules expressed by three different HLA-A2.1 transgenic mouse strains used in this study.

(A) HHD mice¹¹⁹ express a hybrid HLA-A2.1 molecule with a murine $\alpha 3$ domain to facilitate interaction with murine CD8⁺ T cells on a mouse MHC (H-2^b) null background. (B) AAD mice¹⁰⁴ express a hybrid HLA-A2.1 molecule similar to that in HHD mice and also express native H-2^b molecules. (C) AAA mice¹⁰⁵ express fully humanized HLA-A2.1 (i.e. the $\alpha 3$ domain is human) and also express native H-2^b molecules.

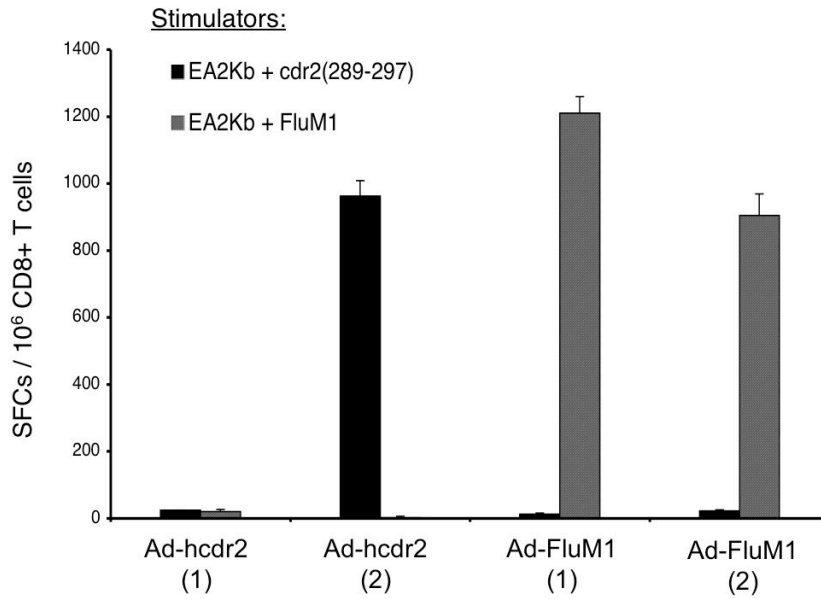


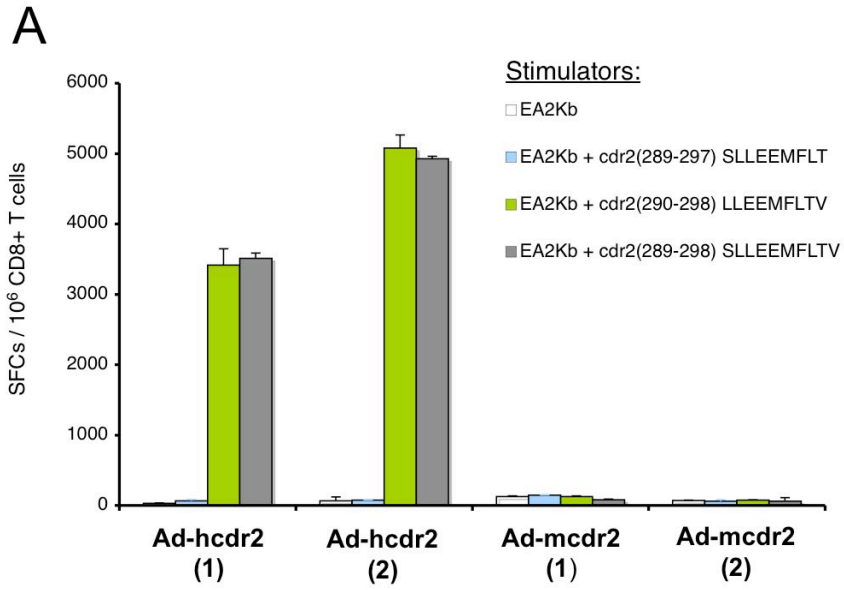
Figure 4. Ex vivo T cell response to human cdr2(289-297) in Ad-hcdr2 immunized HHD mice as determined by IFN- γ ELISPOT assay.

12 d after i.d. immunization of HHD mice with 10^9 pfu of Ad-hcdr2 or Ad-FluM1, CD8+ T cells were purified from splenocytes and cocultured with human cdr2(289-297) or FluM1(58-66) peptide-pulsed (10^{-5} M) EA2Kb cells in an 18 h IFN- γ ELISPOT assay. Values, representing spot forming cells per million CD8+ T cells, are the average of duplicate wells; error bars indicate standard deviation.

Given the inconsistent response of HHD mice to Ad-hcdr2, we undertook several additional experiments immunizing mice from 3 different A2.1 transgenic mouse strains (HHD, AAD, and AAA) with Ad-hcdr2. As represented in Figure 5A and summarized in Figure 5B, we found that peptide cdr2(289-297) failed to elicit specific CD8⁺ T cell responses after immunization when tested directly *ex vivo*, or after splenocyte restimulation *in vitro* with human cdr2(289-297) peptide. Conversely, cdr2(290-298) peptide, an high-scoring A2.1 binding peptide which is nonhomologous to mouse cdr2 (Table 2, Figure 6), elicited CD8⁺ T cell responses in the same experiments. We were also unable to elicit cdr2(289-297)-specific CD8⁺ T cell responses by immunization with peptide emulsified in Titermax Classic[™] adjuvant, while mice immunized with cdr2(290-298) peptide all developed cdr2(290-298)-peptide specific CD8⁺ T cell responses (Figure 7). Human cdr2(289-297) is homologous to murine cdr2(289-297) at all but the position 9 anchor residue (Figure 6), hence a possible implication of the impaired cdr2(289-297)-specific responses in mice was that these T cells could cross react with homologous naturally processed murine peptide on tolerizing thymic or peripheral APCs.

Figure 5. CD8+ T cell responses in Ad-hcdr2 immunized A2.1 transgenic mice.

A2.1 transgenic mice were immunized i.d. with 10^9 pfu of Ad-hcdr2 or Ad-mcdr2. 12 d later, CD8+ T cells were purified from splenocytes and cocultured as indicated with human cdr2(289-297), human cdr2(290-298), human cdr2(289-298) peptide-pulsed (10^{-5} M) EA2Kb cells, or EA2Kb alone in an 18 h IFN- γ ELISPOT assay. (A) Representative direct ex vivo IFN- γ ELISPOT with immunized AAD mice. Values, representing spot forming cells per million CD8+ T cells, are the average of triplicate wells; error bars indicate standard deviation. (B) Summary comparing IFN- γ ELISPOT responses (total number of mice responding/total number of mice immunized) to human cdr2(289-297) and human cdr2(290-298) in A2.1 Tg mice immunized with Ad-hcdr2, either directly ex vivo or post 8 day restimulation with 0.5 μ g/ml of the indicated cognate peptide.



B

	Direct <i>ex vivo</i>	Post restimulation
cdr2(290-298)	16/17	17/17
cdr2(289-297)	1/13	2/13

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cdr2(289-297)
                                123456789
Hu  EKLVPDSLYVPFKEPSQSLLEEMFLTVPESHRKPLKRSSSET
    :::::::::: :::::::::::::::::::: :: ::::::::::::::
Mu  EKLVPDSLFVPFKEPSQSLLEEMFLAAPEAPRKPLKRSSSET

cdr2(290-298)
                                123456789
Hu  EKLVPDSLYVPFKEPSQSLLEEMFLTVPESHRKPLKRSSSET
    :::::::::: :::::::::::::::::::: :: ::::::::::::::
Mu  EKLVPDSLFVPFKEPSQSLLEEMFLAAPEAPRKPLKRSSSET

cdr2(289-298)
                                12345678910
Hu  EKLVPDSLYVPFKEPSQSLLEEMFLTVPESHRKPLKRSSSET
    :::::::::: :::::::::::::::::::: :: ::::::::::::::
Mu  EKLVPDSLFVPFKEPSQSLLEEMFLAAPEAPRKPLKRSSSET

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Figure 6. Sequence homology of murine and human cdr2 surrounding residues 289-298.

Murine and human cdr2 proteins have an overall amino acid sequence identity of 87%.

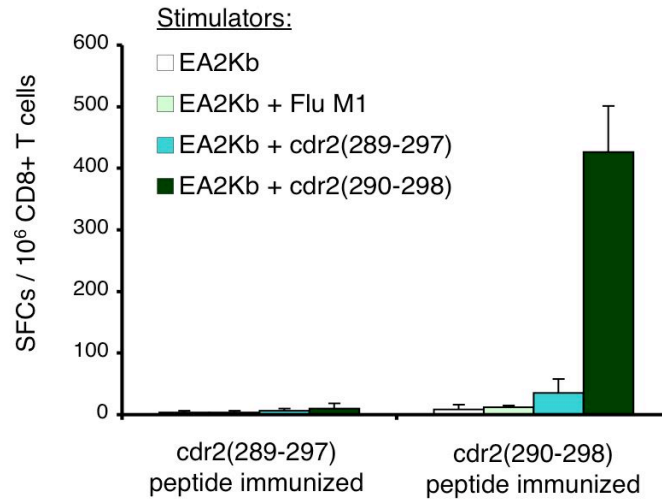


Figure 7. Direct ex vivo CD8+ T cell response to human cdr2(290-298) but not human cdr2(289-297) in peptide immunized AAD mice.

AAD mice were immunized intrafootpad with 100 μ g peptide emulsified in Titermax ClassicTM adjuvant. 7 d later, draining popliteal and inguinal lymph nodes were isolated and CD8+ T cells were purified and cocultured with cdr2(289-297), cdr2(290-298) or FluM1 peptide-pulsed (10^{-5} M) EA2K^b cells as indicated in an 18 h IFN- γ ELISPOT assay. Depicted above are responses from lymph nodes pooled from 2 mice per group. Values, representing spot forming cells per million CD8+ T cells, are the average of triplicate wells; error bars indicate standard deviation. Data are representative of three independent experiments.

HLA-A2.1-restricted CTLs specific for human cdr2(289-297) cross react with the homologous murine peptide cdr2(289-297)

To investigate the possibility of cross recognition of the human and murine cdr2(289-297) peptides, we used the immunized mouse (Ad-hcdr2-2 from Figure 3) that demonstrated a direct *ex vivo* CD8 T cell response to cdr2(289-297), to establish a cdr2(289-297)-specific CTL line (HHD 289) by weekly restimulation with the human cdr2(289-297) peptide (SLLEEMFLI) (Figure 8A). Direct evidence for the immunological cross recognition of murine cdr2 (SLLEEMFLA) was provided by coculturing the HHD 289 CTL line with EA2K^b cells pulsed with either the human cdr2(289-297) or murine cdr2(289-297) peptides. As measured by IFN- γ ELISPOT assay, HHD 289 CTL recognized EA2K^b target cells that had been pulsed with either the human or murine cdr2(289-297) peptides, but not cells alone, or cells pulsed with the irrelevant peptides, FluM1(58-66) or cdr2(290-298) (Figure 8B).

Human and murine cdr2(289-297) are naturally processed and presented

The preceding results demonstrated that synthetic peptides corresponding to the sequences derived from human or murine cdr2(289-297) could be presented by HLA-A2.1 when added to cells. To determine whether these peptides could be naturally processed and presented in association with A2.1 molecules, we infected primary kidney epithelial cells (KECs, that do not express cdr2 protein;

Figure 8. HHD 289 CTL cross react with both human and murine cdr2(289-297) peptides.

(A) Schematic of the generation of the HHD 289 CTL line. Splenocytes from the immunized HHD mouse in Figure 4, which demonstrated a direct ex vivo response to human cdr2(289-297) peptide, were restimulated every 10-14 d with human cdr2(289-297) peptide-pulsed stimulators as described in Materials and Methods (Chapter 2). (B) After 2 rounds of in vitro restimulation, the HHD 289 CTL were tested for recognition of EA2K^b cells pulsed with no peptide, murine cdr2(289-297), human cdr2(289-297), human cdr2(273-281), or FluM1 at 10⁻⁵ M in an 18 h IFN- γ ELISPOT. Values, representing spot forming cells per million CD8⁺ T cells, are the average of triplicate wells; error bars indicate standard deviation. (C) The sequences of the murine and human cdr2(289-297) peptides are identical except for the position 9 anchor residue.

data not shown, and Figure 16) derived from HHD mice with Ad-hcdr2 or Ad-mcdr2. After pretreatment with IFN- γ for 40 hours to upregulate MHC class I expression, KECs infected with either Ad-hcdr2 or Ad-mcdr2, but not Ad-FluM1, were specifically recognized by HHD 289 CTL (Figure 9). These results confirmed that both human and murine cdr2(289-297) can be naturally processed and presented in association with HLA-A2.1 molecules. By IFN- γ ELISPOT assay, we observed that HHD 289 CTL had an approximately 4-fold greater response to endogenously presented murine cdr2 than to human cdr2 (Figure 9). This was not due to significant differences in cdr2 expression levels in the two Ad-infected KEC populations (data not shown) or to significant differences in human versus murine cdr2 peptide binding to A2.1, as assessed by T2 binding assays (Figure 2B). An alternative explanation for these findings is that there is a difference in the functional avidity of HHD 289 CTL for human or murine cdr2(289-297). We tested this possibility by incubating HHD 289 CTLs with EA2K^b stimulator cells that had been pulsed with 10-fold serial dilutions of either peptide in 18h IFN- γ ELIPSOT assays. Significantly, the concentration of murine cdr2(289-297) peptide versus human cdr2(289-297) required to induce half-maximal spot forming cells (SFCs) was \sim 4-fold lower (20.0 nM versus 78.0 nM) (Figure 10). Thus the difference in recognition of endogenous human and murine cdr2 protein is likely a reflection of differences in the functional avidity of the HHD 289 line for the murine and human presented epitopes.

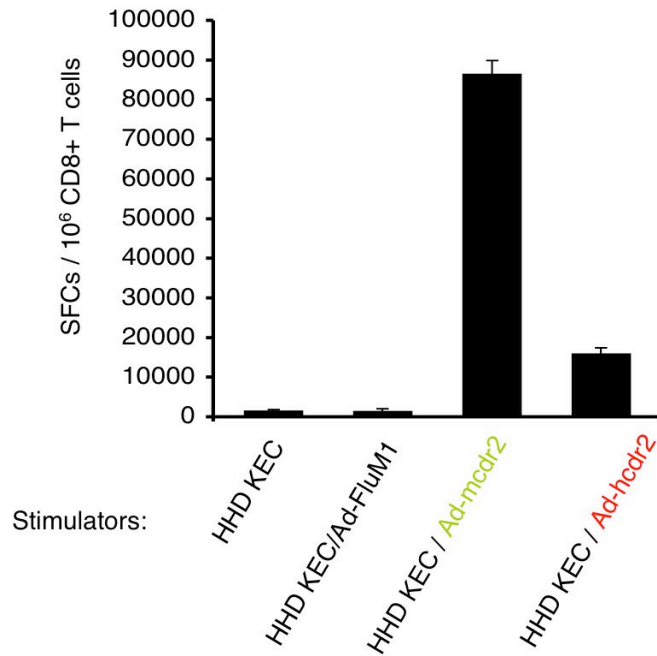


Figure 9. HHD 289 CTL recognize endogenous murine and human cdr2

To test for recognition of endogenous murine and human cdr2, HHD 289 CTL (after 3 or 4 rounds of restimulation) were cocultured with HHD KECs that were uninfected, or infected with Ad-FluM1, Ad-mcdr2, or Ad-hcdr2 in an 18h IFN- γ ELISPOT assay. Values, representing spot forming cells per million CD8+ T cells, are the average of triplicate wells; error bars indicate standard deviation. Data are representative of 2 independent experiments.

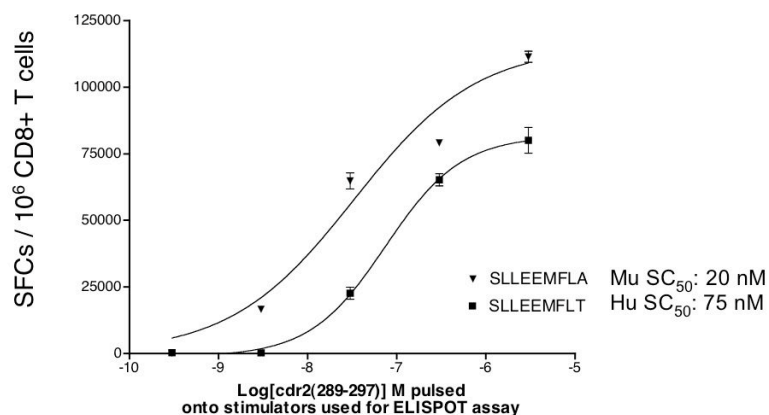


Figure 10. Avidity comparison of HHD 289 CTL for murine and human cdr2(289-297) peptides.

The HHD 289 CTL line was generated as described (Chapter 2, Materials and Methods). CD8⁺ T cells were purified by negative selection and were cocultured with EA2K^b cells that had been pulsed with the indicated concentration of murine (triangles) or human (square) cdr2(289-297) peptide. IFN- γ production was measured in an 18 h ELISPOT assay. Values, representing spot forming cells per million CD8⁺ T cells, are the average of triplicate wells; error bars indicate standard deviation. The SC₅₀ values, or concentration cdr2(289-297) murine or human peptide required for half-maximal IFN- γ production, is the average of three independent experiments.

Minimal binding of A2.1/cdr2 (289-297) tetramers by the HHD 289 CTL line.

As a rule, binding of murine CTL by A2.1 tetramers is CD8 independent, since murine CD8 does not interact with the A2.1 molecule and therefore such binding reflects high avidity TCR-MHC/peptide interactions^{120,121}. To determine whether the HHD 289 CTL line expressed high affinity TCR, we analyzed its ability to bind A2.1/human cdr2(289-297) tetramers. We found that this CTL line contained only a very small subset of CD8⁺ T cells (~0.1%) capable of binding these tetramers (Figure 11). The poor binding exhibited by the HHD 289 CTL line was not the result of a paucity of T cells in this population that are actually specific for this peptide/MHC complex, since approximately 8% (or 80-times more cells) were found to be IFN- γ -producing cdr2(289-297)-specific cells by ELISPOT assay (Figure 8). Moreover, HHD 289 CTL recognize A2.1 KECs engineered to overexpress human or murine cdr2, but demonstrate only minimal recognition of human or murine tumor cells expressing physiologic levels of cdr2 protein (Figure 12). Since the murine and human cdr2(289-297) peptides were demonstrated to be processed endogenously, HHD 289 CTL were likely of too low avidity to allow recognition of lower levels of natural Ag, consistent with at least partial self-tolerance to the cdr2 antigen in A2.1 transgenic mice.

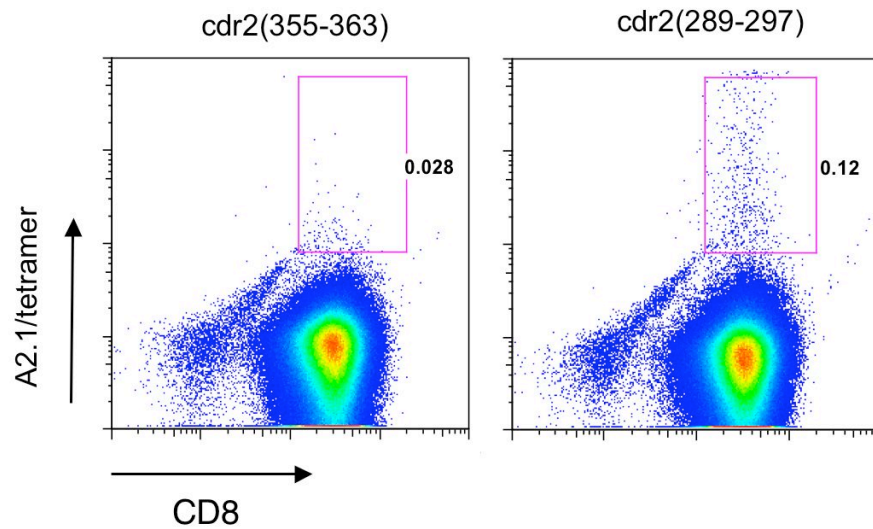


Figure 11. Binding of HHD 289 CTL to A2.1/cdr2(289-297) tetrameric complexes

7 d after the third in vitro restimulation, HHD 289 CTL were purified by CD8 negative selection (MACs; Miltenyi Biotec) and stained as described in Materials and Methods (Chapter 2) with tetrameric PE conjugates for A2.1/cdr2(289-297) or A2.1/cdr2(355-363) as a negative control. Both tetrameric conjugates were provided by the NIH tetramer facility. Staining is representative of 3 independent experiments. The percentage of tetramer binding cells did not change significantly between 3 and 5 in vitro restimulations.

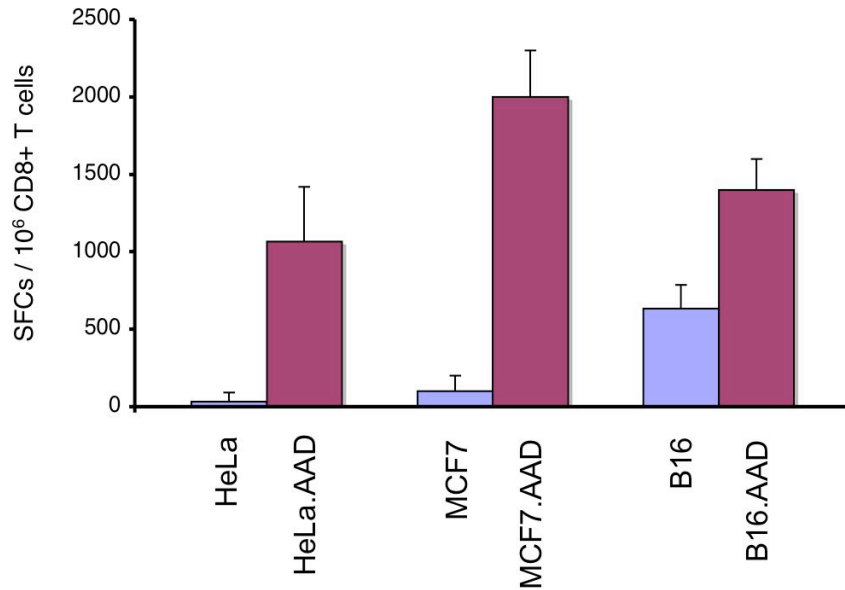


Figure 12. Minimal recognition of physiologic levels of endogenous human and murine cdr2 in tumor cell lines by HHD 289 CTL.

To test for recognition of endogenous cdr2 tumors, HHD 289 CTL were cocultured with HeLa, HeLa.AAD, MCF7, MCF7.AAD, B16, or B16.AAD tumor cells in an 18 h IFN- γ ELISPOT assay. Values, representing spot forming cells per million CD8+ T cells, are the average of triplicate wells; error bars indicate standard deviation.

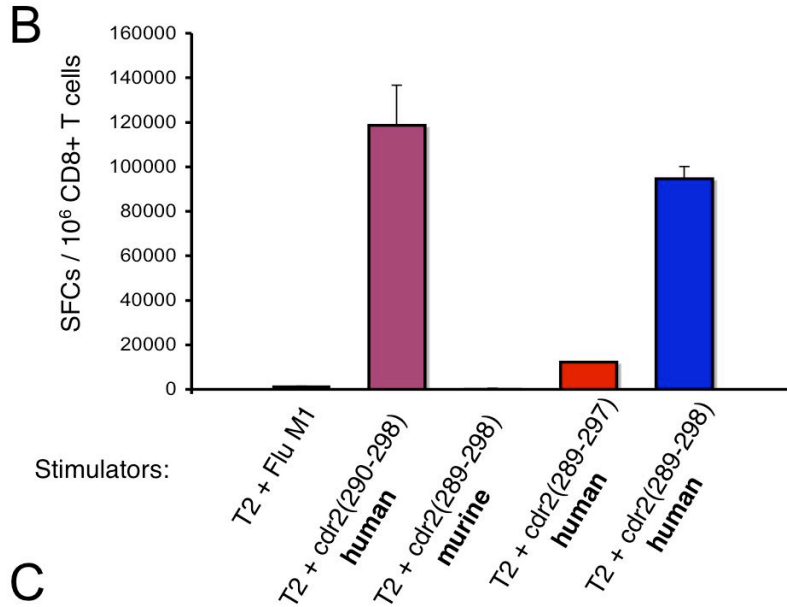
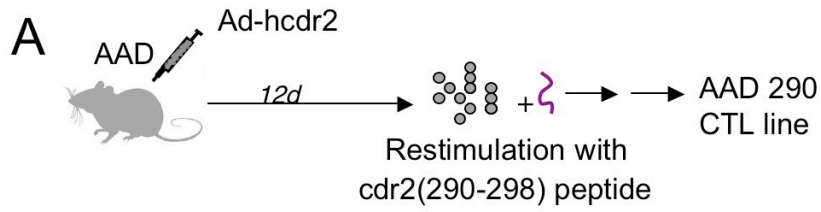
Generation of high affinity human cdr2(290-298)-specific CTLs in A2.1 transgenic mice.

In contrast to the minimal responses to cdr2(289-297) observed in immunized A2.1 transgenic mice, we obtained robust CD8⁺ T cell responses specific for cdr2(290-298) in all mice immunized with either Ad-hcdr2 (Figure 4) or human cdr2(290-298) peptide (Figure 6). cdr2(290-298) has both of the optimal anchor residues for peptide binding to HLA-A2.1 which are leucine and valine at the 2nd and 9th positions of peptide respectively¹²², and had the highest net score in our peptide screen (Table 1). Human cdr2(290-298) LLEEMFLTV is nonhomologous to mouse cdr2(290-298) LLEEMFLAA at position 8, and thus has an amino acid difference at an exposed position in the MHC/peptide complex. This suggested that it might be possible to generate a higher affinity CTL line specific for human cdr2(290-298) because these CTL would not be affected by the potential self-tolerance to mouse self cdr2. AAA A2.1 mice¹⁰⁵, were originally chosen for derivation of A2.1-restricted CTL with the expectation that the presence of a human $\alpha 3$ domain on the A2.1 molecule would facilitate the selection of CTL capable of CD8-independent antigen recognition. AAD¹⁰⁴, rather than AAA A2.1, transgenic mice were ultimately selected for these studies as they responded most vigorously to the cdr2(290-298) peptide (data not shown). We generated a cdr2(290-298) peptide specific CTL line (herein designated AAD 290) from an Ad-hcdr2 immunized mouse. The AAD 290 CTL line recognized human cdr2(290-298) but not murine cdr2(290-298) or FluM1 peptide pulsed T2 cells in IFN- γ ELISPOT assays (Figure 12B). As demonstrated in Figure 13, the

decapeptide cdr2(289-298), which is equivalent to cdr2(290-298) extended NH₂-terminally by one residue, was recognized as efficiently as the original nonamer. We also observed a small but reproducible cross-reactivity to human cdr2(289-297), but this response was 100-fold lower than the response to cdr2(290-298), and was absent at concentrations of peptide lower than 10⁻⁶ M. The AAD 290 CTL line was approximately 10-fold more avid for its cognate antigen pulsed on T2 cells (SC₅₀ = 6.8 nM) than the HHD 289 CTL line was for its antigen pulsed on EA2K^b (SC₅₀ = 78.0 nM) (Figure 13; Figure 10), similar in magnitude to the difference in responses reported in TAA-specific CTL lines derived from WT versus antigen null mice¹²³, further consistent with the idea that mice show tolerance to mcd2. A large proportion (~ 40%) of AAD 290 CTL were high affinity antigen-specific cells, as indicated by their ability to bind to A2.1/cdr2(290-298) tetramers (Figure 15). Taken together these results demonstrate that A2.1 transgenic mice can generate higher avidity T cells for non-self cdr2 epitopes than they can for self cdr2 epitopes, and hence highlight both the limitations for screening homologous self peptides in A2.1 transgenic mice and the possibility of harnessing sequence differences for the purpose of generating high avidity CD8-independent T cell lines from which therapeutic TCRs might be isolated.

Figure 13. Peptide specific response of AAD 290 CTL

(A) Schematic of the generation of the AAD 290 CTL line. Splenocytes from the Ad-hcdr2 immunized AAD mouse with the greatest response to cdr2(290-298) peptide after one round of in vitro restimulation were used to generate the AAD 290 CTL line by restimulation every 10 - 14 d with human cdr2(290-298) peptide-pulsed stimulators as described in Materials and Methods. (B) After 2 rounds of in vitro restimulation, AAD 290 CTL were tested for recognition of T2 cells pulsed with FluM1, human cdr2(290-298), murine cdr2(289-298), human cdr2(289-297), or cdr2(289-298) or at 10^{-5} M in an 18 h IFN- γ ELISPOT. Values, representing spot forming cells per million CD8+ T cells, are the average of triplicate wells; error bars indicate standard deviation. (C) Sequences and alignment of peptides used in the IFN- γ ELIPSOT.



C

cdr2(289-297):

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Hu EKLVPDSLYVPFKEPSQSLLEEMFLTVPESHKPLKRSSSET
  :::::::::: :::::::::::::::::::: :: ::::::::::::::
Mu EKLVPDSLFLVPFKEPSQSLLEEMFLAAPEAPRKPLKRSSSET
  
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cdr2(290-298):

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Hu EKLVPDSLYVPFKEPSQSLLEEMFLTVPESHKPLKRSSSET
  :::::::::: :::::::::::::::::::: :: ::::::::::::::
Mu EKLVPDSLFLVPFKEPSQSLLEEMFLAAPEAPRKPLKRSSSET
  
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cdr2(289-298):

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Hu EKLVPDSLYVPFKEPSQSLLEEMFLTVPESHKPLKRSSSET
  :::::::::: :::::::::::::::::::: :: ::::::::::::::
Mu EKLVPDSLFLVPFKEPSQSLLEEMFLAAPEAPRKPLKRSSSET
  
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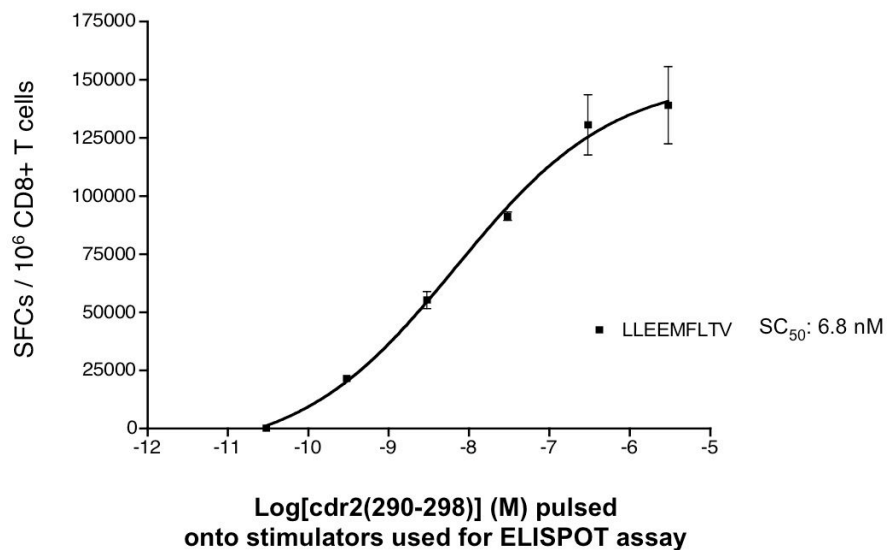


Figure 14. Avidity of the AAD 290 CTL line for human cdr2(290-298) peptide.

The AAD 290 CTL line was generated as described (Chapter 2, Materials and Methods). CD8⁺ T cells were purified by negative selection and were cocultured with T2 cells that had been pulsed with the indicated concentration of cdr2(290-298) peptide. IFN- γ production was measured in an 18 h ELISPOT assay. Responses, depicted as spot forming cells per million CD8⁺ T cells, are the average of triplicate wells; error bars indicate standard deviation. The SD_{50} value, or dose of human cdr2(290-298) required for half-maximal IFN- γ production is an average of two independent experiments.

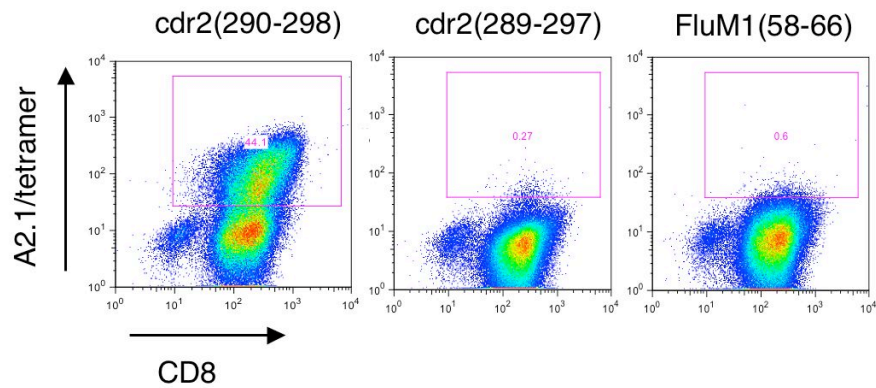


Figure 15. Binding of AAD 290 CTL to A2.1/cdr2(290-298) tetrameric complexes

9 d after the third in vitro restimulation, AAD 290 CTL were purified by CD8 negative selection (MACs; Miltenyi Biotec), Fc blocked, and stained as described in Materials and Methods (Chapter 2) with tetrameric PE conjugates for A2.1/cdr2(290-298), A2.1/cdr2(289-297), or A2.1/FluM1(58-66) or A2.1/cdr2(355-363) (iTAg MHC Tetramer, Beckman Coulter, Fullerton, CA, USA). Staining is representative of 2 independent experiments.

Nonhomologous human cdr2(290-298) peptide is a naturally processed CTL epitope.

While the generation of cdr2(290-298)-specific T cells through immunization with full length human cdr2-expressing adenovirus implied that this epitope was naturally processed. We confirmed this by testing the ability of the AAD 290 CTL line to respond to KECs transduced with Ad-hcdr2 in an IFN- γ ELISPOT assay. As seen in Figure 16, AAD 290 CTL produced IFN- γ specifically in response to coculture with KECs infected with Ad-hcdr2. We also observed that AAD 290 CTL do not cross-react with KECs infected with Ad-mcdr2 or Ad-GFP, thus confirming the antigen specificity of the T cells. Recognition of Ad-hcdr2-infected HHD KECs (Figure 16, III) indicated that this response was A2.1 restricted. Furthermore, AAD 290 CTL were capable of HLA-dependent, CD8-independent recognition of endogenous human cdr2, as demonstrated by the response to Ad-hcdr2 infected KECs derived from AAA mice, which have a human $\alpha 3$ domain (Figure 16, II).

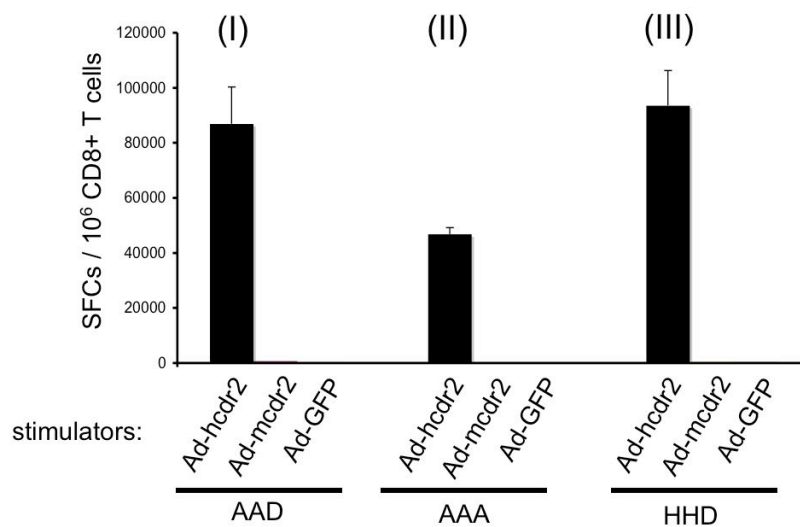


Figure 16. CD8-independent recognition of endogenous cdr2 by AAD 290 CTL

To test for recognition of endogenous cdr2, AAD 290 CTL were cocultured with either AAD (I), AAA (II), or HHD (III) KECs that had been infected with Ad-hcdr2, Ad-mcdr2, or Ad-GFP in an 18h IFN- γ ELISPOT assay. Values, representing spot forming cells per million CD8+ T cells, are the average of triplicate wells; error bars indicate standard deviation. Recognition of AAA KECs is CD8-independent because murine CD8 cannot interact with the human a3 domain on the AAA molecule. Recognition of Ad-hcdr2 infected HHD KECs demonstrates that this response is A2.1 rather than H-2^b-restricted.

Recognition of cdr2(290-298) in tumor cell lines.

Having confirmed that the cdr2(290-298) peptide is a naturally processed CTL epitope, we tested AAD 290 CTL for recognition of physiologic levels of endogenous cdr2 in human tumor cells. We found that cdr2 protein was expressed in a number of tumor cell lines, including the breast cancer cell line MCF7, and the cervical carcinoma cell line, HeLa (Figure 17). With the exception of brain and testis, cdr2 protein is not expressed in normal tissue⁵¹, represented in Figure 17 by primary cultures of kidney epithelial cells. The cdr2-expressing tumor cell lines HeLa (A2.1 negative) and MCF7 (A2.1 positive) were stably transfected with the chimeric A2.1 molecule AAD to facilitate recognition by murine CTL. The resulting cell lines, HeLa.AAD and MCF7.AAD (Figure 18), were used as stimulators in an IFN- γ ELISPOT assay. AAD 290 CTL secreted IFN- γ in response to coculture with HeLa.AAD and MCF7.AAD, but not to HeLa cells alone, and only minimally to MCF7 alone (Figure 19). The IFN- γ ELISPOT response to MCF7 (5700 +/- 1212 / 10⁶ CD8+ T cells) may have represented CD8-independent recognition of cdr2(290-298) presented on the human HLA-A2.1 molecule since this response was significantly greater than the response to A2.1 negative cdr2-expressing HeLa cells (133 +/- 58 SFCs / 10⁶ CD8+ T cells). Specific recognition of all tumor cell targets was greater after pretreatment with IFN- γ (Figure 19), a cytokine known to facilitate target recognition by increasing HLA-A2.1 expression, antigen processing, and induction of ICAM expression^{124,125}.

We also tested whether AAD 290 CTL were also able to specifically kill cdr2-expressing A2.1 + targets. As shown in Figure 20, AAD 290 CTL could specifically lyse both T2 targets pulsed with cdr2(290-298) peptide and the AAD/A2.1+ cdr2+ cell line HeLa.AAD in a 4 hour ⁵¹Cr release assay. In contrast, T2 pulsed with FluM1(58-66) peptide and the parental A2.1 negative cdr2+ cell line HeLa were not lysed, indicating that target cell recognition was both A2.1-restricted and antigen specific. In conclusion, these results demonstrate that AAD 290 CTL are highly specific for endogenous cdr2 expressed in tumor cells, and that human CD8+ CTL of this specificity might be responsible for mediating anti-cdr2 tumor immunity in A2.1+ PCD patients.

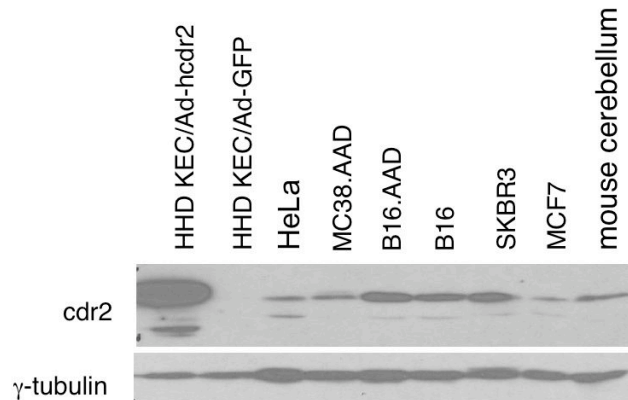


Figure 17. Analysis of cdr2 protein expression tumor cell lines and normal tissue.

Western blot analysis demonstrates specific expression of cdr2 in mouse cerebellum but not in mouse primary kidney epithelial cells. cdr2 protein is expressed in all tumor cell lines tested. High levels of cdr2 are expressed in Ad-hcdr2 transduced kidney epithelial cell cultures. Protein lysates were resolved by SDS-PAGE and blotted onto a PVDF membrane. The blot was labeled with PCD patient antiserum followed by rabbit anti-human HRP, and cdr2 protein was visualized by chemiluminescence. The blot was stripped and reprobed with a mouse mAb to γ -tubulin to verify equivalent protein loading.

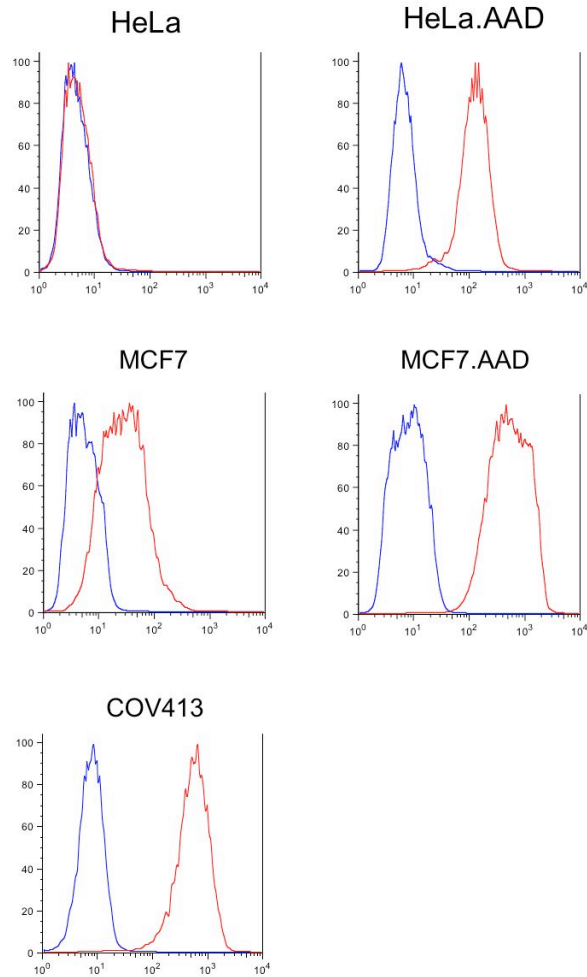


Figure 18. Analysis of HLA-A2.1 expression by human tumor cell lines.

Tumor cell lines were harvested and the level of cell surface expression of native HLA-A2.1 and the transfected chimeric A2.1 molecule AAD was measured using FITC-conjugated A2.1 (clone BB7.2; red line) or isotype control antibody (blue line) by flow cytometry.

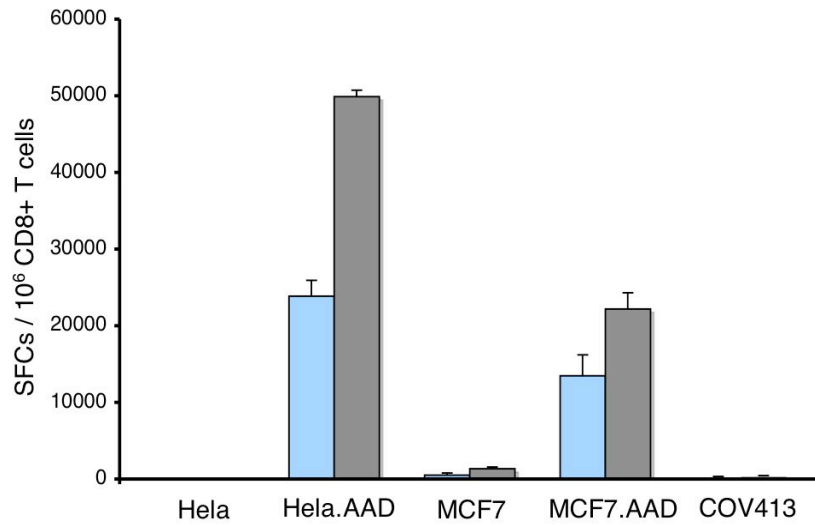


Figure 19. AAD 290 CTL recognize endogenous cdr2 in tumors

To test for recognition of endogenous cdr2 tumors, AAD 290 CTL were cocultured with HeLa, HeLa.AAD, MCF7, MCF7.AAD, or COV413 tumor cells plus (grey) or minus (blue) pretreatment with IFN- γ for 40 h in an 18 h IFN- γ ELISPOT assay. Values, representing spot forming cells per million CD8⁺ T cells, are the average of triplicate wells; error bars indicate standard deviation.

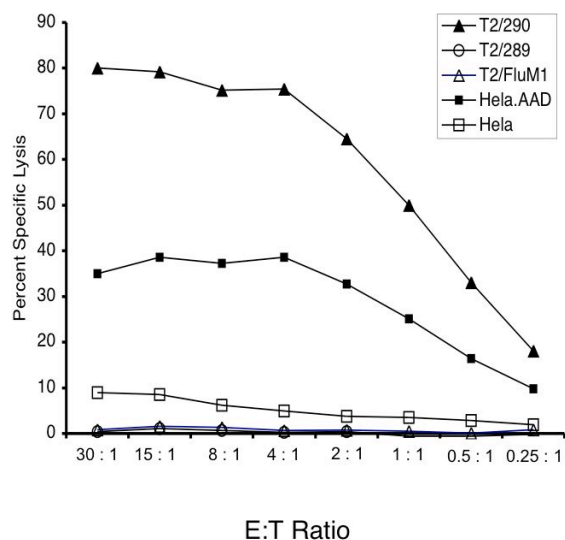


Figure 20. HLA-A2.1-restricted lysis of cdr2-expressing HeLa cells by AAD 290 CTL.

AAD 290 CTL were assayed for killing activity toward T2 cells that had been pulsed (10^{-5} M) with cdr2(290-298)(filled triangles), cdr2(289-297)(open circles), or FluM1(58-66) (open triangles) peptides, or toward IFN- γ pretreated HeLa (open squares) or HeLa.AAD cells (filled squares) in a 4 h ^{51}Cr release assay.

Detection of cdr2(289-297) and cdr2(290-298)-peptide binding CD8+ T cells in the peripheral blood of A2.1+ PCD patients.

Cdr2(289-297) and cdr2(290-298)-specific CTL are reactive against endogenous cdr2 in A2.1+ tumor cells. Could cells of these specificities be responsible for the clinically evident immunity to cdr2-expressing tumors in A2.1 PCD patients? To begin to address this question, we evaluated two A2.1+ PCD patients for the presence of cdr2(289-297) and cdr2(290-298)-tetramer binding peripheral blood CD8+ T cells directly *ex vivo*. For control and comparison, we performed staining analysis in parallel using the following tetramers: 1) commercially available negative tetramers from Beckman Coulter, 2) HLA-A2.1/FluM1(58-66) tetramers, and 3) HLA-A2.1/PSMA(4-12) tetramers. Peripheral blood from A2.1+ PCD patient A (Figure 21A) had peripheral blood T cells which demonstrated significant staining with A2.1 tetramers of the cdr2(290-298) specificity (0.027% of CD8+ T cells) relative to two negative control tetramers, negative tetramer (0% of CD8+ T cells) and the A2.1/PSMA tetramer (0.0013% of CD8+ T cells). We did not observe a significant population of cdr2(289-297)-specific T cells for this population. For comparison, this patient had a discrete population of FluM1-tetramer staining peripheral blood CD8+ T cells (0.072%), which was correlated with a FluM1(58-66) peptide-specific memory CD8+ T cell response as determined by direct IFN- γ ELISPOT assay (data not shown). Conversely, while the peripheral blood contained cdr2(290-298) tetramer-binding cells, we did not observe an IFN- γ ELISPOT response to cdr2(290-298) peptide-pulsed autologous DCs in the same assay (data not shown). Peripheral blood from a second A2.1 +

PCD patient (Figure 21B) contained CD8⁺ T cells that bound specifically to A2.1/cdr2(289-297) tetramers (0.2% of CD8⁺ cells; Figure 20A). A2.1/FluM1 tetramer binding CD8⁺ T cells were not detected in this patient, a finding that was corroborated by the absence of a memory CD8⁺ T cell response specific for FluM1 peptide-pulsed autologous dendritic cells (DCs) by IFN- γ ELISPOT assay (data not shown). The A2.1/cdr2(289-297) tetramer binding cells likely represented a population of anergic or unresponsive cells as determined by the absence of an IFN- γ ELISPOT response to cdr2(289-297) peptide pulsed autologous DCs (data not shown). Among the patients we have analyzed, cdr2 tetramer staining appears to be specific to PCD patients; cdr2(290-298) tetramer did not stain any of several control T cell samples (Figure 22) including one A2.1⁺ normal donors (Figure 22, I), two A2.1⁺ patients with the paraneoplastic Hu syndrome (Figure 22, II and III), and one neurologically normal ovarian cancer patient (Figure 22, IV). Therefore these studies correlate the presence of cdr2(289) or cdr2(290) tetramer-staining peripheral blood T cells with PCD and anti-gynecologic tumor immunity and suggest that cdr2(289-297) and cdr2(290-298) are bone fide tumor associated CTL epitopes.

Figure 21. Detection of cdr2(290-298) and cdr2(289-297)-specific CD8+ T cells in the peripheral blood of HLA-A2.1+ PCD patients.

10⁶ PBMC from two A2.1 + PCD patients were stained with cdr2(289-297), cdr2(290-298), FluM1(58-66), PSMA(4-12), or negative A2.1 tetrameric complexes for 20 min at room temperature plus CD8 antibody during the final 10 minutes. Cells were washed and analyzed immediately on a FACScaliber flow cytometer. Results are gated on CD8+ T cells.

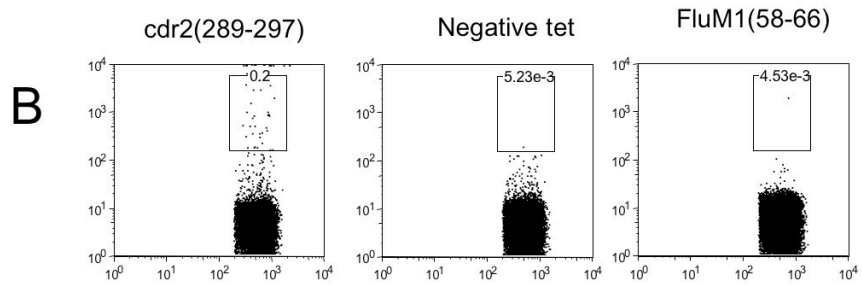
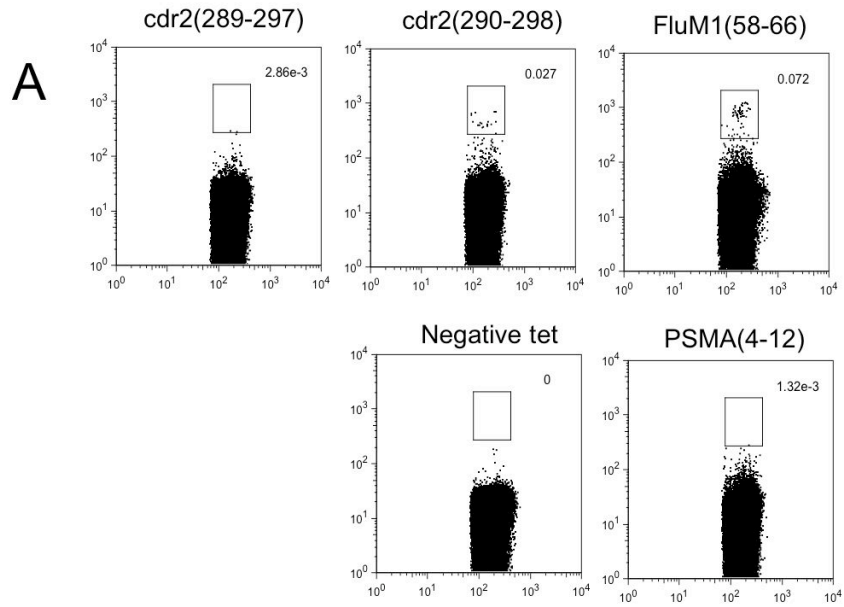
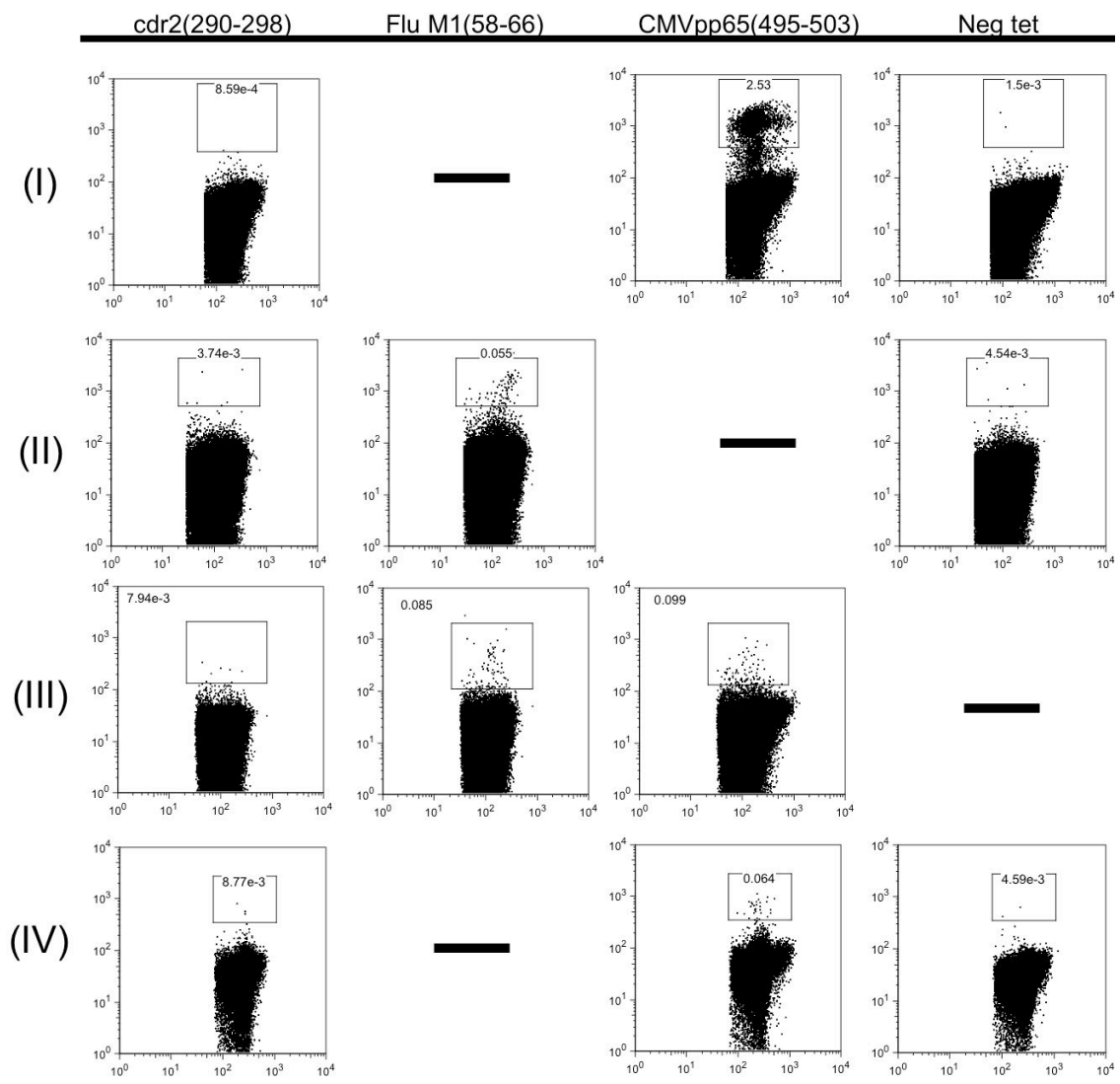


Figure 22. CD8+ T cells from control individuals do not bind to A2.1/cdr2(290-298) tetramer

A2.1/cdr2(290-298) tetrameric complexes did not stain PBMC from a normal donor (I), two patients with the Hu syndrome (II and III), or one neurologically normal ovarian cancer patient (IV). For control and comparison PBMC were stained with FluM1(58-66), CMVpp65(495-503), or negative A2.1 tetrameric complexes Results are gated on CD8+ T cells.



Discussion

We became interested in exploring cdr2 as a tumor rejection antigen for breast and ovarian cancer immunotherapy after two important observations from our laboratory: the discovery of cdr2 peptide-specific CTL in the peripheral blood of individuals with tumor immunity and PCD⁴⁶; and the observation that cdr2 is expressed by a large proportion of gynecologic tumors from individuals who do not develop neurologic disease⁵⁵. These findings, along with clinical data from other PNDs suggesting that tumor immunity and autoimmunity towards these antigens may be able to be uncoupled, encouraged us to identify naturally processed CTL epitopes that would deepen our understanding of T cell responses to this protein in PCD patients and neurologically normal cancer patients, as well as possibly become the basis of cdr2-based immunotherapy.

We chose to focus on CTL responses restricted by the human leukocyte antigen (HLA)-A2.1 molecule because this is the most common class I HLA allele in the Western population, with a prevalence of 30-40%¹²⁶ and because HLA-A2.1 transgenic mice for this allele were readily available to provide us with an unrestricted source of human HLA-restricted T cells. Also, many reports have demonstrated the utility of HLA-A2.1 transgenic mice as an *in vivo* model for identifying peptides presented in association with class I MHC. Independent identifications of epitopes in transgenic mice and humans, confirm that CTL from HLA transgenic mice can respond to the same peptides as humans^{127 128}. Here we have taken a two-step approach to the identification of human cdr2

epitopes: first, screening all possible cdr2-derived nonamer epitopes for HLA-A2.1 binding, followed by screening in HLA-A2.1 transgenic mice to identify those which are naturally processed. Using this approach we have identified two naturally processed and presented HLA-A2.1-restricted CTL epitopes of the human PCD antigen cdr2. Furthermore, we found circulating CD8+ T cells specific for these epitopes in the peripheral blood of 2 A2.1+ PCD patients but not in 4 A2.1 + control individuals, which suggests that cdr2(289-297) and cdr2(290-298) are bona fide tumor epitopes associated with gynecologic tumor immunity.

cdr2(289-297) is a naturally processed A2.1-restricted CTL epitope

Cdr2(289-297) was previously shown to be targeted by PCD patient CTL. The study by Albert et al.⁴⁶ demonstrated that CTL from the peripheral blood of A2.1+ PCD patients could specifically lyse cdr2(289-297) peptide-pulsed T2 cells after 7 days of in vitro restimulation with autologous peptide-pulsed DCs or, in some cases, directly ex vivo. Cdr2 peptide-specific CTL were not detected in 5 HLA-A2.1+ control individuals, suggesting that these responses were specific to PCD patients. However, the ability of these CTL to recognize tumor cells endogenously expressing cdr2 was not tested, thereby leaving unresolved the issue of whether these peptides are bona fide tumor associated CTL epitopes.

By isolating and analyzing a human cdr2(289-297)-specific murine CTL line (HHD 289), we have established that the cdr2(289-297) peptide is naturally

processed and presented by both murine and human cdr2-expressing A2.1+ cells. HHD 289 CTL specifically recognized HHD KECs infected with Ad-hcdr2 and Ad-mcdr2 as well as, to a lesser extent, physiologic levels of cdr2 in the human and murine tumor cell lines HeLa.AAD, MCF7.AAD, and B16.AAD. Therefore, we can conclude that the cdr2(289-297)-specific CD8+ T cells detected in the peripheral blood of PCD patients, both here and in the Albert study, are bona fide tumor reactive lymphocytes.

Evidence for partial tolerance to cdr2

In the process of establishing the HHD 289 CTL line, we obtained evidence of self-tolerance to cdr2. The level of immunologic tolerance to self-proteins appears to be related to the level and location of antigen expression, but is best determined by empiric experimentation. Most identified tumor associated antigens are also expressed at low levels in normal tissues^{3,129} or, due to the autoimmune regulator (AIRE) transcription factor¹³⁰, in the thymus. However, consideration of the robust anti-tumor and autoimmune responses evident in PCD patients and the tight restriction of cdr2 expression to “immune privileged” tissues⁵¹, led us to hypothesize that cdr2 would be treated immunologically as a foreign antigen after it became expressed in tumors. Thus, we expected that the human cdr2(289-297) peptide, which had a relatively high affinity for HLA-A2.1 in our assays, would be immunogenic in HLA-A2.1 transgenic mice, since work from investigators studying non-self antigens such as viral peptides suggests that such a correlation exists¹¹⁴.

Unexpectedly, we observed a lack of responsiveness to human cdr2(289-297) in cdr2-immunized mice. This was not due to poor priming of the A2.1 transgenic mice; we were able to elicit strong responses to both FluM1 and to nonhomologous cdr2 peptides, but not to cdr2(289-297), by the same immunization regimens in 3 different strains of A2.1 transgenic mice.

One effect tolerance can have on the immune system is to purge the repertoire of T cells that recognize self epitopes with high avidity, while sparing those with low avidity for the same epitopes^{131,132,133}. We observed this to be the case with the mouse from which the HHD 289 CTL line was derived. Although we could isolate a cdr2(289-297)-specific CTL line (HHD 289) and demonstrate that these cells recognize endogenous naturally processed overexpressed cdr2 in Ad-infected HHD KECs, we observed only minimal staining with A2.1/cdr2(289-297) tetramers, indicating that only a small percentage of HHD 289 CTL were high avidity cells capable of CD8-independent recognition of cdr2(289-297). Furthermore, HHD 289 CTL demonstrated weak recognition of physiologic levels of endogenous cdr2 in B16.AAD, HeLa.AAD, and MCF7.AAD tumors. A likely reason for the impaired human cdr2(289-297)-specific responses became apparent in our experiments: HLA-A2.1-restricted CTLs specific for human cdr2(289-297) can cross-react with the homologous naturally processed and presented murine peptide. As a result, any tolerance to the murine peptide would preclude the generation of high avidity CTL specific for the homologous human peptide. Given our inability to prime high avidity human cdr2(289-297)-

specific CTL in A2.1 transgenic mice, we conclude that mice are at least partially tolerized to cdr2.

Implications of tolerance to cdr2

Based on the above findings, we can infer that human cdr2 epitope screening in A2.1 transgenic mice will not yield reliable information about immunogenicity in cases where the human and murine sequences are identical, since it will be difficult to determine whether the lack of a CTL response is a consequence of low immunogenicity of the peptide or of self tolerance to cdr2. It will be interesting to determine whether the impact of self tolerance is as pronounced for murine-homologous human cdr2 peptides which bind poorly to HLA-A2.1, such as human cdr2(355-363) peptide. Cdr2(355-363) will be a particularly interesting example to study because it is targeted by A2.1+ PCD patient CTL, but has yet to be confirmed as a naturally processed and presented epitope.

Definitive demonstration of self tolerance to cdr2 will require analysis of T cell responses to cdr2(289-297) in A2.1/cdr2 null mice (currently being made by Graeme Couture and Kevin O'Donovan in our laboratory). These mice will allow us to distinguish immunological tolerance from other mechanisms that may account for the poor immunogenicity of cdr2(289-297) including inefficient antigen processing¹³⁴⁻¹³⁶, or insufficiencies in the T cell repertoire not deriving from central or peripheral tolerance mechanisms. If immunologic tolerance indeed plays a major role in poor reactivity to cdr2, HLA-A2.1/cdr2 null mice

will become invaluable reagents for the generation and isolation of high avidity cdr2(289-297)-specific T cells from which we can isolate potentially therapeutic TCR (as discussed in Chapter 4).

Our observation of possible tolerance to cdr2 in mice suggests that we may need to reconsider the assumptions about how immunity is generated to the cdr2 antigen in PCD patients in particular, and to other onconeural antigens in PND patients in general. The generally accepted dogma is that onconeural antigens such as cdr2 are seen by the immune system as “foreign” proteins when they are expressed in systemic tumors. In this study we have observed that ectopic expression of cdr2 in the context of an inflammatory immunization regimen is not sufficient to trigger a CTL response. This suggests that ectopic expression of cdr2 in a tumor may not be sufficient to trigger immunity to this antigen, a finding that is supported by the apparent widespread expression of cdr2 in breast and ovarian tumors of patients without any apparent tumor immunity. Based on our findings, the reason for the unresponsiveness to cdr2 in neurologically normal cancer patients may be tolerance to this antigen rather than ignorance.

cdr2(290-287) is a naturally processed A2.1-restricted CTL epitope

In contrast to the unresponsiveness of cdr2-immunized A2.1 transgenic mice to human cdr2(289-297), we could consistently prime high avidity human cdr2(290-298)-specific CTL. This was most likely due to the differences between human

cdr2(290-298) peptide and its murine homologue, suggested by the absence of cross recognition of murine cdr2(289-298) by AAD 290 CTL. Taken together, these results demonstrate the importance of targeting human cdr2 peptides that are nonhomologous to mouse/self cdr2 for the purpose of generating high avidity T cell lines from which high affinity TCR might be isolated. AAD 290 CTL were of high enough avidity to recognize cdr2(290-298) in a CD8-independent manner, as determined by their ability to both bind to A2.1/cdr2(290-298) tetrameric complexes and to recognize endogenous overexpressed cdr2 in Ad-hcdr2 infected A2.1 AAA KECs. AAD 290 CTL were also able to recognize and kill A2.1+ tumor cell lines expressing physiologic levels of cdr2. However the efficiency of this recognition of lower (physiologic) levels of cdr2 antigen was dependent on the avidity boost provided by the murine a3 domain on the AAD molecule which can interact with murine CD8, thus highlighting the contribution made by the CD8 coreceptor to target recognition by CTL.

Detection of cdr2(289-297) and cdr2(290-298)-peptide binding CD8+ T cells in the peripheral blood of A2.1+ PCD patients

Having demonstrated that cdr2(289-297) and cdr2(290-298) are naturally processed A2.1 restricted epitopes, we next used A2.1/cdr2 tetramers to examine the peripheral blood of patients with clinically documented anti-gynecologic tumor immunity for cells of this specificity. The use of tetramers is particularly attractive because it allows a direct quantitation of Ag specific cells from the

blood without the need for their *in vitro* expansion^{137,66}. In 2 out of 2 A2.1+ PCD patients tested, we observed significant populations of either cdr2(289-297) or cdr2(290-298)-specific CD8+ T cells suggesting that cdr2(289-297) and (290-298) are bona fide tumor antigens. Interestingly, these tetramer staining CD8+ T cells did not appear to be making IFN- γ , suggesting that they may be anergic. We did not test these cells for antigen specific production of other cytokines such as TNF- α and GM-CSF, and thus cannot rule out the possibility that these are memory T cells producing cytokines other than IFN- γ . Nevertheless, direct IFN- γ ELISPOT assay appears to not be a reliable strategy for detecting cdr2-specific cells in the peripheral blood of patients with PCD. This is true for other PNDs as well (Wendy Roberts, unpublished observation), and corroborates the findings of another group who was unable to detect cdr2(289-297)-specific T cells in A2.1+ PCD patients by IFN- γ ELISPOT assay¹³⁸. These results suggest that perhaps only a fraction of PCD patients, such as those in the acute state of their disease, have CTL that can be detected without an *in vitro* restimulation step⁴⁶ and underscore the value of tetramer reagents for evaluating PCD patients for cdr2-specific CD8+ T cells directly *ex vivo*.

The finding that 60% of ovarian tumors and 25% of breast tumors from neurologically normal individuals express cdr2 suggests that ectopic expression of this “immune privileged” antigen is not sufficient to trigger tumor immunity. Our observation of possible tolerance to this antigen in mice suggests that unresponsiveness to cdr2 in neurologically normal cancer patients may be

due to tolerance to this antigen rather than ignorance. Do neurologically normal breast and ovarian cancer patients have circulating cdr2-specific CD8⁺ T cells and, if so, do they express markers characteristic of naïve or anergic cells? The epitopes that we have identified and the A2.1/cdr2 tetramer reagents that we have validated in this study will allow us to address this important question. In sum, the findings presented in this chapter will allow us to take the next step towards understanding the naturally occurring tumor immunity in PCD and how it might be applied to the general population of cancer patients.

Acknowledgements

Wendy Roberts performed the patient tetramer staining. Ashby Thomas and Travis Williams performed the iTopia peptide screen. Francois Lemonnier provided the HHD mice. Victor Engelhard provided the B16.AAD, MCF7, and COV413 cell lines as well as the AAD plasmid. Mark Dudley provided T cell culture protocols, cloning advice, and invaluable cheerleading.

CHAPTER 4. ISOLATION AND CHARACTERIZATION OF A HUMAN CDR2-SPECIFIC T CELL RECEPTOR

Introduction

Background

A major hurdle for tumor immunotherapy, other than the identification of tumor rejection antigens to target, is the generation of tumor antigen-specific T cells in tumor bearing hosts. These T cells need to be functional, exhibit high avidity^{59,60}, and their numbers must reach levels above the threshold required to mediate regression of established tumors^{61,62}. Generation of memory cells to prevent tumor recurrence is also desirable. However, because most TAA are nonmutated self antigens, the TAA-specific T cell repertoire is generally small and of low avidity. ACT protocols have shown some promise, but one large hurdle for ACT is its reliance on the successful isolation and expansion tumor reactive lymphocytes (usually tumor infiltrating lymphocytes or TIL^{82,83}) pre-existing in the patient. Even in the case of melanoma, there are many patients for whom T cells of the appropriate anti-tumor specificity and avidity cannot be isolated, even after immunization⁸⁴. Isolating TIL or tumor reactive lymphocytes for solid tumors such as breast and ovarian carcinoma is even more difficult.

Replacement of a tolerized endogenous T cell compartment is theoretically possible through the combination of adoptive T cell transfer (ACT) and TCR gene transfer. Potentially therapeutic TCR can be isolated from a number of

sources including patients with the same tumor type who have undergone clinical regression following vaccination or adoptive T cell therapy, an allogeneic T cell repertoire^{94,95} or from HLA-A2.1 transgenic mice^{96,97}, when the autologous repertoire is tolerant⁹⁸.

Advantages of murine anti-human TCR

HLA-A2.1 transgenic mice may be a particularly good source of therapeutic TCRs; high-affinity TCRs specific for self/tumor antigens can be generated in by immunizations which take advantage of nonhomology between human and mouse sequences^{89,96}. Murine TCR are of sufficient structural homology to human TCR that they can be incorporated into the human CD3 complex⁸⁹ and can rescue surface expression in mutant T cells¹³⁹. Gene transfer of HLA-A2.1-transgenic mice-derived TCR into human T cells has been shown to circumvent self tolerance to the tumor-associated self/tumor antigens murine double minute 2 (MDM2)⁸⁹ and p53^{96,98}. Moreover, in the recent study by Kuball et al., it was demonstrated that a murine TCR of high enough affinity to function in a CD8 coreceptor-independent fashion could be used to generate human MHC class I restricted CD4+ helper (Th) T cells⁹⁶. The provision of cognate CD4 help is likely to be of great importance to the success of CD8+ CTL-based immunotherapy^{140,141,142,143}. Although most tumors lack MHC class II, precluding direct attack by CD4 Th cells, antigen specific Th activity has been shown to be important for efficient eradication of malignancies^{144,145}. CD4 Th cells can exert their antitumor effect independently of CD8+ CTL by recruiting innate immune

and nonimmune effectors to the tumor site^{144,146}, by crosstalk to CTL through cytokine release^{140,141}, and by interaction with dendritic cells or other professional APC^{147,148}. Furthermore, the collaboration between CD4+ Th cells and CD8+ CTL seems to be particularly important for the generation of memory T cells¹⁴⁹. The recent study by Kuball et al. demonstrated that genetically engineered MHC class I-restricted CD4+ T cells could act cooperatively and synergistically with CD8+ T cells via dendritic cell intermediates and tumor targets. Therefore, given the relative ease of isolating high-affinity CD8-independent TCR from HLA-A2.1 transgenic mice (taking advantage of either nonhomology between human and murine TAA sequences, or by using TAA null mice), this study presents an innovative way to circumvent a pervasive problem, namely that the generation of tumor antigen-specific CD4+ helper T cells is limited by the paucity of known MHC class II-restricted tumor epitopes and by the lack of MHC class II molecules on tumor cells.

There is also some indication that transferred murine TCR function better in human cells than do their human counterparts. Cohen et al. had observed that transduction of human lymphocytes with p53-specific murine TCR conferred enhanced tumor reactivity compared to other human TCR^{93,97,150}. They hypothesized that this was because the introduced murine TCR are less likely than their human counterparts to mispair with the endogenous human TCR chains. Indeed it has been shown that unlike the generation of TCR-transgenic mice where allelic exclusion limits the formation of such heterodimers^{151,152},

transfer of exogenous TCR chains into mature T cells, which already express endogenous $\alpha\beta$ TCR chains, can lead to mispairing with the pre-existing endogenous TCR chains¹⁵³⁻¹⁵⁵. The major determinants for pairing of the TCR α - and β - subunits are thought to be located within the constant domains and these domains are invariant enough that the assembly machinery is not able to distinguish endogenous and exogenous TCR. The formation of mixed heterodimers could both impede the generation of cells of the desired tumor reactivity and generate cells with new undesired specificity.

A recent study¹⁵⁶ explored the molecular basis of the apparent superior performance of murine TCR in human cells by creating hybrid murine and human TCRs by swapping the original constant regions with either human or mouse ones, respectively. TCR with mouse constant regions functioned better in human cells than those with human constant regions; they were overexpressed on the surface of human lymphocytes relative to the human or humanized TCR and they conferred greater tumor reactivity. They also observed that transfer of murine or “murinized” TCR resulted in increased stability of the TCR/CD3 ζ complex¹⁵⁶. Therefore, this study provides another example of how TAA-specific TCR of murine origin could be superior to human TCR in the clinical setting.

A closer look at TCRs

On a molecular level, the TCR represents the direct link between the effector T cells and the target cell¹⁵⁷. T cell specificity is dictated by two membrane bound

chains, which are 280-310 amino acids in length and linked by a disulfide bond. These chains are denoted as TCR α - and TCR β -subunits in the majority of T cells. Signaling through the TCR depends on the interaction of the variable TCR α - and β -chains with the invariant CD3 $\gamma/\delta/\epsilon$ and CD3 ζ chains. TCR α and β chains consist of a variable (V) segment, a joining (J) segment, and a constant (C) region with the β chain also containing a diversity (D) region. Germline rearrangements occurring within the TCR α and TCR β loci during T cell development randomly join different V-J or V-D-J regions into a single transcriptional unit. The majority of the TCR diversity is the result of the random insertion or deletion of nucleotides at the junctions between the V and J segments for the α chain, and between the V and D and the D and J segments for the β chain. It is these V-J and V-D-J junctions of the α and β chains respectively that encode the third complementarity determining region (CDR3), the part of the heterodimeric TCR α/β complex that specifically recognizes MHC-presented antigen.

The variable domains for the α - and β -chains can be classified into distinct subfamilies which are often used to identify T cell populations. They can be also be used to clone TCRs by creating degenerate primers to cover the whole panel of more than 20 subfamily sequences of each TCR chain. A more straightforward approach that allows amplification and identification of TCR genes in the absence of 5' sequence information is to use linker ligation and rapid amplification of cDNA ends (RACE-PCR). In this approach, an RNA oligonucleotide is first ligated to the 5' end of dephosphorylated, decapped

mRNA to create a universal priming site at the 5' end for reverse transcription to cDNA. PCR is then performed using a primer specific for the ligated oligonucleotide at the 5' end and gene-specific primers for each of the three possible C regions (C α , C β 1, and C β 2) at the 3' end. This is the approach we chose for cloning a cdr2-specific TCR in this chapter because it eliminates the need for multiple V-region-specific primers and also minimizes the likelihood of amplifying truncated products.

Specific Aims of this Chapter

In the previous chapter we identified two tumor associated CTL epitopes for human cdr2. In addition, we demonstrated the importance of targeting human cdr2 peptides that are nonconserved between mouse/self cdr2 for generating a high avidity T cell lines in HLA-A2.1 transgenic mice. We observed that the AAD 290 CTL line, specific for a human cdr2 peptide which is nonhomologous to murine cdr2, was of significantly higher avidity than the HHD 289 CTL line, although both were capable of recognizing naturally processed human cdr2 and both were shown to be bona fide targets in PCD patients. The AAD 290 CTL line was capable of CD8-independent recognition of endogenous cdr2 in Ad-hcdr2 infected AAA A2.1 KECs, which suggested that it may be a good source of cdr2-specific TCRs for human therapy.

For the studies discussed in this chapter, we isolated several cdr2(290-298)-specific CTL clones by limiting dilution of the AAD 290 CTL line. While

literature states the contrary^{158,159}, we observed that the structural avidity of several of the clones did not correlate with functional avidity. We isolated the TCR genes from two cdr2(290-298)-specific clones, clone 11 and clone 12, on the basis of their demonstrating high functional avidity and intermediate structural avidity relative to the other clones. Nucleotide sequence analysis demonstrated that the rearranged TCR genes from these clones were identical. Here we provide a careful functional analysis of the cloned cdr2-specific TCR genes, and demonstrate their ability to transform unresponsive human CD8⁺ T cells into efficient cdr2-specific CTLs. The results presented in this chapter confirm the relevance of circulating CD8⁺ T cells of this receptor specificity in PCD patients, and highlight the therapeutic potential of cdr2(290-298)-specific TCR for breast and ovarian cancer immunotherapy.

Results

The AAD 290 CTL line was cloned by limiting dilution after 4 rounds of in vitro stimulation. Twenty one positive wells from the lowest cloning dilutions were picked. Eleven clones were initially analyzed by IFN- γ ELISPOT with peptide pulsed T2 cells (data not shown), and the eight most reactive clones were expanded for further analysis.

Analysis of structural and functional avidity of CTL clones derived from the AAD290 line.

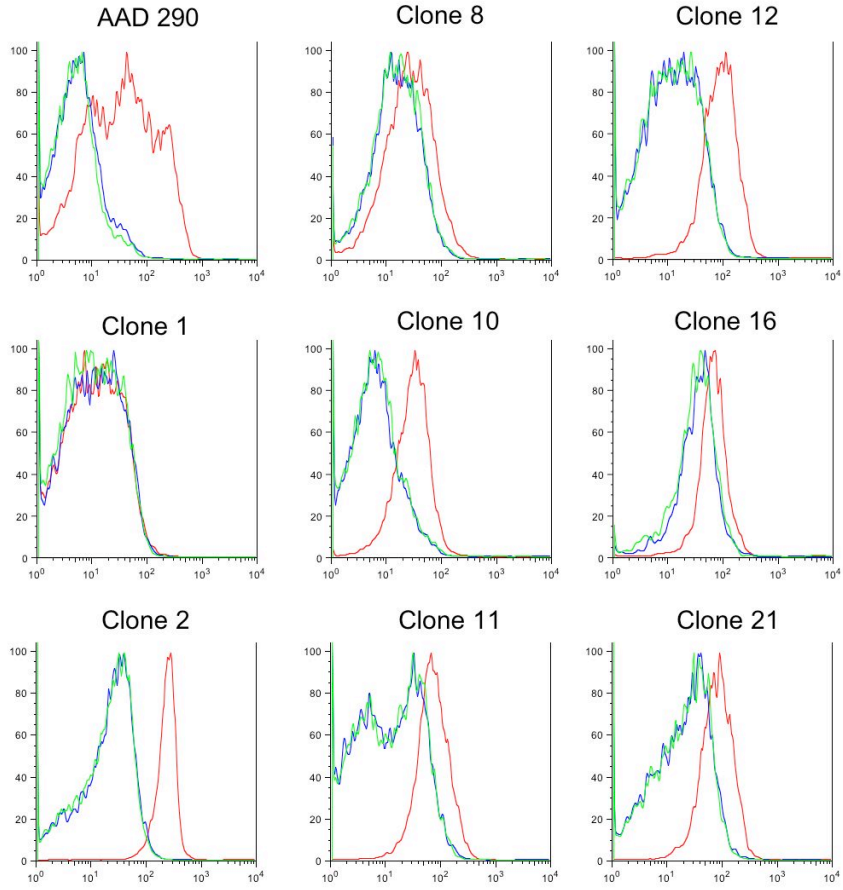
The intensity of tetramer staining has been accepted as a direct measure of the affinity of TCR for the MHC-peptide complex¹⁵⁸⁻¹⁶⁰ and has been extrapolated to be an indication of the actual sensitivity of the CTL response to MHC-peptide antigen density. Following this rationale, we expected to observe a relationship between the staining intensity of the cdr2(290-298) clones with A2.1/cdr2(290-298) tetramers and their ability to secrete IFN- γ in response to coculture with cdr2-expressing targets. The broad tetramer staining intensity range of the parental AAD 290 CTL line observed by FACS analysis (Figure 23) suggested that the daughter clones could have a range of structural and functional avidities. As predicted, a broad range of structural avidities was observed among the 8 clones tested, however, we observed a striking discordance between the tetramer staining profiles of the clones (Figure 23), and their functional avidity as measured by IFN- γ ELISPOT responses to cdr2-expressing tumor cells (Figure 24). The clone exhibiting the highest tetramer staining, clone 2 (Figure 23), responded relatively poorly to cdr2-expressing tumors in an IFN- γ ELISPOT assay (Figure 24). Conversely, clone 1, which exhibited almost no specific tetramer staining (Figure 23), demonstrated the highest functional avidity by IFN- γ ELISPOT (Figure 24). Total RNA was prepared from all eight clones as well as from the parental AAD 290 line. Clones 11 and 12 were chosen for further analysis because they both demonstrated functional avidity greater than the

parental AAD CTL line while still maintaining intermediate tetramer binding (Figures 23 and 24).

Figure 23. Structural Avidity of the AAD290 CTL line and daughter cdr2(290) clones; binding to A2.1 tetramers.

The AAD 290 CTL line (after the 6th in vitro restimulation) and eight daughter CTL clones were rested for 9 days after their last restimulation and CD8+ T cells were purified by negative selection (MACS; Miltenyi Biotec). Following Fc blocking for 20 min on ice, 10⁵ purified cells per condition were stained at room temperature for 20 minutes with A2.1/cdr2(290-298) tetramer (red line) or with the negative control tetramers A2.1/cdr2(289-297) (blue line) and A2.1/FluM1(58-66) (green line), washed with FACS buffer, and read immediately on a FACSCaliber flow cytometer.

— 290
— 289
— FluM1



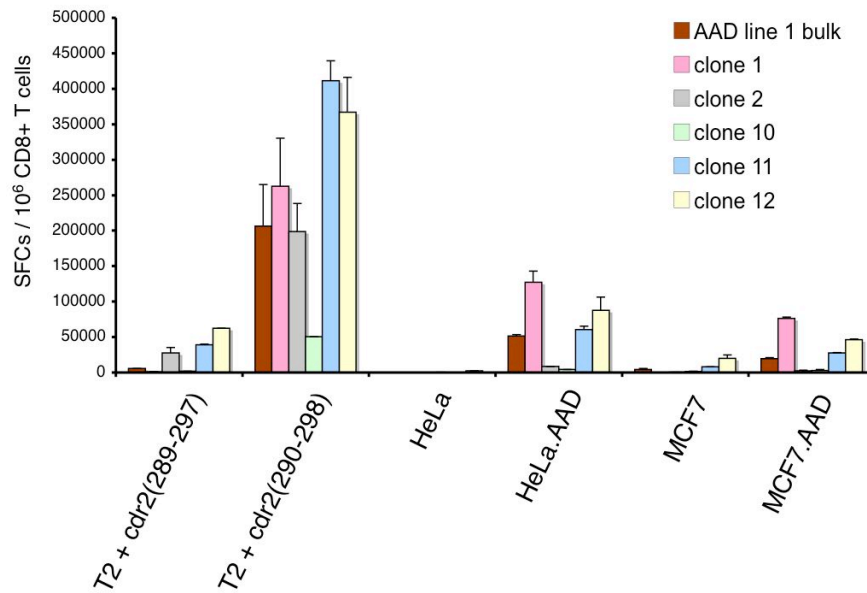


Figure 24. Functional avidity of cdr2(290) clones.

The AAD 290 CTL line (6th restimulation) and 5 daughter clones were rested for 10 days after their last restimulation and CD8⁺ T cells were isolated by negative selection (MACS; Miltenyi Biotec). CD8⁺ T cells were cocultured with the human cdr2(289-297) or cdr2(290-298) peptide-pulsed T2 cells (10⁻⁶ M) or cdr2-expressing tumor cells in an 18 h IFN- γ ELISPOT assay. Values, representing spot forming cells per million CD8⁺ T cells, are the average of triplicate wells; error bars indicate standard deviation.

Isolation and cloning of Murine TCR

We chose a straightforward unbiased approach to cloning the clone 11 and clone 12 TCR genes which was to isolate the full length coding sequences of the TCR genes by rapid amplification of cDNA ends (RACE-PCR). 32 α -chain and 32 β -chain RACE-PCR products, generated from 2 separate PCR reactions for each T cell clone, were sequenced. Sequence analysis demonstrated that clone 11 and clone 12 expressed an identical TCR. Variable (V) region sequence analysis was performed using the IMGT database (IMGT, the international ImMunoGeneTics information system® <http://imgt.cines.fr> (founder and director: Marie-Paule Lefranc, Montpellier, France)¹⁶¹⁻¹⁶³. Analysis revealed that both T cell clones were TRV α 14D-1*01J42*01C α (V α 2.2); CDR3 CAASGASGGSSNAKLTF and TRV β 12-1*01J2-1*01C β 2 (V β 5.2); CDR3 CASSLGGWAEQFF. The full nucleotide and deduced amino acid sequences of the α - and β - TCR genes are presented in Figures 25 and 26, respectively. V β profiling on clone 11 was performed to verify its clonality as well as to provide confirmation of the sequence analysis (Figure 27).

Figure 25. Nucleotide and deduced amino acid sequence of the cdr2 TCR α chain

The constant region is boxed in red. The CDR3 domain of the variable region is underlined in green. The V gene segment (unboxed) was classified according to IMGT, the international ImMunoGeneTics information system® <http://imgt.cines.fr> (founder and director: Marie-Paule Lefranc, Montpellier, France) and was determined to be TRV α 14D-1*01J42*01; CDR3 sequence: CAASGASGGSNAKLTF.

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·   ATG GAC AAG ATT CTG ACA GCA TCA TTT TTA CTC CTA GGC CTT CAC CTA GCT
·   M  D  K  I  L  T  A  S  F  L  L  L  G  L  H  L  A

·   GGG GTG AAT GGC CAG CAG AAG GAG AAA CAT GAC CAG CAG CAG GTG AGA CAA
·   G  V  N  G  Q  Q  K  E  K  H  D  Q  Q  Q  V  R  Q

·   AGT CCC CAA TCT CTG ACA GTC TGG GAA GGA GGA ACC ACA GTT CTG ACC TGC
·   S  P  Q  S  L  T  V  W  E  G  G  T  T  V  L  T  C

·   AGT TAT GAG GAC AGC ACT TTT AAC TAC TTC CCA TGG TAC CAA CAG TTC CCT
·   S  Y  E  D  S  T  F  N  Y  F  P  W  Y  Q  Q  F  P

·   GGG GAA GGC CCT GCA CTT CTG ATA TCC ATA CTT TCA GTG TCC GAT AAA AAG
·   G  E  G  P  A  L  L  I  S  I  L  S  V  S  D  K  K

·   GAA GAT GGA CGA TTC ACA ACC TTC TTC AAT AAA AGG GAG AAA AAG CTC TCC
·   E  D  G  R  F  T  T  F  F  N  K  R  E  K  K  L  S

·   TTG CAC ATC ATA GAC TCT CAG CCT GGA GAC TCA GCC ACC TAC TTC TGT GCA
·   L  H  I  I  D  S  Q  P  G  D  S  A  T  Y  F  C  A

·   GCA AGT GGG GCT TCT GGA GGA AGC AAT GCA AAG CTA ACC TTC GGG AAA GGC
·   A  S  G  A  S  G  G  S  N  A  K  L  T  F  G  K  G

·   ACT AAA CTC TCT GTT AAA TCA AAC ATC CAG AAC CCA GAA CCT GCT GTG TAC
·   T  K  L  S  V  K  S  N  I  Q  N  P  E  P  A  V  Y

·   CAG TTA AAA GAT CCT CGG TCT CAG GAC AGC ACC CTC TGC CTG TTC ACC GAC
·   Q  L  K  D  P  R  S  Q  D  S  T  L  C  L  F  T  D

·   TTT GAC TCC CAA ATC AAT GTG CCG AAA ACC ATG GAA TCT GGA ACG TTC ATC
·   F  D  S  Q  I  N  V  P  K  T  M  E  S  G  T  F  I

·   ACT GAC AAA ACT GTG CTG GAC ATG AAA GCT ATG GAT TCC AAG AGC AAT GGG
·   T  D  K  T  V  L  D  M  K  A  M  D  S  K  S  N  G

·   GCC ATT GCC TGG AGC AAC CAG ACA AGC TTC ACC TGC CAA GAT ATC TTC AAA
·   A  I  A  W  S  N  Q  T  S  F  T  C  Q  D  I  F  K

·   GAG ACC AAC GCC ACC TAC CCC AGT TCA GAC GTT CCC TGT GAT GCC ACG TTG
·   E  T  N  A  T  Y  P  S  S  D  V  P  C  D  A  T  L

·   ACT GAG AAA AGC TTT GAA ACA GAT ATG AAC CTA AAC TTT CAA AAC CTG TCA
·   T  E  K  S  F  E  T  D  M  N  L  N  F  Q  N  L  S

·   GTT ATG GGA CTC CGA ATC CTC CTG CTG AAA GTA GCC GGA TTT AAC CTG CTC
·   V  M  G  L  R  I  L  L  L  K  V  A  G  F  N  L  L

·   ATG ACG CTG AGG CTG TGG TCC AGT
·   M  T  L  R  L  W  S  S

```

Figure 26. Nucleotide and deduced amino acid sequence of the cdr2 TCR β chain

The constant region is boxed in red. The CDR3 domain of the variable region is boxed in green. The V gene segment (unboxed) was classified according to IMGT, the international ImMunoGeneTics information system® <http://imgt.cines.fr> (founder and director: Marie-Paule Lefranc, Montpellier, France) and was determined to be TRV β 12-1*01J2-1*01; CDR3 sequence: CASSLGGWAEQFF. The constant region is C β 2.

```

·   ATG TCT AAC ACT GTC CTC GCT GAT TCT GCC TGG GGC ATC ACC CTG CTA TCT
·   M S N T V L A D S A W G I T L L S

·   TGG GTT ACT GTC TTT CTC TTG GGA ACA AGT TCA GCA GAT TCT GGG GTT GTC
·   W V T V F L L G T S S A D S G V V

·   CAG TCT CCA AGA CAC ATA ATC AAA GAA AAG GGA GGA AGG TCC GTT CTG ACG
·   Q S P R H I I K E K G G R S V L T

·   TGT ATT CCC ATC TCT GGA CAT AGC AAT GTG GTC TGG TAC CAG CAG ACT CTG
·   C I P I S G H S N V V W Y Q Q T L

·   GGG AAG GAA TTA AAG TTC CTT ATT CAG CAT TAT GAA AAG GTG GAG AGA GAC
·   G K E L K F L I Q H Y E K V E R D
·
·   AAA GGA TTC CTA CCC AGC AGA TTC TCA GTC CAA CAG TTT GAT GAC TAT CAC
·   K G F L P S R F S V Q Q F D D Y H

·   TCT GAA ATG AAC ATG AGT GCC TTG GAA CTG GAG GAC TCT GCT ATG TAC TTC
·   S E M N M S A L E L E D S A M Y F

·   TGT GCC AGC TCT CTC GGA GGA TGG GCT GAG CAG TTC TTC GGA CCA GGG ACA
·   C A S S L G G W A E Q F F G P G T

·   CGA CTC ACC GTC CTA GAG GAT CTG AGA AAT GTG ACT CCA CCC AAG GTC TCC
·   R L T V L E D L R N V T P P K V S

·   TTG TTT GAG CCA TCA AAA GCA GAG ATT GCA AAC AAA CAA AAG GCT ACC CTC
·   L F E P S K A E I A N K Q K A T L

·   GTG TGC TTG GCC AGG GGC TTC TTC CCT GAC CAC GTG GAG CTG AGC TGG TGG
·   V C L A R G F F P D H V E L S W W

·   GTG AAT GGC AAG GAG GTC CAC AGT GGG GTC AGC ACG GAC CCT CAG GCC TAC
·   V N G K E V H S G V S T D P Q A Y

·   AAG GAG AGC AAT TAT AGC TAC TGC CTG AGC AGC CGC CTG AGG GTC TCT GCT
·   K E S N Y S Y C L S S R L R V S A

·   ACC TTC TGG CAC AAT CCT CGA AAC CAC TTC CGC TGC CAA GTG CAG TTC CAT
·   T F W H N P R N H F R C Q V Q F H

·   GGG CTT TCA GAG GAG GAC AAG TGG CCA GAG GGC TCA CCC AAA CCT GTC ACA
·   G L S E E D K W P E G S P K P V T

·   CAG AAC ATC AGT GCA GAG GCC TGG GGC CGA GCA GAC TGT GGA ATC ACT TCA
·   Q N I S A E A W G R A D C G I T S

·   GCA TCC TAT CAT CAG GGG GTT CTG TCT GCA ACC ATC CTC TAT GAG ATC CTA
·   A S Y H Q G V L S A T I L Y E I L

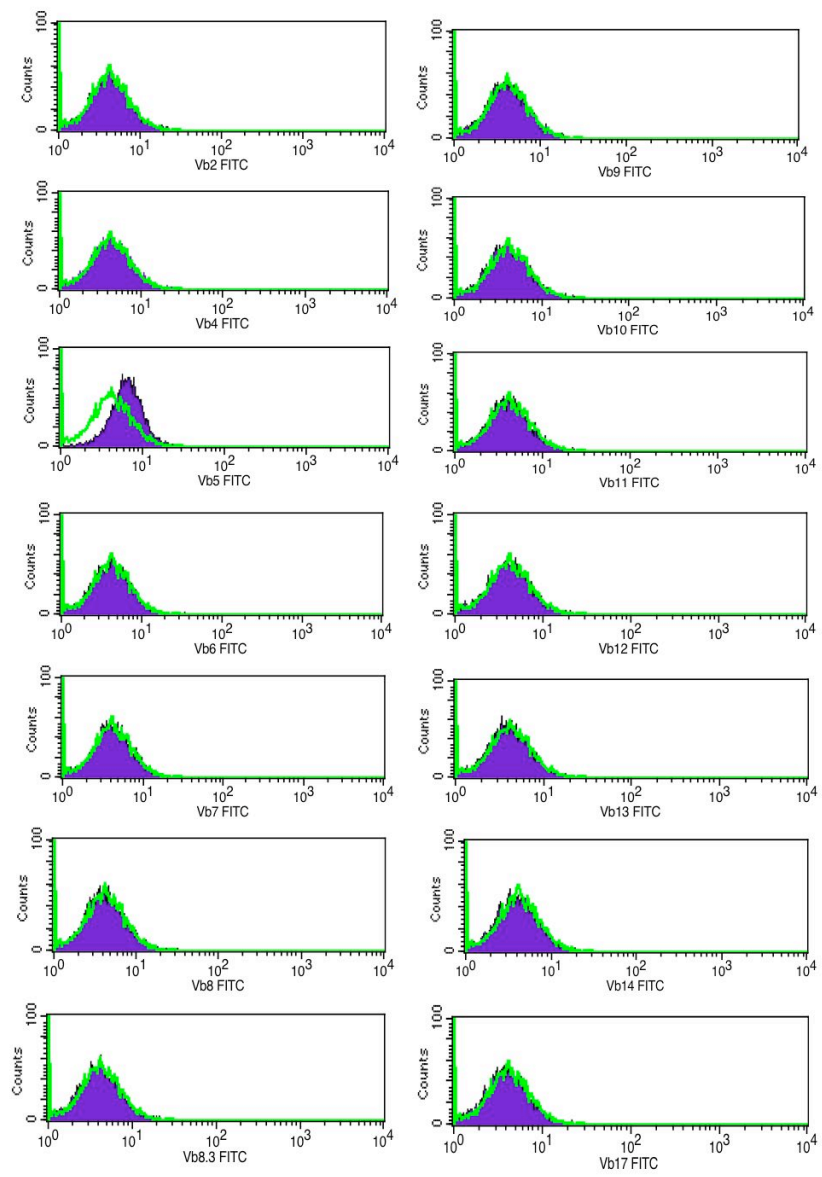
·   CTG GGG AAG GCC ACC CTA TAT GCT GTG CTG GTC AGT GGC CTG GTG CTG ATG
·   L G K A T L Y A V L V S G L V L M

·   GCC ATG GTC AAG AAA AAA AAT TCC TGA
·   A M V K K K N S *

```

Figure 27. Assessment of the TCR V β composition in Clone 11 CTL by flow cytometry

To confirm the TCR sequence analysis, CD8-purified clone 11 CTL were analyzed by staining with a panel of V β -subfamily domain antibodies (purple filled) or isotype control antibody (green line) followed by flow cytometry. Clone 11 CTL were determined to express only V β 5, thus providing independent confirmation of both the clonality of the population and the results of the sequencing.

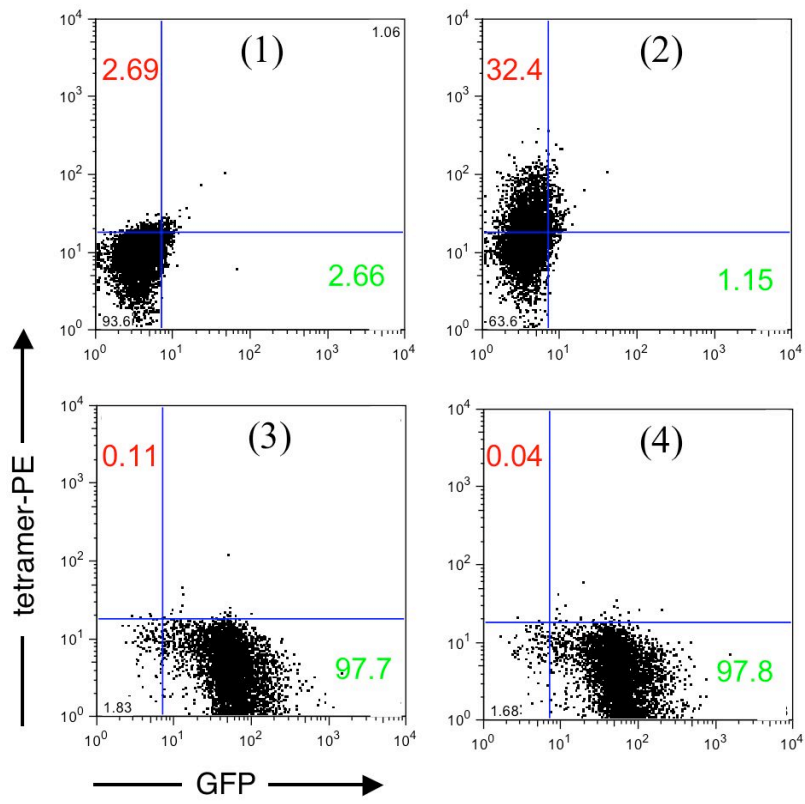


Recognition of peptide-pulsed and human cdr2-transduced cells by TCR-electroporated human CD8+ T cells.

We tested the ability of the cloned α - and β - chains to form functional TCR $\alpha\beta$ heterodimers by electroporating in vitro transcribed RNA into primary human lymphocytes, a method previously demonstrated to yield high transfection efficiencies with low transfection-related toxicity¹⁶⁴. Over 32% of CD8+ human T cells co-electroporated with α - and β -chain mRNA formed cell surface heterodimers as measured by staining with HLA-A2.1/cdr2(290-298) tetramers 12 hours after electroporation (Figure 28). This probably represented close to the maximum transfection efficiency since over 97% of CD8+ human T cells electroporated in parallel with GFP mRNA expressed GFP protein by flow cytometry in the same time period. Negative control tetramer did not stain either of the electroporated T cell populations, and GFP electroporated cells did not stain with the HLA-A2.1/cdr2(290-298) tetramer.

Figure 28. Efficiency of RNA electroporation of Human CD8+ T cells.

PBLs stimulated with OKT3 Ab plus IL-2 for 3 days underwent CD8 purification (MACs, Miltenyi Biotec) followed by electroporation 10 days later with in vitro-transcribed RNA at $2 \mu\text{g}/1 \times 10^6$ cells. cdr2 TCR α - and β - chain and GFP RNAs were generated by in vitro transcription of PCR-amplified templates bearing T7 promoter and poly(A) tail at the 5' and 3' end, respectively. Twelve hours after electroporation, cdr2 TCR or GFP expression was determined by FACS analysis. Human CD8+ T cells that had been coelectroporated with the α - and β - chains of the cdr2 TCR were stained at room temperature for 20 min in human FACS buffer containing 0.02% sodium azide, with a non-specific negative tetramer (1), or with HLA-A2.1/cdr2(290-298) tetramer (2). GFP RNA electroporated T cells in (3) and (4) served as a positive control for RNA electroporation and as a negative control for staining with the negative tetramer (3) and HLA-A2.1/cdr2(290-298) tetramer (4). The percentage of cells staining with tetramer (red) or expressing GFP (green) was as shown.



To determine whether TCR-electroporated lymphocytes could mediate the release of the effector cytokine IFN- γ , CD8⁺ T cells were electroporated with the cdr2 TCR α - and β -chain RNAs and then cocultured with T2 cells that had been pulsed with different concentrations of specific (cdr2(290-298) and cdr2(289-298) or control (FluM1 and cdr2(289-297)) peptides to assess the avidity of the electroporated PBL. cdr2(290-298) and cdr2(289-298)-specific IFN- γ release was detected in cocultures with T2 cells pulsed with the specific cdr2 epitopes but not in cocultures with the control peptides (at 1 μ M) or no peptide (Figure 29). No significant IFN- γ (<15 pg/ml) was observed in cocultures with GFP-electroporated T cells. The cdr2 TCR electroporated human CD8⁺ T cell populations were capable of releasing IFN- γ 20-fold above background at cdr2(290-298) LLEEMFLTV concentrations as low as 0.5 pM, with half maximal IFN- γ secretion occurring at peptide concentrations of 35 pM, a more than 3-log improvement in avidity from the original AAD 290 CTL line, and was similar to previous studies that have demonstrated that expression of an A2.1 transgenic mouse-derived TCR in CD8⁺ human T lymphocytes results in human CD8⁺ T cell with an enhanced functional avidity relative to the parental mouse clone⁹⁶. Similar to the results seen with the parental AAD 290 CTL line, we observed cross reactivity of the TCR electroporated CD8⁺ human T cells to the decamer peptide cdr2(289-298) (Figure 29, squares). We can conclude that this is a result of promiscuous recognition at the molecular level since human CD8⁺ T cells acquired specificities for both peptides after electroporation with a single cdr2-TCR.

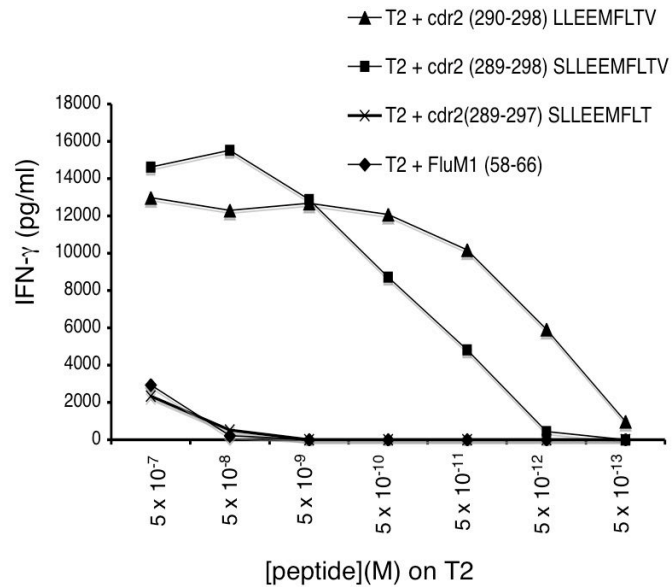


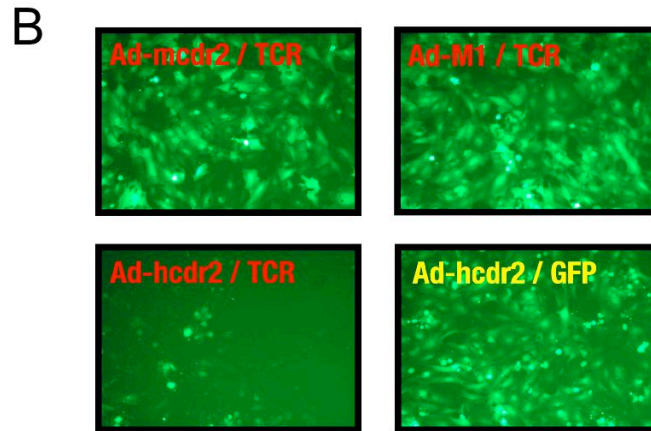
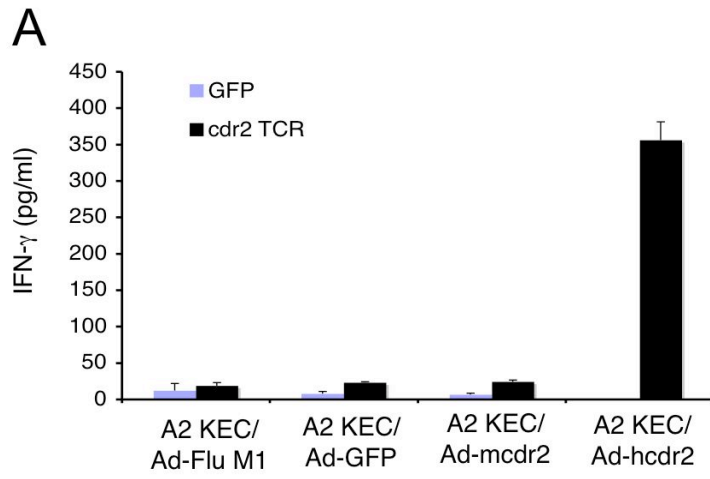
Figure 29. Functional analysis of cdr2 TCR: recognition of specific cdr2 peptides.

Following three-day stimulation and CD8 purification, human CD8⁺ T cells were electroporated 12 d later with in vitro transcribed cdr2 TCR α - and β - chain RNA and cocultured for 18 h with T2 cells pulsed with different concentrations of specific cdr2 peptide (290-298; triangles) or (289-298; squares), or nonspecific peptides cdr2(289-297, crosses), or FluM1 (diamonds). For this assay 10^5 electroporated T cells were cocultured with 10^5 peptide-pulsed T2 cells in a total volume of 0.2 ml. The concentration of IFN- γ secreted into the medium was measured by ELISA. Values are the average of duplicate wells. IFN- γ secretion in cocultures with GFP electroporated CD8⁺ T cells was <15 pg/ml for all peptides (data not shown).

Having confirmed the ability of the cloned α - and β - TCR chains to respond to cdr2 peptide-pulsed stimulators, we next investigated the reactivity of electroporated PBL to endogenously processed cdr2. We transduced cultures of primary kidney epithelial cells derived from AAA A2.1 (with a human α 3 domain) transgenic mice with Ad-hcdr2 or with control vectors (Ad-mcdr2, Ad-FluM1, and Ad-GFP). Twenty four hours later, when over 98% of the KECs were transfected, they were cocultured with TCR or control (GFP)-electroporated CD8⁺ T cells. IFN- γ production was detected only in the cocultures with Ad-hcdr2 transduced cells; there was no significant IFN- γ production detected with any of the control transduced cells or with any of the transduced KECs cocultured with GFP-electroporated PBL (Figure 30A). In the same assay, we were able to observe the dramatic specific killing ability accompanying the specific IFN- γ production of our TCR-electroporated T cells. Specific lysis of Ad-hcdr2 KECs but not control KECs could be visualized by loss of GFP expression cells by fluorescence microscopy (Figure 30B).

Figure 30. Functional analysis of cdr2 TCR; recognition of human cdr2-transduced cells.

Following three-day stimulation and purification, human CD8⁺ T cells were electroporated with in vitro transcribed cdr2 TCR α - and β - chain RNA or GFP RNA and cocultured for 18 h with AAA A2.1 kidney epithelial cells (KECs) that had been transduced with Ad-hcdr2, Ad-mcdr2, Ad-GFP, or Ad-FluM1. For this assay, 10^5 electroporated T cells were cocultured with 10^5 transduced KECs in a total volume of 0.2 ml. (A) The concentration of IFN- γ secreted into the medium as measured using ELISA. In this same assay, we were able to observe the lytic activity of the TCR-electroporated T cells due to the fact that our adenovirus constructs contained dual CMV promoters driving GFP expression along with transgene expression in the transduced cells. (B) 15 h after the initiation of the coculture, the wells in (A) were assessed for cell lysis by fluorescence microscopy.



CD8⁺ T cell mediated killing has historically been assessed by standard chromium release assay¹⁶⁵. This assay does not directly examine the CD8⁺ T cells that mediate the killing; rather, it examines the death of the target cells. On the other hand, since one way that CTL mediate killing of target cells is via a granule-dependent (perforin/granzyme) pathway, analysis of degranulation through direct measurement of cumulative exposure of the granular membrane proteins CD107a and CD107b on the surface of responding antigen specific T cells can provide a direct determination of functional reactivity on a per cell basis¹⁶⁶. In order to directly examine the effector status of our TCR electroporated T cells, we performed a CD107a mobilization assay (Figure 31). In this experiment, the bulk population of TCR or mock electroporated human CD8⁺ T cells were cocultured with either Ad-hcdr2 or Ad-GFP infected AAA KECs. Data in Figure 30 demonstrate that while few (2%) of the cdr2 TCR-electroporated cells mobilized CD107a after 5 hours of coculture with control (GFP)-transduced KECs, 35% became positive for surface CD107a expression upon coculture with hcdr2-transduced KECs. Mock electroporated CD8⁺ T cells did not show significant mobilization of CD107a in response to coculture with either Ad-hcdr2 or Ad-GFP infected KECs. The percentage of TCR transduced T cells that expressed CD107a after coculture with hcdr2-infected KECs was roughly equivalent to the percentage of cdr2(290-298) tetramer positive cells (32%, Figure 28) for this experiment, suggesting a correlation between proper heterodimerization of the transferred TCR chains and the acquisition of specific killer T cell activity.

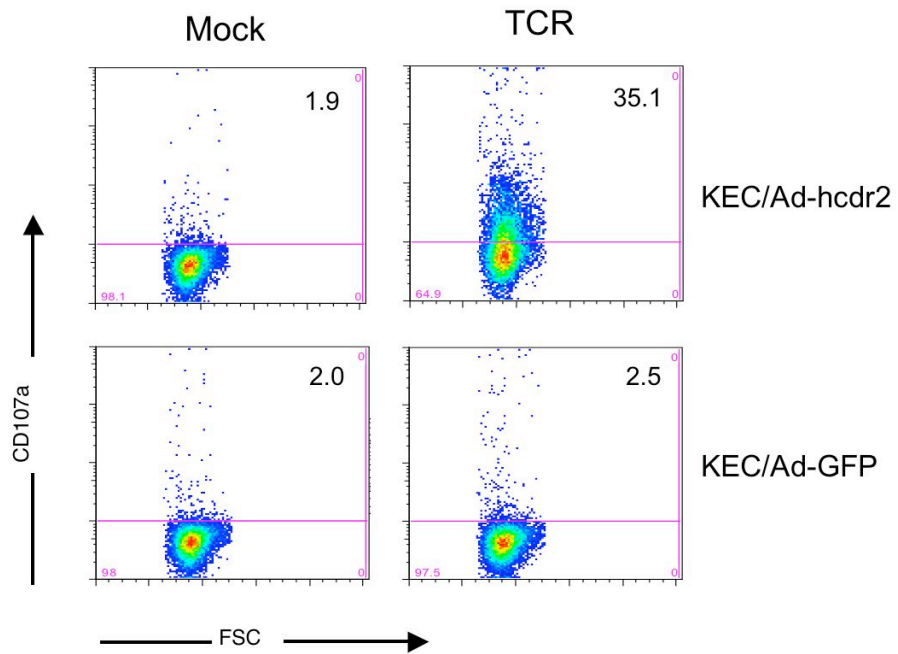


Figure 31. CD107a mobilization by TCR electroporated human CD8+ T cells.

FACS analysis for the degranulation marker protein CD107a was performed after 5 hour coculture of mock- or TCR-electroporated human CD8+ T cells with Ad-hcdr2 or Ad-GFP-transduced AAA (A2.1) KECs as indicated. Gating was performed on live lymphocytes based on FSC and SSC characteristics. The percentages of cells staining for CD107a were as indicated.

cdr2 TCR-expressing human CD8⁺ T cells recognize *cdr2*-expressing gynecologic tumor cells.

It has been reported that up to 60% of ovarian tumors and 25% of breast tumors express *cdr2*. To determine whether this *cdr2*-specific murine TCR might be suitable for the targeting of *cdr2* in human tumors, we evaluated the ability of TCR electroporated human CD8⁺ T cells to recognize physiologic levels of endogenous *cdr2* in tumor cell lines. TCR-modified but not GFP-modified human CD8⁺ T cells were able to recognize malignant A2.1⁺ targets expressing physiologic levels of endogenous *cdr2* by INF- γ ELISPOT assay (Figure 32). Significant recognition of HeLa cells that had been stably transfected with A2.1 (HeLa.A2.1) but not the parental A2.1 negative cell line was observed. A significant, but lower, number of TCR-transfected CD8⁺ T cells produced INF- γ specifically in response to the A2.1⁺ *cdr2*⁺ breast cancer cell line MCF7. This response was lower than that seen with both the parental clone response to MCF7.AAD may have been due to reduced levels of A2.1 expression, since we did not pre-treat any of the target cells with INF- γ for this assay. Finally, while the A2.1⁺ *cdr2*⁺ ovarian tumor line COV413 was only minimally recognized by the parental murine AAD 290 CTL line, human CD8⁺ T cells electroporated with the murine *cdr2* TCR became capable of robust recognition of COV413 cells. These experiments demonstrate that we have isolated murine TCR sequence capable of reprogramming human T lymphocytes for specific recognition of the human *cdr2* antigen in the context of breast and ovarian tumors and suggest that

there may be a clinical application for cdr2(290-298)-specific TCR in breast and ovarian cancer immunotherapy.

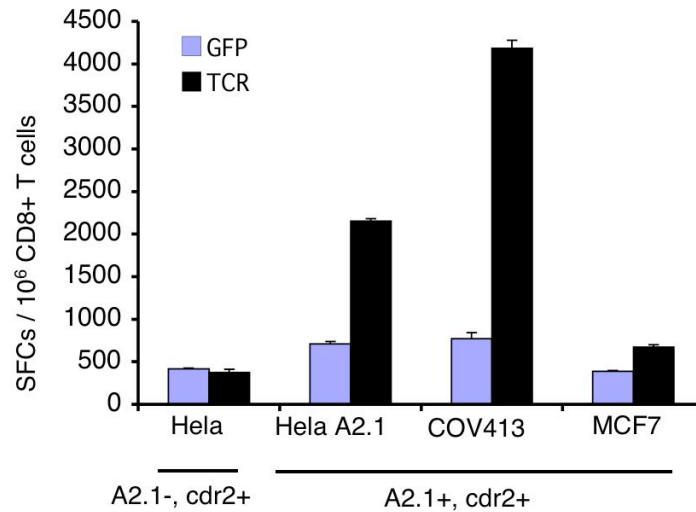


Figure 32. Recognition of cdr2-expressing gynecologic tumor cells by cdr2 TCR-electroporated CD8+ T cells.

Following three-day stimulation and purification, human CD8+ T cells were electroporated with in vitro transcribed cdr2 TCR α - and β - chain RNA (black bar) or GFP RNA (blue bar) and cocultured (10^5) with either HeLa, HeLa.A2.1, COV413, or MCF7 tumor cells (5×10^4) in an 18 h IFN- γ ELISPOT assay. Values, representing spot forming cells per million CD8+ T cells, are the average of duplicate wells; error bars indicate standard deviation. Data are representative of 2 independent experiments.

Discussion

TCR gene transfer is an attractive technology for production of antigen-specific T cells for adoptive immunotherapy. Cloned TCR genes can serve as generic “off the shelf” reagents that can be combined with adoptive T cell transfer for the treatment of patients with malignancies expressing the TCR-recognized antigen. This approach bypasses the need for laborious isolation and expansion of pre-existing autologous tumor-reactive lymphocytes that often do not even exist patients. To date, TCR genes have been isolated for a several human tumor associated antigens. These include the human-derived MART-1⁹³, NY-ESO-1¹⁶⁷, and gp-100¹⁵⁰ TCRs, and the murine-derived MDM2⁸⁹ and p53⁹⁶ TCRs. To this armamentarium we have added a murine-derived human cdr2-specific TCR whose isolation and characterization we have described in this chapter.

Here, we have demonstrated that RNA electroporation of human CD8+ T cells with a cdr2-specific TCR can reprogram them toward specific recognition of human breast and ovarian tumor cell lines expressing physiologic levels of endogenous cdr2. Notably, the TCR-electroporated lymphocytes exhibited antigen specific lytic function as well as cytokine-production. Furthermore, we found that the fine specificity of the clones was maintained through the TCR isolation and transfer: TCR-electroporated CD8+ T cells maintained the ability to specifically recognize both the cdr2(290-298) and cdr2(289-298) peptides. An interesting consequence of this cross-reactivity is that we cannot determine from our current studies whether one or both of these epitopes is being presented by

A2.1 on cdr2-expressing tumors. Further studies using acid extraction combined with reverse-phase-high-performance-liquid-chromatography (RP-HPLC) and mass spectrometry will have to be performed to determine the relative contributions of the nonamer and decamer peptides to the observed tumor recognition. Furthermore, by transferring a murine-derived TCR into a human CD8⁺ T cell, we observed in two ways the dramatic effect that such TCR recontextualization has on A2.1⁺ tumor recognition. First, we observed a more than 3-log improvement in functional avidity (recognition of peptide-pulsed T2 cells) from the original AAD 290 CTL line. Second, while the original AAD 290 CTL line demonstrated minimal recognition of the A2.1⁺ COV413 ovarian carcinoma cell line, cdr2 TCR-electroporated human CD8⁺ T cells demonstrated robust recognition of these cells. Both of these results are presumably due to the boost to TCR/MHC-peptide interaction provided by the human CD8 molecule. Our findings are similar to previous studies that have demonstrated that expression of an A2.1 transgenic mouse-derived TCR in CD8⁺ human T lymphocytes results in human CD8⁺ T cell with an enhanced functional avidity relative to the parental mouse clone⁹⁶. It is surprising that we did not observe a similar boost in structural avidity, as gauged by tetramer staining, relative to the parental mouse clone. We are currently trying to determine the reason for this discrepancy, but it may be related to our lower TCR transfection efficiency, or that we did not pick the highest affinity TCR (as discussed below). Nevertheless, these studies demonstrate that TCR of this specificity could to have clinical application to the adoptive immunotherapy of breast and ovarian cancer.

How to pick the “best” TCR

Although many cellular components are potentially responsible for the avid recognition of TAA, the TCR is the main component of this activity^{155,168}. We therefore wanted to choose a CTL clone with highly avid anti-tumor activity as TCR starting material. However, the best criteria for screening our CTL clones for this purpose was not immediately obvious. Several factors influence the avidity of interactions between the TCR and MHC I/peptides complexes, including the density and stability of TCR/MHC class I peptide complexes¹⁶⁹⁻¹⁷¹, the colocalization of TCR and CD8 molecules in the immune synapse^{169,172-174}, and the binding of CD8 molecules.¹⁷⁵ Studies have shown that the intensity of staining by tetramers^{158,159} and the stability of tetramer binding to T cell clones^{176,177} can be used as a direct measure of the affinity of TCR for the MHC-peptide complex and can be extrapolated to be an indication of the actual sensitivity of the CTL response to MHC-peptide antigen density. Following this rationale, we expected to observe a direct relationship between the staining intensity of the cdr2(290-298) clones with A2.1/cdr2(290-298) tetramers and their ability to secrete IFN- γ in response to coculture with cdr2-expressing targets. Instead we found an inverse relationship between tetramer staining and IFN- γ secretion in 2 out of 5 clones. While we ended up isolating the TCR genes from two clones that did not share this puzzling discrepancy, the question still remains: Which are the best criteria for isolating a high affinity TCR?

Closer examination of the literature reveals some experimental evidence opposing the view that the stability of the TCR/MHC-peptide contact correlates with high avidity T cell-target interactions^{178,179}. Studies with human cells have found that even clones with identical TCRs may have different relative avidity for peptide loaded APC targets; similar results have been reported in animal models where T cells sharing the same TCR have markedly different avidities¹⁸⁰. The latter case highlights the importance of the CD8 coreceptor for T cell target recognition. During antigen recognition, peptide/MHC complexes engage both the TCR and the CD8 coreceptor via the $\alpha 3$ domain of MHC. CD8 molecules recruit the kinase p56^{lck} to the CD3 complex and this mediates T cell activation¹⁸¹. Classically, CD8 is described as a T cell membrane $\alpha\beta$ heterodimer, but a CD8 $\alpha\alpha$ homodimer form has also been described which is significantly less efficient than the $\alpha\beta$ heterodimers at binding to MHC and p56^{lck} and at producing T cell activation¹⁸⁰. This highlights two factors that can have an impact on functional avidity of a T cell independent of structural avidity: the amount of CD8 on the surface of a T cell and the ratio of CD8 $\alpha\beta$ to CD8 $\alpha\alpha$.

We did not look for differences in CD8 expression on the AAD 290 T cell clones, nor did we examine the possibility that individual clones might have different levels of other molecules involved in TCR signaling such as the p56^{lck} and p59^{fym} kinases or the CD3 ζ chain^{182,183}. All of these factors could affect apparent functional avidity. Therefore, it will be interesting to see if clone 2, which had the highest structural avidity but low functional avidity, demonstrates poor function

only in the context of its current cellular environment. With the highest structural avidity of all the clones (at least a half log higher staining than the others), it may have a TCR which can confer superior functionality when transferred to a new host cell.

We tried to isolate cdr2(290)-specific clones on the basis of CD8-independent recognition of cdr2 antigen with the expectation that that this would lead to the isolation of a CD8-independent TCR. Having a murine TCR capable of CD8-independent recognition would be beneficial for two reasons. First, while all murine TCR that are transferred into human CD8+ T cells get an avidity boost from the human CD8 molecule, leading to more efficient recognition of human HLA-A2.1-associated antigens, this effect is even more pronounced when the murine TCR is CD8-independent to start with. Most importantly, a CD8-independent TCR can be used to turn CD4+ T cells into class I-restricted T helper cells. Given the role that CD4+ Th cells play in supporting cross-priming and the generation of memory T cell responses, we believe this will be a crucial parameter to the success of gene transfer and adoptive T cell therapy. Future experiments will evaluate whether our cloned TCR is capable of conferring recognition of cdr2 to CD4 +T cells.

In sum, we have demonstrated the overall robustness of the technology of TCR gene transfer for conferring recognition of antigens that might be subject to self tolerance in the majority of tumor-bearing hosts. Most importantly, we have

demonstrated that this cloned murine TCR has the same specificity (binds the same A2.1/cdr2(290-298) tetramer) as the peripheral blood CD8⁺ T cells from an A2.1⁺ PCD patient. Taken together, these data confirm the biological relevance of cdr2(290-298)-specific T cells in PCD, and suggest that cdr2(290-298)-specific TCR may hold therapeutic promise for breast and ovarian cancer immunotherapy.

Acknowledgements

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CHAPTER V – GENERAL DISCUSSION

Summary

The results presented in this thesis represent the identification and careful characterization of two naturally processed A2.1-restricted epitopes of the breast and ovarian cancer-associated PCD antigen cdr2: cdr2(289-297) and cdr2(290-298). Using HLA-A2.1 transgenic mice, we have generated A2.1-restricted CTL lines specific for each of these peptides and demonstrated that these CTL can target cells expressing endogenous human cdr2. We have validated that these are bona fide tumor associated CTL epitopes by demonstrating the presence of CD8+ T cells specific for both cdr2(289-297) and cdr2(290-298) in peripheral blood from A2.1+ PCD patients, but not from normal controls, by tetramer staining. Thus we have correlated the presence of T cells specific to these epitopes, which we now know represent natural cdr2 epitopes, with PCD and effective anti-gynecologic tumor immunity.

Human and murine cdr2 have 87% identity at the amino acid level. A single amino acid difference between human and murine cdr2(290-298) at position 8 facilitated the isolation of several high avidity CTL clones and a high affinity human cdr2-specific TCR from HLA-A2.1 transgenic mice. We have demonstrated that transferring this mouse-derived TCR into human CD8+ T cells turns the lymphocytes into efficient cdr2-specific CTLs. We conclude that gene transfer of TCR specific for cdr2(290-298) could provide the basis for potent breast and ovarian cancer immunotherapies.

Before we can contemplate any therapeutic use however, we need a better understanding of what induces neurologic disease in PCD. Until now we have not addressed the neuronal autoimmunity that co-occurs with this effective tumor immunity in PCD patients. In this final chapter, we will consider the evidence for and against T cell mediated pathogenesis of the neuronal degeneration in PCD and then discuss a preclinical animal model that will enable us to evaluate whether there is an exploitable therapeutic window between the tumor immunity and neuronal autoimmunity in addition to addressing more basic questions about neuro-immune interactions.

Unclear role for CTL in neuronal autoimmunity in PCD

While it is apparent that cdr2-specific CTL can mediate efficient tumor destruction and that the cdr2 onconeural antigen is the target of the immune mediated pathology in the nervous system, it remains unclear whether T cells are responsible for the pathogenesis of the neuronal degeneration. In the animal model experimental autoimmune encephalomyelitis (EAE), T cells are activated in the periphery by an injection of a specific protein or peptide, such as myelin basic protein (MBP), and then traffic across the BBB where they induce inflammation and pathology similar to that observed in patients with multiple sclerosis (MS). PCD is hypothesized to have a similar pathogenesis: a systemic anti-tumor immune response activates T cells which then are able to cross the BBB into the brain parenchyma where they induce autoimmune neurodegeneration after coming into contact with cdr2-expressing Purkinje cells.

The main pathological change in PCD is the loss of Purkinje neurons in the cerebellar cortex¹⁸⁴. There are reports of occasional lymphocytic infiltrates in the cerebellum, although not generally in the Purkinje cell layer. This may be due to the long time lapse between neuronal degeneration and autopsy, since the neurologic disease itself is generally not fatal. Recently, Albert et al. found activated $\alpha\beta$ T cells in the CSF of PCD patients and thus provided the first correlation between CSF T cells, an ongoing peripheral anti-tumor CTL response, and clinically evident neuronal destruction¹⁸⁵. Moreover, the brain is not considered to be as profoundly isolated by its “immune privileged” status as was once thought. While the passage of antibodies and cells, including resting lymphocytes, into the brain is restricted, activated T cells and monocytes are able to traffic across the blood brain barrier (BBB) and through the brain parenchyma^{186,187}. Neuronal MHC class I molecules may be constitutively expressed, and/or induced by cytokines, making them potential CTL targets^{188,189}. Recently we have obtained preliminary evidence of cdr2(289-297) and cdr2(290-298)-specific cells in the CSF of A2.1+ PCD patients by tetramer staining, thus associating cdr2-specific CD8+ T cells and the neuronal autoimmunity. These are exciting preliminary results, but further studies need to be done with more controls to determine whether these populations are enriched relative to other tetramer staining populations found in the peripheral blood.

Still, there are some problems with the idea that T cells are solely responsible for the elimination of cdr2-expressing neurons in PCD. Some uncertainty is raised by

the work of Michael Oldstone and colleagues. Work using a viral model system has demonstrated that LCMV antigen-specific CD8+ T cells are unable to kill LCMV-infected neurons in mice, even when they are engineered to overexpress MHC class I¹⁹⁰. More recent work by others suggests that neurons are protected from perforin-dependent CTL attack due to neuronal expression of Fas ligand (FasL/CD95L/Apo-1 ligand) which reduces granule release by CTL¹⁹¹. Furthermore, neurons have recently been shown to have the ability to convert activated T cells into disease-suppressing regulatory T cells¹⁹², which raises the question of whether some of the CD3+CD25+ “activated” T cells previously observed in the CSF of PCD patients¹⁸⁵ were, in fact, CD25-expressing PND-suppressing regulatory T cells.

Finally there is the issue of Yo antibodies, which are present at high titers in PCD patient CSF, and which recognize the leucine zipper region of cdr2, an important functional domain through which interactions with c-myc occur¹⁹³. It has been hypothesized that all onconeural antigens, including cdr2, have critical physiologic functions whose disruption by antibodies may trigger neuronal death leading to neurologic disease¹⁹⁴. There is evidence that autoantibodies can and do penetrate living cells both in culture¹⁹⁵ and in the intact nervous system¹⁹⁶. In the later case, cross-linking of cellular prion protein in vivo with monoclonal antibodies was found to trigger rapid and extensive neuronal death¹⁹⁶. Still, attempts to reproduce PCD in mice, either by passive transfer of Yo antibody¹⁹⁷

or active immunization with recombinant cdr2 protein¹⁹⁸ have not been able to recapitulate the disease.

A humanized animal model based on cdr2(289-297)-specific CTL

We currently lack an animal model of PND. Therefore we do not know which immune system components are necessary and sufficient for the induction of neuronal autoimmunity versus the killing of a systemic tumor. The work described herein provides the framework for generating a PCD animal model based on adoptive cell transfer. Having determined that the human and murine cdr2(289-297) are nearly identical and are both naturally processed and HLA-A2.1-restricted, we can create a humanized animal model in HLA-A2.1 transgenic mice based on cdr2(289-297)-specific T cells that will moreover provide information that can be usefully incorporated into the design of clinical trials using these epitopes. One possibility would be to clone a high affinity TCR from the HHD 289 CTL line by sorting tetramer positive cells for TCR isolation. Alternatively, we could clone a TCR of this specificity from tetramer-sorted CSF-infiltrating PCD patient T cells (the idea being that these might be analogous to TIL). Either of these TCR could then be used to create a human-mouse chimeric TCR-HLA-AAD transgenic¹⁹⁹ or a “retrogenic”²⁰⁰ mouse. Using adoptive transfer of cdr2-TCR transgenic T cells, we can evaluate whether there is a therapeutic window between the treatment of a cdr2-expressing tumor such as B16.AAD, HeLa.AAD, or MCF7.AAD (the later two in NOD-SCID A2.1 mice), and the induction of neurologic disease.

As discussed earlier, in the case of the Hu syndrome, there appear to be many more patients with cancer harboring tumor immunity than there are patients that develop neurologic disease, suggesting that a second event may be necessary for the development of neurologic disease in such circumstances. Our preliminary studies suggest that it is possible to induce cdr2-specific T cells in HLA-A2.1 transgenic mice, and notably these primed animals do not demonstrate evidence of cerebellar degeneration. Therefore we hypothesize that the line from tumor immunity to autoimmunity is not crossed simply by the generation of T cells with this specificity, but rather may be a consequence of a greater magnitude and persistence of the T cell response (perhaps via the provision of CD4 help), abnormal access to the CNS, or local immune dysregulation (or any combination of these). Autoimmune destruction of melanocytes (vitiligo and, in some cases, uveitis) is a relatively common occurrence in patients and mice undergoing successful melanoma immunotherapy by adoptive cell transfer or vaccination. Since the success of adoptive cell transfer in particular has been shown to depend on the depletion of immune regulatory components and the dose and persistence of the tumor-specific lymphocytes in vivo, it is possible that autoimmunity is largely a numbers game.

If the adoptive transfer of a large number of cdr2 TCR-specific T cells into cdr2 tumor-bearing A2.1 transgenic mice mediates tumor regression without leading to autoimmunity, we will have important evidence of preclinical safety of cdr2 TCR gene transfer for gynecologic tumor immunotherapies. In the case that this

protocol generates neuronal autoimmunity, we will have established the first animal model of PND, from which we can explore neuro-immune interactions as well as possible treatments for PND patients. If we transfer a high affinity cdr2-specific TCR into CD4+ Tregs by lentiviral transduction (thus generating class I restricted Tregs), will the co-administration of these cells ameliorate neurologic disease? Will blocking lymphocyte migration across the blood brain barrier with Natalizumab, an $\alpha 4\beta 1$ and $\alpha 4\beta 7$ integrin inhibitor recently approved for the treatment of multiple sclerosis²⁰¹, reestablish a therapeutic window between tumor immunity and neuronal autoimmunity?

It is still unclear what cellular function cdr2 confers onto tumor cells. That cdr2 is consistently and selectively expressed in a large percentage of breast and ovarian tumors underscores its importance for tumor biology and suggests that it may be an especially good immunotherapy target. By the insights gleaned from this animal model based on the cdr2(289-297)-specific CTL response, we hope to 1) better define the pathogenesis of the PNDs 2) elucidate fundamental differences between tumor immunity and neuronal autoimmunity; and 3) validate safe and effective strategies for the treatment of cancer patients.

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