2008

The Hu Syndrome: At the Intersection of Cancer and Autoimmunity

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A thesis presented to the faculty of

The Rockefeller University

in partial fulfillment of the requirements for

the degree of Doctor of Philosophy

by

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June 2008

The Hu Syndrome: At the Intersection of Cancer and Autoimmunity

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Paraneoplastic neurologic diseases (PNDs) arise when systemic malignancies express proteins normally restricted to neurons. Abnormal expression of a neuronal protein by tumor cells in the periphery results in an autoimmune response that then targets both the tumor and the nervous system. These diseases offer a unique opportunity to gain insight into the mechanisms behind both tumor immunity and neuronal autoimmunity. The Hu syndrome is an example of PND that affects patients with small cell lung cancer (SCLC). Hu patients are diagnosed by the presence of antibodies in the blood that recognize the HuD antigen. HuD is normally restricted in expression to neurons of both the peripheral and central nervous systems, but ectopically expressed by SCLCs. Despite its expression in virtually all SCLCs, only a small fraction of SCLC patients go on to develop neurologic disease. These patients mount an impressive immune response to their cancer that results in remarkable tumor immunity to this typically aggressive malignancy. Although antibodies to HuD are important diagnostic criteria for the disease, they are not sufficient for disease pathogenesis. Because HuD is an intracellular protein, CD8 T cells are more prone to mediate the destruction of HuD-expressing SCLC cells and neurons. We

previously demonstrated that patients with paraneoplastic cerebellar degeneration (PCD), another form of PND, harbor CD8 T cells specific for the onconeural antigen Cdr-2, suggesting that CD8 T cells mediate tumor immunity and neuronal degeneration in these patients. To study the CD8 T cell response to HuD, we performed an exhaustive screen of the entire HuD peptide library to identify the immunodominant murine CD8 T cell epitope of the protein. We showed that mice are peripherally tolerized to this neuron-specific protein, which could help to explain why most SCLC patients remain neurologically intact despite tumor expression of HuD. In addition, HuD-specific CD8 T cells were able to traffic to the central nervous system in an adoptive transfer model, however these cells were not sufficient to induce neurologic degeneration. To translate these results to the clinic, we screened the HuD peptide library over 8 human MHC I alleles in order to define HLA-restricted epitopes of the protein. This lead to the discovery of two human CD8 T cell epitopes of HuD. Using tetramers specific for these HLA-restricted epitopes, we demonstrated that patients with the Hu syndrome harbor cytotoxic HuD-specific CD8 T cells in their blood. By combining our results from the clinic with the mouse system we are closer to understanding how the immune system is able to mediate both tumor immunity and neuronal degeneration in patients with the Hu syndrome.

For Mom and Dad, my most valuable mentors

Acknowledgements

First and foremost, I would like to thank my thesis advisor Bob Darnell, for his support and enthusiasm. He has let me grow into my own as a scientist by providing continual encouragement and advice. I especially appreciate our trips to the RU Hospital. I have a great deal of admiration for him as a physician and as a scientist, and I know that his mentorship will continue to guide me throughout my career.

Many thanks to all the members of the Darnell lab for making the past five years as fun and exciting as possible. Most especially, thank you to Ashby Thomas, Travis Williams, Patricia Foo, Emily Conn, Kevin O' Donovan, Graeme Couture, Matteo Ruggiu, Jack Fak, Jennifer Darnell, Mithila Jegathesen, Elvira Lugo, Randy Longman, Brad Rosenberg, Salina Parveen, Teresa Ramirez-Montegut, Julia Kaufman, Mayu Frank and Noreen Buckley.

Thank you to Jeff Smith. I would not have made it through to the end without Jeff. He has been a supportive, entertaining, interesting and funny person to work with every day. He has helped me overcome my computer challenges, and so much more. If there were a stronger way to say thank you, I would use it here.

Thank you to James Okano for his advice over the past few years, and for providing the HuD knockout mice and recombinant adenoviruses. Thank you to Nishath Rehman and Gulayse Ince-Dunn for their help breeding and genotyping the HuD knockout mice.

I owe many thanks to my thesis committee members, Bruce McEwen and Nina Papavisiliou for their advice and support. And I am extremely grateful to Joan Goverman for coming all the way from the west coast to participate as my outside committee member.

Thanks to Dean Strickland, Kristen Cullen, Marta Delgado, Cristian Rosario, and Sue Ann Chong for making my time at Rockefeller as easy and comfortable as possible.

A special thanks to Bianca Santamasso and Nathalie Blachere for their mentorship. Together, they have taught me most of what I know about mouse immunology. I owe them so many thanks for their continued support and sound advice.

Thank you to Wendy Roberts, for her friendship and guidance. Her strength and encouragement have helped me make important decisions, and her wit and personality have made my experience in the lab so much

fun. She has picked me up and dusted me off during my most difficult times and for that I am very thankful.

I want to thank my family and friends for their love and support. They have enthusiastically let me share my work with them and that has made my experience at Rockefeller so much sweeter. A special thanks to Elizabeth, for her advice on all things scientific and otherwise. Thank you to Daniel, for his patience, understanding and humor. Having him in my corner has meant a lot. Finally, and most importantly, a very special thanks to my mom and dad. They have been my biggest source of strength and my most enthusiastic supporters from the beginning.

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CHAPTER I. INTRODUCTION

Paraneoplastic neurologic degenerations (PNDs)

Paraneoplastic neurologic degenerations (PNDs) offer a unique opportunity to gain insight into the mechanisms behind tumor immunity and neuronal autoimmunity. These diseases arise when systemic malignancies express proteins normally restricted to neurons. Abnormal expression of a neuronal protein by tumor cells in the periphery results in an autoimmune response that then targets both the tumor and the nervous system. PNDs are arguably the most well documented examples of naturally occurring tumor immunity, most commonly associated with breast, ovarian and lung tumors. The tumor immunity generated is so effective that PND patients often present first with neurologic symptoms before their cancer is diagnosed. It is important to note that the majority of cancer patients never mount a detectable immune response to their tumor, but in this small subset of PND patients (0.01%) the immune system responds to the cancer, resulting in both remarkable tumor immunity as well as autoimmune neurologic degeneration (Fig 1).

The immune response to neuronal proteins ectopically expressed by tumor cells in PND patients serves to connect their tumor immunity and neuronal degeneration. Evidence for an immune-mediated disease came

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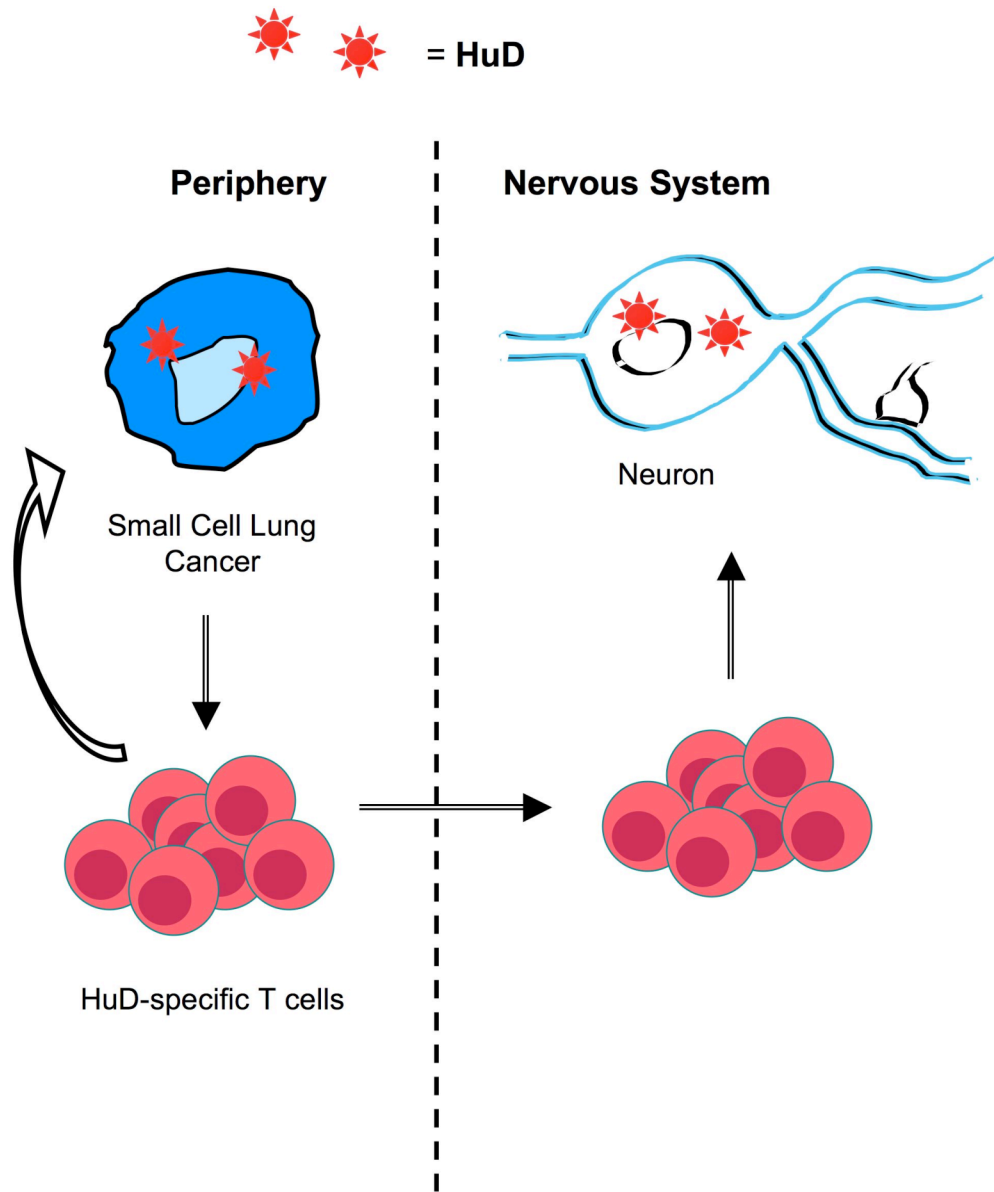
Fig 1. Model of anti-Hu syndrome. The presence of a small cell lung cancer tumor in the periphery that expresses HuD results in an immune response to the HuD protein that is able to respond to the tumor, resulting in tumor immunity, and traffic to the nervous system to target HuD-expressing neurons, resulting in neuronal degeneration.

from the identification of high titer antibodies in the blood and cerebrospinal fluid of PND patients (Graus, Elkon et al. 1986; Furneaux, Reich et al. 1990). Patient antibodies were used to identify so-called onconeural proteins aberrantly expressed by tumors and naturally found in the nervous system.

Although the presence of high titer autoantibodies in PND patients serves as a diagnostic tool, their role in disease pathogenesis remains unclear. Onconeural antigens are intracellular proteins and therefore not easily accessible to circulating antibodies. Furthermore, attempts to establish animal models of disease based on the generation of high titer antibodies to onconeural antigens have failed (Sakai, Gofuku et al. 1995; Sillevis Smitt, Manley et al. 1995). Given the intracellular nature of the PND antigens, it seems likely that T cells play an important role in disease pathogenesis. This is evidenced by the presence of lymphocytic infiltrates into relevant regions of the CNS in PND brains obtained at autopsy (Jean, Dalmau et al. 1994; Verschuuren, Chuang et al. 1996). Clear evidence for T cell involvement in disease pathogenesis has been demonstrated for paraneoplastic cerebellar degeneration (PCD), a particular type of PND that affects women with breast and ovarian cancer. Patients with PCD have high titer antibody to Cdr2, an intracellular protein normally expressed exclusively by Purkinje cells in the cerebellum. Freshly isolated CD8 T cells from the blood of PCD

patients have been shown to kill Cdr2-expressing targets in a cytotoxic T lymphocyte (CTL) assay, and activated T cells are found in the cerebrospinal fluid of PCD patients (Albert, Darnell et al. 1998; Albert, Austin et al. 2000). Taken together, this evidence suggests that T cells play a critical role in the pathogenesis of PCD.

Anti-Hu Syndrome

Like PCD, anti-Hu syndrome (also referred to as subacute sensory neuropathy/encephalomyelitis) is a particular form of PND characterized by neuronal loss and inflammatory infiltrates of the nervous system (Henson, Hoffman et al. 1965; Horwich, Cho et al. 1977; Anderson, Cunningham et al. 1987). Patients with anti-Hu syndrome develop neurologic symptoms that affect discrete areas of the nervous system including the dorsal root ganglia, limbic system, cerebellum, brainstem, motor or autonomic nervous system. Most patients go on to develop multifocal neuronal degeneration and die from neuronal causes, on average, seven months after diagnosis (Dalmau, Graus et al. 1992). Antisera from patients recognize the Hu antigen, which is normally restricted to neurons of both the peripheral and central nervous systems. Anti-Hu syndrome is associated with Small Cell Lung Cancer (SCLC) and patients are able to generate impressive tumor immunity to this typically aggressive malignancy. SCLCs from anti-Hu patients are usually limited to single nodules, which are discovered only after presentation of

neurologic symptoms (Dalmau, Graus et al. 1992).   Although very few SCLC patients go on to develop PND (0.01%), all SCLCs express the Hu antigen (Manley, Smitt et al. 1995). The presence of high titer antibody to Hu allows for diagnosis of anti-Hu syndrome and is correlated with natural tumor immunity. Apart from the small cohort of SCLC patients with the Hu syndrome, 15% of SCLC tumors generate a low titer anti-Hu antibody response in the absence of neurologic disease (Dalmau, Furneaux et al. 1990). Patients with low titer antibodies have limited stage tumors and better prognoses compared to SCLC patients with no detectable Hu antibodies (Dalmau, Furneaux et al. 1991; Graus, Dalmou et al. 1997).   Uncovering the differences between high titer, neurologically affected PND patients and low titer, neurologically normal SCLC patients will undoubtedly improve our understanding of the mechanisms behind both tumor immunity and neuronal autoimmunity.

The Hu antigen recognized by patient antisera is part of a protein family consisting of four members: HuA, HuB, HuC, and HuD. Hu family members are highly homologous to a *Drosophila* nuclear protein ELAV. Deletion mutants of *elav* are embryonic lethal due to abnormal neuronal development (Robinow, Campos et al. 1988; Robinow and White 1991). The sequence of Hu genes is highly conserved between individual Hu family members within and across species (Okano and Darnell 1997). The Hu proteins bind to RNA, and are found in the nuclei of neurons of

both the peripheral and central nervous systems (Ma, Cheng et al. 1996; Keene 1999). HuA is the ancestral Hu gene, and unlike HuB, HuC, and HuD, is ubiquitously expressed throughout the body (Ma, Cheng et al. 1996; Okano and Darnell 1997). HuB, HuC, and HuD are restricted to neurons, and all neurons contain one or more of the Hu proteins; for example, the dorsal root ganglia contain all three Hu proteins whereas Purkinje cells in the cerebellum contain HuC exclusively (Okano and Darnell 1997). Antisera from patients with the Hu syndrome react with HuB, HuC, and HuD however only HuD is expressed by SCLCs (Manley, Smitt et al. 1995). HuD, then, appears to be the antigen responsible for initiating an autoimmune response to SCLC in the periphery. This HuD-specific immune response is then able to traffic to the nervous system and target neurons expressing the Hu antigen. Patients with the Hu syndrome present first with neurologic disease affecting one region of the nervous system but go on to develop multifocal neurologic degeneration. If the initial autoimmune attack is directed against HuD specifically, it may spread to other Hu family members and target neurons expressing various combinations of the Hu proteins.

There is no difference between the HuD gene expressed by SCLCs and by neurons (Carpentier, Voltz et al. 1998). Histologic characteristics of tumors found in PND patients are no different from those in neurologically normal SCLC patients except that they may be heavily

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infiltrated with inflammatory cells (Rosenblum 1993). Thus, the initiation of an autoimmune response to HuD does not rely on some deviation in the protein or tumor in anti-Hu patients. Importantly, patients with the Hu syndrome do not succumb to peripheral autoimmunity and tissues outside of the nervous system are never attacked. If the ultimate autoimmune response is able to recognize other Hu family members in addition to HuD, it should not recognize HuA, as this would lead to massive peripheral autoimmunity given HuA's ubiquitous expression pattern.

The autoimmune response to Hu is evidenced by the presence of autoantibodies in the blood and cerebrospinal fluid of anti-Hu patients, however given that the Hu proteins are sequestered within the nuclei of neurons, it seems unlikely that antibodies play a dominant role in disease pathogenesis. Rather, T cells specific for HuD are more apt to generate anti-tumor immunity and neuronal degeneration. Cell mediated autoimmunity is suggested by the presence of activated T cell in the cerebrospinal fluid of anti-Hu patients. Moreover, the immune response in patients is Th1-skewed, with Th1 CD4 helper T cells and IgG_1 antibodies present in circulation (Jean, Dalmau et al. 1994; Benyahia, Liblau et al. 1999). Attempts to create mouse models of the disease by immunizing with whole HuD protein fail to induce neuronal autoimmunity despite the generation of high titer antibodies to Hu

(Sillevis Smitt, Manley et al. 1995). For these reasons, T cells appear to play an essential role in the Hu syndrome, and understanding the mechanisms behind tumor immunity and neuronal autoimmunity should focus on the T cell response to the Hu antigen.

Anti-Hu syndrome is an aggressive illness. Prognosis is usually poor because disease involves irreversible neuronal loss and evolves subacutely so that intervention is delayed. When the syndrome is diagnosed, treatment of this presumably T cell mediated PND relies on an immunosuppressant like tacrolimus and elimination of the underlying SCLC tumor (Albert, Austin et al. 2000). To date, there is no known immunogenic epitope of the HuD protein, which severely limits our ability to examine T cell responses to HuD in patients with the Hu syndrome. In order to more effectively diagnose and treat patients with this aggressive autoimmune disease, we must identify immunogenic epitopes of HuD so that we can isolate and study T cells specific for this antigen.

Tolerance mechanisms to self-antigens

HuD is a self-protein naturally expressed by neurons. To understand how the body generates autoimmunity against HuD, it is important to understand the mechanisms responsible for tolerance induction. Tolerance is the body's defense against reactivity to self. Lack or loss of

tolerance leads to immune responses against self-proteins, eventually resulting in clinical autoimmune disease. The induction and maintenance of tolerance involves interactions between antigen presenting cells (APC) and lymphocytes (Pugliese 2004). Central tolerance happens in the thymus and is responsible for purging the T cell repertoire of self-reactive clones (Gallegos and Bevan 2004) (Starr, Jameson et al. 2003). Ideally, any lymphocyte capable of recognizing self-peptide is eliminated during development, sculpting an immune system that is armed with T cells specific only for foreign antigens. The thymic architecture is designed to ensure that mature T cells emerge from the thymus with the ability to recognize self-MHC molecules but not self-peptides. Cortical thymic epithelial cells (cTECs) are responsible for positive selection of lymphocytes whereas medullary thymic epithelial cells (mTECs) delete autoreactive cells. This process is imperfect, however, and self-reactive clones escape into the periphery where there is potential recognition of self-tissue (Gallegos and Bevan 2006) (Bouneaud, Kourilsky et al. 2000). In order to silence these self-specific T cells and avoid autoimmune disease, peripheral tolerance exists. The mechanisms responsible for peripheral tolerance are varied and can act directly on T cells, through their deletion or inactivation, or through accessory cells, namely regulatory T cells.

Central tolerance

The role of central tolerance in preventing autoimmunity was highlighted by the discovery of AIRE, a transcription factor expressed in the thymus that controls the ectopic expression of tissue-specific antigens by mTECs (Holmdahl 2007). Thymic stromal cells do not endogenously express proteins normally restricted in expression to particular organs. AIRE, and perhaps other undiscovered transcription factors are responsible for driving expression of these tissue-specific antigens in the thymus for deletion of autoreactive T cell clones. AIRE was first identified as the underlying gene responsible for the human autoimmune disorder termed APECED (Peterson, Nagamine et al. 1998). Patients with APECED (for autoimmune polyendocrinopathy candidiasis ectodermal dystrophy) develop multiorgan autoimmune disease due to a lack of functional AIRE protein. As in humans, loss of AIRE in mice leads to the development of multiorgan autoimmune disease that is dependent on the absence of AIRE from thymic stromal cells (Liston, Lesage et al. 2003) (Anderson, Venanzi et al. 2002). The ectopic expression of tissue-restricted antigens by AIRE in the thymus is necessary for the deletion of self-specific T cell clones and protection against autoimmune disease. Recently, it was suggested that the role of AIRE in preventing autoimmunity be extended to include deletion of autoreactive T cells in the periphery. AIRE was shown to be expressed in lymph node stromal cells in a transgenic mouse model of tolerance induction. Ectopic

expression of tissue-restricted antigens in the lymph nodes resulted in elimination of self-reactive T cell clones (Lee, Epardaud et al. 2007). In this model, a cytosolic form of ovalbumin (OVA) under the control of a tissue-specific promoter was endogenously processed and presented onto MHC I molecules by a stromal cell population in the lymph node cortex. Endogenous presentation of OVA in the lymph node was an effective means of inducing tolerance in naïve transgenic OVA-specific CD8 T cells.

Peripheral tolerance

Peripheral tolerance is designed to suppress the activity of self-specific T cell that have escaped thymic deletion. The mechanisms responsible for peripheral tolerance can be distinguished between those that act on responding T cells directly and those that rely on additional cell types, namely regulatory T cells. T-cell intrinsic mechanisms include ignorance, anergy, and activation-induced cell death (Walker and Abbas 2002). Ignorance is achieved by sequestering tissue-specific antigens in areas not easily accessed by lymphocytes (Alferink, Tafuri et al. 1998). Limiting the amount of antigen in the periphery is also an important means of maintaining T cell ignorance, since there is a threshold below which T cells are unable to respond (Kurts, Miller et al. 1998). Anergy is the functional inactivation of a T cell upon encounter with antigen. Inhibitory receptors, most notably CTLA-4 and PD-1, are responsible for

inducing anergy in response to T cell receptor (TCR) ligation. CTLA-4 on the surface of T cells binds to the co-stimulatory molecules B7-1 and B7-2, and was shown to be critical for anergy induction *in vivo* (Perez, Van Parijs et al. 1997). PD-1 is highly expressed on the surface of anergic T cells, and mice lacking this receptor develop autoimmune disorders (Nishimura, Nose et al. 1999). Activation-induced cell death and anergy depend on T cell recognition of MHC-peptide complexes on APCs so that APCs, particularly dendritic cells, play an important role in maintaining tolerance in the periphery.   Activation-induced cell death is arguably the most effective means of preventing autoimmunity since it results in the elimination of self-specific T cell clones from the repertoire. Because self-antigens naturally persist instead of being cleared from the body, autoreactive T cells that repetitively engage their TCR may be subject to apoptosis via activation-induced cell death.   Ligation of the Fas death receptor is critical to this pathway, and defects in Fas signaling are associated with human autoimmune lymphoproliferative syndrome (ALPS) (Fisher, Rosenberg et al. 1995).

Experimental evidence has shown that bone marrow derived APCs and not the peripheral tissue itself are responsible for inducing peripheral tolerance (Kurts, Heath et al. 1996; Kurts, Kosaka et al. 1997). Recognition of MHC-peptide complexes on APCs by T cells specific for tissue-restricted antigens results in tolerization, either though deletion or

anergy (Heath, Kurts et al. 1998) (Redmond and Sherman 2005). Using a transgenic model of peripheral tolerance induction in which OVA is expressed in the pancreas and kidney, it was shown that naïve transgenic OVA-specific CD8 T cells undergo tolerance in the form of deletion as a result of OVA presentation in the periphery. Deletion was preceded by an initial period of activation and proliferation that did not translate into effective priming (Kurts, Kosaka et al. 1997). Like CD8 T cells, CD4 T cells are tolerized in the periphery by APCs that have acquired exogenous antigen (Heath, Kurts et al. 1998). In transgenic mice that express hemagglutinin (HA) in various parenchymal cells, HA-specific CD4 T cells are anergized by presentation of HA on bone marrow derived APCs (Adler, Marsh et al. 1998). Bone marrow derived APCs play a pivotal role in maintaining tolerance in the periphery to both CD4 and CD8 T cell epitopes. Experimental evidence indicates that myeloid DCs in particular are responsible for tolerance induction (Albert, Jegathesan et al. 2001). DCs are exceptionally adept at antigen processing and presentation, with the capacity to take up antigens and present them to T cells as peptides bound to MHC I and II molecules (Mellman and Steinman 2001). Compared to other antigen presenting cells, DCs are more effective at stimulating naïve T cells and shaping the quality of immune responses. The types of antigens captured and presented by DCs include both foreign and self-proteins. Activation of T cells to foreign antigens is required for effective clearance of invading

pathogens. Conversely, suppression of T cells specific for self-antigens is necessary for the prevention of autoimmunity. DCs do not endogenously express tissue-specific proteins and must therefore capture peripheral tissue for processing and presentation of self-peptides onto MHC I and MHC II molecules. To this end, DCs utilize a pathway termed cross presentation that allows exogenous antigen to be presented on MHC I as well as MHC II molecules to CD8 and CD4 T cells, respectively (Mellman and Steinman 2001). The factors that determine whether DC epitope presentation will result in priming or tolerization are still unclear. For CD8 T cell priming via cross-presentation by a DC, it was shown that cognate CD4 T cells are required for the generation of a productive immune response (Albert, Jegathesan et al. 2001). The absence of cognate CD4 T cell help resulted in tolerance of CD8 T cells. A coordinated response by CD8 and CD4 T cells is therefore necessary for the initiation of an effective immune response, and activation of cognate CD4 T cells is an important checkpoint for CD8 T cell priming.

The maintenance of peripheral tolerance depends on a persistent source of antigen. Both transgenic and non-transgenic systems have been used to study the mechanisms behind peripheral tolerance induction. The non-transgenic model of peripheral tolerance to the murine ovarian-specific antigen ZP3 highlights the requirement for a constant source of antigen in the periphery (Garza, Agersborg et al. 2000). Females are

naturally tolerized to ZP3, requiring 100-fold more antigen to elicit an immune response compared to males. Tolerance in females was shown to depend on the presence of endogenous ZP3, as removal of the ovaries from neonates converted the female immune response into that of males. Moreover, exposure to neonatal antigen was insufficient for the maintenance of tolerance, which required a constant source of antigen throughout adulthood. When the ovaries were removed from adult females that then received syngeneic ovarian implants followed by immunization to ZP3, the mice developed overt ovarian autoimmune disease. Normal females given ovarian implants and immunized to ZP3 maintained tolerance to the antigen and did not exhibit autoimmunity.

Regulatory T cells

Regulatory T cells are a specialized population of cells critical to the maintenance of peripheral tolerance. Peripheral tolerance induction by regulatory T cells is achieved by active suppression of effector T cells. Most regulatory T cells are classified as $CD4^+CD25^+$ cells that express the transcription factor FoxP3, and in both humans and mice represent less than 10% of the total CD4 T cell population (Akbar, Vukmanovic-Stejic et al. 2007). There are two distinct classes of regulatory T cells, distinguished by their developmental course. Natural regulatory T cells emerge from the thymus as $FoxP3^+$ cells with the intrinsic ability to inhibit the proliferation of activated T cells, whereas induced or adaptive

regulatory T cells are converted from activated effector or memory CD4 T cells in the periphery (Itoh, Takahashi et al. 1999; Akbar, Vukmanovic-Stejic et al. 2007). Production of natural regulatory T cells occurs through presentation of self-peptides in the thymus. The precise signals leading to the generation of natural regulatory T cells, as opposed to the deletion of self-specific thymocytes, are not well understood (Picca and Caton 2005) (Apostolou, Sarukhan et al. 2002). Both pathways require recognition of self-peptide in the thymus by developing lymphocytes, however the type of APC responsible for antigen presentation, as well as the strength of the TCR-MHC interaction, may help to determine the fate of self-reactive T cells.

A peripheral source of self-antigen is required for the maintenance of effective regulatory T cells from thymic emigrant precursors (Picca and Caton 2005). Natural regulatory T cells emerge from the thymus with an intrinsic ability to suppress effector T cells, however their survival depends on continual exposure to antigen in the periphery. This has been demonstrated in a non-transgenic model of autoimmune thyroiditis (Seddon and Mason 1999). Autoimmune thyroiditis results in high titer antibodies against thyroglobulin and extensive leukocytic infiltration of the thyroid glands. Development of disease can be prevented by reconstitution with natural regulatory T cells. In order to study the dependency of these regulatory T cells on peripheral antigen, the thyroid

was ablated in utero before the emergence of T cells from the thymus so that no peripheral source of antigen existed. Peripheral CD4 T cells or thymocytes from athyroid animals were transferred into thyroiditis-prone hosts. Whereas thymocytes from athyroid animals were able to function effectively as regulatory T cells and prevent thyroiditis, peripheral CD4 T cells from athyroid animals were ineffective suppressors and could not inhibit the development of autoimmunity. Peripheral CD4 T cells were presumably ineffective due to their lack of exposure to antigen in the periphery in athyroid animals. In addition, the ineffectiveness of peripheral CD4 T cells proved to be antigen-specific, as these cells were able to prevent the development of other forms of autoimmunity such as diabetes.

There are various pathways that lead to the production of regulatory T cells in the periphery from effector or memory CD4 T cells. Tr1 cells are a type of adaptive regulatory T cell, defined by their dependence on IL-10 (Roncarolo, Gregori et al. 2006). IL-10 is required for the induction of Tr1 cells in *vivo*, and is responsible, in part, for Tr1-mediated suppression. Suppression by Tr1 cells is carried out by secretion of the anti-inflammatory cytokines IL-10 and TGF-β. IL-10 was initially discovered by its ability to block cytokine secretion from Th1 cells (Moore, Vieira et al. 1990). Its immunosuppressive properties have been extended to include down regulation of costimulatory molecules and

inhibition of pro-inflammatory cytokine production by APCs (Roncarolo, Gregori et al. 2006). TGF-β acts to inhibit proliferation and differentiation of both CD4 and CD8 T cells (Li, Wan et al. 2006). Adaptive regulatory T cells, in addition to natural regulatory T cells, have been shown to play a role in preventing autoimmunity. Defects in Tr1 cells have recently been discovered in patients with multiple sclerosis (MS) (Astier, Meiffren et al. 2006). MS is an autoimmune disease characterized by inflammation in the CNS and destruction of myelin. Tr1 cells from MS patients were hyporesponsive to stimulation *in vitro* and secreted lower levels of IL-10 compared to Tr1 cells from healthy donors (Astier, Meiffren et al. 2006).

The immune system is responsible for protecting the body from foreign invaders. It is therefore essential that the immune repertoire be armed with receptors capable of identifying any foreign substance or organism. To ensure a sufficient amount of diversity among lymphocytes, the immune system randomly arranges receptors to create a repertoire that consists of billions of different clones. Given the randomness and magnitude of lymphocyte diversity, a portion of the immune system will unavoidably be able to recognize self-tissue. Tolerance is therefore essential to protect the body from its own defenses. Maintaining immunologic tolerance is tantamount to generating effective immune responses against foreign pathogens. The multiple, and sometimes

redundant mechanisms behind tolerance induction reflect the importance of preventing self-reactivity. Anti-Hu syndrome offers a unique opportunity to study tolerance to a self-protein expressed exclusively in neurons. The fact that all SCLCs express the HuD protein, but only 15% of SCLC patients mount a detectable immune response to HuD, suggests that the immune system is normally tolerized to HuD, and that tolerance is broken in patients with anti-Hu antibodies. Of the 15% of patients with anti-Hu antibodies, a very small population goes on to develop neuronal autoimmunity. This implies an additional requirement besides the loss of tolerance to HuD for the generation of brain disease. The uniqueness of the central nervous system, and the properties that distinguish it from other tissues in the body, undoubtedly contribute to the rare incidences of autoimmune mediated neuronal degeneration.

Immunosurveillance and inflammation of the central nervous system

The CNS has historically been considered an immune privileged site. Immune privilege signifies the inability of the immune system to access and survey certain anatomical sites. Surveillance of the CNS was considered impractical due to a lack of lymphatic drainage from the CNS parenchyma, the absence of traditional antigen presenting cells (APCs), and the presence of the blood-brain barrier (Ransohoff, Kivisakk et al.

2003).   These factors represented physical and functional barriers that combined to make the CNS unreachable by the immune system.   We now understand that the immune system is not excluded from the CNS, although access is more carefully monitored relative to other tissues.

The blood-brain barrier

The CNS consists of the brain and spinal cord, covered by three layers of membrane and encased in bone.   Cerebrospinal fluid (CSF) is generated locally by specialized secretory cells of the choroids plexuses, which are located within individual ventricles, or cavities, of the brain.   After formation in the ventricles, CSF circulates through the brain and spinal cord before being reabsorbed into venous blood.   Recent studies indicate that CSF also drains into cervical lymph nodes, making it a unique form of lymph specific to the CNS (de Vos, van Meurs et al. 2002; Karman, Ling et al. 2004).   The extracellular fluid that bathes the parenchyma of the brain has access to CSF so that soluble proteins from CNS grey and white matter can readily reach lymphoid tissues (Ransohoff, Kivisakk et al. 2003).   Evidence of CSF flow into cervical lymphatics makes it clear that there is appreciable immune surveillance of soluble CNS antigens.

Movement of cells into the CNS is a tightly regulated process. Capillaries of the CNS vasculature protect the parenchyma by excluding circulating cells and macromolecules on account of endothelial tight junctions.

Tight junctions seal the gap between the blood and CNS, preventing movement of lymphocytes across the endothelium. Movement of solutes is also precluded by the inability of endothelial cells to pinocytose. Together, these unique features of the endothelium are referred to as the blood-brain barrier. In a healthy state, the blood-brain barrier is an effective impediment to lymphocyte migration across the CNS endothelium. However, experimental evidence indicates that lymphocytes are able to gain access to the CNS under inflammatory conditions (Hickey 2001). TNFα is a proinflammatory cytokine that causes marked changes in the blood-brain barrier when produced either locally or systemically (Hickey 2001). In both *in vitro* and *in vivo* models, TNFα has been shown to increase the permeability of the blood-brain barrier, thereby enhancing the passage of material between the blood and CNS (Mark and Miller 1999) (Dickstein, Moldofsky et al. 2000). Other proinflammatory substances such as LPS and IFNγ influence the permeability of the blood-brain barrier by activating endothelial cells of the CNS vasculature, resulting in upregulation of adhesion molecule expression (Hickey 2001).

Inflammation in the CNS

Although the CNS is normally protected from the development of harmful immune responses, inflammatory reactions can occur behind the blood-brain barrier. Whereas naïve lymphocytes are rarely found in the

parenchyma of the brain, activated T cells readily traverse the CNS regardless of antigen specificity (Karpus and Ransohoff 1998; Becher, Bechmann et al. 2006).   Antigen specificity becomes important for retention of T cells behind the blood-brain barrier and the subsequent initiation of an inflammatory immune response.   Inflammation requires antigen-specific stimulation of activated T cells that have crossed into the brain, which in turns depends on the presence of an antigen presenting cell (APC) in the CNS (Becher, Bechmann et al. 2006) (Perry 2004). Antigen-specific stimulation of T cells in the CNS is a complicated process given the multiplicity of APCs with the potential to function as T cell stimulators.   There are both endogenous and peripheral APCs present in the inflamed CNS.   Peripheral macrophages and DCs are recruited as part of the inflammatory response whereas microglia are resident APCs with the capacity to express MHC and costimulatory molecules.   In a normal, nonpathologic state, microglia express insufficient levels of MHC molecules and are therefore incapable of functioning as APCs (Aloisi 2001).   MHC expression is quickly upregulated under inflammatory or neurodegenerative conditions so that microglia become important participants in the CNS immune response (Aloisi 2001; Carson, Doose et al. 2006).   Despite their role in the development of CNS inflammation, microglia do not appear to be the cell type responsible for initiating T cell responses behind the blood-brain barrier.   Rather, peripheral DCs are sufficient for the induction of CNS

inflammation (Carson, Doose et al. 2006) (Lauterbach, Zuniga et al. 2006) (Greter, Heppner et al. 2005). The prevailing model for the generation of CNS pathology begins with the induction of an inflammatory response as a result of CNS antigen-specific T cell stimulation by peripheral DCs behind the blood-brain barrier, followed by infiltration of mononuclear cells and additional T cells into the CNS parenchyma where microglia are able to direct T cell effector function (Ransohoff, Kivisakk et al. 2003; Carson, Doose et al. 2006).

Experimental autoimmune encephalomyelitis

The most commonly studied example of CNS inflammation is experimentally induced autoimmune encephalomyelitis (EAE). EAE is a mouse model of multiple sclerosis (MS) characterized by autoimmunity directed against components of the myelin sheath. Disease is commonly mediated by myelin-specific CD4 T cells that have infiltrated the CNS (Becher, Bechmann et al. 2006). To generate EAE, mice are immunized with myelin peptides or proteins (most commonly MOG, PLP or MBP) in order to prime myelin-specific CD4 T cells in the periphery. The pathology is initiated when activated myelin-specific T cells traffic to the CNS and induce inflammation, characterized by peripheral APC recruitment to the brain and spinal cord, upregulation of MHC and costimulatory molecules on resident microglia, and eventual demyelination (Becher, Bechmann et al. 2006). Peripheral APCs that

have been recruited to the CNS are necessary and sufficient to stimulate activated myelin-specific CD4 T cells behind the blood-brain barrier (Greter, Heppner et al. 2005). Although microglia do not seem to play a role in the initiation of CNS inflammation, they participate in the ongoing inflammatory reaction by modifying T-cell effector function within the parenchyma (Carson, Doose et al. 2006). Using irradiation bone marrow chimeras, it was shown that expression of CD40 on microglia is necessary for the exacerbation of EAE (Becher, Durell et al. 2001). Microglia are capable of phagocytosing antigen when activated under inflammatory conditions, and experimental evidence suggests that processing and presentation of myelin epitopes to infiltrating T cells by resident microglia is important for the progression of CNS disease (Prineas, Kwon et al. 2001) (Carson 2002).

Immunosuppression in the CNS

Inflammatory responses in the CNS can cause irreparable damage, as evidenced by autoimmune diseases like EAE and PND. In order to prevent immune-mediate neurodegeneration, the CNS has designed ways to mitigate immune responses behind the blood-brain barrier. Regulation of infiltrating lymphocytes by resident brain cells is an active process designed to limit or suppress CNS inflammation. Constitutive expression of FasL in the CNS promotes apoptosis of activated T cells, and immunosuppressive cytokines, most notably TGF-β, act to inhibit

inflammation (Bechmann, Mor et al. 1999; Vitkovic, Maeda et al. 2001). Microglia have the capacity to promote inflammation and tissue damage in some circumstances, but also function to preserve homeostasis in the CNS (Minghetti and Levi 1998; Streit 2002). Neuroprotection is demonstrated by the role of microglia in the regeneration of motor neuron axons after facial nerve axotomy (Streit 2002). Facial nerve axotomy involves transecting or severing the axons of the facial nerve, and is a widely used model of degeneration and regeneration of the nervous system *in vivo* (Moran and Graeber 2004). There is effective communication between neurons and microglia, as evidenced by the activation of microglia within the CNS subsequent to transection of axons in the periphery (Streit 2002) (Bessis, Bechade et al. 2007). Activated, phagocytic microglia surround dying neurons and neuronal debris, and following axotomy, function to remove synaptic terminals from the cell body, a process that has been termed "synaptic stripping" (Moran and Graeber 2004) (Raivich, Jones et al. 1998). The activation, proliferation and ensheathment of neurons by microglia in response to neuronal injury are vital to the regeneration of motor axons, and highlight the role of microglia as neuroprotective cells in the CNS.

CD8 T cell mediated disease in the CNS

The apparent interactions between microglia and neurons reflect a dynamic CNS environment that promotes communication amongst

resident cells. For antigen-specific T cell mediated neurodegeneration, there is a requirement for contact between neurons and infiltrating T cells. In our model of anti-Hu syndrome, disease pathogenesis depends on the targeting of HuD expressing neurons by activated HuD-specific CD8 T cells. This in turn requires MHC I expression on neurons, which then serve as targets for CD8 T cells. Whereas peripheral tissues readily express MHC I molecules in order to guard against intracellular infection, healthy neurons have undetectable surface expression of MHC I, supporting the perception of the CNS as an immune privileged site. Under pathologic conditions, however, when the CNS environment switches from being immunosuppressive to inflammatory, neurons upregulate MHC I expression. Upregulation of MHC I is achieved by silencing electrical activity in combination with exposure to pro-inflammatory cytokines, most notably IFNγ (Neumann, Cavalie et al. 1995; Neumann, Schmidt et al. 1997). After induction of MHC I expression, neurons are susceptible to antigen-specific lysis by activated CD8 T cells (Medana, Gallimore et al. 2000). CD8 T cells can kill neurons by perforin-induced pore formation or through induction of apoptosis by Fas ligand (Rensing-Ehl, Malipiero et al. 1996; Medana, Gallimore et al. 2000). Studies of CD8-mediated neuronal death have relied on *in vitro* systems, using cultured neurons treated with cytokines or chemicals to induce MHC I expression. There is still a need for

conclusive evidence of CD8 T cell mediated killing of neurons *in vivo,* under pathologic conditions.

EAE is generally considered a CD4 T cell mediated illness, since adoptive transfer of myelin-specific CD4 T cells is sufficient to induce disease in naïve animals. However, CD8 T cells are equally capable of initiating autoimmune demyelination. Demyelination can be generated by CD8 T cells specific for myelin basic protein (MBP), and depends on the production of the inflammatory cytokine IFNγ (Huseby, Liggitt et al. 2001). Adoptively transferred MBP-specific CD8 T cells are able to traffic to the CNS and induce autoimmune neurologic disease in recipient animals. Evidence from studies of MS patients supports the idea that CD8 T cells play an important role in autoimmune demyelination (Ji and Goverman 2007). CD8 T cells outnumber CD4 T cells by nearly ten-fold in MS brains (Booss, Esiri et al. 1983). In addition, CD8 T cells specific for a protein expressed in oligodendrocytes were detected in the blood of patients with MS but not control subjects (Niland, Banki et al. 2005). These T cells were shown to lyse oligodendrocytes expressing the appropriate MHC I allele *in vitro,* which suggests a possible mechanisms for CD8 T cell mediated demyelination in patients.

CD8 T cell mediated neurologic disease occurs spontaneously in transgenic mice with constitutive expression of the costimulatory

molecule B7.2/CD86 on microglia (Zehntner, Brisebois et al. 2003; Brisebois, Zehntner et al. 2006).   Expression of B7.2/CD86 on resident CNS microglia creates a permanent immunostimulatory environment for infiltrating T cells that naturally leads to the development of autoimmunity, highlighting the importance of inflammation behind the blood-brain barrier to the generation of CNS autoimmune disease.   In this model, CD8 T cells are the effector cells responsible for disease pathogenesis, whereas CD4 T cells play an inhibitory role in disease development.   In the absence of CD4 T cells, B7.2 transgenic mice succumb to neurologic disease more rapidly, suggesting a protective role for CD4 T cells in the activation of myelin-specific CD8 T cells (Brisebois, Zehntner et al. 2006).   Regulatory CD4 T cells may help to suppress CD8 T cells specific for CNS self-antigens, so that depletion of the CD4 T cell repertoire in the B7.2 transgenic model serves to eliminate this important inhibitory population, resulting in accelerated disease.

Regulatory T cells in the CNS

It is unclear how regulatory T cells react to inflammation in the CNS. Pathologic inflammatory responses behind the blood brain barrier are apparently able to overcome immune suppression induced by regulatory T cells. With regard to designing better therapeutic approaches, it is important to determine whether regulatory T cells fail to traffic to the target organ altogether, or if their mode of suppression is inhibited in the

midst of an inflammatory response.   In mice that were immunized with MOG to induce EAE, it was found that regulatory T cells persisted in the CNS during the course of disease but were unable to prevent demyelination by MOG-specific effector CD4 T cells (Korn, Reddy et al. 2007).   Regulatory T cells from the inflamed CNS effectively blocked proliferation of effector CD4 T cells isolated from the spleen but could not suppress proliferation of CNS effector T cells.   These results suggest that during EAE, regulatory T cells in the periphery are functional suppressors that traffic to the target organ.   Once inside the inflamed CNS, however, these cells are incapable of preventing autoimmune mediated demyelination.   The failure on the part of CNS regulatory T cells to suppress disease seemed to be due, in part, to the cytokine milieu.   Effector CD4 T cells isolated from the CNS secreted large amounts of pro-inflammatory cytokines, namely IL-6 and TNFα (Korn, Reddy et al. 2007).   When peripheral regulatory T cells were exposed to these two cytokines *in vitro*, their suppressive effect was abrogated. This suggests that regulatory T cells in the inflamed CNS are ineffective at limiting autoimmunity due to the production of pro-inflammatory cytokines by effector cells within the target organ.

Inhibition of regulatory T cells within the inflamed CNS during EAE can happen as a result of the local cytokine environment (Korn, Reddy et al. 2007).   Pro-inflammatory cytokines function to reverse the suppressive

properties of regulatory T cells and allow for the escalation of an effective immune response behind the blood brain barrier. Normally, the brain is bathed in TGF-β, a potent anti-inflammatory cytokine. TGF-β acts to maintain tolerance through regulation of lymphocyte proliferation and survival, but it can also drive the differentiation of CD4 T cells into regulatory T cells (Li, Wan et al. 2006). Differentiation of CD4 T cells into regulatory T cells via TGF-β is inhibited when inflammatory cytokines, namely IL-6, are present. In the presence of both IL-6 and TGF-β, CD4 T cells differentiate into Th_{17} cells (Veldhoen, Hocking et al. 2006) (Bettelli, Carrier et al. 2006). Th_{17} cells contribute to many inflammatory immune responses, and have been implicated in the development of autoimmunity (Kikly, Liu et al. 2006). Regulatory T cell development and Th_{17} differentiation were shown to be mutually exclusive pathways for naïve CD4 T cells (Bettelli, Carrier et al. 2006). The presence of IL-6, and perhaps other inflammatory cytokines, is therefore critical to shaping the CD4 T cell immune response. Exposure to inflammatory cytokines in the CNS, where there is constitutive expression of TGF-β, would serve to promote the development of effector rather than inhibitory CD4 T cells.

Tolerance to CNS antigens

The CNS is considered an immune privileged site due to the presence of the blood-brain barrier, which is designed to limit or restrain the initiation of inflammatory immune responses. Inflammation of the CNS does occur, necessitating protection from autoimmunity via tolerance induction to CNS antigens. CNS proteins are not hidden or ignored by the immune compartment, and therefore mechanisms must exist to safeguard against autoimmune neurologic disease. Experimental evidence of tolerance to CNS antigens has focused on the myelin proteins, since EAE is the most prevalent model of CNS autoimmunity. EAE is induced by priming T cells to myelin self-antigens, such as myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), and myelin basic protein (MBP). All three proteins are components of myelin, but tolerance to each is maintained in different ways.

Tolerance to the myelin proteins

Central tolerance ensures that PLP-specific T cells do not escape from the thymus. Thymic expression is restricted to a shorter splice variant of PLP called DM20, so that T cells specific for epitopes within DM20 are deleted during development. C57BL/6 mice are resistant to PLP-induced EAE because DM20 expression in the thymus is sufficient for the elimination of PLP-specific clones. SJL/J mice, conversely, are highly susceptible to PLP-induced EAE since PLP-specific CD8 T cells in

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this strain respond to an epitope not found in DM20 and are not deleted during development (Anderson, Nicholson et al. 2000; Klein, Klugmann et al. 2000).

MOG is a minor component of CNS myelin, distinguished from other myelin autoantigens by its immunodominance. Tolerance to MOG is weak, despite the generation of MOG-specific regulatory T cells in the thymus (Korn, Reddy et al. 2007). The frequency of MOG-specific natural regulatory T cells is so low that these cells are unable to suppress the activation of an immune response to MOG upon immunization (Delarasse, Daubas et al. 2003; Korn, Reddy et al. 2007). MOG-deficient animals mount an immune response to the protein that is identical to that of their wild type counterparts, and MOG-specific T cells from either wild type or MOG-deficient mice induce comparable disease upon adoptive transfer (Delarasse, Daubas et al. 2003).

Tolerance to MBP happens both in the thymus and in the periphery. Thymic expression is limited to a non-classical form of MBP (called golli-MBP) that contains stretches of sequence identical to peripheral MBP (Tranquill, Skinner et al. 1996; Cabbage, Huseby et al. 2007). Because certain epitopes of classic-MBP are missing, tolerance is also mediated by thymic APCs that have acquired MBP from the periphery (Huseby, Sather et al. 2001). Tolerance to MBP has been studied with regard to MBP-

specific CD4 and CD8 T cells. CD4 T cells specific for an immunogenic epitope not found in golli-MBP ($MBP_{121-140}$) are deleted during development through cross presentation of exogenous MBP by thymic APCs (Huseby, Sather et al. 2001).   Negative selection does not completely eliminate MBP-specific CD4 T cells from the repertoire, however, due to developmentally regulated expression of MBP in the periphery. In young mice (< 3 weeks old), a population of MBP-specific CD4 T cells escapes thymic selection as a result of low levels of MBP protein expression during the first 2 to 3 weeks of life (Huseby, Sather et al. 2001).   In order to keep these self-reactive T cells in check, MBP-specific CD4 T cells are suppressed in the periphery by regulatory T cells (Cabbage, Huseby et al. 2007).

Pathogenic MBP-specific CD8 T cells that recognize an epitope contained within golli-MBP (MBP_{79-87}) are also tolerized (Huseby, Ohlen et al. 1999; Perchellet, Stromnes et al. 2004). To study the mechanisms behind tolerance induction of MBP-specific CD8 T cells, two distinct T cell receptor (TCR) transgenic mice were created, both with specificity for MBP_{79-87}. Despite their inherent similarities, these two T cell populations were subject to very different forms of tolerance induction. CD8 T cells from one mouse were subject to central and peripheral tolerance induction.   The majority of these cells were deleted in the thymus during development, and those that escaped were inactivated in the

periphery. CD8 T cells from the second TCR transgenic mouse bypassed both forms of tolerance, as there was no deletion in either the thymus or periphery. The most striking observation was the capacity of this second T cell line to rescue other MBP-specific CD8 T cells from tolerance induction. Based on their experimental results, the authors purpose a model whereby certain MBP-specific CD8 T cells are able to strip APCs of MBP peptide as a means of inducing tolerance (Perchellet, Stromnes et al. 2004).

Tolerance to neuronal proteins?

Tolerance to myelin antigens is maintained by a variety of mechanisms, and understanding how the immune system responds to myelin epitopes is of great clinical significance with regard to designing better therapies for the treatment of MS. HuD is similar to the myelin proteins in that it is specific to the nervous system. Unlike the myelin proteins, HuD is an intracellular protein expressed exclusively by neurons. To date, there is no evidence of tolerance to neuronal antigens. The recognition of Hu-expressing neurons by autoreactive T cells appears to be responsible for neuronal degeneration in patients with the Hu syndrome. Since all SCLCs express the HuD antigen but only a fraction of patients mount a detectable immune response to the protein, there are apparent mechanisms responsible for preventing autoimmunity directed against this antigen. Immune privilege to HuD could potentially occur through

deletion of HuD-specific T cells from the repertoire. If this were true, patients with HuD-specific immune responses would necessarily avoid central tolerance induction. Alternatively, HuD-specific T cells that circulate in the periphery may be ignorant of the protein, given the lack of neuronal MHC expression under non-inflammatory conditions. Reliance on immunologic ignorance is risky, however, as neurons are able to upregulate MHC I expression during inflammation at the same time as activated T cells are allowed entry to the CNS. If HuD-specific T cells avoid thymic deletion, suppression in the periphery via regulatory T cells or anergy induction may serve to prevent the development of neuronal autoimmunity. To date, there is no evidence of tolerance to neuronal antigens, nor a model to explain how such tolerance could be generated.

Specific Aims

Patients with the Hu syndrome mount an immune response to HuD that is evidenced by the presence of high titer antibodies to the protein in the blood and CSF. Hu patients have impressive tumor immunity coupled with severe neurologic disease, presumably mediated by HuD-specific T cells that are able to target both tumor cells and neurons expressing the Hu antigen.

CD8 T cells are designed to monitor the intracellular compartment. Given the intracellular location of the HuD antigen, HuD-specific CD8 T cells are expected to be responsible for recognition and destruction of HuD expressing cells in patients with the Hu syndrome. To better understand the mechanisms behind CD8 T cell mediated neurodegeneration, we sought to generate a mouse model of disease. An exhaustive screen of the entire HuD peptide library was performed in C57BL/6 in order to identify immunogenic epitopes of the HuD protein. Potential epitopes were then assessed for their ability to be naturally processed and presented by the immune system, which allowed for the identification of the immunodominant CD8 T cell epitope of HuD. HuD-specific CD8 T cells were subjected to phenotypic and functional assays in order to determine if these cells behave as typical cytotoxic T cells capable of lysing HuD expressing targets. In order to understand how HuD-specific CD8 T cells mediate neurodegeneration, we established an adoptive transfer model that enabled us to study HuD-specific CD8 T cell trafficking to the CNS.

Maintaining tolerance is critical to protecting the body from destructive immune responses against self-antigens. Studies on the mechanisms behind tolerance induction have focused primarily on peripheral antigens. How the body maintains tolerance to neuronal proteins is poorly understood. To better understand this process, we sought to

investigate tolerance induction to HuD. We also compared tolerance to HuD versus its related family member HuA. HuA shares high sequence homology with HuD, however the protein is not neuron-specific but ubiquitously expressed. The difference in expression between HuD and HuA suggested that the two proteins might be subjected to different forms of tolerance induction. We hypothesized that tolerance to HuA would be stricter compared to HuD since the protein is present in all tissues and not restricted in expression to neurons.

Finally, we sought to identify clinically relevant human HuD epitopes in order to better understand and diagnose anti-Hu syndrome. Prior to this study, no definitive proof of the presence of HuD-specific CD8 T cells in patients with the Hu syndrome existed. In order to determine if HuD-specific CD8 T cells are expanded and activated during disease pathogenesis, we sought to identify human immunogenic CD8 T cell epitopes of HuD. A comprehensive screen of the entire HuD peptide library was performed over 8 human HLA MHC I alleles. In this way, two human CD8 T cell epitopes of HuD were discovered. Based on these results, we designed HuD-specific tetramers in order to identify and characterize HuD-specific CD8 T cells in patients.

CHAPTER II. MATIERALS AND METHODS

Peptides.

The peptide library for iTopia screening was purchased from Jerini Peptide Technologies (Berlin, Germany). All other peptides were purchased from American Peptide Company.

Mice.

Wild type C57BL/6, Thy1.1 (stock no. 000406), and Rag1$^{-/-}$ (stock no. 00216) mice were purchased from The Jackson Laboratory. HuD$^{-/-}$ mice were obtained from H. Okano (ref PNAS paper). A2.1 transgenic HHD mice were provided by Dr. Francois Lemonnier (Pasteur Institute). A2.1 transgenic AAD mice were purchased from The Jackson Laboratory. AIRE mice were purchased from The Jackson Laboratory (stock no. 004743).

HuD Peptide Screen.

C57BL/6 mice were injected in the right footpad with a single HuD peptide emulsified in Titermax adjuvant. To form the peptide emulsion, 75ul of HuD peptide at 10mg/mL in 50% DMSO was added to 75ul ddH$_2$0. This solution was then added to a sterile eppendorf tube containing 150ul Titermax adjuvant. The tube was then vortexed for 30 minutes on high to form an emulsion. The emulsion was drawn into an

insulin syringe and 50ul (approximately 125ug of peptide) was injected per animal. After 7 days, the animal was sacrificed and the right popliteal and inguinal lymph nodes were removed. Lymph nodes were ground between two sterile frosted glass slides to obtain a single cell suspension. CD8 T cells were isolated by MACS purification (Miltenyi Biotec) and plated in a 20 hour IFNγ ELISPOT assay at 2×10^5 CD8 T cells per well. EL4 cells were added as stimulators at 5×10^4 per well with 10uM peptide.

Immunizations.

For immunization with recombinant adenovirus, 6-8 wk old mice were injected with 100ul purified adenovirus (10^9 PFU/mL) i.d. and treated with Pertussis Toxin (Sigma) at days 0 and 2.

For EAE induction, 6-8 wk old C57BL/6 mice were immunized with 100ug MOG_{35-55} in CFA and treated with Pertussis Toxin on days 0 and 2.

Preparation of cells from the brain.

Brains were removed from mice perfused with 50 mL cold heparinized saline and mechanically dissociated using a dounce homogenizer (VWR) in HBSS w/ Ca and Mg (Gibco) containing 500ug/mL Collagenase D (Roche), 5mg/mL Dispase II (Roche), 20U/mL DNase I (Roche) and 10mM HEPES (Gibco). The dissociated tissue in solution was incubated for 15

minutes on a rotating neutator and then left to stand for an additional 15 minutes. The resulting cell suspension was washed once in R-10 media (RPMI 1640 supplemented with 10% FBS, nonessential amino acids, sodium pyruvate, glutamine, 22-ME, gentamicin) and fractionated on a 30/37/70% percoll gradient at 1760rpm for 30 minutes. Cells were collected from the 37/70% interface.

Preparation of primary murine kidney cells.

Kidneys from adult mice were mashed using the back of a syringe and pipetted until a single cell suspension was obtained. The cell suspension was passed over a 70um cell strainer and washed in D-10 (DMEM supplemented with 10% FBS, nonessential amino acids, sodium pyruvate, glutamine, 22-ME, gentamicin) before plating in 10cm dishes. Cultures were fed by replacing D-10 on days 4 and 7. On day 7, recombinant mouse IFNγ (R & D Systems) was added at 10U/mL. On day 8, 10ul recombinant adenovirus at 10^9 PFU/mL was added to each dish and the cells were harvested on day 9 for use in an IFNγ ELISPOT assay.

Adenovirus production.

Recombinant adenoviruses expressing GFP, β-galactosidase, or HuD-GFP was made by H. Okano. Recombinant adenovirus expressing HuA-GFP was made by inserting the full-length HuA gene into the pAdenoTrack

vector as previously described (He, PNAS 1998). Virus was produced by transduction of HEK293 cells (ATCC) and purified using the Adenopure Kit (Puresyn) according to the manufacturer's instructions. Viral titers were assessed by infection of HEK293 cells with serial dilutions of purified virus followed by determination of the number of virally infected cells after 20 hours by fluorescence or XGal staining.

Retrovirus production.

Retrovirus was produced by transfection of ecotropic Phoenix cells (a gift from the DeLange Lab, Rockefeller University) with retroviral constructs containing either HuD-GFP or GFP. 48 hours post transfection, viral supernatant was harvested and used directly to infect target cells. 10mL viral supernatant was added per 10cm dish of targets with polybrene (5mg/mL).

Elispot assay.

CD8 cells were isolated from spleens or lymph nodes of immunized mice using MACS purification (Miltenyi Biotec). T cells were added to IFNγ ELISPOT plates at the indicated concentrations along with stimulators and incubated for 20. Cells were washed out of the ELISPOT plate using a mild detergent followed by incubation with 1ug/mL biotin-conjugated anti-IFNγ mAb. Wells were developed using the Vectastain Elite Kit according to the manufacturer's instructions (Vector Laboratories).

Colored spots represent IFNγ-secreting cells and are enumerated as spot forming cells (SFCs) per 10^6 cells. The ELISPOT plate evaluation was performed by an independent evaluation service (Zellnet Consulting) using an automated ELISPOT reader (Carl Zeiss).

Mouse dendritic cell preparation.

Bone marrow-derived DCs were prepared as previously described (Inaba 1992 JEM). Briefly, bone marrow from tibia and femurs was lysed of RBC and cultured in 6 well plates in R-10 in the presence of GM-CSF (ref for J558L). Fresh GM-CSF supplemented medium was added to the wells on days 2, 4, and 6. On day 7, DCs were matured for 2 days with recombinant mouse TNFα (R&D Systems) by harvesting and re-plating at 10^6/mL in 6 well plates with GM-CSF supplemented medium plus recombinant mouse TNFα (125ng/mL).

Mouse antibody titers.

Sera from mice were diluted in 5% Dry Carnation Milk (Carnation Co.) in .1% Tween/PBS and incubated with PVDF membrane strips containing identical amounts of recombinant HuD antigen. The membrane was then washed thoroughly in .1%Tween/PBS and incubated with horseradish peroxidase-conjugated secondary antibody. Signal was visualized with a chemiluminescent substrate and a 35kD band indicated the presence of anti-HuD antibody.

Western blot with cell lysates.

10^6 cells were lysed in 100ul of passive lysis buffer (Promega). 25ul of lysate (or 1ug rHuD) was run on a 10% SDS-polyacrilamide gel and transferred to a PVDF membrane. The membrane was blocked in 5% Dry Carnation Milk (Carnation Co.) and incubated with Anti-Hu patient sera (at 1:1000). The membrane was then washed thoroughly in .1%Tween/PBS and incubated with horseradish peroxidase-conjugated secondary antibody. Signal was visualized with a chemiluminescent substrate and a 35kD band indicated the presence of HuD protein.

Mouse CD8 T cell stimulations.

For *in vitro* stimulation, 2.5-3 x 10^7 splenocytes from adenovirus-immunized mice were incubated at 37°C in upright T25 culture flasks (Corning) in R-10 (half of a spleen per flask) with .5uM peptide for 7 days. For further rounds of restimulation, splenocytes were plated in 24 well plates (2-6 x10^5 splenocytes per well) with peptide-pulsed feeder cells in R-10 with 50 CU/mL recombinant human IL-2 (Chiron). Feeder cells were prepared from spleens of naïve syngeneic mice by pulsing with .5uM peptide for 1 hour at room temperature and irradiating at 3,000 Rads before plating (one spleen per 24 well plate).

Adoptive transfer.

For adoptive transfer experiments, mice received i.v. injections of *in vitro* stimulated CD8 T cells (5 x 10^6 CD8/mouse) and DCs pulsed with peptide (2 x 10^6 DC/mouse) along with IL-2 (6 injections of 10^5 CU/mouse i.p. every 12 hours) and Pertussis Toxin (400ng/mouse i.p. on days 0 and 2). *In vitro* stimulation of CD8s was performed as described. After 7 days of stimulation, CD8s were isolated by negative selection using a CD8 T cell isolation kit (Miltenyi Biotec). The cells were washed twice and resuspended at 2.5 x 10^7/mL in RPMI 1640DCs for i.v. injection. DCs were prepared from bone marrow as described. For the peptide pulse, TNFα-matured DCs were resuspended at 10^7 DCs/mL in R-10 with 100uM peptide and incubated at room temperature for 1 hour, shaking every 15 minutes. The cells were then washed twice and resuspended at 10^7/mL in RPMI 1640 for i.v. injection.

In Vivo CTL assay.

Mice received a single i.v. injection of target cells. The targets consisted of two populations of cells in a 1:1 ratio: irrelevant peptide-pulsed syngeneic splenocytes labeled with .5uM CFSE (CFSE^{lo}) and relevant peptide-pulsed syngeneic splenocytes labeled with 5uM CFSE (CFSE^{hi}). To prepare target cells, spleens from syngeneic mice were mashed with the back of a syringe, decanted into two 15mL conical tubes, and pelleted. To lyse erythrocytes, each pellet was resuspended in 5mL ACK

lysis buffer (Gibco) and left at room temperature for 4 minutes. Lysis was stopped by adding 8mL cold PBS to each tube. The cell suspensions were combined in a 50mL conical tube, pelleted, resuspended in 10mL warm R-10 and passed through a 70um cell strainer. To pulse with peptide, the cells were divided into two equal populations at a final concentration of 10^7/mL in R-10. One population was pulsed with 10uM cognate peptide and the other with 10uM irrelevant peptide at 37°C for 90 minutes, shaking every 15 minutes. After 90 minutes, excess peptide was removed by washing the cells three times in PBS. The last wash was done in room temperature PBS. Cells were counted and resuspended at 5 x 10^7 in warm (37°C) PBS. The irrelevant peptide-pulsed population was labeled with .5uM CFSE (CFSE^{lo}) and the relevant peptide-pulsed population with 5uM CFSE (CFSE^{hi}). To label the cells, CFSE was added to the tubes, which were then inverted 6 times and placed at 37°C for 10 minutes. Labeling was stopped by adding ice cold PBS to the tubes. The cells were washed once in cold PBS, and then twice in room temperature PBS. The cells were resuspended at 10^8/mL and combined in a 1:1 ratio. Mice received a single i.v. injection of 200ul (10^7 CFSE^{lo} and 10^7 CFSE^{hi} targets). 5-18 hours after target injection, the spleens were removed and the amount of CFSE^{lo} versus CFSE^{hi} targets was quantitated by flow cytometry.

Flow cytometry.

All surface antibodies were purchased from Beckton Dickinson. 10^6 cells were resuspended in 100ul staining buffer (PBS containing 1% pooled human serum, 1% FBS and 5% goat serum) and Fc blocked for 10 minutes using 1ug of anti-CD16/CD32 (FcγR11/III). 1ug of each specific Ab was added directly to the cells and incubated for 15 minutes at 4°C. Cells were then washed twice and analyzed using a BD FACSCalibur instrument.

Tetramer staining

Surface antibodies were purchased from Beckton Dickinson. Tetramers were obtained from Beckman Coulter Immunomics. 10^6 CD8 T cells were resuspended in 100ul staining buffer (PBS containing 1% pooled human serum, 1% FBS and 5% goat serum) and Fc blocked for 10 minutes using 1ug of anti-CD16/CD32 (FcγR11/III). Cells were then incubated with 1:50 dilution of tetramer for 20 minutes at room temperature. 1ug of antibody to CD8 was added for an additional 10 minutes. Cells were washed and analyzed immediately using a BD FacsCaliber.

In Vitro CTL Assay.

Splenocytes from immunized animals were restimulated in 24 well plates (one spleen divided evenly among 24 wells) for 6 days with .5uM peptide

in R-10. On day 6, cells were harvested and resuspended at 5×10^6/mL in R-10. For target cell preparation, 10^6 EL4 cells in 100ul of R-10 were labeled with 10uM peptide plus 100uCi Cr^{51} for 1 hour at 37°C, shaking every 10 minutes. EL4 cells were then washed 3 times in 1mL R-10 and resuspended at 10^5/mL. 5×10^3 EL4 cells were plated per well in a round bottom plate with serial dilutions of T cells, beginning with 5×10^5 T cells per well (100 T cells to 1 target cell) and ending at 3.125×10^4 T cells per well (6.25 T cells to 1 target cell). Plates were spun at 300rpm for 5 minutes and incubated at 37°C for 4.5 hours. To harvest and measure radioactivity from the CTL assay, 100ul of supernatant from each well was pipetted into a sample plate for gamma measurement with 100ul gamma scintillation fluid.

RMA/S Assay.

RMA/S cells were plated in T-75 flasks at 5×10^5/mL in D-10 at 37°C for 9 hours and then placed at room temperature overnight with caps tightly sealed. RMA/S cells were harvested and resuspended in R-10 at 5×10^6/mL.   50ul of RMA/S cells were added to 50ul of peptide stock. Peptides stocks were made at 100uM, 10uM, 1uM, 100nM, 10nM. Duplicate tubes of RMA/S cells with peptide were incubated at room temperature for 45 minutes. 4mL of room temperature PBS was added to each tube and placed at either room temperature or 42°C for 5 hours. For FACS staining, cells were resuspended in 50ul staining buffer (PBS

containing 1% pooled human serum, 1% FBS and 5% goat serum) and Fc blocked for 10 minutes at room temperature using 1ug of anti-CD16/CD32 (FcγR11/III). 1ul of biotin anti-mouse H-2D^b antibody at.5mg/mL (BD Pharmingen, clone #28-14-8) was added and cells were incubated at room temperature for 15 minutes. Cells were washed in 4mL room temperature PBS and resuspended in 100ul staining buffer with 1ul streptavidin-PE at .5mg/mL (BD Pharmingen) and incubated for 10 minutes at room temperature. Cells were washed in 4mL room temperature PBS and analyzed by FACS.

iTopia screen.

Peptides were dissolved in 10mM DMSO and stored in aliquots at -20°C. Screening assays were carried out according to the manufacturer's instructions using the provided reagents (Beckman Coulter). In the initial binding assay, peptides were added individually at .1mM to plates coated with recombinant HLA molecules. β2 microglobulin and FITC-labeled anti-HLA class I were also added and the plates were then incubated for 18 hours at 21°C before being washed and read by a fluorescent plate reader (Ex 490nm, Em 520nm). For affinity measurements, peptides were assayed as described above over eight serial dilutions. For off-rate analysis, peptides were added to HLA coated plates as described above, incubated at 21°C for 18 hours and then washed and placed at 37°C for 0, .5, 1, 2, 4, 6, and 8 hours before

being read. Data from each assay was analyzed in Prism using iTopia software and iScores were generated from a proprietary formula that takes into account measurements from all three assays.

Peripheral blood isolation.

Blood cells were collected by leukapheresis under a Rockefeller University IRB-approved protocol with informed consent. PBMC were isolated by density gradient centrifugation over Ficoll-Hypaque (Pharmacia), and separated into T cell enriched (ER+) and T cell depleted (ER-) fractions by rosetting with neuraminidase-(Calbiochem, La Jolla, CA) treated sheep red blood cells (Colorado Serum Company, Denver, CO). These cell fractions were cryopreserved by freezing in 10% human serum albumin/ 10%DMSO/RPMI.

Human dendritic cell culture.

Human dendritic cells (DC) were generated from peripheral blood mononuclear cells (PBMC). Briefly, ER- cells were cultured in the presence of 100U/ml IL-4 (R&D Systems) and 100U/ml GM-CSF (Immunex) for 6 days to generate immature DC. DC were harvested on day 6 and plated with PGE2 and TNFa to induce maturation. After 2 days mDC were harvested and used for T cell stimulation.

Human CD8 T cell isolation.

Isolation of T cell fractions has been described (Albert, Jegathesan et al. 2001). Briefly, ER+ PBMC fractions were thawed incubated with anti-CD8 MACS beads (Miltenyi Biotech, Auburn, CA) in 5%FBS/PBS, washed and run through a magnetic column to isolate antibody bound cells. After washing the column, the positive cells were eluted by plunging the column. For experiments with adenovirus-immunized mice, spleens were harvested on day 13 and CD8 T cells were purified in a manner similar to the human protocol using anti CD8a beads (Miltenyi).

In vitro stimulation of human peripheral blood T cells.

CD8 T cells were incubated with autologous mDC at a 30:1 ratio in 24 well plates with 10ug/ml peptide in AIM-V medium (Invitrogen) supplemented with 5%FBS. After two days of culture recombinant human IL-2 (Chiron, CA) was added at 50IU/ml.

Tetramer staining on human T cells.

All HLA-A0201 tetramers were obtained from Beckman Coulter Immunomics. 1.0×10^6 patient PBMC were incubated with 1:20 dilution of tetramer for 20 minutes at room temperature. Antibody to CD8 was then added to the cells for an additional 10minutes, washed and analyzed immediately using a BD FacsCaliber. Cells were gated on the CD8+ population.

Quantitative western blot with human sera.

For confirmation of Hu antibody titers in patients, quantitative western blot analysis with patient serum and recombinant HuD (rHuD) protein was performed as described previously (Dalmau, Furneaux et al. 1990). Briefly, 20ug of nickel-column purified his-tagged rHuD was loaded into a large, single well of a SDS-polyacrylamide gel and transferred to PVDF membrane. The blocked membrane was incubated with dilutions of patient serum and horseradish peroxidase-conjugated secondary antibody and the signal visualized with a chemiluminescent substrate. The presence of reactivity to HuD, a 35kD protein, at a dilution greater than 1:1000 indicates a high titer Hu patient. Sera from normal donors were used as a negative controls and known positive patient samples as positive controls.

Tetramer+ T cell sorting.

CD8 T cells were purified by positive selection (Miltenyi) from the frozen T cell fractions of patient leukapheresates. 1 x10^6 CD8 T cells were plated per well of a 24 well plate with 6000 peptide-pulsed, autologous mDC in AIM-V medium (Invitrogen) containing 5% FBS. 24 hours after initiation of the culture, IL-2 was added at 10U/ml. After 8-12 days of culture the cells were harvested and incubated at 20 x 10^6/ml with a 1:20 dilution of tetramer. The tetramer-positive population was sorted on a FACS Aria instrument (BD BioSciences). After sorting, cells were placed into

culture in 96-well plates with autologous, peptide-pulsed PBMC and 50U/ml IL-2. After a recovery period of 8 days in culture, T cells were used in ELISPOT and CTL assays.

CHAPTER III. IDENTIFICATION OF THE IMMUNODOMINANT H-2^b CD8 T CELL EPITOPE OF HUD

Introduction

In order to study the mechanisms responsible for both T cell mediated neurologic degeneration and neuronal tolerance induction, we first sought to identify and characterize HuD-specific CD8 T cell clones.   A comprehensive screen of the entire HuD peptide library was performed in C57BL/6 mice, which lead to the identification of the immunodominant CD8 T cell epitope of HuD. HuD-specific CD8 T cells were subjected to phenotypic and functional assays in order to characterize these cells and assess their ability to lyse HuD expressing targets *in vitro* and *in vivo*.

Results

Comprehensive screen to identify potential mouse H-2^b HuD epitopes

To determine if HuD-specific CD8 T cells are present in the mouse H-2^b repertoire, we performed a comprehensive screen of the entire HuD peptide library in C57BL/6 mice. C57BL/6 mice were chosen both for their ability to preferentially generate cell mediated rather than humoral immune responses and because most immunologically relevant transgenic strains are bred onto the C57BL/6 background. The HuD peptide library consisted of 386 overlapping nonamers, including peptides derived from each known splice variant of the protein. In order

to ensure that all HuD peptide-specific CD8 T cells present in the H-2^b T cell repertoire were identified, we chose an aggressive immunization strategy that allowed for the expansion of peptide-specific CD8 T cells without the need for an APC or CD4 T cell help. Mice were immunized with each individual HuD peptide emulsified in Titermax adjuvant. After 7 days, CD8 T cells were isolated from the draining lymph nodes and assessed for their ability to secrete IFNγ in an ELISPOT assay with cognate peptide-pulsed syngeneic stimulators. As a negative control, syngeneic stimulators were pulsed with an irrelevant peptide. The secretion of IFNγ in response to cognate peptide indicated that there were HuD-specific CD8 T cells present in the T cell repertoire. After screening the entire peptide library in this fashion, we identified 7 potential HuD CD8 T cell epitopes (Fig 1a).

Identification of the immunodominant H-2^b HuD epitope

We next sought to identify naturally processed and presented HuD CD8 T cell epitopes from these 7 potential peptides. The presence of CD8 T cells specific for a given peptide in the H-2^b repertoire does not by itself demonstrate natural processing and presentation. In order to show natural processing and presentation, mice were immunized with a replication-deficient recombinant adenovirus expressing full length HuD to allow for *in vivo* processing and presentation of HuD epitopes onto MHC I molecules and subsequent CD8 T cell priming. To further

Fig 1. p321 is the immunodominant CD8 T cell epitope of HuD. (a) The entire HuD peptide library was screened in C57BL/6 mice. 7 peptides (in bold) were identified as potential CD8 epitopes. (b) C57BL/6 mice were immunized with AdVHuD plus PTx. 13 days after immunization, splenocytes were divided into 8 in vitro stimulation cultures and stimulated with each of the 7 HuD peptides or the immunodominant Bgal epitope p96. CD8 T cells were purified from stimulation cultures and plated (10^4 T cells/well) with cognate or irrelevant peptide-pulsed irradiated EL4 cells (5 x 10^4/well) in an IFNg ELISPOT assay. (c) As a control for in vitro priming, C57BL/6 mice were immunized with AdVBgal + PTx and stimulated in vitro with each of the 7 potential HuD epitopes or Bgal p96 and assayed for IFNg secretion as in (b).

Fig. 1

(a)

HuD Protein

MEWNGLK**MIISTMEPQ**VSNGPTSNTSNGPSSNNRNCPSPMQTGAATDDSKTNLI**VNYLPQ
NMT**QEEFRSLFGSIGEIESCKLVRDKITGQSLGYGFVNYIDPKDAEKAINTLNGLRLQTKTIKV
SYARPS**SASIRDANL**YVSGLPKTMTQKELEQLFSQYGRIITSRILVDQVTGVSRGVGFIRFDK
RIEAEEAIKGLNGQKPSGATEP**ITVKFANNP**SQKSSQALLSQLYQSPNRRYPGPLHHQAQRF
RLDNLLNMAYGVKRLMSGPVPPSACPPRFSPITIDGMTSLVGMNIPGHTGT**GWCIFVYNL**SP
DSDESVLWQLFGPF**GAVNNVKVIRDFNTN**KCKGFGFVTMTNYDEAAMAIASLNGYRLGDR
VLQVSFKTNKAHKS

(b)
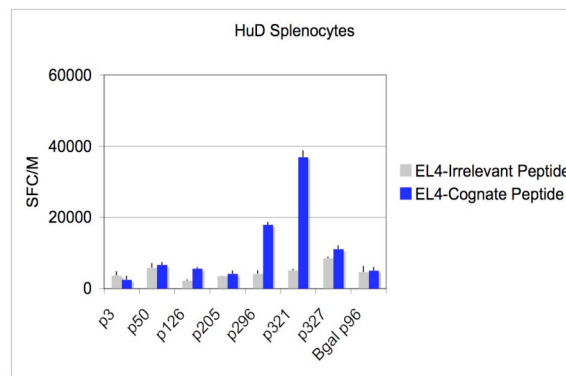
(c)
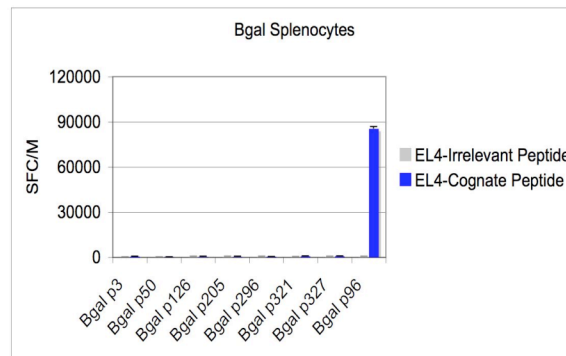
expand the population of peptide-specific CD8 T cells after adenovirus immunization, splenocytes from immunized animals were stimulated *in vitro* for 7 days with each of the 7 potential HuD epitopes. CD8 T cells were then isolated from stimulation cultures and T cell activation was assessed in an IFNγ ELISPOT assay using cognate peptide-pulsed cells as stimulators (Fig 1b). In order to ensure that a positive CD8 T cell response was the result of *bona fide* natural processing and presentation *in vivo* and not due to *in vitro* priming, we immunized a second group of mice with a replication-deficient recombinant adenovirus expressing the neo-antigen β-galactosidase (β-gal). Splenocytes from β-gal immunized mice were stimulated *in vitro* with each of the 7 potential HuD epitopes and T cell activation was assessed in an IFNγ ELISPOT assay using cognate peptide-pulsed cells as stimulators (Fig 1c). Of the 7 potential HuD epitopes, peptides 296 and 321 were able to prime CD8 T cells *in vivo* after adenovirus-HuD immunization. Neither of these peptides elicited IFNγ secretion after immunization with adenovirus-β-gal, excluding the possibility of *in vitro* priming. Based on the strength of the immune response to each of these two peptides, we concluded that peptide 321 (p321) represents the immunodominant epitope of HuD.

(a)
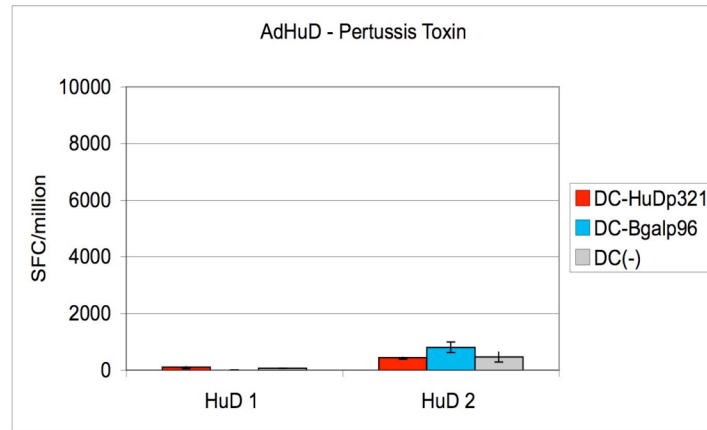
(b)

Fig 2.   Pertussis toxin (PTx) is required for HuD-specific CD8 T cell priming.   C57BL/6 mice were immunized with AdVHuD alone (a) or AdVHuD plus PTx (b). 13 days after immunization, splenoc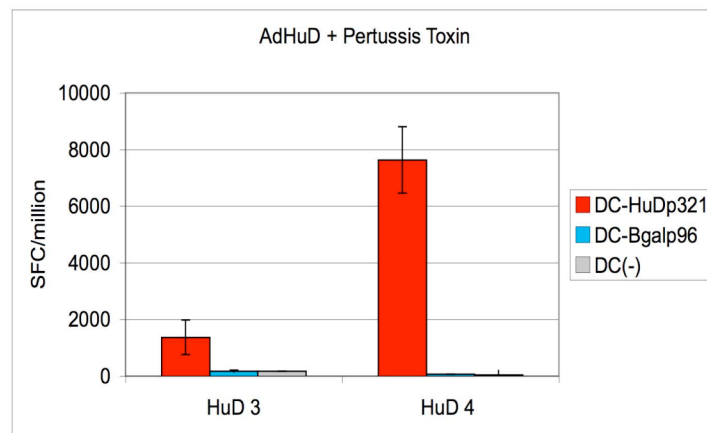ytes were stimulated in vitro with p321. CD8 T cells were purified from stimulation cultures and plated (10^4 T cells/well) with cognate or irrelevant peptide-pulsed dendritic cells (7×10^3/well) in an IFNg ELISPOT assay.

Pertussis Toxin is required for the generation of IFNγ-secreting HuD-specific CD8 T cells

Immunization with adenovirus-HuD followed by *in vitro* stimulation with peptide proved to be an effective priming regiment for HuD-specific CD8 T cells only if pertussis toxin was injected together with adenovirus. Immunizing with adenovirus-HuD alone did not generate IFNγ-secreting HuD-specific CD8 T cells even after p321 stimulation *in vitro* (Fig 2). Historically, pertussis toxin (PTx) has been used in the induction of EAE. EAE is arguably the most studied example of autoimmunity in the CNS, and thus it seemed logical to look to this model of disease for guidance in the generation of an immune response to HuD.   PTx was historically thought to act on EAE by serving to break down the blood brain barrier. We now understand that PTx increases adhesion molecule expression, thereby initiating leukocyte infiltration into the brain (Kerfoot, Long et al. 2004).   Recent work has shown that PTx also induces maturation of dendritic cells, which promotes the differentiation of effector T cells (Hou, Wu et al. 2003).   In our attempt to generate cytotoxic CD8 T cells specific for HuD, this last effect was especially relevant.   In order to determine if PTx could assist in the production of HuD-specific effector T cells, mice were immunized with adenovirus-HuD alone or in combination with PTx. Mirroring the EAE induction protocol, we injected PTx intraperitoneally (i.p.) on day 0 and day 2.   Splenocytes from immunized mice were then stimulated with p321 *in vitro* and assessed for

their ability to secrete IFNγ in an ELISPOT assay.   Mice that received adenovirus-HuD alone were unable to produce IFNγ-secreting CD8 T cells specific for HuDp321, whereas mice receiving PTx in combination with adenovirus-HuD generated a robust peptide-specific response (Fig 2).

D^b MHC I molecules present p321

In order to further characterize HuDp321-specific CD8 T cells, we defined their MHC restriction. This was an important issue to address with regard to designing tetramers for these cells. A tetramer consists of a fluorochrome-tagged complex of four MHC I:peptide molecules and is able to bind specifically to the T cell receptor on a CD8 T cell. Initially, we were able to predict the MHC I specificity of p321 by using publicly available prediction algorithms of peptide-MHC interactions. Based on its amino acid sequence, both the Bioinformatics and Molecular Analysis Section of the National Institutes of Health (BIMAS) and the University of Tubingen (SYFPEITHI) predicted p321 to bind D^b MHC I. To formally prove its MHC I restriction, we took advantage of a transgenic mouse strain that does not express K^b but retains expression of D^b MHC I. H2^{bm1} mice are deficient in K^b expression due to a point mutation at the H-2 K^b locus.   Primary kidney cells derived from either wild type C57BL/6 mice or H2^{bm1} mice were pulsed with peptide in order to determine the MHC I restriction of p321.   As a positive control, we pulsed these same sets of stimulators (wild type versus H2^{bm1} kidney

cells) with the β-gal epitope p96, which binds to K^b MHC I molecules. Peptide-pulsed kidney cells were used as stimulators in an IFNγ ELISPOT assay with 3x-restimulated HuDp321-specific and β-galp96-specific CD8 T cells (Fig 3). As expected, β-galp96-specific CD8 T cells responded to wild type p96 pulsed stimulators but not to $H2^{bm1}$ pulsed stimulators. HuDp321-specific CD8 T were able to respond equally well to wild type and $H2^{bm1}$ p321 pulsed stimulators, confirming the prediction that p321 is a D^b restricted CD8 T cell epitope.

Characterization of HuDp321-specific T cells

Next, we assessed the affinity of HuDp321-specific CD8 T cells. Using twice re-stimulated HuDp321-specific CD8 T cells, we measured the ability of these cells to recognize serial dilutions of peptide pulsed onto syngeneic dendritic cells in an IFNγ ELISPOT assay (Fig 4). HuDp321-specific CD8 T cells were able to secrete IFNγ in response to 10^{-9} M peptide, but below this concentration the response was lost. Twice re-stimulated β-galp96-specific CD8 T cells were also able to recognize 10^{-9} M peptide (data not shown). Thus, HuDp321-specific CD8 T cells are relatively high affinity T cells. Finally, we asked if HuDp321-specific CD8 T cells display a characteristic cell surface phenotype upon activation. We chose to look at CD25, CD69 and CD62L as markers of T cell activation. CD25 is the IL-2 receptor α chain and is up-regulated on activated T cells. CD62L (or L-selectin) is a member of the selectin

WT Stimulators

Bgalp96 CD8 T cells   HuDp321 CD8 T cells

K^b -/- Stimulators
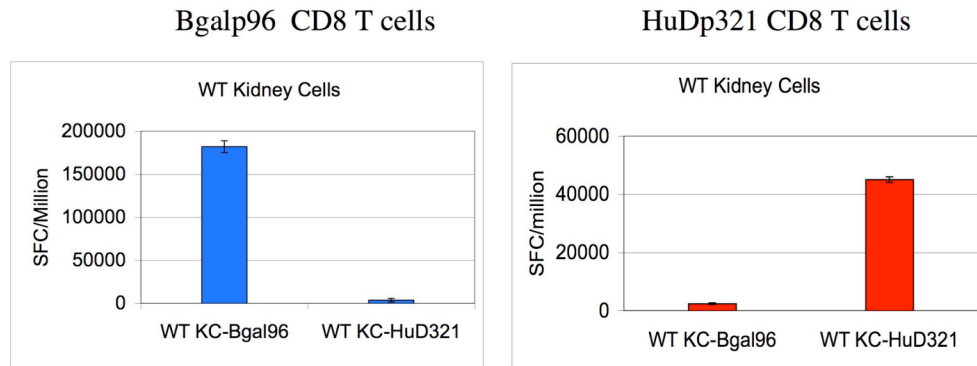
Bgalp96 CD8 T cells   HuDp321 CD8 T cells
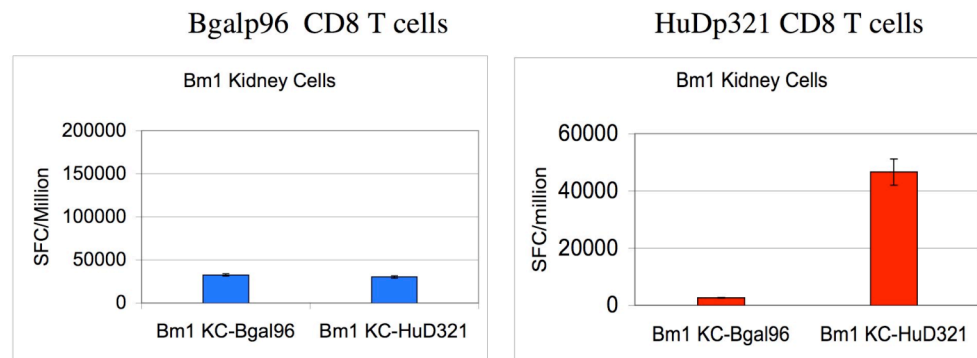
Fig 3.   HuD p321 is presented on Db MHC I. (a) Primary kidney cells from C57BL/6 mice (Db+/ Kb+) or transgenic Bm1 mice (Db+/ Kb-) were irradiated and pulsed with p321 or p96 and used as stimulators in an IFNg ELIPOST assay (5 x 10^4/well) with 3x restimulated HuD p321-specific or Bgal p96-specific CD8 T cells (10^4/well).
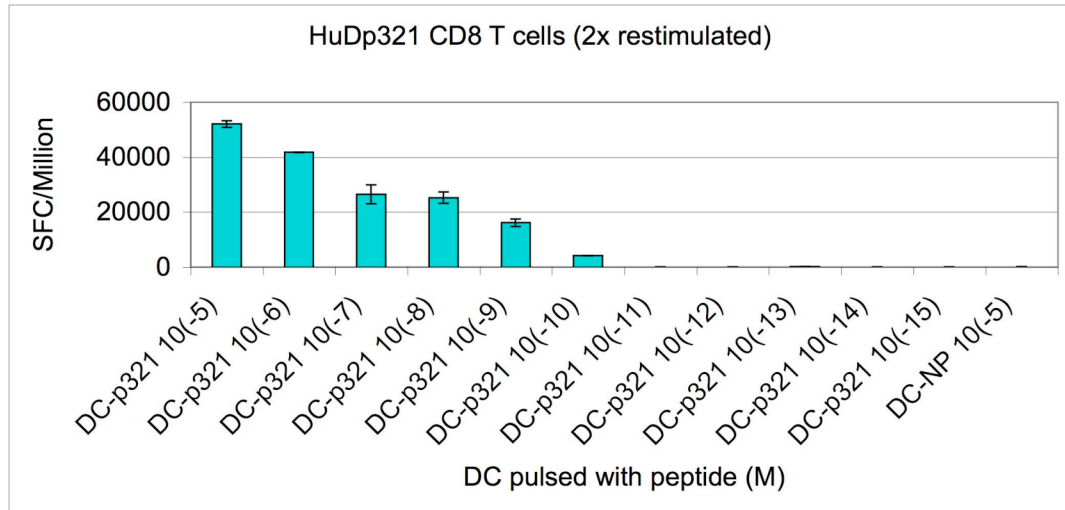
Fig 4. HuDp321-specific CD8 T cells are high affinity T cells. HuDp321-specific CD8 T cells (2x restimulated) were plated in an IFNg ELISPOT assay (1.5 x 10^4/well) with C57BL/6 DC pulsed with serial dilutions of cognate peptide or NP as a negative control (10^4/well).

family of cell adhesion molecules and plays an important role in lymphocyte homing to peripheral lymph nodes. It is a glycoprotein expressed on naïve T cells and down-regulated upon activation-induced differentiation. CD69 is an early activation antigen expressed by T cells. After stimulating HuDp321-specific CD8 T cells *in vitro* with cognate peptide for 7 days, we looked at expression of these three surface markers by flow cytometry (Fig 5). As expected, both CD25 and CD69 were up-regulated whereas CD62L was down-regulated on activated HuDp321-specific T cells. In comparison, naïve T cells expressed no CD25 or CD69 but had high expression of CD62L, whereas activated β-galp96-specific CD8 T cells displayed an identical cell surface phenotype to activated HuDp321-specific T cells (Fig 5). Thus, HuDp321-specific CD8 T cells display a characteristic cell surface phenotype upon activation.

Cytolytic function of HuDp321-specific T cells in vitro

Having established that HuDp321-specific cells are relatively high affinity CD8 T cells with a characteristic activated cell surface phenotype, we next asked if they could function as effector CD8 T cells capable of lysing targets expressing cognate peptide. The ability of CD8 T cells to lyse target cells in an antigen-dependent manner is a critical function of the immune system and an important part of the body's defense against intracellular pathogens. Activated CD8 T cells are aptly referred to as
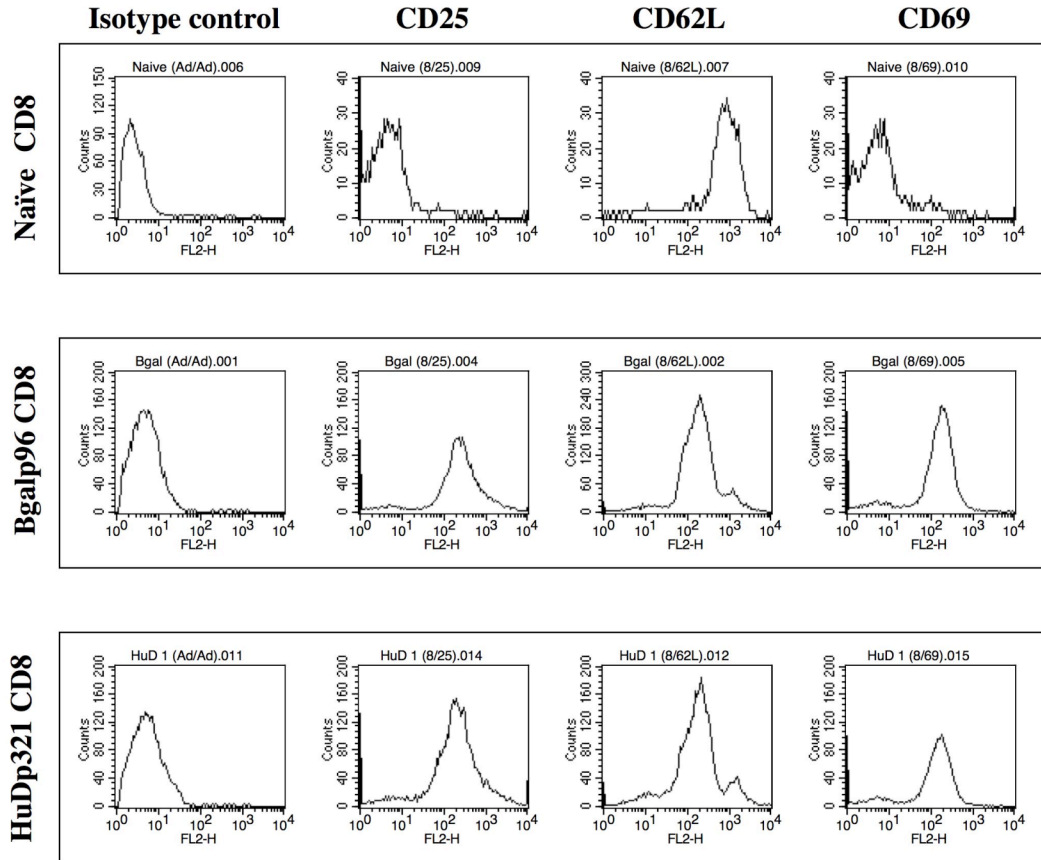
Fig 5. Stimulated HuDp321-specific CD8 T cells display a characteristic cell surface phenotype. In vitro stimulated HuDp321-specific or Bgalp96-specific CD8 T cells were stained on day 7 for CD25, CD62L, and CD69. Naïve T cells from the spleen of a C57BL/6 were used as a control. Plots are gated on CD8+ cells.

cytotoxic T lymphocytes (CTL).   We first examined the lytic ability of HuDp321-specific cells *in vitro*, using chromium labeled peptide-pulsed syngeneic cells as targets (Fig 6).   Splenocytes from adenovirus-HuD immunized animals were stimulated *in vitro* with p321.   Activated T cells were then cocultured with targets pulsed with cognate or irrelevant peptide.   For comparison, β-galp96-specific T cells were assessed for their lytic ability in an identical assay (Fig 6).   Both HuDp321-specific T cells and β-galp96-specific T cells were able to lyse target cells expressing cognate peptide.   β-galp96-specific T cells had a greater percent lysis compared to HuDp321-specific T cells, however this is explained by the larger number of p96-specific T cells present after *in vitro* stimulation. Stimulating splenocytes from adenovirus-HuD immunized animals *in vitro* with p321 results in approximately 1% p321-specific CD8 T cells (Fig 7).   In contrast, after *in vitro* stimulation of adenovirus-β-gal splenocytes with p96, 5-10% of the CD8 T cells are specific for p96 (Fig 7).   For our *in vitro* CTL assay, bulk cells from *in vitro* stimulation cultures were counted and plated and thus there were roughly 10-fold more p96-specific CD8 T cells than p321-specific CD8 T cells per well. Given the results of the *in vitro* CTL assay, we can conclude that HuDp321-specific T cells are functional effectors capable of lysing target cells in an antigen-dependent manner.
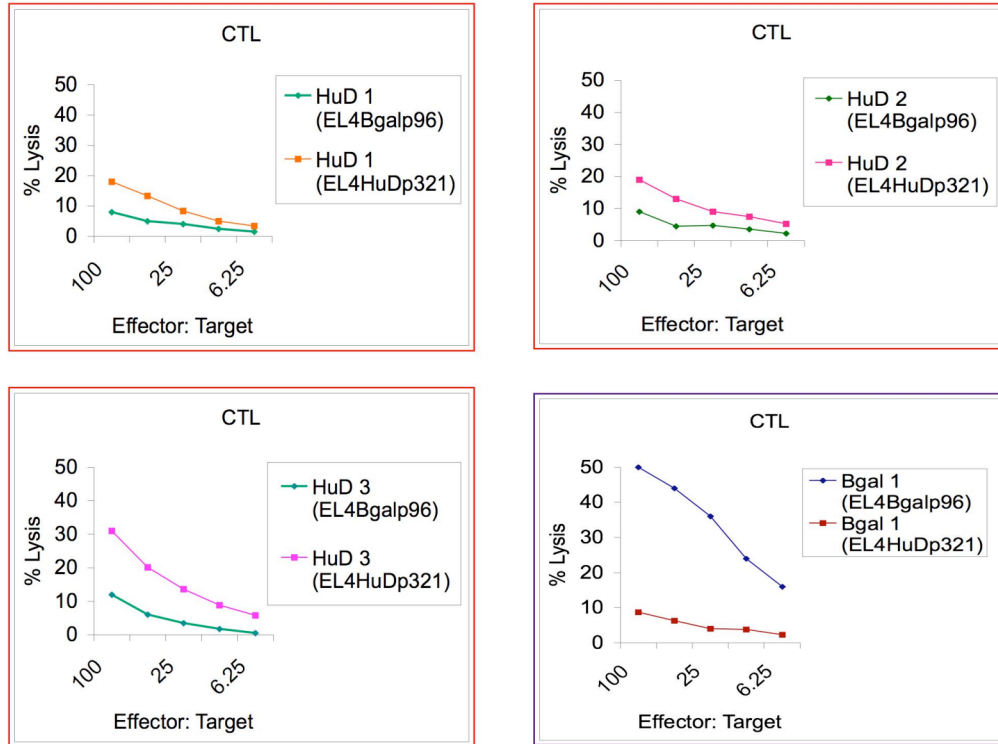
Fig 6. HuDp321-specific CD8 T cells can kill *in vitro*. C57BL/6 mice were immunized with AdVHuD plus PTx or AdVBgal plus PTx. 13 days after immunization, splenocytes were stimulated in vitro with cognate peptide (HuDp321 or Bgalp96). On day 6, cells from stimulation cultures were plated with chromium labeled peptide-pulsed EL4 cells at the indicated E:T ratios. EL4 cells were pulsed with 10uM Bgalp96 or 10uM HuDp321. In red, three individual AdVHuD-immunized mice. In blue, one representative AdVBgal-immunized mouse.

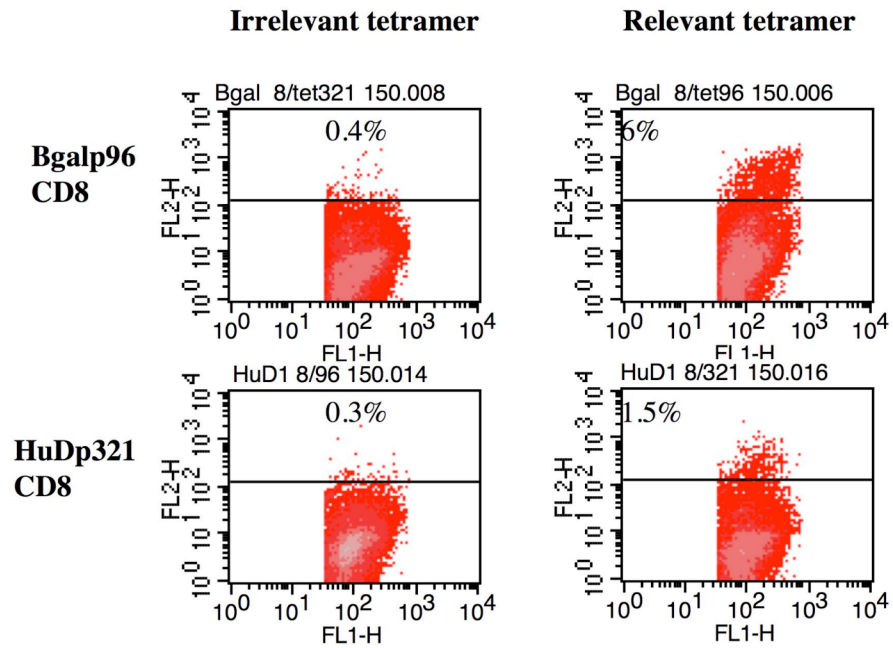**Fig 7.   Frequency of *in vitro* stimulated CD8 T cells.** C57BL/6 mice were immunized with AdVHuD plus PTx (a) or AdVBgal plus PTx (b). 13 days after immunization, splenocytes were stimulated in vitro for 7 days with cognate peptide (HuDp321 or Bgalp96). CD8 T cells were purified from stimulation cultures and stained with Bgalp96 tetramer or HuDp321 tetramer. Plots are gated on CD8 T cells.

Cytolytic function of HuDp321-specific T cells in vivo

If HuD-specific CD8 T cells are responsible for generating neuronal autoimmunity in anti-Hu syndrome, these cells must be capable of functioning as cytotoxic T cells *in vivo*. In order to examine the lytic ability of HuD-specific cells *in vivo,* we adoptively transferred T cells into a syngeneic host and measured their ability to lyse a population of peptide-pulsed target cells. Targets consisted of C57BL/6 splenocytes labeled with carboxyfluoroscein succinimidyl ester (CFSE) and pulsed with peptide. Two populations of target cells were injected into the host, a cognate peptide-pulsed population and an irrelevant peptide-pulsed control population. These two populations were distinguished by their CFSE intensity (p321-pulsed targets were labeled with a high concentration of CFSE [CFSE^{hi}] and p96-pulsed targets were labeled with a low concentration of CFSE [CFSE^{lo}]) and were mixed at a 1:1 ratio before being injected intravenously. 6 to 18 hours after target injection, the host animal was sacrificed and the amount of each target population remaining *in vivo* was measured by flow cytometry (Fig 8). In a host that received HuDp321-specific CD8 T cells, the p321-pulsed CFSE^{hi} population was reduced, demonstrating *in vivo* antigen-dependent lysis by HuDp321 T cells. Similarly, in a host containing β-galp96-specific T cells, the p96-pulsed CFSE^{lo} population was eliminated. Mirroring the results of our *in vitro* CTL assay, β-galp96-specific T cells had a greater amount of specific lysis compared to HuDp321-specific cells, again due

to the increased numbers of p96-specific cells after *in vitro* stimulation. In order to enhance the effector function of our transferred T cells, we injected mature C57BL/6 dendritic cells (DC) pulsed with p321 along with stimulated HuDp321-specific CD8 T cells. We reasoned that this peptide-pulsed DC population would provide an *in vivo* re-stimulation for HuDp321-specific T cells, thereby increasing their numbers and enhancing the amount of lysis. Host animals were injected with *in vitro* stimulated HuDp321-specific cells in combination with DCs pulsed with p321 or, as a control, DCs pulsed with p96. Similarly, a second group of animals was injected with stimulated β-galp96-specific T cells together with peptide-pulsed DCs. The addition of cognate peptide-pulsed DCs increased the amount of lysis by both HuDp321-specific T cells and β-galp96-specific T cells (Fig 8). This increase was not due to non-specific stimulation of T cells by DCs, as the addition of irrelevant peptide-pulsed DCs did not increase the lytic ability of our T cells. We chose to assess target lysis by T cells *in vivo* in two different strains of mice: wild type C57BL/6 mice and transgenic Rag$^{-/-}$ animals that are deficient for the Rag-1 gene and therefore lack mature T cells and B cells. Rag$^{-/-}$ mice were used because we were concerned that the endogenous T cell repertoire, which includes regulatory T cells, could potentially serve to inhibit our transferred cells and suppress their effector function. Our initial *in vivo* CTL experiment used the Rag$^{-/-}$ strain in order to provide an empty host devoid of endogenous T cells (Fig 8). Transferred

HuDp321 CD8

+ DC-p96   **+ DC-p321**

Bgalp96 CD8

+ DC-p321   **+ DC-p96**
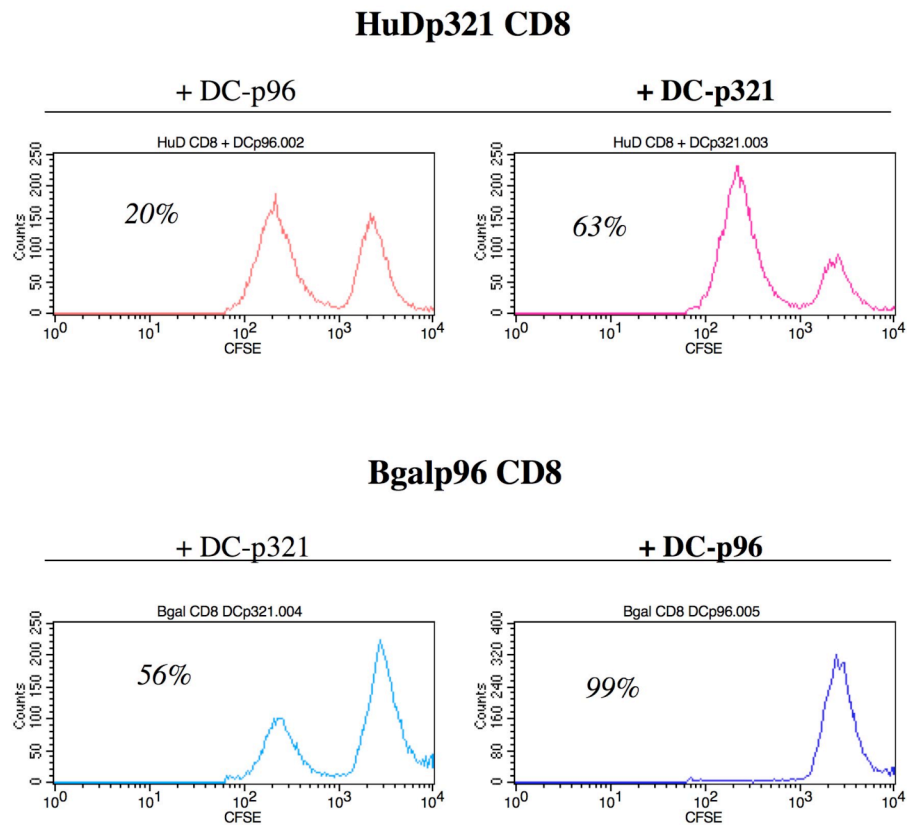
Fig 8. DC pulsed with cognate peptide enhance killing by HuDp321-specific CD8 T cells *in vivo*. 5 x 10^6 HuDp321-specific or Bgalp96-specific *in vitro* stimulated CD8 T cells were adoptively transferred into Rag$^{-/-}$ mice with 2 x 10^6 C57BL/6 bone marrow derived DC pulsed with cognate or irrelevant peptide. Mice also received PTx and IL-2. Nine days post transfer, mice were injected with CFSE-labeled syngeneic splenocytes pulsed with p321 or p96. p321-pulsed targets were labeled with a high fluorescence intensity and p96-pulsed targets were labeled with a low fluorescence intensity. 7 hours after target injection, splenocytes were analyzed by FACS for *in vivo* target cell lysis

HuDp321-specific CD8 T cells were efficient at lysing targets in a Rag$^{-/-}$ host.   In order to examine the issue of suppression by the endogenous C57BL/6 T cell repertoire, we performed a second experiment, comparing the lytic ability of HuDp321-specific T cells in a Rag$^{-/-}$ versus a wild type host (Fig 9).   Our transferred cells were equally potent cytotoxic effectors in either strain of mice.   We concluded that once activated, HuDp321-specific CD8 T cells are able to function as cytotoxic effectors in an immune-competent host.

Summary

We have shown that there are HuD-specific CD8 T cells present in the H-2^b repertoire, and that peptide 321 represents the immunodominant epitope of the HuD protein.   Peptide 321 is a D^b restricted epitope, and HuDp321-specific CD8 T cells are high affinity cells that display a characteristic cell surface phenotype upon *in vitro* stimulation. Immunization with recombinant adenovirus-HuD followed by *in vitro* peptide stimulation allowed for the activation of HuDp321-specific CD8 T cells.   Pertussis toxin was also required for successful priming *in vivo*, since immunization with adenovirus-HuD alone did not result in HuDp321-specific T cell activation, even after *in vitro* stimulation.   *In vitro* stimulated HuD-specific CD8 T cells were shown to be functional cytotoxic T cells capable of secreting IFNγ and lysing target cells in an antigen-dependent manner.   There was no loss of lytic ability *in vivo*

HuDp321 CD8 + DCp321
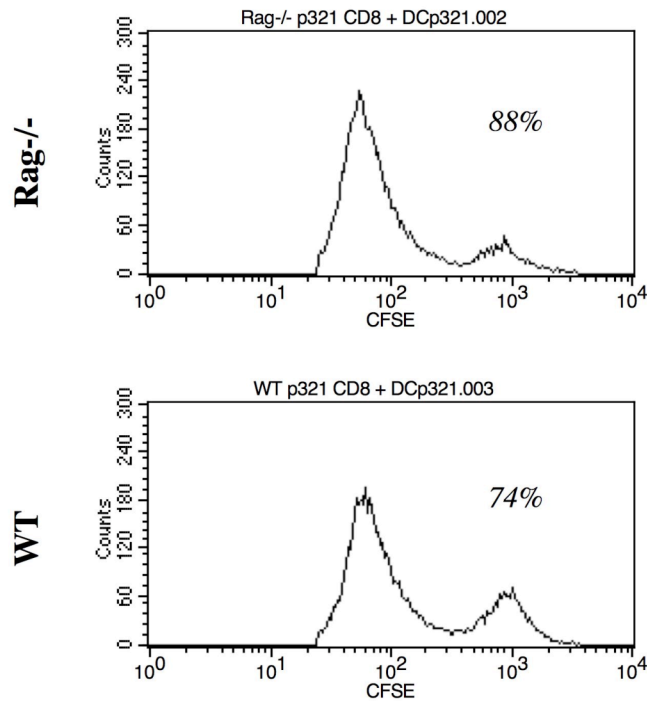
Fig 9. HuD p321-specific CD8 T cells can kill *in vivo*. (a) 5 x 10⁶ HuD p321-specific *in vitro* stimulated CD8 T cells were adoptively transferred into Rag⁻/⁻ mice with 2 x 10⁶ C57BL/6 bone marrow derived DC pulsed with p321. Mice also received PTx and IL-2. Eight days post transfer, mice were injected with CFSE-labeled syngeneic splenocytes pulsed with p321 or p96. p321-pulsed targets were labeled with a high fluorescence intensity and p96-pulsed targets were labeled with a low fluorescence intensity. 6 hours after target injection, splenocytes were analyzed by FACS for *in vivo* target cell lysis. (b) as in (a) except that WT C57BL/6 mice were used as recipients of adoptively transferred HuDp321-specific CD8 T cells.

when stimulated HuD-specific CD8 T cells were transferred into a wild type host, suggesting that once expanded and activated, these cells are able to overcome any form of immune suppression.

CHAPTER IV. TOLERANCE TO HUD IN THE MOUSE

Introduction

The immune system is designed to distinguish self proteins from foreign antigens.   Effective immune responses to self tissues are necessarily avoided in order to guard against the development of deleterious autoimmune disease.   The central nervous system is not ignored by the immune system, as activated T cells are allowed access to the CNS in order to defend against infection.   Immunosurveillance of the CNS poses the risk of developing autoimmune neurodegeneration mediated by neuron-specific T cells. Tolerance induction to neuronal antigens is poorly understood.   Patients with anti-Hu syndrome have aggressive neuronal autoimmunity, presumably mediated by HuD-specific T cells. The fact that a majority of SCLC patients fail to mount an immune response to HuD despite the presence of this protein in all tumors suggests that tolerance induction to HuD is normally maintained.   In patients with the Hu syndrome, tolerance would necessarily be broken to allow autoreactive lymphocytes to target HuD expressing neurons. Having identified and characterized HuD-specific CD8 T cells in mice, we sought to investigate tolerance induction to HuD.   Our initial experiments showed that *in vitro* stimulation with peptide subsequent to immunization with full-length protein and pertussis toxin was required to detect activated HuD-specific CD8 T cells.   The need for *in vitro*

stimulation and PTx suggested that HuD-specific CD8 T cells are subject to tolerance induction. In order to formally show tolerance to HuD, we took advantage of HuD-deficient transgenic mice. In addition, we sought to compare tolerance induction to HuD versus HuA. HuA is a closely related Hu family member with high sequence homology to HuD, however the protein is not neuron-specific but ubiquitously expressed. Given the difference in expression between HuD and HuA, we reasoned that the two proteins might undergo different forms of tolerance induction. We hypothesized that tolerance to HuA should be stricter compared to HuD since the protein is present in all tissues and not restricted in expression to neurons.

Results

HuDp321 specific T cells do not secrete IFNγ ex vivo after adenovirus-HuD immunization

IFNγ secretion is a hallmark of T cell activation. We have already shown that HuDp321-specific CD8 T cells are capable of secreting IFNγ upon immunization with recombinant adenovirus, however these results relied on pertussis toxin treatment and *in vitro* stimulation with peptide. Immunization followed by stimulation *ex vivo,* outside of the host's normal immune system, serves to release cells from any inhibition felt *in vivo.* We have shown activation of HuD-specific CD8 T cells subsequent to removing these cells from their *in vivo* environment (*in vitro* stimulated

HuDp321-specific CD8 T cells secrete substantial amounts of IFNγ in response to cognate peptide and are able to lyse target cells in an antigen-dependent manner), however we have yet to assess the activation of these cells directly after immunization with adenovirus-HuD. An inability to activate HuD-specific CD8 T cells *in vivo* would imply immune tolerance to the HuD protein. In order to determine if HuDp321-specific T cells can be activated *in vivo*, we immunized mice with adenovirus-HuD together with pertussis toxin and isolated CD8 T cells from the spleen. CD8 T cells were plated directly in an IFNγ ELISPOT assay with cognate peptide-pulsed stimulators (Fig 1). As a control, mice were immunized with adenovirus-β-gal. β-gal is a neo-antigen and therefore not subject to tolerance induction *in vivo*. After immunization with adenovirus-β-gal without *in vitro* p96 stimulation, we were able to show robust IFNγ secretion by β-galp96-specific CD8 T cells. In contrast, we saw no IFNγ secretion by HuDp321-specific CD8 T cells after immunization with adenovirus-HuD (Fig 1). The inability to activate HuD-specific T cell *in vivo* after immunization suggests that mice are tolerized to the HuD protein.

HuDp321 specific T cells expand in vivo after immunization with HuD
Because we can isolate T cells from mice immunized with adenovirus-HuD and generate IFNγ-secreting CD8 T cells after p321 stimulation *in vitro*, we can be certain that a population of HuD-specific CD8 T cells

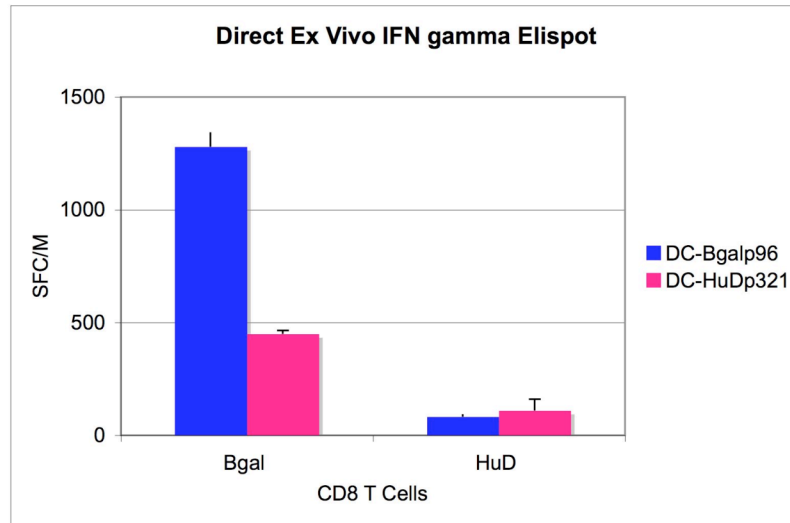**Fig 1.** **HuD p321-specific CD8 T cells do not secrete IFNg directly** *ex vivo* **after adenovirus-HuD immunization.** C57BL/6 mice were immunized with AdVHuD or AdVBgal (i.d.) + PTx . 13 days later, CD8 T cells were isolated from the spleen and plated in an IFNg ELISPOT assay (2 x 10^5/well) with mDC pulsed with 10uM peptide (7 x 10^3/well).

exists in the H-2^b repertoire. The fact that we cannot see IFNγ secretion directly *ex vivo* after immunization implies that there are tolerance mechanisms acting to suppress HuD-specific T cell activation. Both central and peripheral tolerance mechanisms protect the body from auto-reactive immune responses. Central tolerance serves to delete any self-reactive T cell clones from the repertoire during development in the thymus, whereas peripheral tolerance actively suppresses those self-reactive T cells that escape thymic deletion to circulate in the periphery. Because we were unable to detect HuDp321-specific T cells by IFNγ secretion directly after immunization, we instead used tetramer to visualize and quantify these cells in mice after adenovirus-HuD immunization. For comparison, a second group of mice was immunized with influenza virus (e.g. flu). We measured the number of HuDp321-specific CD8 T cells or NP-specific CD8 T cells by tetramer directly *ex vivo* and after *in vitro* stimulation (Fig 2). NP is a D^b restricted CD8 T cell epitope of influenza. Splenocytes for adenovirus-HuD or flu immunized animals were stained directly *ex vivo* with both HuDp321 tetramer and NP tetramer. We observed the expected frequency of NP-specific T cells (1.5% of the total CD8 T cell repertoire), and we could expand this population by *in vitro* stimulation with NP. In comparison, after adenovirus-HuD immunization we observed .04% HuDp321-specific CD8 T cells. This population could be expanded *in vitro* by p321 stimulation. Expansion of HuDp321-specific T cells depended on the presence of HuD
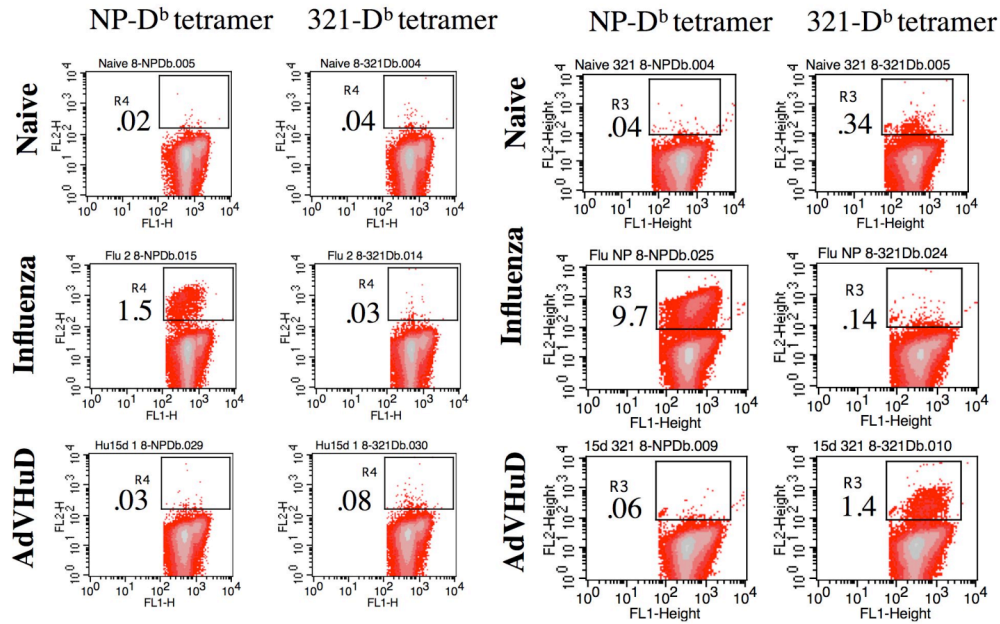
Fig 2. HuDp321-specific CD8 T cells expand *in vivo* after immunization with adenovirus-HuD. C57BL/6 mice were immunized with either AdVHuD + PTx or influenza virus (naïve mice were left untreated). 15 days after immunization, CD8 T cells were isolated from the spleen and stained directly *ex vivo* with anti-CD8 antibody and tetramer. Each population of CD8 T cells was stained with NP-D[b] tetramer and p321-D[b] tetramer separately. Splenocytes from each mouse were also stimulated *in vitro* with cognate peptide for 7 days. Naïve mice were stimulated with p321. CD8 T cells from *in vitro* stimulation cultures were stained with anti-CD8 antibody and tetramer.

in vivo, as *in vitro* stimulation of naïve splenocytes with p321 did not generate a population of HuDp321-specific cells (Fig 2).   Thus, there is visible, albeit slight, response *in vivo* of HuDp321-specific CD8 T cells after immunization, but this population is readily expanded upon *in vitro* stimulation with antigen.

HuAp268-specific T cells are absent from the H-2^b repertoire

To better understand the mechanisms behind tolerance to the Hu proteins, we compared the immune response to HuDp321 versus its homologous epitope in HuA, p268. HuAp268 differs from HuDp321 at a single amino acid position (Fig 3a). The anchor residues that stabilize the peptide-MHC interaction are conserved between the two peptides so that both should bind MHC I with similar affinity. In order to ensure that HuAp268 binds efficiently to MHC I molecules, we assessed the affinities of p268 versus p321 using RMA/S cells incubated with serial dilutions of peptide (Fig 3b). HuAp268 had a higher affinity for D^b MHC I compared to HuDp321, which bound D^b with similar affinity compared to the positive control peptide NP.   Unlike HuD, HuA is not confined to neurons but is ubiquitously expressed. We hypothesized that immune tolerance to this protein would be stricter compared to a neuron-specific protein like HuD.   To determine if HuAp268-specific CD8 T cells are present in the H-2^b repertoire or deleted as a result of central tolerance induction, we immunized C57BL/6 mice with Titermax-peptide and

(a)

HuA	---GPF**GAVTNVKVI**RDF---
HuB	---GPF**GAVTNVKVI**RDF---
HuC	---GPF**GAVTNVKVI**RDF---
HuD	---GPF**GAV**N**NVKVI**RDF---

(b)

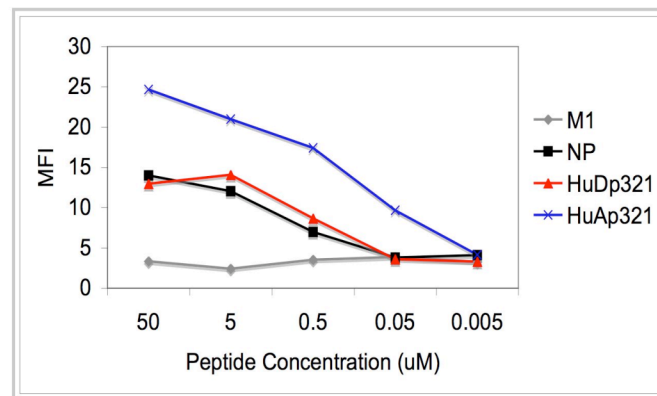**Fig 3. HuA p268 binds with high affinity to D^b MHC I.** (a) HuDp321 and homologous peptides in HuA, HuB, HuC. (b) RMA/S cells were incubated with serial dilutions of peptide and stained for Db MHC I. HuD p321 and its homologous epitope in HuA (p268) were assayed. The A2.1 epitope of influenza (M1) was used as a negative control. The Db epitope of influenza (NP) was used as a positive control.

looked for IFNγ secretion by CD8 T cells in an ELISPOT assay (Fig 4a). Whereas HuDp321-specific and NP-specific T cells were clearly present in the repertoire, HuAp268 cells were absent. The absence of HuAp268-specific T cells suggests that these cells were centrally deleted. Given the high affinity of p268 for MHC I, and its sequence homology to p321, we can assume that this peptide is naturally processed and presented. The complete absence of HuAp268 cells from the T cell repertoire is indicative of central tolerance to HuA.

HuDp321 specific T cells do not cross-react with HuA

Patients with the Hu syndrome succumb to autoimmunity directed against the nervous system but not against peripheral tissues, implying that the immune response to HuD does not cross-react with HuA. To investigate cross-reactivity to HuA, we asked if HuDp321-specific CD8 T cells are able to respond to primary kidney cells infected with adenovirus-HuA (Fig 4b). HuDp321-specific CD8 T cells were restimulated three times *in vitro* and plated in an IFNγ ELISPOT assay with kidney cells infected with either adenovirus-HuA, adenovirus-HuD, or a control adenovirus expressing GFP. Only kidney cells infected with adenovirus-HuD were able to elicit IFNγ secretion from HuDp321-specific CD8 T cells. The lack of cross-reactivity to HuA is in accordance with clinical data and supports our model of autoimmunity directed exclusively against neurons.

(a)
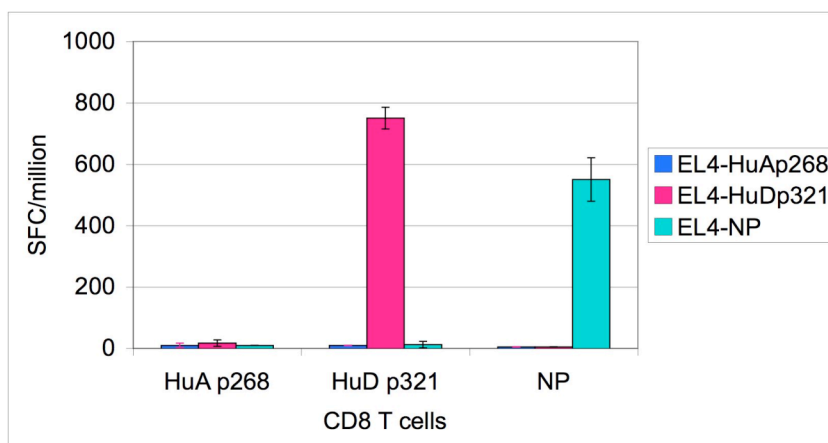
(b)
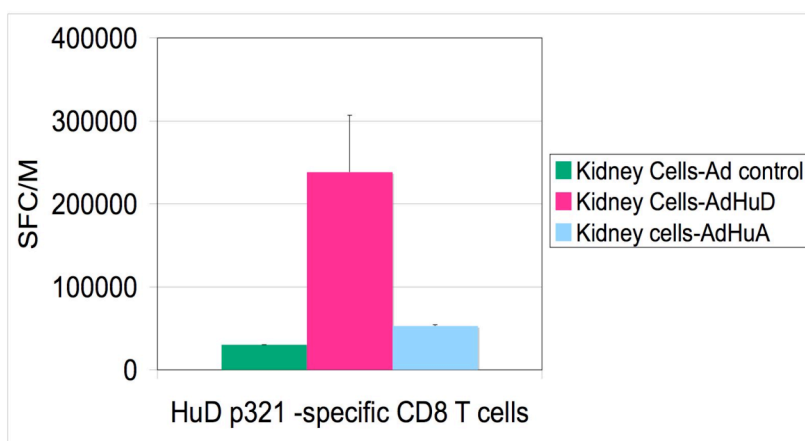
Fig 4.   HuA p268-specific CD8 T cells are absent from the H-2^b repertoire. (a) C57BL/6 mice were immunized with individual peptides (NP, HuA p268, or HuD p321) in titermax adjuvant. 7 days later, draining lymph node CD8 T cells were plated in an IFNg ELISPOT assay (2×10^5/well) with peptide-pulsed EL4 cells (5×10^4/well).   (b) HuD p321-specific CD8 T cells (3x restimulated) were plated in an IFNg ELISPOT assay (10^4/well) with primary kidney cells infected with recombinant adenovirus (5×10^4/well).

Mice are tolerized to HuD

We have shown that HuD-specific CD8 T cells exist in the H-2^b repertoire, although there appears to be tolerance to the protein since *in vitro* stimulation is required for their activation. To definitely prove tolerance to HuD, we took advantage of the transgenic HuD-deficient mouse (Akamatsu, Fujihara et al. 2005). HuD-/- mice were generated by targeted disruption of the HuD gene. Phenotypically, HuD-/- pups are indistinguishable from their wild type littermates. By 4-8 weeks of age, a 70-80% of HuD-/- mice exhibit an abnormal clasping of the hind limbs when suspended by the tail, suggesting cortical and/or basal ganglia defects. Immunologically, the absence of HuD *in vivo* would prevent any form of tolerance to the protein. If HuDp321-specific CD8 T cells experience tolerance induction, then elimination of the HuD protein should result in efficient expansion and activation of these cells *in vivo.* To assess tolerance to HuD, we immunized HuD-/- versus wild-type mice with adenovirus-HuD and measured IFNγ secretion by HuDp321-specific CD8 T cells directly *ex vivo* (Fig 5a). HuDp321-specific CD8 T cells from HuD-deficient mice exhibited a strong response *ex vivo*, whereas HuD+/+ mice were unable to generate activated HuDp321-specific cells directly after immunization (Fig 5a). To ensure that the lack of an *ex vivo* response in wild type mice was not due to an ineffective immunization, portions of the spleens from individual mice were stimulate *in vitro* with p321 (Fig 5b). Wild type mice were able to

(a)

Ex vivo

(b)

In vitro simulation
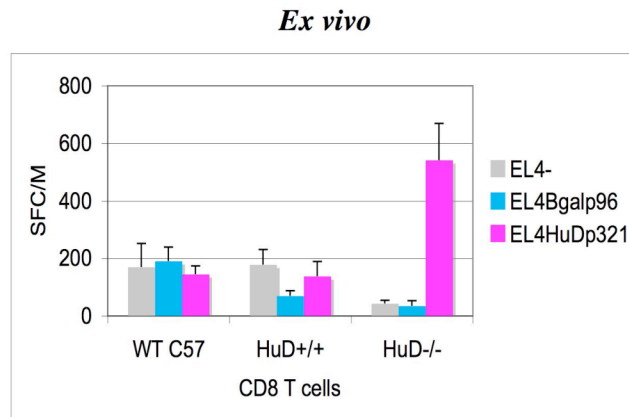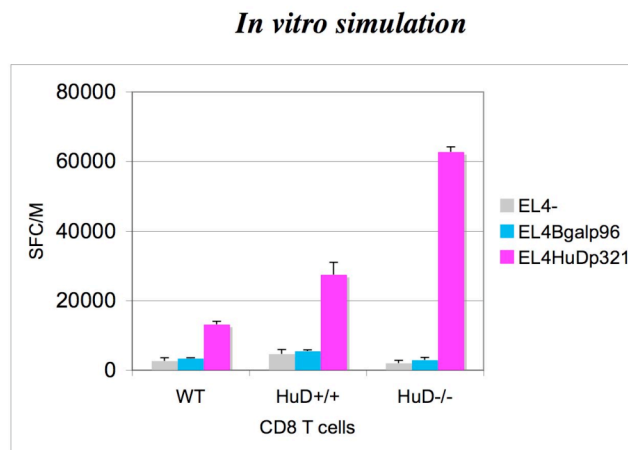
Fig 5. C57BL/6 mice are tolerized to HuD. (a) HuD$^{-/-}$, HuD$^{+/+}$ and WT C56BL/6 mice were immunized with AdVHuD + PTx. 13 days after immunization, CD8 T cells were isolated from the spleens and plated in an IFNg ELISPOT assay (2 x 10^5/well) with peptide-pulsed EL4 cells (5 x 10^5/well). (b) half of the spleens from mice immunized in (a) were stimulated *in vitro* with p321. After 7 days, CD8 T cells were isolate from stimulation cultures and plated in an IFNg ELISPOT assay (10^4/well) with peptide pulsed EL4 cells (5 x 10^4/well).

generate activated HuDp321-specific CD8 T cells after *in vitro* stimulation. Taken together, these results show that HuD-specific CD8 T cells are subject to tolerance induction *in vivo*.

HuC-deficient mice remain tolerized to HuD

Tolerance can be achieved by the generation of CD4 regulatory T cells. If regulatory T cells are responsible for tolerance induction to HuD, these cells may be specific for CD4 T cell epitopes shared between HuD and other family members. In order to determine if tolerance to HuD was dependent on the presence of HuD protein specifically or induced by other Hu family members, we took advantage of the HuC knockout mouse. The HuD and HuC proteins are closely related, however the HuC homolog of HuDp321 differs by a single amino acid (Fig 3a). In fact, the amino acid sequence of p321 is not found in any other Hu family member. If tolerance depends on other T cell epitopes aside from HuDp321, then deletion of the HuC gene could result in a loss of tolerance to HuD. In order to investigate tolerance induction by other Hu family members, we immunized mice deficient for HuC with adenovirus-HuD and asked if HuDp321-specific CD8 T cells were activated *in vivo* (Fig 6). HuC-deficient animals showed no response to HuDp321 directly *ex vivo* (Fig 6a). After stimulating splenocytes *in vitro* with p321, however, HuDp321-specific CD8 T cells were able to secrete IFNγ, ensuring an effective immunization (Fig 6b). We can conclude that

Ex vivo

(b)

In vitro simulation
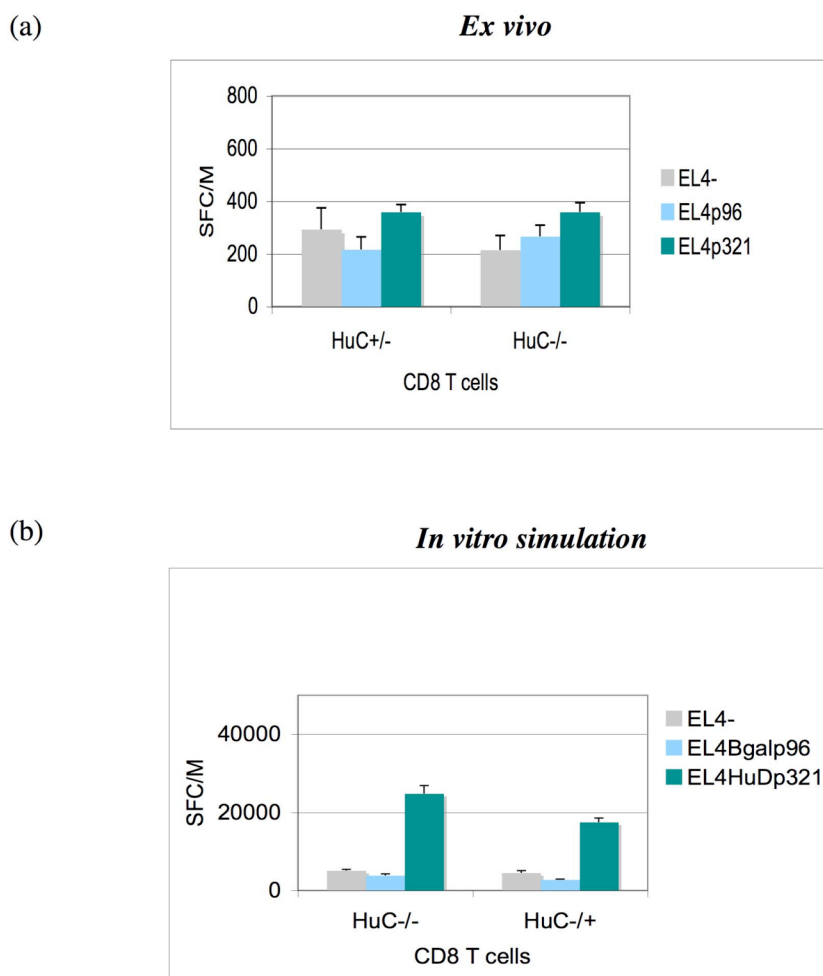
Fig 6.   HuC-/- mice remain tolerized to HuD. (a) HuC-/- and HuC+/- mice were immunized with AdVHuD + PTx. 13 days after immunization, CD8 T cells were isolated from the spleens and plated in an IFNg ELISPOT assay (2×10^5/well) with peptide-pulsed EL4 cells (5×10^4/well). (b) splenocytes from mice immunized in (a) were stimulated in vitro with p321. After 7 days, CD8 T cells were isolate from stimulation cultures and plated in an IFNg ELISPOT assay (10^4/well) with peptide pulsed EL4 cells (5×10^4/well).

tolerization of HuDp321-specific CD8 T cells does not require HuC, but is dependent on HuD specifically.

CD4 depletion does not overcome tolerance to HuD

Peripheral tolerance induction can depend on active suppression of autoreactive clones by regulatory T cells.　In order to investigate the role of regulatory T cells in maintaining tolerance to HuD, we depleted the CD4 T cell population *in vivo* prior to adenovirus-HuD immunization (Fig 7).　CD4 depletion was achieved by injecting mice with anti-CD4 antibody, followed by immunization with adenovirus-HuD or adenovirus-β-gal. We measured activation of HuDp321-specific CD8 T cells in an IFNγ ELISPOT assay.　To assess the efficacy of our depletion regiment, splenocytes were analyzed for the presence of CD4 and CD8 T cells. There were no CD4 T cells remaining in the depleted mice two weeks after immunization with adenovirus-HuD (Fig 7). The absence of CD4 T cells did not overcome tolerance to HuD, as we saw no IFNγ secretion directly *ex vivo* in response to p321. It is important to note that CD4 depletion does not specifically target regulatory T cells but instead eliminates the entire CD4 T cell population.　The absence of CD4 T cells did not effect T cell priming by immunization with recombinant adenovirus, since CD4-depleted mice immunized with adenovirus-β-gal were able to generate activated β-gal-specific CD8 T cells (Fig 7).　Still, β-
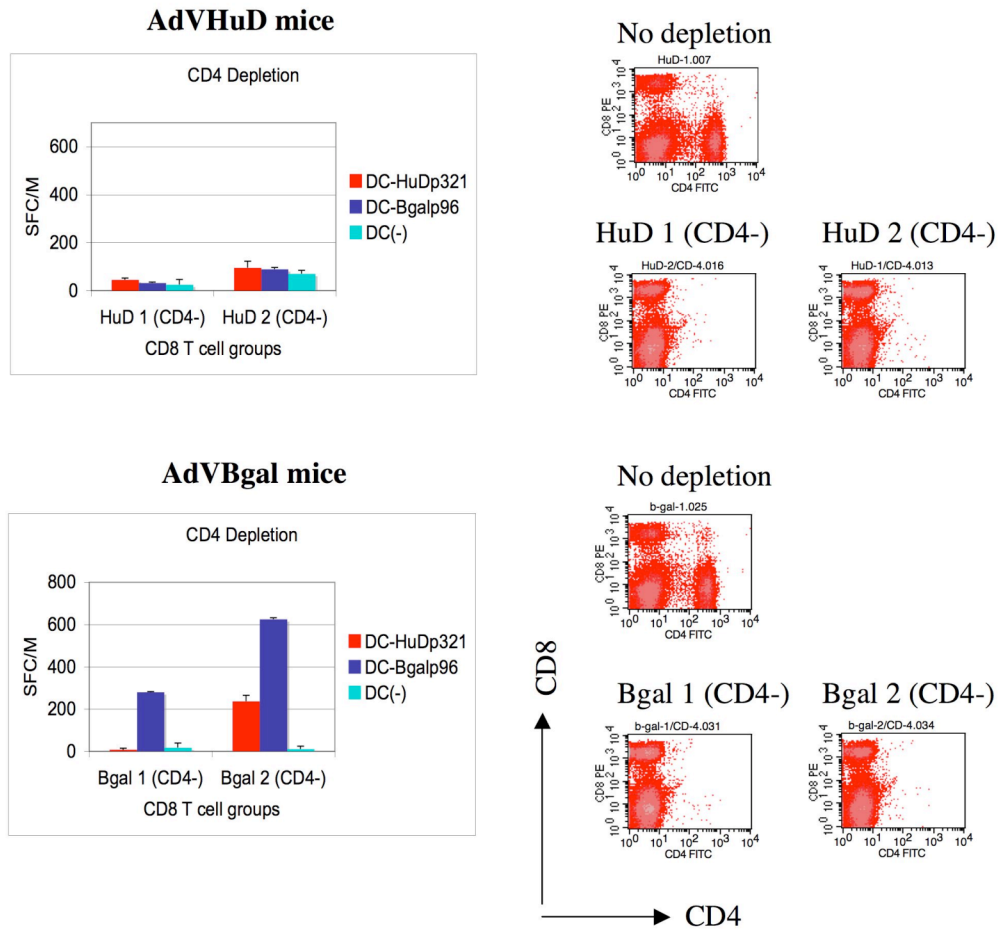
Fig. 7. CD4 depletion does not overcome tolerance to HuD. C57BL/6 mice were injected with anti-CD4 antibody GK1.5 (500ug/mouse) i.p. on days -7 and -4. On day 0, mice were immunized with AdVHuD or AdVBgal + PTx (2 mice per group). 13 days after immunization, CD8 T cells were isolated from the spleens and plated in an IFNg ELISPOT assay (2×10^5/well) with peptide-pulsed DC (7×10^3/well). Splenocytes from each mouse were analyzed on day 13 by FACs to ensure CD4 depletion. Splenocytes from control mice immunized with AdVHuD or AdVBgal + PTx were analyzed by FACs (No depletion).

gal is not an autoantigen like HuD, so that CD8 T cell priming to HuD may require CD4 T cells whereas CD8 T cell priming to β-gal does not.

AIRE does not regulate tolerance to HuD

In order to explore the role of AIRE in mediating tolerance to HuD, we immunized AIRE-deficient mice with adenovirus-HuD and assessed HuDp321-specific CD8 T cell activation directly *ex vivo*. We reasoned that if AIRE were to regulate tolerance to HuD, then AIRE-deficient animals should mount an immune response similar to that of HuD-deficient mice. After immunization of an AIRE-deficient mouse and its wild-type littermate with adenovirus-HuD, we saw no response directly *ex vivo* to HuDp321 in either animal (Fig8a). A portion of the spleen from each animal was stimulated *in vitro* with p321 in order to ensure that our immunization was successful and that AIRE mice did not have gross immunologic abnormalities that prevented HuD CD8 T cell priming. After *in vitro* stimulation, both the AIRE-deficient mouse and its wild type littermate generated a typical HuDp321-specific CD8 T cell response (Fig 8b). We can conclude that AIRE does not influence tolerance induction to HuD.

Summary

HuDp321-specific CD8 T cells were not detectable by IFNγ secretion directly *ex vivo* after immunization with adenovirus-HuD but could be

Ex vivo

In vitro simulation
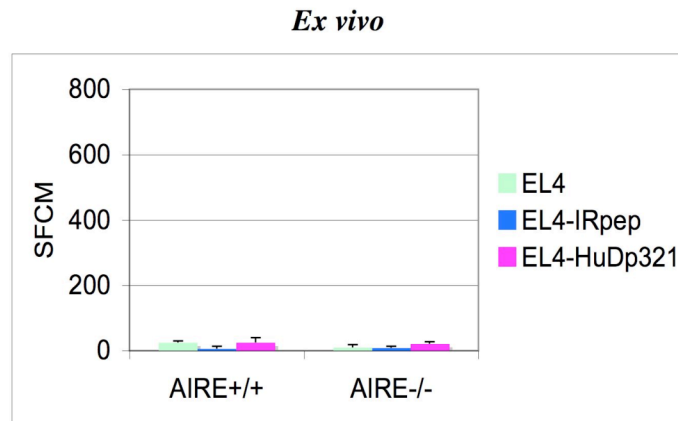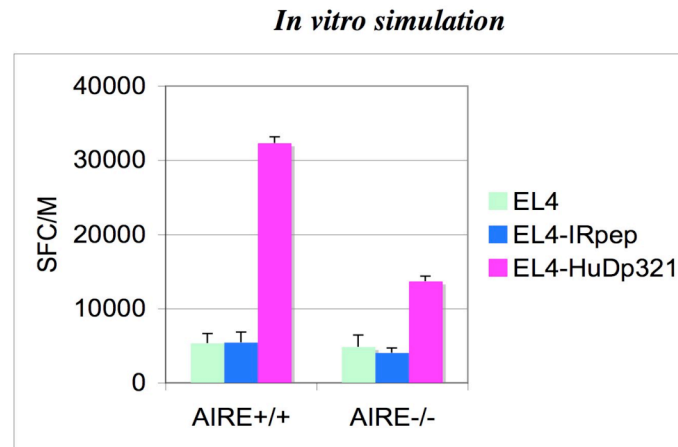
Fig 8.   AIRE does not regulate tolerance to HuD. (a) AIRE-/- and AIRE+/+ mice were immunized with AdVHuD + PTx.   13 days after immunization, CD8 T cells were isolated from the spleens and plated in an IFNg ELISPOT assay (2 x 10^5/well) with peptide-pulsed EL4 cells (5 x 10^4/well). (b) splenocytes from mice immunized in (a) were stimulated in vitro with p321. After 7 days, CD8 T cells were isolate from stimulation cultures and plated in an IFNg ELISPOT assay (10^4/well) with peptide pulsed EL4 cells (5 x 10^4/well).

expanded and activated *in vitro* upon stimulation with peptide. Tolerance to HuD is normally maintained, since immunization of HuD-deficient mice with adenovirus-HuD generated a robust *ex vivo* response from HuDp321-specific CD8 T cells that did not require *in vitro* stimulation. Aggressive immunization of wild type mice with peptide 321 emulsified in Titermax adjuvant elicited a strong *ex vivo* IFNγ response from HuDp321-specific CD8 T cells, demonstrating that these cells exist in the repertoire and are not subject to central tolerance induction. In comparison, HuAp268-specific CD8 T cells were not detectable after immunization with Titermax-peptide, which is indicative of thymic deletion. HuAp268 binds with higher affinity to D^b MHC I compared to HuDp321, and amino acid residues flanking the two peptides are conserved, suggesting that both are naturally processed and presented in a similar manner. There is a single amino acid difference between the two peptides at the fourth residue, which is thought to affect the interaction with the T cell receptor. Central deletion of HuAp268 reactive T cells is critical to prevent peripheral autoimmune disease, given the ubiquitous expression pattern of the protein. CD8 T cells specific for a neuronal antigen like HuD escape central tolerance induction and are instead subjected to regulation in the periphery. The fact that patients with the Hu syndrome never succumb to peripheral autoimmunity suggests that the HuD-specific immune response is unable to cross-react with HuA. We have formally shown that HuDp321-specific CD8 T cells do not recognize

processed and presented HuA.   Thus, the immune response to HuD is neuron-specific, and tolerance to this neuronal antigen is critical to protecting the nervous system from autoimmune degeneration.

CHAPTER V. HUD-SPECIFIC CD8 T CELLS IN THE CNS

Introduction

Patients with the Hu syndrome develop neurologic symptoms that affect discrete areas of the nervous system including the dorsal root ganglia, limbic system, cerebellum, brainstem, motor or autonomic nervous system. Most patients go on to develop multifocal neuronal degeneration and die from neuronal causes, on average, seven months after diagnosis. The disease is characterized by neuronal loss and inflammatory infiltrates of the nervous system. The presence of high titer antibodies to Hu serves as a diagnostic tool, however the role of antibodies in disease pathogenesis remains unclear. Given the intracellular nature of HuD, it seems likely that CD8 T cells play an important role in disease pathogenesis. We have identified the immunodominant CD8 T cell epitope of HuD in order to model CD8 T cell mediated neuronal degeneration in mice. Upon adoptive transfer of HuD-specific CD8 T cells into recipient mice, we sought to assess the ability of these cells to traffic to the CNS. In addition, we measured recruitment of peripheral APCs and upregulation of MHC I and MHC II molecules on resident microglia, as these are hallmarks of inflammatory CNS diseases. In order to assess neuronal loss, we sent tissue sections from the CNS to an independent pathologist for review.

Results

Adoptive transfer of HuD-specific CD8 T cells

In order to study the ability of HuD-specific CD8 T cells to traffic to the central nervous system and target HuD expressing neurons, we developed a system that relied on the adoptive transfer of labeled CD8 T cells into a host animal (Fig 1a). Based on the results of our *in vivo* CTL experiments, we opted to transfer stimulated CD8 T cells along with syngeneic dendritic cells pulsed with cognate peptide. Host animals also received pertussis toxin and exogenous IL-2. IL-2 was used for its ability to augment the function of transferred T cells. In a published model of tumor regression mediated by adoptive transfer of self-reactive CD8 T cells, it was shown that IL-2 increases the number and function of self-specific T cells (Overwijk, Theoret et al. 2003). Given these results, we reasoned that IL-2 could support the expansion and cytolytic ability of transferred HuD-specific CD8 T cells. In order to distinguish transferred CD8 T cells from the host repertoire, we transferred stimulated CD8 T cells derived from the congenic Thy1.1 mouse strain. Thy1.1 is an isoform of the Thy1 glycoprotein found exclusively on T cells; wild-type C56BL/6 mice carry the Thy1.2 isoform, which is easily differentiated from Thy1.1.

HuDp321-specific CD8 T cells can traffic to the central nervous system

In our initial adoptive transfer experiment we used Rag$^{-/-}$ mice as the recipients of CD8 T cells. Rag$^{-/-}$ mice received β-galp96-specific or HuDp321-specific CD8 T cells together with cognate peptide-pulsed DCs, pertussis toxin and IL-2. To assess the purity of transferred CD8 T cells, we stained a representative sample of cells for Thy1.1 and CD8 T cell markers (Fig 1b). Roughly 90% of the transferred cells were positive for Thy1.1 and CD8. To assess CD8 T cell trafficking to the nervous system, we sacrificed recipients one month after transfer and isolated cells from brain (Fig 2). For comparison, we measured CD8 T cell trafficking to the spleen. Both Thy1.1 β-gal-specific CD8 T cells and Thy1.1 HuD-specific CD8 T cells were found in the spleens of recipients one month post-transfer, ensuring that our adoptive transfer strategy allowed for the survival and persistence of transferred T cells. Thy1.1 T cells in the spleens expressed high levels of the CD8 co-receptor, as expected. When we examined the brains of recipient animals, we detected Thy1.1 transferred T cells, indicating that these cells could indeed traffic to the central nervous system. Trafficking to the brain was not dependent on the presence of antigen behind the blood brain barrier, as both β-gal-specific CD8 T cells and HuD-specific CD8 T cells were found in the CNS. Thy1.1 cells in the brain had down-regulated the CD8 co-receptor (Fig 2). Down-regulation of CD8 was not antigen-dependent but perhaps a result of the brain's cytokine milieu. The CD8

(a)

Thy1.1 in vitro stimulated CD8 T cells

(HuD CD8 v. Bgal CD8)

+ PTX and IL-2

DC pulsed with cognate peptide

(b)

HuD THY1.1/8.006   *92%*

Bgal THY1.1/8.005   *88%*

CD8
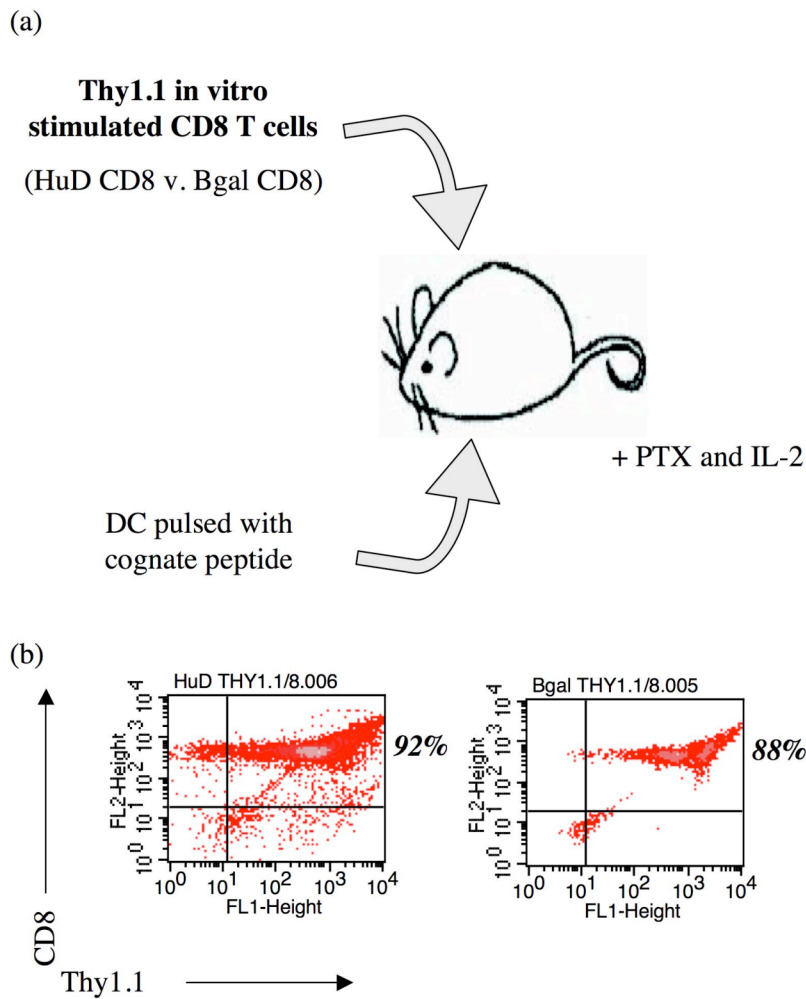
Thy1.1

Fig 1. **Adoptive transfer of HuDp321-specific CD8 T cells.** (a) for adoptive transfer experiments, C57BL/6 mice (WT or Rag-/-) received 5 x 10^6 in vitro stimulated Thy1.1 HuDp321-specific or Bgalp96-specific CD8 T cells (i.v.) with 2 x 10^6 cognate peptide-pulsed DC (i.v), PTx, and IL-2. (b) to assess the purity of transferred CD8 T cells, transferred cells were stained for Thy1.1 and CD8 T cell markers.
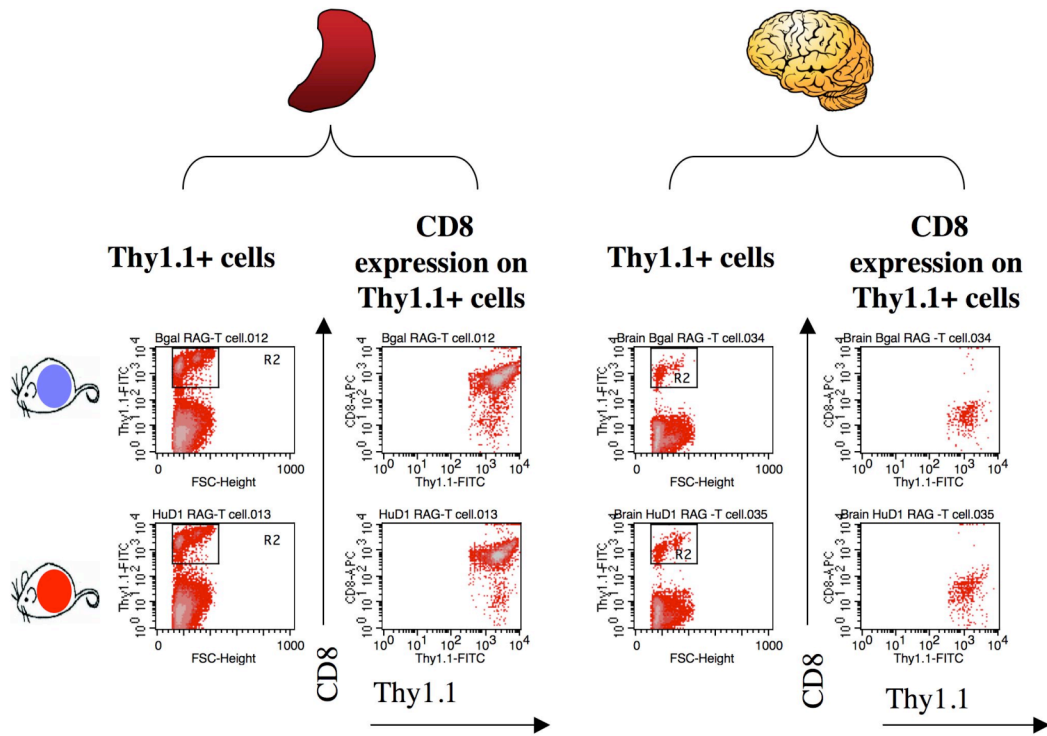
Fig 2. **Transferred CD8 T cells traffic to the CNS.** Rag-/- mice received an adoptive transfer of HuD-specific (red mouse) or Bgal-specific (blue mouse) Thy1.1 CD8 T cells as described in Fig 1a. One month later, cells from the spleens and brains of perfused recipients were stained for Thy1.1 and CD8 T cells markers.

co-receptor is an important component of the T cell receptor signaling pathway that affects proliferation and activation of CD8 T cells. Down-regulation of the CD8 molecule in the CNS may be a means of suppressing T cells and thereby inhibiting potentially damaging immune responses.

HuDp321-specific CD8 T cells from the central nervous are capable of secreting IFNγ in vitro

Given that transferred CD8 T cells in the brain down-regulate the CD8 co-receptor, we asked if these cells were permanently suppressed as a result of trafficking to the CNS. IFNγ secretion was used as an indication of T cell activation. CD8 T cells isolated from the brain were assessed for the ability to secrete IFNγ in response to cognate peptide in an ELISPOT assay (Fig 3). As a positive control, we isolated cells from the spleens of recipients and measured IFNγ secretion. Both β-gal-specific CD8 T cells and HuD-specific CD8 T cells isolated from the spleen were capable of producing IFNγ in response to cognate peptide. Cells from the brain were also able to secrete IFNγ, demonstrating that suppression in CNS was not permanent but easily reversed *ex vivo*.

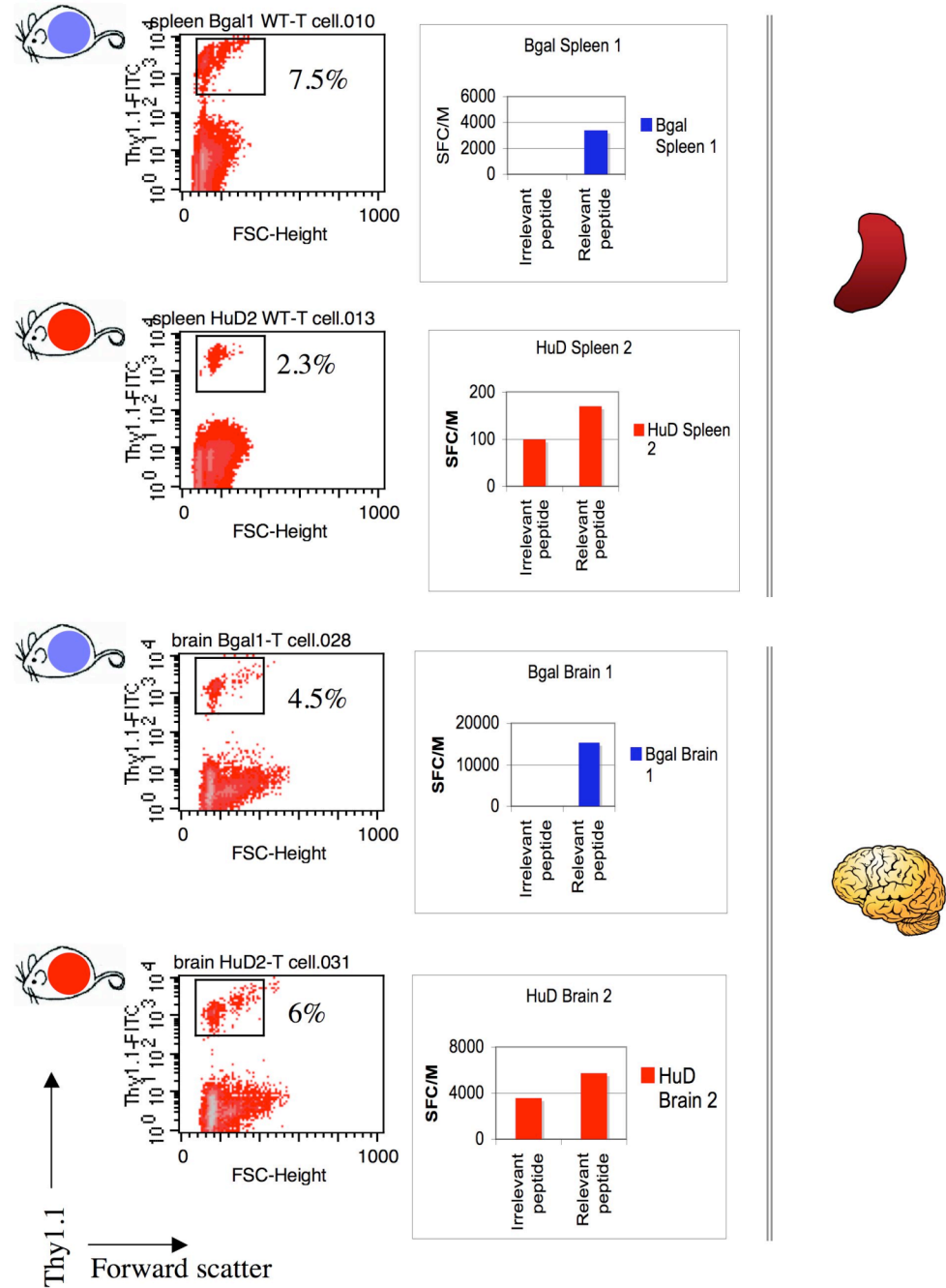**Fig 3. Transferred CD8 T cells in the CNS are not anergic.** WT C57BL/6 mice received an adoptive transfer of Thy1.1 HuD-specific (red mouse) or Bgal-specific (blue mouse) CD8 T cells as described in Fig 1a. One month later, bulk cells from the spleens and brains of perfused recipients were plated in an IFNg ELISPOT assay with 1ug cognate or irrelevant peptide. Cells were also stained for Thy1.1.

HuDp321-specific CD8 T cells in the central nervous system are not sufficient for the induction of inflammation and neurologic degeneration

From our previous results we can conclude that CD8 T cells specific for HuD are able to traffic to the CNS after adoptive transfer but are unable to cause gross neurologic degeneration despite the presence of cognate antigen in the brain. Clearly, there is an additional requirement apart from HuD-specific CD8 T cell entry into the brain that is required for autoimmune destruction of HuD expressing neurons. In EAE, which is characterized by CD4 T cell mediated autoimmune destruction of myelin in the CNS, T cell infiltration is accompanied by inflammation behind the blood brain barrier. Hallmarks of this inflammatory response are recruitment of peripheral APCs to the brain and up-regulation of MHC I and MHC II molecules on resident microglia. Because there were no apparent signs of neuronal loss in our adoptive transfer recipients, we asked if this was do to a lack of inflammation in the CNS. As a measure of inflammation in the CNS, we assessed recruitment of peripheral APCs to the brain in recipient mice. Both microglia and peripheral APCs are CD11b$^+$ myeloid-derived cells that are distinguished by their levels of CD45 expression. Microglia are a defined CD11b$^+$CD45^{intermediate} population whereas peripheral macrophages/DC are CD11b$^+$CD45^{high} cells. As a positive control for CNS inflammation, we analyzed APC recruitment to the CNS in a symptomatic EAE animal (Fig 4). Here, we saw a significant number of peripheral APCs in the brain, in addition to

Asymptomatic "EAE" Brain (clinical score 0)
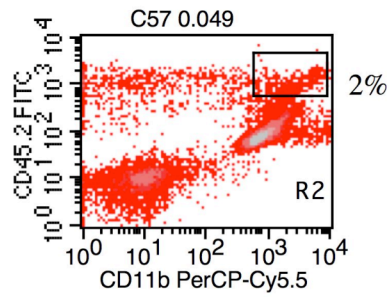
Symptomatic EAE Brain (clinical score 2.5)
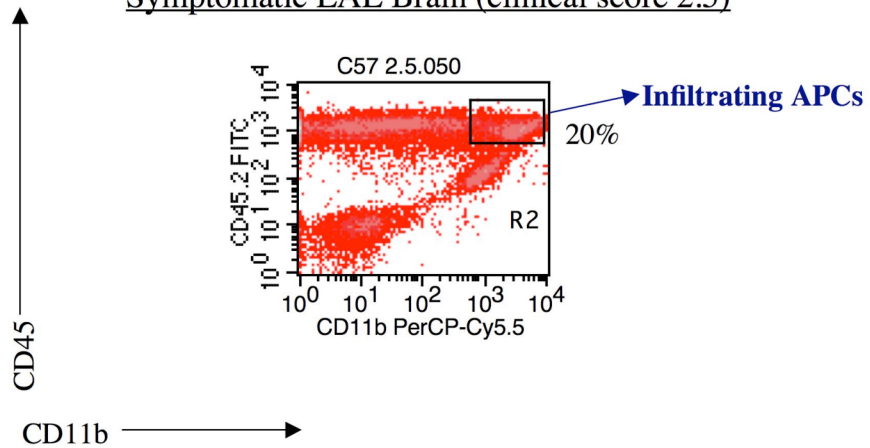
Fig 4. **Peripheral APC are recruited to the CNS in EAE.** C57BL/6 mice were immunized with MOG_{35-55} in CFA + PTx to induce EAE. Brain cells from an asymptomatic mouse (score 0) and a symptomatic mouse (score 2.5) were stained for CD45 and CD11b.

resident microglia. In comparison, an immunized asymptomatic mouse with no visible signs of EAE did not exhibit recruitment of antigen presenting cells from the periphery (Fig 4). When we examined the brains of adoptive transfer recipients, we saw little recruitment of macrophages or DCs from the periphery (Fig 5). These results demonstrate a lack of inflammation in the CNS of our adoptive transfer mice, despite the presence of HuD-specific CD8 T cells and cognate antigen behind the blood-brain barrier. Trafficking of activated T cells to the brain is not antigen-dependent, as activated T cells are readily allowed access to this compartment, presumably as a means of monitoring the CNS for infection. Initiation of an inflammatory response behind the blood brain barrier requires the recruitment of peripheral antigen presenting cells and up-regulation of antigen processing and presentation machinery.

HuDp321-specific CD8 T cells in the inflamed CNS are not sufficient for the induction of neuronal degeneration

HuD-specific CD8 T cells were unable to induce inflammation and neuronal degeneration. In order to generate an inflammatory environment in the CNS, we induced EAE in recipient animals before the adoptive transfer of HuD-specific CD8 T cells (Fig 6). C57BL/6 mice were immunized with MOG peptide to initiate EAE. Prior to the onset of clinical disease, HuD- or β-gal-specific CD8 T cells were adoptively
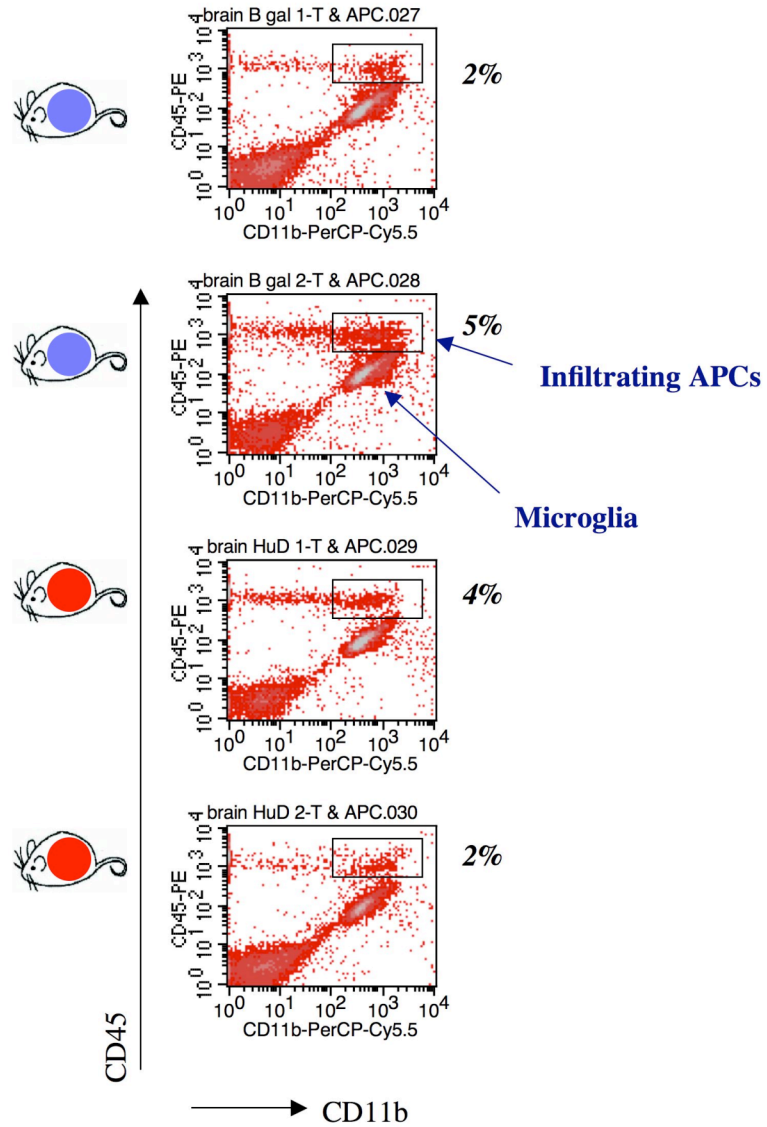
Fig 5. Peripheral APC are not recruited to the CNS after adoptive transfer of HuD-specific CD8 T cells. Rag-/- mice received an adoptive transfer of Thy1.1 HuD-specific (red mice) or Bgal-specific (blue mice) CD8 T cells (as in Fig 1a). One month later, cells from the brains of perfused recipients were stained for CD45 and CD11b. Numbers indicate percentages of CNS infiltrating APCs.

EAE Induction: MOG$_{35-55}$/CFA + PTx

Thy1.1 *in vitro* stimulated CD8 T cells

(HuD CD8 v. Bgal CD8)

+

DC pulsed with cognate peptide

+ PTX and IL-2
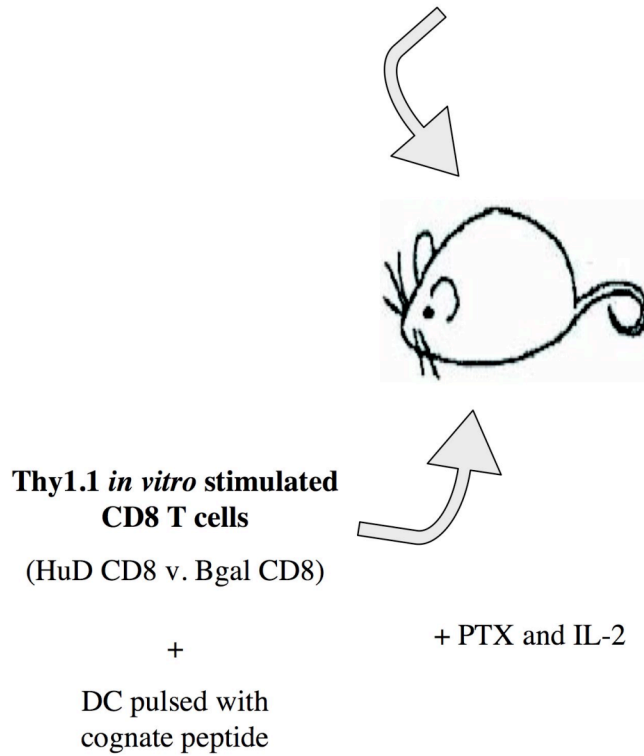
Fig 6. **Adoptive transfer of HuDp321-specific CD8 T cells into EAE mice.** C57BL/6 mice were immunized with MOG$_{35-55}$ in CFA + PTx to induce EAE. 6 days later, mice received 5×10^6 in vitro stimulated Thy1.1 HuDp321-specific or Bgalp96-specific CD8 T cells (i.v.) with 2×10^6 cognate peptide-pulsed DC (i.v), PTx, and IL-2.

transferred into MOG-immunized recipients. Mice exhibited clinical symptoms of demyelination, with no exacerbation or mitigation of disease by transferred HuD-specific T cells. When brain and spinal cord sections were examined by histology, there were visible signs of inflammation and infiltration by immune cells, however no neuronal damage was observed (Fig 7). Brain cells from recipients exhibited marked up-regulation of MHC I and MHC II molecules on resident microglia and infiltrating peripheral APCs (Fig 8). Induction of EAE was effective at generating inflammation in the CNS, however this was not sufficient for the initiation of neuronal degeneration by HuD-specific CD8 T cells.

Summary

Upon adoptive transfer, HuD-specific CD8 T cells were able to traffic to the CNS. Recruitment to the brain was not antigen-dependent, as both HuD- and βgal-specific CD8 T cells were present behind the blood brain barrier. Transferred T cells isolated from spleens of recipient animals were positive for both Thy1.1 and CD8 markers. When lymphocytes from the brain were analyzed, Thy1.1 transferred T cells were lacking in expression of the CD8 coreceptor. Downregulation of the CD8 coreceptor was possibly due to the immunosuppressive environment in the CNS, which is designed to limit the generation of inflammatory immune responses. Any suppression of transferred CD8 T cells within the CNS was reversed after removal of these cells from the brain followed

Mice with HuD-specific CD8 T cells
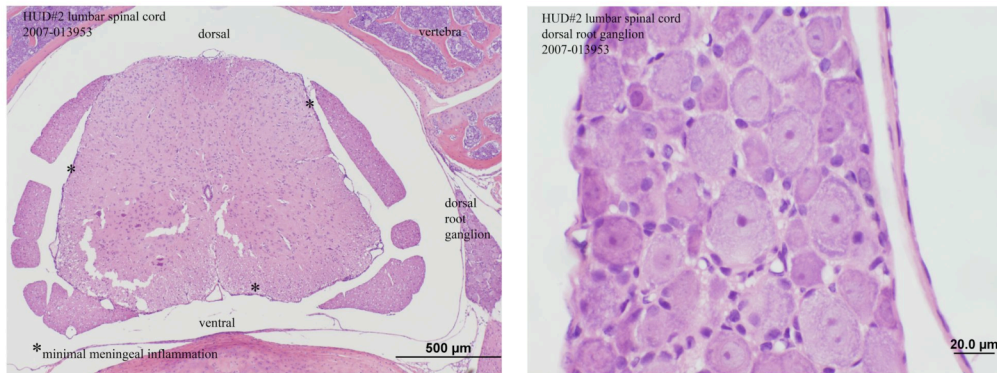
Mice with Bgal-specific CD8 T cells
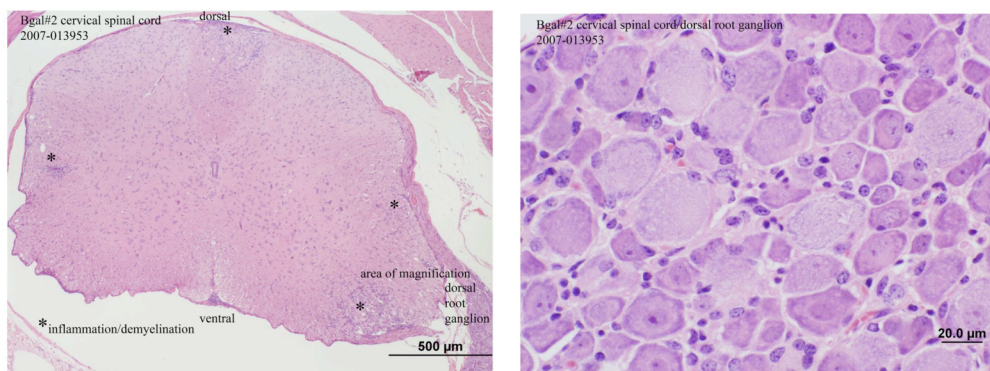
Fig 7. **Adoptive transfer of HuDp321-specific CD8 T cells into EAE mice does not result in neuronal degeneration.** EAE was induced in C57BL/6 mice before adoptive transfer of Thy1.1 HuD-specific or Bgal-specific CD8 T cells (as described in Fig 6). Mice were sacrificed when disease reached a clinical score of 2 (partial hind limb paralysis) and perfused with PBS followed by paraformaldehyde. Brain and spinal cord sections were fixed in formalin. Fixed tissues were sent to Charles River for analysis. (a) H & E staining on a representative mouse that received HuD-specific T cells. (b) H & E staining on a representative mouse that received Bgal-specific T cells.

Microglia　　　　　　　**Peripheral APC**
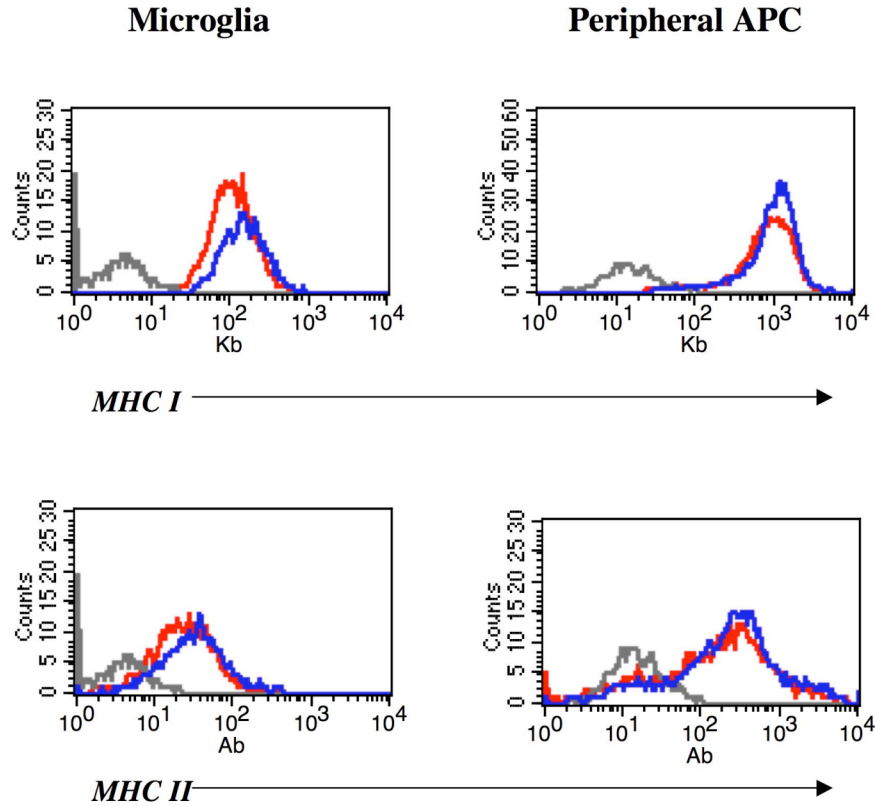
Fig 8. **MHC I and II are upregulated in the CNS during EAE.** EAE was induced in C57BL/6 mice before adoptive transfer of Thy1.1 HuD-specific or Bgal-specific CD8 T cells (as described in Fig 6). Brain cells from two representative mice, both with a clinical score of 2.5, were stained for MHCI and MHC II expression. Histograms show MHC I and MHC II expression on microglia cells (CD11b$^+$CD45^{int}) and peripheral APC (CD11b$^+$CD45^{hi}). In red is a mouse that received HuD-specific T cells, in blue is a mouse that received Bgal-specific T cells.

by stimulation with cognate peptide. Both HuD- and βgal-specific CD8 T cells isolated from the CNS were able to secrete IFNγ in response to cognate peptide in an ELISPOT assay. Although HuD-specific CD8 T cells were able to traffic to the brain when transferred into Rag$^{-/-}$ recipients, we saw no signs of inflammation or neuronal loss in the CNS, despite the presence of HuD antigen and the absence of regulatory T cells in these hosts. Restimulation of infiltrating lymphocytes is an important step in the generation of CNS inflammation, and depends on peripheral APC recruitment and upregulation of MHC molecules on resident microglia. The inability of HuD-specific CD8 T cells to induce disease was likely due to a lack of restimulation behind the blood brain barrier. We saw no recruitment of APCs to the CNS, and no upregulation of MHC molecules on microglia in our adoptive transfer recipients. In order to artificially create an inflammatory environment for transferred HuD-specific T cells, we generated EAE in recipient mice. These mice displayed characteristic clinical signs of demyelinating disease, and exhibited high expression of MHC I and MHC II molecules on both microglia and peripheral APCs in the CNS. Examination of CNS tissue revealed a lack of neuronal degeneration but obvious areas of infiltration, and mice did not appear visibly affected by the transfer to activated HuD-specific CD8 T cells.

CHAPTER VI. IDENTIFICATION OF HUMAN HLA CD8 T CELL EPITOPES OF HUD

Introduction

We sought to identify clinically relevant human HuD epitopes in order to better understand and diagnose anti-Hu syndrome. Our immediate goal was to use the results of this comprehensive peptide screen to design HuD-specific tetramers that would allow us to visualize and characterize HuD-specific CD8 T cells in the blood and cerebrospinal fluid of patients. Using a novel approach to epitope discovery developed by Beckman Coulter, we screened the entire HuD peptide library over 8 human HLA MHC I alleles. Each HuD peptide was evaluated based on its affinity and off-rate for individual MHC alleles. Based on the results of this screen, peptides were given a numerical score and ranked, with higher scores signifying more likely CD8 T cell epitopes. Data for all 8 human HLA MHC alleles were acquired. We chose to evaluate peptides restricted to the HLA A2.1 and HLA A3.1 alleles, since these are two of the most prevalent MHC alleles in the western population. Tetramers were designed for each potential epitope, which were used to analyze patient blood for the presence of HuD-specific CD8 T cells. This provided irrefutable evidence of HuD-specific CD8 T in the blood of patients with the Hu syndrome.

Results

A comprehensive screen to identify human HuD epitopes

To identify human epitopes of HuD, we generated a complete HuD peptide library consisting of 386 overlapping nonamers, including peptides derived from all known splice variants of the protein. Each peptide was screened for its ability to bind to recombinant HLA MHC I molecules. We screened the entire HuD peptide library over 8 different HLA alleles, including HLA A2.1 and HLA A3.1. Together, HLA A2.1 and HLA A3.1 are carried by roughly 40% of the Caucasian population, making them two of the most common MHC I alleles. For this reason, our studies focused on epitopes presented by these specific MHC molecules, although future work will extend to all 8 alleles examined in the screen. Peptides were ranked according to a final iScore, which was generated based on binding, affinity and off-rate measurements. We identified 39 HLA A2.1-restricted peptides with significant iScores. To narrow down this list of potential HLA A2.1 HuD epitopes, we reasoned that pathologically relevant HuD-specific CD8 T cells should recognize peptides that are not shared between HuD and its closely related family member HuA. As previously discussed, HuA is a ubiquitously expressed protein found throughout the body. Moreover, patients with the Hu syndrome generate an immune response that targets the nervous system exclusively, leaving peripheral tissues unaffected. This lack of peripheral autoimmunity suggests that relevant disease epitopes are not common to

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HuA.   Of the 39 HLA A2.1-restricted peptides, 20 were different in sequence from HuA and therefore chosen for further evaluation.

HuDp157 is a naturally processed A2.1 epitope

In order to determine which of these 20 potential HuD HLA A2.1-restricted peptides are naturally processed and presented epitopes, we took advantage of the AAD transgenic mouse that expresses human HLA A2.1 molecules.   Because HuD is highly conserved, with only two amino acid differences between the mouse and human proteins, we were able to immunize A2.1 transgenic mice with adenovirus-HuD to assess natural processing and presentation without fear of priming responses to neo-epitopes.   AAD mice were immunized with adenovirus-HuD in combination with pertussis toxin to allow for natural processing and presentation of HuD epitopes onto HLA A2.1 molecules and subsequent CD8 T cell priming *in vivo*.   CD8 T cells from immunized mice were stimulated *in vitro* with each of the 20 peptides and assessed for their ability to secrete IFNγ in response to cognate peptide in an ELISPOT assay (Fig 1).   Of the 20 potential epitopes, 11 were able to elicit IFNγ secretion from CD8 T cells in an antigen-dependent manner.   It seemed unlikely that all 11 peptides represented true HuD epitopes, and we therefore chose to assess natural processing and presentation in a more stringent assay. We re-screened CD8 T cells from immunized mice in an ELISPOT assay using syngeneic stimulators infected with adenovirus-
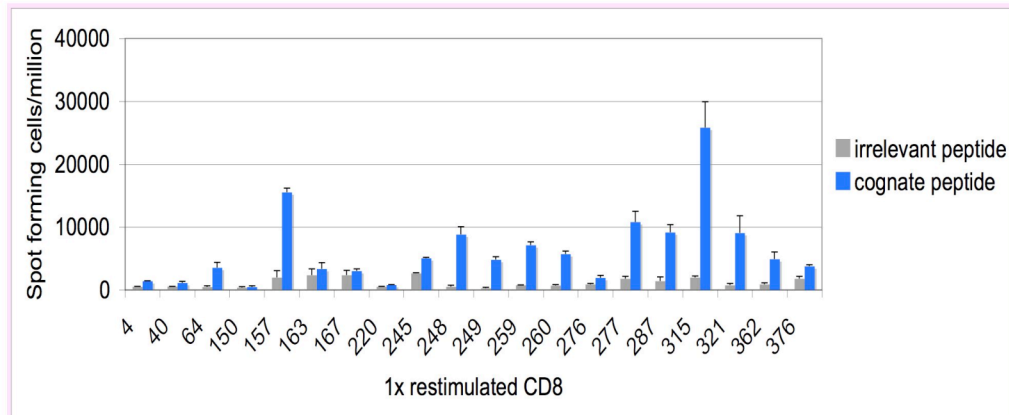
Fig. 1.   Screening HLA A2.1 restricted HuD peptides in AAD mice.
Spleen cells from AAD mice immunized with AdVHuD + PTx were stimulated *in vitro* with the peptides indicated on the x-axis. After ten days of stimulation, purified CD8 T cells were plated in an IFNγ ELISPOT with EL4-A2.1 cells pulsed with relevant or irrelevant peptide.

HuD (Fig 2). In this way, HuD epitopes were naturally processed and presented onto HLA A2.1 molecules both for priming *in vivo* and for antigen-specific IFNγ secretion *in vitro*. Because AAD mice retain expression of mouse H-2^b MHC I molecules, we used three sets of syngeneic stimulators in the ELISPOT assay to ensure that IFNγ secretion was in response to peptides presented on HLA A2.1 rather than mouse D^b or K^b molecules. Syngeneic stimulators consisted of kidney cells from AAD, HHD, or C57BL/6 mice. Again, AAD mice express both mouse MHC I molecules as well as human A2.1, whereas HHD mice are deficient for mouse MHC I and express only human A2.1. C57BL/6 mice are wild type H-2^b mice that express K^b and D^b molecules. Kidney cells derived from each of these three strains were infected with either adenovirus-HuD or, as a control, adenovirus-GFP. A true HLA A2.1-restricted epitope of HuD should elicit a response from HuD-specific CD8 T cells only when presented by either AAD or HHD kidney cells infected with adenovirus-HuD. IFNγ secretion should not be seen in response to C57BL/6 kidney cells infected with adenovirus-HuD, as this would imply presentation by mouse H-2^b MHC I. Of the 11 peptides that screened positive in the initial ELISPOT assay, only two elicited IFNγ secretion in response to AAD and HHD kidney cells infected with adenovirus-HuD (Fig. 2). Peptides 157 and 287 appeared to be true A2.1-restricted HuD epitopes, and we therefore sought to generate tetramers for both peptides. Due to technical difficulties, p287-specific tetramers were
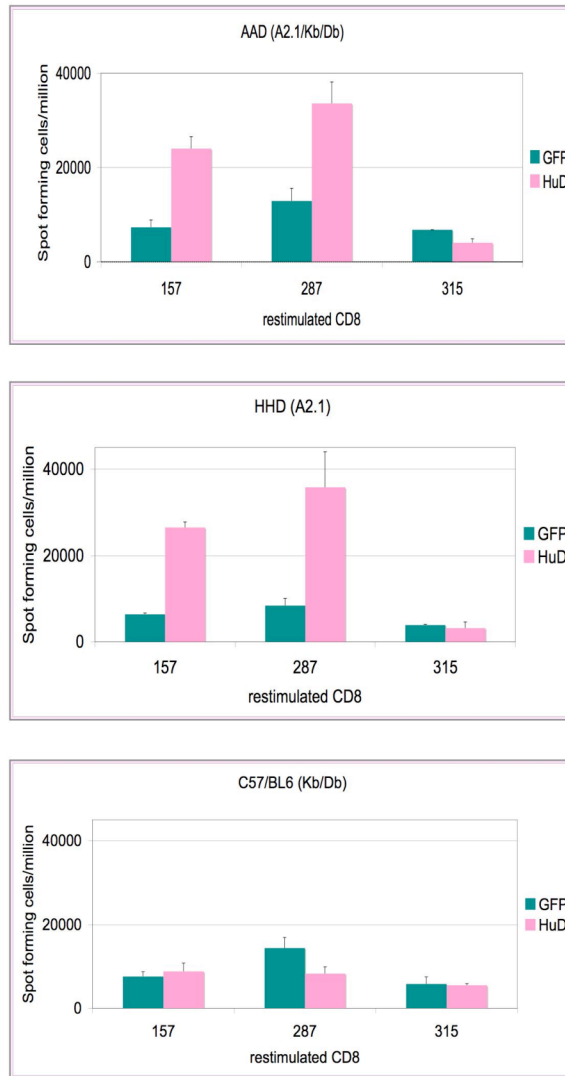
Fig. 2. **HuDp157 is a naturally processed A2.1-restricted epitope.** CD8 T cells purified from spleen stimulation cultures were tested for their ability to recognize whole HuD protein processed and presented by primary kidney cells infected with AdVHuD (or AdVGFP as a negative control). CD8 T cells specific for HuDp157, HuDp287, or HuDp315 were plated in an IFNg ELISPOT with infected kidney cells from AAD mice (top panel), HHD mice (middle panel), or wild type mice (bottom panel).

impossible to create and after several rounds of discussion with iTopia consultants from Beckman Coulter, we chose to discount p287 as a real HuD epitope.

HuDp157 specific T cells are present in the blood of Hu patients

We designed HLA A2.1-restricted tetramers specific for HuDp157 in order to screen the blood of patients with the Hu syndrome for the presence of HuDp157-specific CD8 T cells.   After a single round of *in vitro* stimulation with p157, we detected a population of HuDp157-specific CD8 T cells from an HLA A2.1+ Hu patient but not from HLA A2.1+ normal donors (Fig 3).   Normal donors exhibited an expanded population of CD8 T cells specific for common viral epitopes of influenza or CMV (Fig 3).   This was the first direct proof of HuD-specific T cells in the blood of an Hu patient. Taken together, these results support the hypothesis that CD8 T cells play an important pathological role in autoimmune neurologic degeneration.

HuDp157-specific CD8 T cells from patients are functional cytotoxic T cells

 Our model of disease pathogenesis depends on the destruction of HuD-expressing tumor cells and neurons by effector HuD-specific CD8 T cells. To show that CD8 T cells isolated from Hu patients are capable of lysing HuDp157-expressing targets, we performed a CTL assay with tetramer-sorted HuDp157-specific T cells.   We chose to tetramer-sort T cells from

Fig 3. HuDp157-specific T cells are present in Hu patient blood.
Peripheral blood T cells from one Hu patient and four normal donors
were expanded *in vitro* with HuDp157 and stained with HuDp157-specific
or irrelevant peptide-specific (negative) tetramer.   As a control,
peripheral blood T cells were expanded with M1 or CMV peptide and
stained with relevant tetramer.
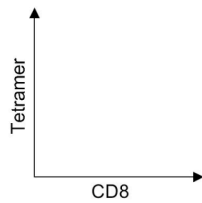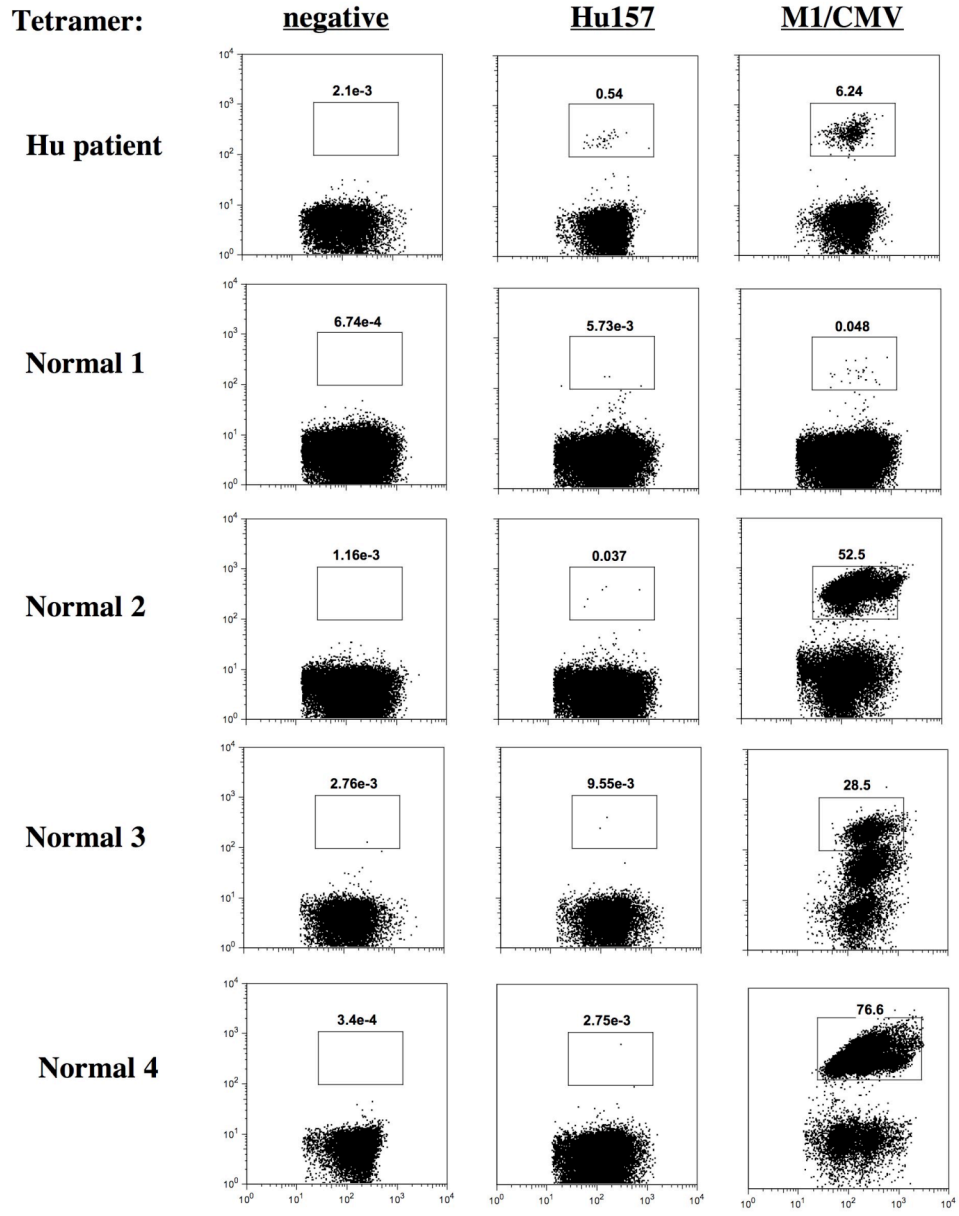
Fig. 3

patients because previous attempts to assess the function of HuD-specific T cells using bulk T cell cultures were unsuccessful. We reasoned that the frequency of HuD-specific T cells isolated from patient blood was too low for detection in our assays. To overcome this limitation, we stimulated patient T cells *in vitro* with p157 prior to sorting tetramer-positive CD8 T cells from bulk T cell stimulation cultures. Tetramer-sorted HuDp157-specific CD8 T cells from two individual Hu patients were used in CTL assays with cognate or irrelevant peptide-pulsed targets. HuDp157-specific CD8 T cells from both patients were able to lyse target cells in an antigen-dependent manner (Fig 4). These results strengthen our model of CD8 T cell mediated disease pathogenesis, and mimic the results from our mouse system. Both human and mouse HuD-specific CD8 T cells are able to act as cytotoxic T cells, but require isolation and *in vitro* stimulation in order to expand and acquire effector function.

HLA A3.1 restricted HuD-specific CD8 T cells from patients are functional cytotoxic T cells

To identify clinically relevant HLA A3.1 restricted epitopes of HuD, we used the five top-scoring peptides from the iTopia screen in patient experiments. Because there were fewer candidate peptides for A3.1 than

for A2.1, we chose to move directly to A3.1+ patient assays. T cells from

four A3.1+ Hu patients were used to screen the A3.1-restricted candidate

(a)
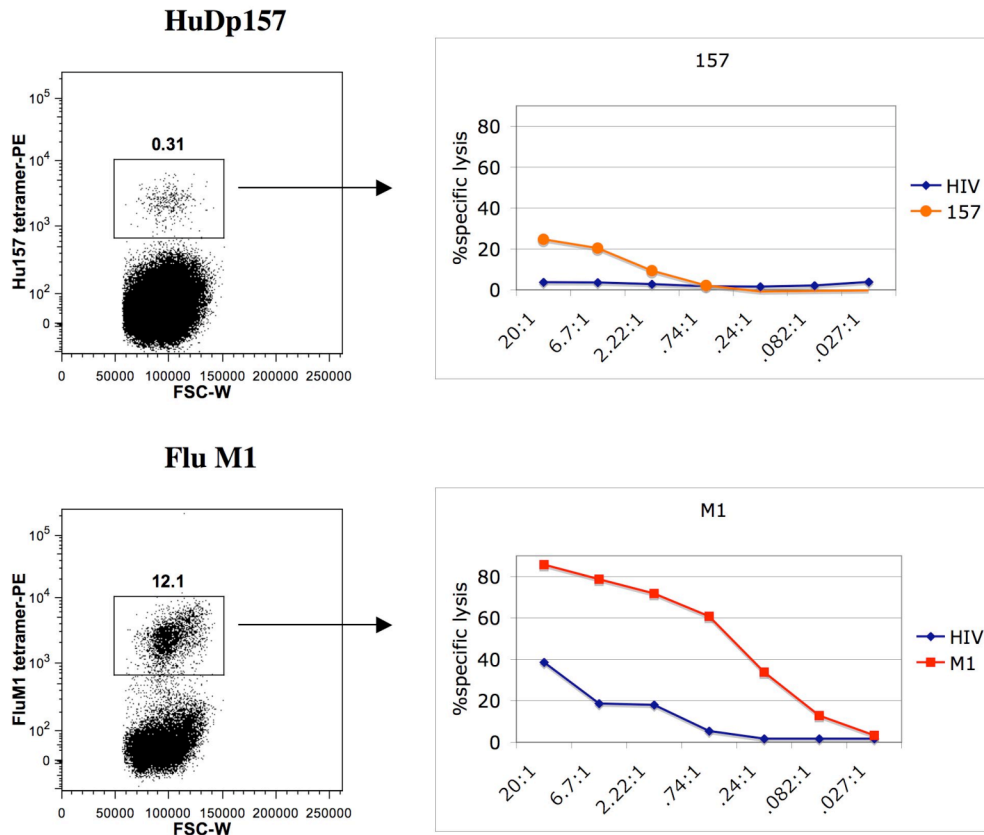
Fig. 4.   HuDp157-specific T cells from patients are functional cytotoxic T cells. (a) HuDp157 and influenza M1 tetramer+ T cells from an Hu patient were expanded *in vitro* with peptide, followed by FACS sorting of the tetramer+ population.   Tetramer+ cells were allowed to recover with irradiated, peptide-pulsed, autologous PBMC and IL-2. After recovery, T cell lysis was measured in a CTL assay with peptide-pulsed T2 cells.   (b) lytic functions of HuDp157 and influenza M1 tetramer+ T cells from a second Hu patient were measured as in (a).

(b)

HuDp157
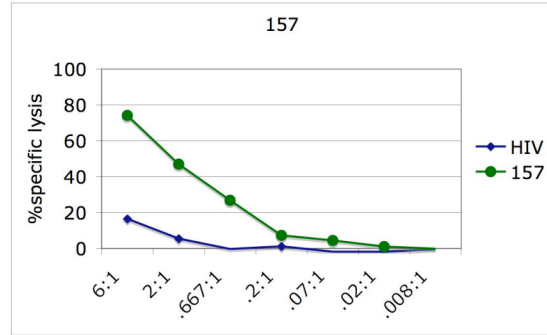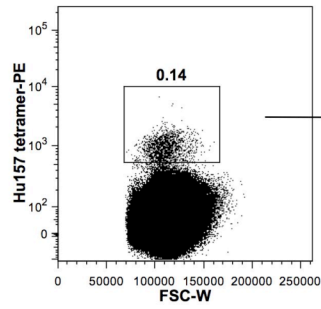
Flu M1
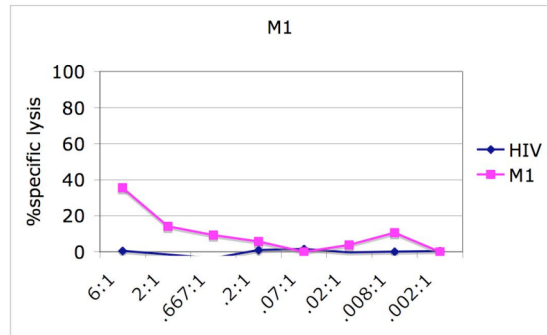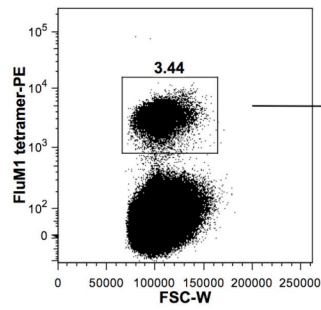
peptides. CD8 T cells were cultured for 8 days with autologous peptide-pulsed DCs to expand antigen specific T cells before their use in tetramer and CTL assays. Of the five A3.1 candidate peptides, HuDp133 was the only peptide capable of expanding CD8 T cells from patient blood (Fig 5). One out of four Hu patients exhibited expansion of HuDp133 specific T cells (Fig 5). In comparison, none of the neurologically normal A3.1+ control patients had an expanded HuDp133 population, but all demonstrated expansion of influenza specific T cells. The lack of HuDp133-specific CD8 T cells in three of the four A3.1+ Hu patients is not surprising, considering that these patients have other HLA MHC I alleles in addition to A3.1, and may generate HuD-specific responses to epitopes restricted to these other alleles. Expanded HuDp133-specific CD8 T cells were assessed for the ability to lyse target cells expressing cognate peptide (Fig. 6). Bulk T cells were stimulated *in vitro* with each of the five A3.1-restricted HuD peptides before use in a CTL assay. HuDp133-specific CD8 T cells were able to lyse targets in an antigen-dependent manner, demonstrating that HLA A3.1-restricted HuD-specific T cells are functional effectors present in Hu patient blood.

Summary

CD8 T cells are the effector cells responsible for the destruction of neurons and tumor cells in our model of the Hu syndrome. In order to provide evidence in support of CD8 T cell mediated disease, we sought to

Fig. 5. HuDp133 is an HLA A3.1-restricted epitope of HuD. Tetramer staining of T cells from four different HLA A3.1 Hu patients and two normal donors after expansion with five A3.1-restricted HuD peptides. T cells were stained with tetramer specific for cognate A3.1 peptide or an irrelevant tetramer. As a positive control, T cells were expanded with the HLA A.3.1-restricted epitope of influenza nucleoprotein (NP) and stained with NP-specific tetramer.
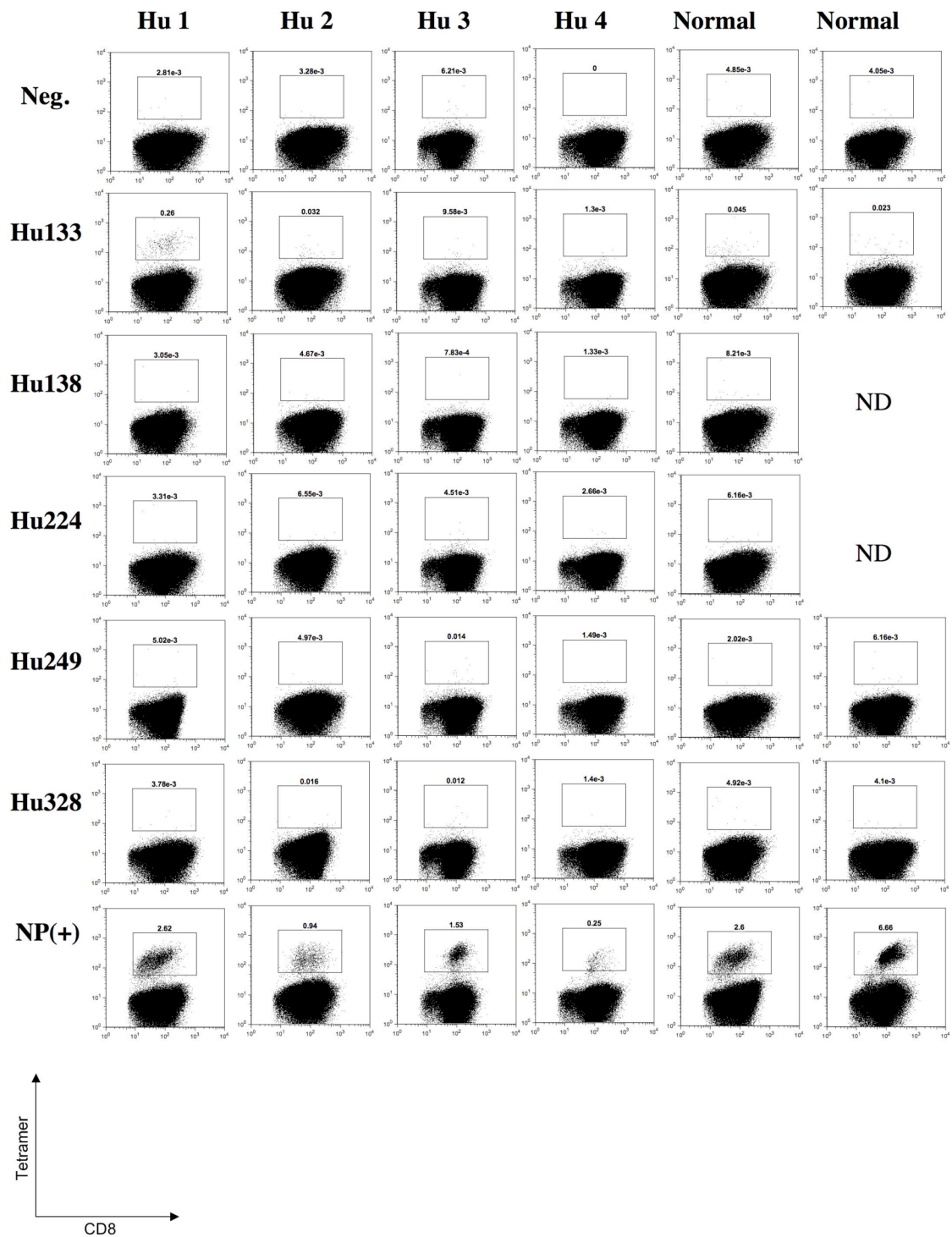
Fig. 5

Fig. 6. HuDp133-specfic T cells are functional cytotoxic T cells.   T cells from an A3.1+ Hu patient (Hu #1 in figure 5) were expanded *in vitro* with five A3.1-restricted HuD peptides.   After expansion, cells were stained with tetramer specific for cognate HuD peptide or irrelevant peptide. Lytic function of the expanded T cells was measured in a CTL assay with T2 cells pulsed with cognate HuD peptide or irrelevant peptide.
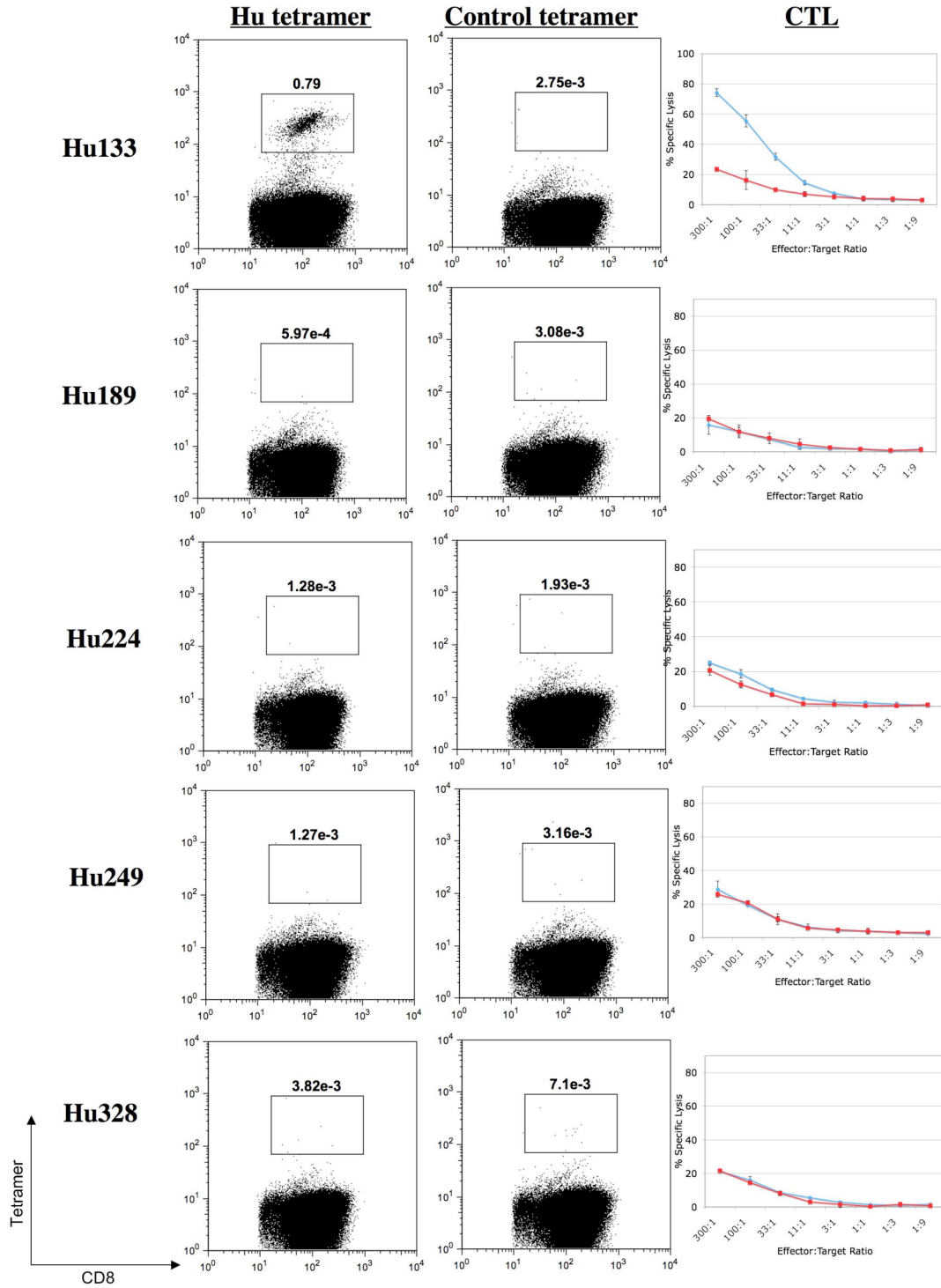
Fig. 6

isolate and characterize HuD-specific CD8 T cells from patients. This required the identification of human CD8 T cell epitopes of the protein. To this end, we performed a comprehensive screen of the entire HuD peptide library over 8 human HLA alleles. A list of potential epitopes was generated from the screen, which lead to the discovery of two human CD8 T cell epitopes of HuD. Tetramers for these two epitopes were used to examine the blood of Hu patients for the presence of HuD-specific CD8 T cells. We found an expanded population of HuD-specific CD8 T cells in Hu patients but not in normal donors, indicating that these T cells play a role in disease development. Tumor immunity and neurodegeneration in the Hu syndrome require lysis of HuD-expressing cells by cytotoxic T cells. We therefore assessed the lytic ability of HuD-specific CD8 T cells isolated from the blood of Hu patients. We found that A2.1- and A3.1-restricted HuD-specific CD8 T cells from patients were efficient at destroying target cells that expressed cognate HuD peptide. This provided clear evidence in support of our model of disease pathogenesis.

CHAPTER VII. DISCUSSION

Development of neuronal autoimmunity in patients with SCLC appears to depend on a loss of tolerance to the onconeural antigen HuD (Fig 1). This destructive immune response against HuD-expressing neurons is also able to mediate impressive tumor immunity. Tumor immunity and neuronal autoimmunity are not always coupled, since a significant number of SCLC patients mount an immune response to their cancer that correlates with limited stage tumors and better prognoses in the absence of neurologic disease (Dalmau, Furneaux et al. 1990). Understanding the mechanisms responsible for tolerance induction to HuD, and how tolerance is broken in the presence of an HuD-expressing tumor, are of great clinical significance with regard to designing better therapies for SCLC.

In order to study tolerance to HuD in an animal model, we first identified the immunodominant CD8 T cell epitope of the protein in C57BL/6 mice through an exhaustive screen of the entire HuD peptide library. We found CD8 T cells specific for 7 peptides of the protein, and went on to determine which of these represented naturally processed and presented epitopes. This allowed for the identification of the immunodominant CD8 T cell epitope of HuD, p321. HuDp321-specific CD8 T cells were characterized in functional and phenotypic assays. We found that these
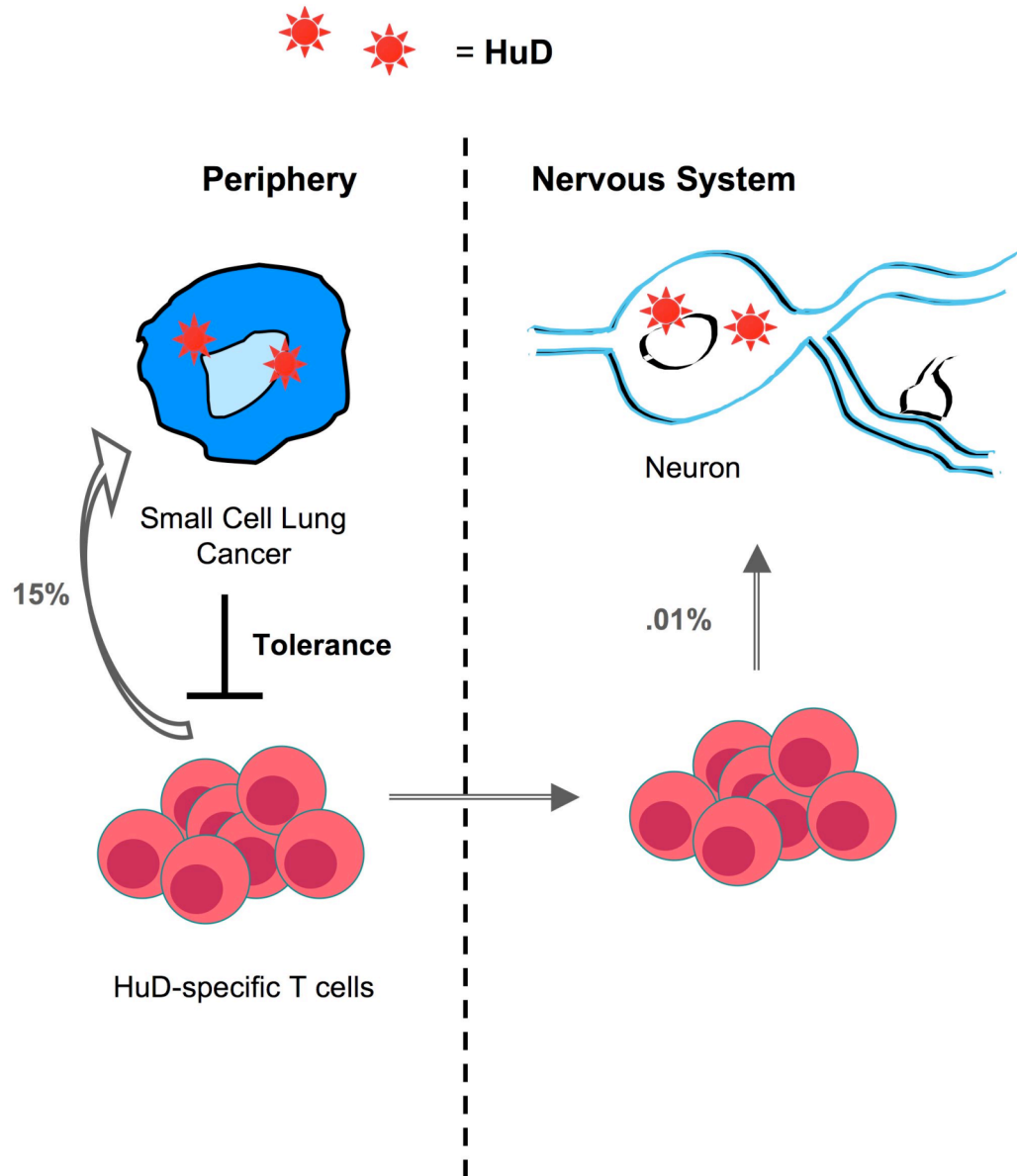
Fig 1. Revised model of anti-Hu syndrome. HuD-specific T cells are normally tolerized to the HuD protein. The presence of a small cell lung cancer tumor that expresses HuD is able to break tolerance to the HuD antigen in 15% of patients, resulting in an immune response against the cancer. In 01% of patients, this immune response is also able to traffic to the nervous system to mediate destruction of HuD expressing neurons.

cells, once activated *in vitro*, were high affinity T cells capable of lysing targets in an antigen dependent manner. The inability to detect these cells directly after immunization with recombinant adenovirus was the first indication that HuD-specific CD8 T cells were subject to tolerance induction. Priming an effective immune response required pertussis toxin in combination with adenovirus-HuD followed by *in vitro* stimulation with peptide. *In vitro* stimulation allowed for the expansion of HuD-specific CD8 T cells, and served to eliminate any suppression felt *in vivo*. The role of pertussis toxin in our system was less clear. It obviously acted as an adjuvant, enhancing the immune response to HuD *in vivo* in order to allow for further expansion *in vitro*. Immunization with adenovirus-HuD together with *in vitro* stimulation in the absence of pertussis toxin failed to generate a detectable population of functional, IFNγ-secreting HuD-specific CD8 T cells, indicating that pertussis toxin served to quantitatively or qualitatively change the T cell response *in vivo*. Still, pertussis toxin alone was not sufficient to generate detectable IFNγ-secreting HuD-specific cells, since T cells isolated from mice immunized with pertussis toxin and adenovirus-HuD did not secrete IFNγ in response to antigen directly *ex vivo*, in the absence of *in vitro* stimulation. The need for pertussis toxin together with *in vitro* stimulation for the generation of HuD-specific CD8 T cells may be the result of low precursor frequency in conjunction with peripheral tolerance induction. Pertussis toxin might relieve some of the suppression on HuD-specific T cells in

vivo, but additional expansion by *in vitro* stimulation may be necessary due to low precursor frequency.

Pertussis toxin has been shown to act as an adjuvant by inducing maturation of dendritic cells, resulting in the expansion of effector T cells and IFNγ production (Hou, Wu et al. 2003). It has also been implicated in blocking the function of regulatory T cells, thereby promoting productive immune responses (Chen, Winkler-Pickett et al. 2006). The means of inducing peripheral tolerance to HuD are still unclear. Anergy induction or inhibition by regulatory T cells are both possible mechanisms of suppression of HuD-specific T cells, either of which would be overcome by pertussis toxin. Our preliminary experiments have shown that depletion of CD4 T cells followed by adenovirus-HuD immunization failed to generate a detectable HuD-specific response directly *ex vivo*, implying that regulatory T cells are not responsible for tolerance induction. However, CD4 T cell depletion is not a specific means of eliminating regulatory T cells, since the entire CD4 T cell population is removed, which includes helper CD4 T cells that may be required to assist in HuD-specific CD8 T cell priming. Regulatory T cells may be responsible, in part, for inhibiting HuD-specific immune responses, given that HuD-specific CD8 T cells escape central tolerance to circulate in the periphery with relatively high affinity for antigen. Regulatory T cells could act to inhibit HuD-specific CD4 T cells, which

would in turn prevent the activation of HuD-specific CD8 T cells. A coordinated response from both the CD4 and CD8 T cell arms of the immune system is almost certainly required for the generation of HuD-specific autoimmunity, so that inhibition of HuD-specific CD4 T cells would be an important means of inducing tolerance in the periphery. Given that HuD is an intracellular antigen, cross-presentation of exogenous protein is required for the initiation of HuD-specific CD8 T cell responses. CD4 T cells are necessary for effective cross-presentation to CD8 T cells, making their inhibition imperative to the prevention of HuD-specific CD8 T cell-mediated neurodegeneration (Albert, Jegathesan et al. 2001).

Anergy induction is a potential mechanism of HuD-specific CD8 T cell suppression, which would depend on peripheral presentation of HuD to circulating T cells. Presentation of HuD could involve expression of the protein by a non-neuronal cell type, for example a lymph node stromal cell, which would result in anergy induction. We have yet to find HuD protein or RNA expression in any tissue outside of the nervous system, however it may be expressed at extremely low levels, making detection difficult. The transcription factor AIRE has been implicated in regulating the expression of tissue-specific proteins by lymph node stromal cells for the purpose of inducing tolerance, so we investigated its role in controlling ectopic expression of HuD. However, HuD expression did not

appear to be governed by AIRE. This does not rule out the possibility that unidentified transcription factors exist in order to regulate expression of HuD and other tissue-specific proteins for the purpose of inducing tolerance. In the absence of, or in addition to the ectopic expression of HuD by a non-neuronal cell type, HuD-expressing neurons may be cross-presented via DCs, resulting in cross-tolerization of HuD-specific CD8 T cells. However, neurons do not normally turn over, which makes reliance on cross-presentation for tolerance induction potentially dangerous. It is possible that only pieces of neurons, in the form of membrane-bound blebs, are taken up by DCs for cross-tolerization of HuD-specific CD8 T cells.

We found it useful to compare tolerance induction to HuD versus its related family member HuA. Given the ubiquitous expression of HuA, we reasoned that tolerance to this protein should be stricter compared to HuD. Patients with the Hu syndrome develop autoimmunity that is restricted to the nervous system but never directed against peripheral tissues, suggesting that effector CD8 T cells responsible for disease pathogenesis are unable to cross react with HuA. Furthermore, patient sera react with HuB, HuC and HuD but not with HuA. We hypothesized that HuA-specific T cells should be centrally tolerized, since their presence in circulation could lead to the generation of massive peripheral autoimmunity. If HuA is subject to central tolerance induction, there

should not be T cells specific for any of the shared epitopes between HuD and HuA in the repertoire. We compared all 7 of the potential HuD epitopes initially identified in our peptide screen for sequence homology to their corresponding HuA epitopes. Of the 7 potential epitopes, 6 were different in HuA, supporting the idea that HuA is centrally tolerized. Of these 7 potential epitopes, only one, HuDp321, proved to be naturally processed and presented by the immune system. We compared this immunodominant HuD CD8 T cell epitope to its related epitope in HuA, peptide 268. HuA p268 differs from HuD p321 at a single amino acid position. The fourth residue is converted from an asparagine in HuD to a threonine in HuA. This is a non-conserved amino acid difference, found at a position that is known to interact with the T cell receptor but that does not affect MHC binding affinity. In order to show that HuA p268 binds with similar affinity to MHC I compared to HuD p321, we performed an MHC I stabilization assay. HuA peptide bound MHC I with higher affinity than p321. When mice were immunized with either the HuA peptide or HuD peptide emulsified in titermax adjuvant, there was a strong response from HuDp321-specific CD8 T cells, however we did not detect any HuAp268-specific T cells. The absence of HuA-specific T cells strongly suggests that mice are centrally tolerized to the HuA protein. Conversely, the existence of T cells specific for HuD in the repertoire indicates that central tolerance does not apply to HuD. Also, we have demonstrated that AIRE, a transcription factor involved in the induction

of central tolerance, does not regulate expression of HuD in mice. Nevertheless, we cannot exclude the possibility that HuD is subject to central tolerance induction via an AIRE-independent pathway, and that a portion of HuD-specific T cell clones are able to escape thymic deletion, resulting in a small precursor frequency in the periphery.

The initial immune response in patients with the Hu syndrome is directed against HuD, since this is the only Hu family member expressed in SCLC. Patients present with neurologic symptoms that affect discrete areas of the nervous system but often go on to develop multifocal neuronal degeneration. Although all Hu patients share the characteristic anti-Hu antibody response that is correlated with tumor immunity and neuronal autoimmunity, the regions of the nervous system affected vary widely. The disease begins with an acute phase and can either plateau to reach a state of chronic but not degenerating illness, or progressively worsen from the time of diagnoses until death. These differences among patients reflect diverse pathways of disease progression that may be due to genetic and environmental factors. Every individual has a different group of MHC molecules, referred to as the haplotype, which determines the spectrum of peptides presented to the immune system. MHC haplotype can strongly influence disease susceptibility and immunity, and almost certainly affects the onset and development of the Hu syndrome. The fact that most patients progress from neurologic

symptoms targeted to discrete areas of the nervous system to multifocal neuronal degeneration suggests that there may be epitope spread to generate immune responses against other Hu family members during the course of the disease. The initial immune response to HuD in the periphery via ectopic expression of this neuronal antigen by a SCLC tumor might be expanded to include immune responses against other Hu family members. For instance, HuD-specific CD8 T cell-mediated destruction of neurons that express both HuD and HuC could result in the activation of HuC-specific T cells, which would then mediate neuronal degeneration of HuC expressing neurons, resulting in multifocal disease.

Given the high sequence homology between the Hu proteins, there may be a shared CD4 T cell epitope between Hu family members that facilitates epitope spread. The generation of effector CD8 T cell responses to HuB or HuC would almost certainly depend on cognate CD4 T cell help, since cross presentation by an APC is required. If the initial immune response to HuD-expressing SCLC cells in the periphery results in the activation of CD4 T cells that recognize a shared Hu epitope, these same CD4 T cells would be primed to assist in the generation of an immune response against other Hu family members. In Hu patients, we detect anti-Hu antibodies that recognize HuB, HuC, and HuD, indicating that the B cell epitope of the protein is conserved among family members.

Whether regulatory T cells, as opposed to helper CD4 T cells, recognize a shared epitope is still unclear. We have shown that HuC expression is not required for tolerization of HuD-specific CD8 T cells. When HuC-deficient animals were immunized with adenovirus-HuD, there was no detectable HuD-specific response directly *ex vivo*, whereas HuD-deficient mice showed a strong *ex vivo* response to HuDp321. It would seem efficient to generate regulatory T cells specific for a shared Hu epitope in order to maintain tolerance to the Hu protein family, however our results do not support this hypothesis. What is clear is the requirement for HuD expression for tolerization of HuD-specific CD8 T cells. Either HuD expression serves to generate regulatory T cells specific for a CD4 epitope that is unique to the protein, or is directly responsible for inducing anergy in HuD-specific CD8 T cells. The CD8 T cell epitope of HuD is not shared by other Hu family members, so that anergy induction would necessarily require expression of HuD but not other Hu family members.

The presence of a SCLC tumor in the periphery that expresses HuD appears to be the initial trigger for the generation of an anti-Hu immune response. This pocket of antigen, outside of the nervous system, is able to break tolerance to HuD and elicit effective anti-tumor immunity. The steps required for the loss of tolerance are still unknown. Certain cytokines or growth factors produced by SCLCs may help to overcome suppression and prime HuD-specific lymphocytes. Genetic differences

may also determine which SCLC patients are able to generate anti-Hu antibodies and tumor immunity. Given that 15% of SCLC patients manufacture anti-Hu antibodies, the loss of tolerance is not a particularly rare event. What is rare is the generation of neuronal autoimmunity, as only .01% of SCLC patients succumb to neurologic disease. There is obviously an additional, and more infrequent event that distinguishes patients with autoimmune neurodegeneration from those with low titer antibody and better tumor immunity in the absence of neurologic disease. Perhaps there are multiple mechanisms of inducing tolerance to HuD in order to safeguard against neuronal degeneration. One form of tolerance may be overcome in neurologically normal SCLC patients with anti-Hu antibodies, whereas most or all are broken in anti-Hu patients, allowing for the development of neuronal autoimmunity. The fact that Hu patients generate high titer antibodies to Hu, whereas neurologically normal patients produce low titer antibodies, indicates a qualitative or quantitative difference in the immune response in these two groups. Since both sets of patients are able to generate tumor immunity in the periphery, the distinguishing mechanism or event that translates to neurologic disease would be likely to occur in the nervous system. HuD is expressed in peripheral neurons as well as in the CNS, so it is unclear if this distinguishing event necessarily occurs behind the blood brain barrier. It may instead happen near peripheral neurons, and could require neuronal death or

inflammation in order to prime a strong and lasting immune response to HuD. Patients with Hu antibodies generate a primary response to HuD in the periphery that seems to depend on the ectopic expression of this neuronal protein by a SCLC tumor. The subsequent neuronal autoimmunity in Hu patients should depend on the recognition of HuD in the nervous system via cross presentation by a professional APC. A concurrent infection that generates inflammation would help to overcome tolerance to the protein, resulting in a degenerative immune response against HuD expressing neurons.

Patients with SCLCs that are able to generate natural tumor immunity in the absence of neurologic disease offer invaluable insight into how the body can recognize and destroy cancer. In order to understand and harness this natural tumor immunity for treatment of SCLC, it is imperative that we understand the differences between neurologically normal SCLC patients and patients with the Hu syndrome to ensure that therapy does not cause autoimmune neurodegeneration. Understanding the mechanisms behind autoimmune neurodegeneration requires the creation of a mouse model of CNS disease mediated by HuD-specific T cells. To this end, we designed an adoptive transfer system to study T cell mediated neuronal degeneration, using *in vitro* stimulated HuDp321-specific CD8 T cells. We found that upon adoptive transfer into syngeneic recipients, HuDp321-specific CD8 T cells persisted up to one

month post transfer and were able to traffic to the CNS. However, trafficking to the CNS was not antigen-dependent, as β-galactosidase-specific CD8 T cells were also found in the brain. It has been shown in other experimental systems that activated T cells routinely traffic to the CNS in order to monitor this compartment for infection. Once inside the CNS, both HuD-specific and βgal-specific CD8 T cells downregulated the CD8 co-receptor. Downregulation of the CD8 co-receptor is arguably a means of suppressing activated T cells within the brain in order to protect against inflammation and tissue damage. HuD-specific and βgal-specific CD8 T cells in the CNS were not permanently suppressed, however, since these cells could secrete IFNγ in response to peptide in an ELISPOT assay. Despite the presence of both activated HuD-specific CD8 T cells and cognate antigen in the CNS, we saw no neurologic disease in our experimental system. The lack of disease appeared to be due, in part, to a lack of inflammation behind the blood brain as there was no recruitment of peripheral APCs or upregulation of MHC molecules in the brain. As a means of artificially creating CNS inflammation in our adoptive transfer recipients, we induced EAE in host animals prior to the transfer of HuD-specific CD8 T cells. This resulted in clinical signs of demyelination and characteristic recruitment of peripheral APCs together with upregulation of MHC I and II expression on resident microglia. Nevertheless, recipient animals exhibited no signs of neuronal degeneration by histology. The most obvious explanation for

the lack of disease is the absence of HuD-specific CD4 T cells in this model.

Many models of brain disease involve recruitment of peripheral APCs to the CNS. Reactivation of T cells via DCs behind the blood brain barrier is an initiating step in the development of CNS inflammation (Becher, Bechmann et al. 2006). Microglia are also activated during the inflammatory response by upregulating expression of MHC molecules in order to assist in the maintenance of inflammation. Given that HuD is an intracellular neuronal antigen, cross presentation by an APC is presumably required for reactivation of T cells behind the blood brain barrier in order to initiate an inflammatory response that ultimately results in destruction of HuD-expressing neurons. Cross-presentation to CD8 T cells requires CD4 T cell help, so that the presence of an HuD-specific CD4 T cell in the brain is necessary for disease pathogenesis. Even after the initial reactivation of T cells behind the blood brain barrier, subsequent stages of disease depend on presentation of antigen by microglia, which become active participants in the inflammatory reaction. Exacerbation of disease by microglial antigen presentation to HuD-specific CD8 T cells in the parenchyma would also require cross presentation and CD4 T cell help. The need for CD4 T cells in CNS disease pathogenesis is indicated by the spontaneous development of autoimmune-mediated demyelination in transgenic mice that

constitutively express the costimulatory molecule B7.2 on resident microglia. In this model, microglia are permanently activated in the CNS, which leads to spontaneous CD8 T cell-mediated demyelination (Brisebois, Zehntner et al. 2006). Constitutive expression of B7.2 on microglia may eliminate the need for CD4 T cells if CD4 T cells are normally important for the expression of costimulatory molecules on microglia. In this model, antigen-specific CD4 T cell recognition of MHC-peptide complexes on resident microglia results in upregulation of costimulatory molecule expression on microglia. Activated microglia are then able to productively interact with CD8 T cells in the CNS and maintain a state of inflammation behind the blood brain barrier. In transgenic mice that constitutively express B7.2 on microglia, the need for CD4 T cells is eliminated since costimulatory molecule expression is permanently induced.

Our model of autoimmune-mediated neurologic disease does not include a CD4 T cell component. The CD4 T cell epitope of HuD is currently unknown, and therefore the isolation and characterization of HuD-specific CD4 T cells is difficult. In order to generate autoimmune-mediated neurologic disease, it may be necessary to transfer HuD-specific CD8 T cells together with cognate CD4 T help. In addition, the production of a transgenic mouse expressing the HuDp321-specific T cell receptor will be an invaluable reagent. It may be possible to bypass the

need for CD4 T cells by transferring a sufficient number of clonal HuD-specific CD8 T cells that have been stimulated *in vitro*. Transfer of CD8 T cells 2 days after *in vitro* stimulation, when the cells are at their peak of activation, may also help to eliminate the need for cognate CD4 T cell help. Ultimately, the generation of brain disease by HuD-specific T cells that have been primed by a tumor expressing HuD in the periphery will be the most informative model of autoimmune-mediated neuronal degeneration. To date, there is no naturally occurring HuD-expressing tumor line. It is possible to transfect a tumor line with the HuD gene, however protein expression will most likely exceed physiological levels, which may affect T cell priming *in vivo*. In addition, transfection with the HuD gene is problematic, since HuD is a biologically active protein and most tumor cells will not tolerate its expression.

The ultimate goal of our studies on the Hu syndrome is to design better diagnostic and therapeutic tools for the treatment of this aggressive form of autoimmune neurodegeneration. In our model of disease pathogenesis, destruction of HuD expressing tumor cells and neurons requires antigen-specific recognition and lysis by HuD-specific CD8 T cells. Isolation and characterization of HuD-specific CD8 T cells from patients required the identification of human CD8 T cell epitopes of the protein. To this end, we performed a comprehensive screen of the entire HuD peptide library over 8 human HLA alleles. A list of potential

epitopes was generated, which lead to the discovery of two human CD8 T cell epitopes of HuD. Tetramers for these two epitopes were used to examine the blood of Hu patients for the presence of HuD-specific CD8 T cells. We found an expanded population of HuD-specific CD8 T cells in Hu patients but not in normal donors, indicating that these T cells play a role in disease development. Tumor immunity and neurodegeneration require lysis of HuD-expressing cells. We therefore assessed the lytic ability of HuD-specific CD8 T cells isolated from the blood of Hu patients in a CTL assay. We found that HuD-specific CD8 T cells from patients were efficient at destroying target cells that expressed cognate HuD peptide. These results support our model of disease pathogenesis and allow us to ask the important question of whether there are HuD-specific CD8 T cells in patients with low titer antibody that are able to generate tumor immunity without neurologic disease. If these patients are found to harbor HuD-specific CD8 T cells, this would suggest that tolerance is broken in both groups of patients with anti-Hu antibodies, regardless of their neurologic state. Breaking tolerance to HuD would allow for an effective immune response to SCLC in the periphery and subsequent tumor immunity. The development of neurologic disease in Hu patients would require an additional step that translates the peripheral response against HuD expressing tumor cells into an inflammatory autoimmune response against HuD expressing neurons.

SCLC is a disease that affects heavy smokers. It would also be extremely informative to examine the blood of tumor-free long-term heavy smokers for the presence of HuD-specific CD8 T cells. If these cells were present in this population, we could infer that HuD-specific CD8 T cells provided effective protection from the growth of SCLC. Our goal is to harness the natural tumor immunity generated in patients with anti-Hu antibodies for the treatment of SCLC tumors, all of which express the HuD antigen. If HuD-specific CD8 T cells are expanded in heavy smokers and in neurologically normal patients with Hu antibodies, this would provide a strong impetus for the generation of SCLC immunotherapy based on HuD-specific CD8 T cells.

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