A T Cell Receptor Transgenic Approach to the Study of Thymocyte Development and Autoimmune Disease

by
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B.A. Biochemistry
Rutgers University, 1992

Submitted to the Department of Biology
In partial fulfillment of the requirements
For the degree of

DOCTOR OF PHILOSPHY
in Biology
at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY
FEBRUARY 1999

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Abstract

$CD8^+$ T lymphocytes recognize short peptide fragments when bound to self-class I major histocompatibility proteins. On a mature lymphocyte, such recognition results in activation of the T cell. On an immature thymocyte, such recognition may result in either positive selection, allowing the cell to mature, or negative selection, eliminating that T cell clone from the host's repertoire. The parameters that determine thymocyte fate have remained unclear. An experimental study was carried out to determine these parameters.

A variety of peptide ligands were tested for their influence on the positive and negative selection of T cell receptor-transgenic thymocytes. It was found that peptides with limited amino-acid sequence homology and vastly different affinity values for the T cell receptor could promote positive and negative selection of T cells in a dose dependent manner. It was found that the outcome of selection was also influenced by the type of antigen presenting cell present. The levels of surface expression of class I MHC proteins was significantly different on these cells types and correlated with their physiological roles in positive and negative selection.

Mice that co-express a transgenic T cell receptor and a cognate-ligand developed an autoimmune disease, the pathology of which was reminiscent of psoriasis. The disease was strictly dependent on the presence of both the T cell receptor and antigen. Interestingly, only certain inbred strains of mice developed the disease. The phenotype of transgenic cells differed from affected to unaffected animals. Analysis of these mice could reveal novel genetic factors that influence T cell responses. Furthermore, these mice may also develop into a useful model for human autoimmune psoriasis or other disorders of the skin.

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List of Abbreviations

Ab.....antibody
APC.....antigen presenting cell
CLIP.....class II-associated invariant chain peptide
DC.....dendritic cell
dGuo.....deoxyguanosine
DN.....double negative
DP .....CD4 CD8 double positive
EAE.....experimental autoimmune encephylomyelitis
ER .....endoplasmic reticulum
FACS .....fluorescent activated cell sorting
FTOC .....fetal thymic organ culture
IDDM.....insulin-dependent diabetes melitis
IFN .....interferon
Ig .....immunoglobulin
IL.....interleukin
LN .....lymph node
mAb.....monoclonal antibody
manLN .....mandibular lymph node
MBP .....myelin basic protein
mesLN.....mesenteric lymph node
MHC .....major histocompatibility complex
RAG1 .....recombinase activating gene-1
RTOC.....reaggregated thymic organ culture
SD .....standard deviation
SP .....single positive
SPC .....splenic dendritic cell
SPR .....surface plasma resonance
TAP .....transporter associated with antigen processing
TCR.....T cell receptor
TDC.....thymic dendritic cell
TEC .....thymic epithelial cell
TGF .....transforming growth factor
TNF .....tumor necrosis factor
2Cd.....descendants from the mating of 2C transgenic mice with DBA/2 mice

Chapter 1 Introduction to the immune system

The four hallmarks of the vertebrate adaptive immune response are specificity, diversity, memory and self-tolerance. In 1949, Mcfarlane Burnet unified these elements in his theory of clonal selection, which consists of four premises. First, each lymphocyte bears a single type of receptor of unique specificity. Second, interactions between a foreign molecule and the lymphocyte receptor lead to lymphocyte activation, growth and differentiation. Third, the differentiated cells bear the same receptor as the parental cell. Finally, lymphocytes bearing self-reactive receptors are eliminated at an early stage of development and are therefore absent from the repertoire of lymphocytes found in the adult animal (1).

The idea of clonal selection was preceded by and emerged from a large number of puzzling observations. Edward Jenner in 1798 introduced to Europe the technique of inoculating with cowpox to protect against smallpox. Nearly 100 years later, Louis Pasteur expanded this idea to include inoculations against cholera and anthrax. In 1890, Emil von Behring and Shibasaburo Kitasato demonstrated that the transfer of serum from an animal previously immunized with diphtheria to a naïve animal resulted in the transfer of diphtheria resistance as well. In 1883, Elie Metchnikoff observed that phagocytic white blood cells responded to infection and did so more aggressively in animals previously immunized (2). Taken together, these observations exemplify the concepts of memory, specificity and diversity. The immune system remembers the prior encounter with a pathogen and adapts in anticipation of a future encounter, rendering the host immune to subsequent challenges. Interestingly, the immune system was found not only to respond to pathogenic microorganisms, but also to "harmless" antigens that were

synthesized in the laboratory or that were expressed on the surface of erythrocytes or other cells or tissue from allogeneic or xenogeneic organisms (1, 3).

During the second World War, a physician named Peter Medawar made efforts to treat burn victims with transplants of skin. He observed that while he could transplant skin from one area of a patient to another, he could never successfully transplant skin to a recipient from another donor, even from one as closely related as a brother. He also observed that a second attempt at such a transplant resulted in a stronger and faster rejection of the grafted material. Histological analysis of the rejected grafts revealed that they were subject to massive infiltration by host white blood cells. Through these observations and subsequent experimental work on animals, he concluded that graft rejection resulted from an immunological response to the foreign material.

While seemingly any foreign substance could induce an immune response, the host's own tissues were obviously not attacked by the immune system, at least not in healthy individuals. Owen et al. observed in cattle that dizygotic twins sometimes shared blood group antigens due to placental mixing of their blood. Even though the siblings were not genetically identical, they could tolerate tissues grafts from each other. This observation suggested that probably early in development self-reactivity is eliminated from the immune system.

A completely independent line of research on tissue transplantation had been initiated at the beginning of the century with no apparent relationship to immunology. Oncologists tried to establish tumor cell lines by serial transplantation in mice. These initial attempts were unsuccessful, and it was assumed that the tumor cells were rejected by the immune system because their malignant transformation was associated with the

expression of tumor antigens (4). However, this turned out not to be the case. When inbred mice that had been generated for nonscientific reasons were used, serial transplantation of tumors became possible (5). This finding led to the development of inbred mouse strains for scientific purposes and to the discovery of the transplantation antigens of the major histocompatibility complex (MHC) (6, 7). Genes in this complex not only encoded transplantation antigens, but curiously also controlled immune responses to synthetic peptides (*Ir*-genes). These findings have puzzled immunologists for many years and remained unexplained until the discovery of T cells and of the way these cells recognize antigens (see below).

Failing to see how the immune system could anticipate all the possible antigens that it might confront, some researchers believed that recognition was achieved by instructive mechanisms. The instructional theory speculated that antigen-recognition molecules existed that would undergo conformational changes upon encounter with foreign antigens (1). Such "induced-fit" models allowed for the recognition of a nigh infinite number of antigens with a limited repertoire of receptors, but did not satisfactorily explain how self-tolerance might be achieved. Selection theories suggested that the antigen-receptor repertoire is determined prior to any encounter with antigens. During development, therefore, the repertoire could be shaped by the elimination of cells bearing self-reactive receptors. One of the conceptual stumbling blocks of the selection theory was how diversity is achieved. Since the notion of "one gene–one protein" was dogma to many biologists, the genome was insufficiently large to accommodate one gene for every antigen-receptor. This dilemma was eventually resolved through discovery by Susumu Tonegawa of the recombinatorial events responsible for diversity. Antigen receptors of

lymphocytes were found to be generated by recombination of variable (V), diverse (D) and joining (J) gene segments (8). In this way, a vast diversity of receptor genes can be generated from a few hundred gene segments.

Receptor diversity is generated in two major classes of lymphocytes, B cells and T cells. While B cells produce antibodies, T cells only recognize antigens, usually small peptides, that are bound to MHC proteins. The T cell receptors (TCR) involved in this recognition come in two forms that are close relatives of immunoglobulins: α/β heterodimers and γ/δ heterodimers (9). T cells expressing the α/β TCR are the predominant T cell class in humans and mice. $CD4^+$ α/β T cells and $CD8^+$ α/β T cells are major subclasses. The former are helper cells that guide the defense by B cells and other leukocytes such as macrophages and various types of granulocytes. The latter are also known as cytotoxic T lymphocytes (CTL) and are responsible for the destruction of cells bearing target antigens. When Rolf Zinkernagel and Peter Doherty studied the killing of virus-infected target cells by CTL, they noted that for target cells to be recognized and lysed they had to not only express viral antigens but also the same MHC class I proteins that were expressed by the mice from which the CTL were derived. Thus, CTL generated in vaccinia-virus-infected $H\text{-}2^d$ mice lysed vaccinia-virus-infected target cells expressing $H\text{-}2^d$ MHC proteins but not virus-infected target cells expressing $H\text{-}2^b$ or $H\text{-}2^k$ MHC proteins. The MHC regions relevant for target cell recognition were mapped to the MHC class I loci K and D (and later L). Zinkernagel and Doherty coined the term "MHC restriction" for the surprising ability of T cells to recognize antigen only in association with particular MHC proteins (10, 11). The discovery of MHC restriction represents a

major breakthrough in the study of T cell specificity and function. Subsequent studies led to several important discoveries.

MHC restriction applies to all T cells. While $CD8^+$ CTL are restricted to class I proteins, $CD4^+$ T cells are restricted to MHC class II proteins. T cells expressing γ/δ TCR may be restricted not only by MHC class I and class II proteins, but also by so called non-classical MHC class I proteins, which are much less polymorphic than the classical MHC proteins and are able to present non-peptide antigens.

MHC restriction is acquired during T cell development. In other words, T cells "learn" to recognize the MHC proteins that they require for antigen recognition. The most convincing evidence for this type of learning was provided by Michael Bevan and collaborators through the study of bone marrow chimeras. Chimeric mice are generated by the transfer of bone marrow cells from donor mice into allogeneic mice whose own haemopoietic tissue has been destroyed by irradiation. The mice that recover from this treatment are called chimeric because the genotype of their haemopoietic cells differs from the genotype of all other cells in the body. Bevan found that T cells from A×B→A and A×B→B chimeras (an A or B mouse reconstituted with A×B bone marrow cells) were restricted only to the MHC of the recipient (A or B) while normal A×B mice had both T cells restricted to MHC A and others restricted to MHC B (12, 13). Studies involving transplantation of thymic epithelial rudiments showed that the learning of MHC restriction was dependent on the MHC proteins expressed by the thymic epithelial cells (14). Subsequent studies by Harald von Boehmer using TCR-transgenic mice showed that the learning of MHC restriction was the result of positive selection of maturing T cells in the thymus. Von Boehmer and colleagues used mice whose T cells

express a transgenic TCR specific for the minor transplantation antigen H-Y in the context of the H-2 D^b protein (9). H-Y is a gender-specific antigen that is present and absent in male and female mice respectively. T cells bearing the H-Y specific T cell receptor matured, i.e. were positively selected, in female H-2^b mice but not in female H-2^k mice (15). In male H-2^b mice that expressed both H-2 D^b and the H-Y antigen, T cells bearing the H-Y specific TCR were eliminated in the thymus, a phenomenon known as negative selection (16).

The molecular basis of MHC restriction was clarified by the work of Emil Unanue and later by X-ray structural analysis of MHC proteins (17-19). MHC proteins were found to associate with short peptides. The MHC-peptide complexes are recognized by T cells on the surface of antigen-presenting cells and target cells. Most class I binding peptides are generated from the cleavage of cytoplasmic proteins and are then transported via the TAP1-TAP2 (Transporter associated with Antigen Processing) complex into the endoplasmic reticulum (ER) in an ATP dependent manner (20-25). In the ER, a single peptide and a β_2-microblobulin protein noncovalently associate with a nascent class I molecule to form a stable complex that is then transported to the cell surface (26, 27). In contrast, most class II binding peptides are generated from proteins engulfed during endocytosis. These proteins are proteolytically digested in acidified vesicles that eventually merge with vesicles carrying nascent class II MHC proteins (28). An MHC class II-associated invariant chain (Ii) both targets the class II MHC proteins to the endosomal compartment and prevents premature binding of peptides. Once in the proteolytic environment of the endosomal compartment, the invariant chain is digested and the portion of the Ii that blocks the peptide binding groove (known as the CLIP

peptide) is cleaved or displaced to allow the peptide derived from engulfed material to bind the class II MHC proteins (29-31).

As indicated above, most but not all peptides presented by MHC class I molecules are derived from endogenously synthesized proteins such as viral proteins. However, some peptides derived from endocytosed proteins get into the cytoplasm and, via the TAP1-TAP2 transporter, into the ER where MHC class I loading occurs. Similarly, some peptides presented by MHC class II proteins are derived from endogenously synthesized proteins. These proteins, however, must be translocated from the cytoplasm through the endosomal membrane into the MHC class II loading site. It should be noted that antigen processing and presentation do not discriminate between self- and foreign- antigens. No mechanism is present to prevent the incorporation and presentation of peptides derived from self-proteins. In fact, a common source of class II binding peptides in uninfected individuals is class II MHC proteins that have presumably misfolded and are therefore no longer resistant to digestion (32, 33).

About 30 years after Burnet's proposal of clonal selection, the first two properties of lymphocytes envisioned by Burnet, specificity and diversity, have been confirmed and are now well understood. This, however, is not the case for the two other important properties of the immune system, memory and tolerance. While there is no doubt that memory must be mediated by long-lived lymphocytes, the molecular requirements for the induction of such cells and their maintenance remain ill-defined. There is a continuing debate about the longevity of such cells and whether their maintenance requires repeated exposure to antigen (34).

Tolerance towards self-antigens, the most impressive ability of the immune system, is achieved in part by the elimination of self-reactive cells, as originally proposed by Burnet and further elaborated by Gustav Nossal and Joshua Lederberg (35). Elimination of self-reactive T cells and B cells at an immature stage of their development has been convincingly demonstrated by several research groups using mice that were genetically altered to express transgenic antigen receptors (16, 36, 37). This phenomenon is referred to as central tolerance since it takes place in the bone marrow and the thymus, i.e., those sites where B and T cells are generated. It is clear, however, that the mechanisms of central tolerance do not eliminate all self-reactive T cells. T cell responses can easily be induced by immunization with self-proteins such as myelin basic protein, collagen type II and others. Accidental induction of such responses, for example by cross-reactive microbial antigens, may lead to chronic inflammatory autoimmune diseases such as rheumatoid arthritis, multiple sclerosis or insulin-dependent diabetes mellitus.

Central tolerance eliminates T cells that are reactive to self-antigens that are expressed on the surface of professional antigen presenting cells (APC). Such cells are present in the thymus where they mediate negative selection of maturing T cells. T cells that express receptors for antigens that are expressed in peripheral tissues but not in the thymus are not eliminated. The lack of T cell responses to such antigens in healthy animals is referred to as peripheral tolerance. It appears that peripheral tolerance is based on several distinct mechanisms. First, peripheral antigens may be present in immunologically privileged sites that are not normally subject to immune surveillance or that provide an environment that attenuates or redirects an immune response along a less destructive course. Second, peripheral antigens are largely ignored by T cells if they are

never expressed on the surface of professional APCs. Third, cells that recognize a peripheral antigen in the absence of costimulation, such as that provided by professional APCs, sometimes either become inactivated by "clonal anergy" or simply self-destruct. The term anergy refers to a state in which the T cell survives, but has been rendered unresponsive. Lastly, situations in which responsive (non-anergic) T cells persist in the periphery in spite of the presence of self-antigen has led to the suggestion of active, cell-mediated suppression mechanisms against these T cells.

Experimental evidence exists for each of these mechanisms. Tissue grafts carefully placed in the brain or anterior chamber of the eye, for example, normally do not induce graft rejection. In the former case, this is thought to be due to the isolated nature of the site. The anterior chamber of the eye, while also a sequestered site, normally contains high levels of the cytokine TGF-β, which promotes a less destructive (humoral) immune response. T cells from animals that have been immunized following protocols that usually involve large doses of antigen fail to respond to that same antigen both *in vivo* and *in vitro*, if the cells are not eliminated outright in the animal. Specific suppresser T cells have not been identified, but evidence for their existence comes from experiments in which tolerance to allo-antigens follows the transfer of cells from one animal to another (38).

Animal models have been developed in which immunization with self-antigens such as myelin basic protein or type II collagen can induce an autoimmune response directed at tissues bearing these proteins. Immunization of rodents with myelin basic protein results in experimental autoimmune encephalomyelitis (EAE) which is similar to the human disease multiple sclerosis, whereas immunization with type II collagen results in joint

lesions similar to those observed in the human disease rheumatoid arthritis. The inductive events in humans that mimic immunization of rodents with these proteins have not been determined. Bacterial or viral antigens may resemble these self-antigens and result in an inadvertent cross-reaction. Damage as a result of inflammation, infection, fever or injury may expose an otherwise sequestered epitope to immune attack (39).

An important goal of experimental biology is to provide better animal models with which to study autoimmune diseases. To accomplish this, some researchers have made mice that are transgenic for TCR genes cloned from T cells that respond to an antigen associated with an autoimmune disease. Such mice allow for easier study of the autoreactive cells and sometimes show an increase in incidence of the disease. Mice transgenic for a TCR specific for a myelin basic protein (MBP) epitope are usually asymptomatic despite the disproportionate number of anti-MBP T cells, unless they are immunized with antigen and adjuvant. However, mice that are both transgenic for the anti-MBP TCR and deficient for *RAG1* suffer a 100% incidence of EAE (40). In another example, researchers crossed mice that express genes for a TCR that recognizes an epitope of bovine pancreatic ribonuclease with mice that express A_β^{G7}, an MHC allele whose protein product is also recognized by this TCR. Apparently both central and peripheral mechanisms of tolerance fail to protect TCR-transgenic, A_β^{G7+} mice from developing an inflammatory joint disease remarkably similar to rheumatoid arthritis (41).

By presenting this brief introduction, I had hoped to put my research in perspective to some of the fundamental questions in immunology, namely those of TCR-specificity, MHC restriction, clonal deletion, and peripheral self-tolerance. My initial goal was to acquire a better understanding of the TCR–MHC interactions that either enable a T cell's

progress to maturity or signal its demise. Not surprisingly, however, the results sometimes directed my work in alternate directions. Chapter 2 was work produced under Dr. Philip Ashton-Rickardt, formally of Professor Susumu Tonegawa's lab, and represents my introduction to experimental immunology. During that research, I learned the techniques described therein and, more importantly, the cryptic language that immunologists like to speak. Chapter 2 has been previously published (42) and is reproduced here largely as published, except for the some minor corrections and the reformatting of citations. Chapter 3 reports the development of the 2C TCR-transgenic system for the *in vitro* study of positive and negative selection and presents data regarding the qualitative and quantitative aspects of the peptide–MHC ligand involved in positive and negative selection. The data in Chapter 4 show that the results demonstrated with the manipulation of peptide–MHC ligands can be mimicked with analogous manipulations of the TCR. Chapter 5 offers evidence of the physiological relevance of some of the observations made in the previous two chapters and has been published (43). It is also reproduced here as published, but with reformatted citations. Chapter 6 is a report on the observation that the 2C TCR-transgenic, H-2^d-expressing F2 mice from the cross between 2C-tg mice and DBA/2 inbred mice spontaneously develop an inflammatory autoimmune-type skin disease.

Chapter 2 The differential avidity model of positive selection

Introduction

The repertoire of antigen specificities of T cells of an individual animal is shaped by two types of cellular selection that take place in the thymus. Positive selection (12, 44) ensures that T cells leaving the thymus are capable of major histocompatibility complex (MHC)-restricted recognition of antigen; that is, they possess the capacity to recognize antigen presented by products of self-MHC (10, 45). Positive selection is thought to involve engagement of T cell receptors (TCRs) on immature $CD3^+$ low $CD4^+$ $CD8^+$ thymocytes with self-MHC products expressed on the surface of thymic epithelial cells (46): only those thymocytes expressing TCRs with sufficient affinity for self-MHC are thought to be given the signal to avoid programmed cell death and to mature (15, 47). Engagement of TCRs with MHC class I molecules leads to the differentiation into $CD4^-$ $CD8^+$ (hereafter referred to as $CD8^+$) cytotoxic T cells, whereas engagement of TCRs with MHC class II molecules leads to differentiation into $CD4^+$ $CD8^-$ (hereafter referred to as $CD4^+$) T helper cells (48). Negative selection eliminates through clonal deletion those T cells that are potentially autoreactive (49, 50). Negative selection is also thought to involve engagement of TCRs on immature thymocytes; however, in this process self-peptide–MHC complexes expressed on the surface of bone marrow-derived thymic stromal cells are thought to be recognized (macrophages and dendritic cells). Those thymocytes expressing TCRs with relatively high affinity for the self-peptide–MHC complexes are thought to be given the signal to undergo programmed cell death. One of the central questions that remains to be solved is how the similar interactions that take

place between TCRs and self-MHC or self-peptide–MHC complexes trigger two very different cell fates during the two types of selection.

It is well established that in MHC-restricted recognition of antigen, a TCR interacts not only with an MHC product but also with an antigen-derived peptide entrenched in the groove of the MHC product (17, 19, 51). Since a peptide is essential for the stable expression of an MHC class I molecule on the surface of cells (52-55), it is expected that the self-MHC class I molecules expressed on thymic epithelial cells and recognized by TCRs during positive selection also contain peptides. Indeed, the analyses of $CD8^+$ T cell development in mice expressing mutant class I molecules with altered peptide-binding grooves suggested a role of peptides in determining the specificity of TCR–MHC interaction during the positive selection of $CD8^+$ T cell precursors (56-59).

To address this issue more directly, we exploited *TAP1* (for transporter associated with antigen processing 1) mutant mice (60) that were produced using embryonic stem cell technology (61, 62). The *TAP1* gene encodes an ATP-dependent peptide pump (25), which translocates peptides from the cytosol into the lumen of the endoplasmic reticulum. Peptides then associate with newly synthesized class I heavy chain and β_2-microglobulin to form stable class I molecules that are then transported to the cell surface (27, 52-54). In *TAP1* mutant mice, this major route of peptide loading of class I molecules is blocked. Consequently, surface expression of MHC class I molecules is reduced, and positive selection of $CD8^+$ T cells is severely hampered. Only a few "empty," ill-folded, and heat-sensitive class I molecules appear on the surface of cells in *TAP1* mutant mice (62). These empty class I molecules could be loaded and stabilized with peptides that were provided extracellularly in the presence of β_2-microglobulin. Thus, addition of peptides to

cultured fetal thymi from *TAP1* mutant mice rescued class I expression on thymic stromal cells, but only some of these peptides promoted the positive selection of $CD8^+$ T cell precursors (60). Furthermore, as observed in a fetal thymus organ culture (FTOC) system (63) from β_2-microglobulin-deficient mice (64), complex mixtures of class I-binding peptides (synthetic or thymus derived) were more efficient than single peptides in promoting the positive selection of $CD8^+$ T cells (60). These data confirmed that peptides contribute to the specificity of positive selection of $CD8^+$ T cells in the thymus.

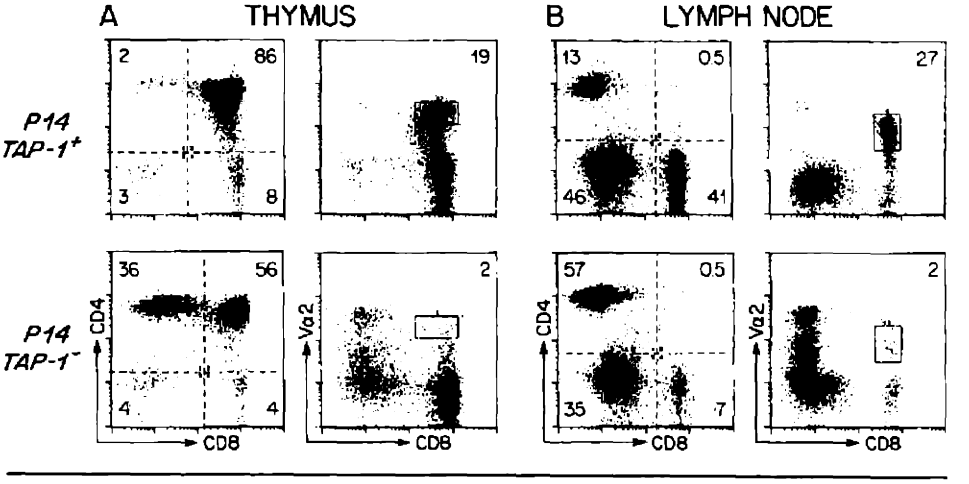
In the present study, we crossed *TAP1* mutant mice (62) with a TCR α and β transgenic mouse (P14) to study the role of peptides in thymic selection in greater detail. The P14 TCR recognizes a peptide from the lymphocytic choriomeningitis virus (LCMV) glycoprotein (amino acids 33-41) presented by H-$2D^b$ molecules (65, 66). As expected, positive selection of $CD8^+$ T cells expressing the P14 TCR was hampered in mice deficient in *TAP1*. Analyses of cultured fetal thymi from *TAP1*$^-$ P14 transgenic and *TAP1*$^+$ P14 transgenic mice that were supplemented by various H-$2D^b$–binding peptides allowed us to draw two major conclusions. First, peptides determine the specificity of TCR–MHC interaction during positive selection, most probably by being directly recognized by TCRs. Second, the critical parameter that controls the fate of thymocytes is the number of TCRs engaged with peptide–MHC complexes that is determined by the avidity between these two cell surface components; regardless of the intrinsic affinity of the TCR–peptide/MHC interaction, when the number of engaged TCRs is moderate, positive selection takes place, and when the number is high, negative selection occurs.

Results

Positive selection of P14 transgenic T cells is impaired in TAP1*-deficient mice*

We have shown previously that positive selection of $CD8^+$ T cells, but not $CD4^+$ T cells, is severely hampered in the thymus of *TAP1* mutant mice (60, 62). Transgenic mice (P14) expressing a TCR (Vα2, Vβ8.1) specific for the LCMV glycoprotein peptide (33-41) (hereafter referred to as LCMV peptide or nominal antigen peptide) presented by H-$2D^b$ (65, 66) were crossed to *TAP1*-deficient mice. We analyzed thymocytes and lymph node cells derived from the P14 *TAP1*$^-$ mice as well as the cells from the control P14 *TAP1*$^+$ mice by flow cytometry. Typical results are shown in Figure 2-1. The thymi of P14 *TAP1* mice contained as many thymocytes as those of P14 *TAP1*$^+$ mice. However, the percentage of $CD8^+$ cells was reduced in P14 *TAP1*$^-$ mice (n = 4; range, 3%–4%)

Figure 2-1 Positive selection of CD8+ transgenic P14 cells is impaired in *TAP1*$^-$ mice



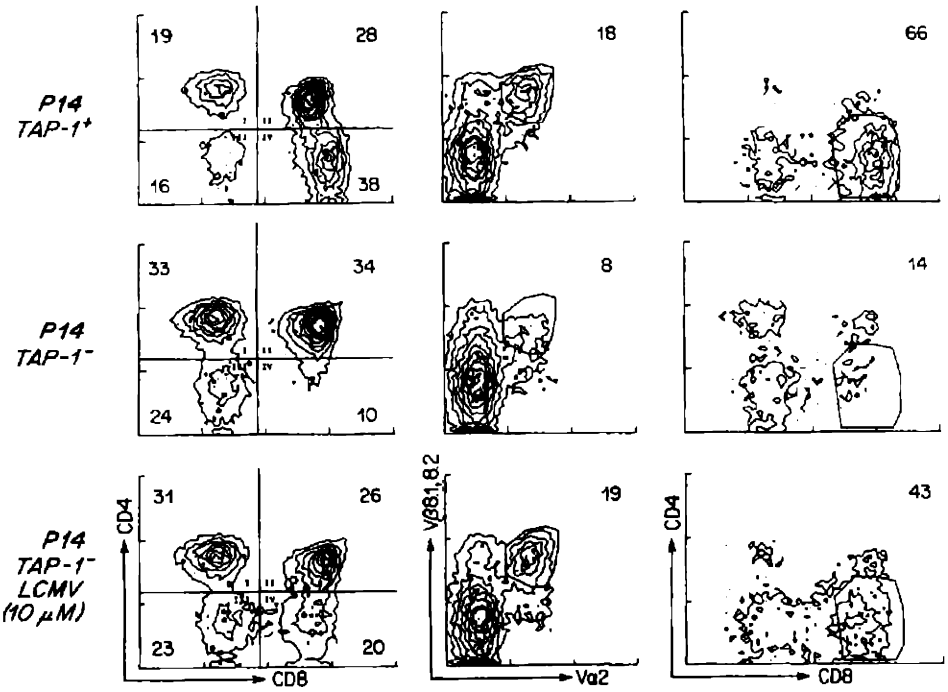
Thymocytes and mesenteric lymph node cells (1×10^5) were prepared from P14 positive *TAP1*$^+$ or *TAP1*$^-$ mice and stained (1.5×10^4 cells) with anti-CD8-FITC and anti-CD4-PE MAbs or with anti-Vα2-PE and anti-CD8-FITC MAbs and then analyzed by FACS. The percentages of cells in each quadrant or window are indicated.

compared with P14 *TAP1*$^+$ mice (range, 7%–10%). In contrast, the percentage of CD4+ cells was augmented in P14 *TAP1*$^-$ mice (range, 30%–40%) compared with P14 *TAP1*$^-$ (range, 2%–4%). Most of the few remaining CD8$^+$ thymocytes in the P14 *TAP1*$^-$ mice did not express the P14 TCR as indicated by the low percentage of Vα2$^+$ CD8$^+$ thymocytes for P14 *TAP1*$^-$ mice (range, 2%–4%) compared with P14 *TAP1*$^+$ mice (range, 19%–23%). Similar results were obtained with peripheral T cells. The mesenteric lymph nodes of P14 TAP$^-$ mice also contained as many cells as those of P14 TAP$^+$ mice. However, a substantial reduction in the percentage of CD8$^+$ cells was observed in P14 *TAP1*$^-$ mice (range, 5%–8%) compared with P14 *TAP1*$^+$ control mice (range, 40%–45%). Here again, the percentage of CD4$^+$ cells was increased in P14 *TAP1*$^-$ mice (range, 55%-46%) compared with P14 *TAP1*$^+$ mice (range, 11%–14%). Most of the remaining CD8$^+$ cells in the P14 *TAP1*$^-$ mice did not express the P14 TCR, as indicated by the relatively low percentage of Vα2$^+$ CD8$^+$ cells (range, 2%–3%) compared with P14 *TAP1*$^+$ control mice (range, 26%–29%). These data indicate that the development of CD8$^+$ transgenic P14 cells is severely impaired in the *TAP1*$^-$ background. Most of the CD4$^+$ thymocytes or lymph node T cells express non-P14 (i.e., nontransgenic) TCR (data not shown). The proportional increase of CD4$^+$ cells in P14 *TAP1*$^-$ mice compared with P14 *TAP1*$^+$ mice can be explained by preferential selection of these cells by class II MHC molecules whose cell surface expression is not affected by the lack of TAP1.

When we cultured day 16 fetal thymic lobes from P14 *TAP1*$^-$ mice (hereafter referred to as *TAP1*$^-$ FTOC) or P14 *TAP1*$^+$ mice (*TAP1*$^+$ FTOC) for 9 days, we also observed a reduction in numbers of CD8$^+$ P14 thymocytes recovered from *TAP1*$^-$ thymi compared with similarly cultured *TAP1*$^+$ thymi. Figure 2-2 shows a representative result of multiple

(n = 8) experiments. We determined the proportion of $CD8^+$ P14 cells by multiplying the percentage of $V\alpha2^+$ high $V\beta8.1$,8.2^+ high cells (which are virtually equivalent to P14

Figure 2-2 Positive selection of $CD8^+$ transgenic P14 cells is impaired in *TAP1*⁻ FTOC



P14-positive *TAP1*⁺ or *TAP1*⁻ thymic lobes were cultured with or without peptide as indicated for 9 days in RPMI, 10% fetal calf serum, and then thymocyte suspensions were prepared by mechanical disaggregation (typically 2 x 10^5 cells per lobe). Cells (4 x 10^4, unless otherwise indicated) were stained with anti-CD8 FITC, anti-CD4-allophycocyanin (APC), anti-TCR Vα2-PE, and anti-TCR Vβ8.1 8.2-biotin-streptavidin-Texas red MAbs and then analyzed by FACS. The percentages of cells falling into the quadrants resulting from the analysis of CD4 and CD8 staining are indicated. Stained cells were gated for high expression of Vα2 and Vβ8.1,8.2 (P14) (percentage shown next to window) and then displayed for log fluorescence intensify resulting from staining for CD4 and CD8. The percentage of this gated population positive for CD8 and negative for CD4 is shown next to the window. The overall percentage of cells determined as $V\alpha2^+$ high $V\beta8.1$ 8.2+ high ($CD8^+$ P14) was calculated by multiplying the percentage of cells $V\alpha2^+$ high $V\beta8.1,8.2^+$ high by the percentage of $CD8^+$ cells of the gated population.

cells) among the total recovered thymocytes with the percentage of $CD8^+$ cells among the P14 cells. Thus, for the experiment shown in Figure 2-2, in *TAP1*⁺ FTOC the percentage of $CD8^+$ P14 cells was 12% ($V\alpha2^+$ high $V\beta8.1,8.2^+$ high [18%] x $CD8^+$ [66%], and in

TAP1$^-$ FTOC, the percentage was 1% (Vα2$^+$ high Vβ8.1 ,8.2$^+$ high [8%] x CD8$^+$ [14%]). In all experiments performed, the percentage of CD8$^+$ P14 cells ranged from 1%–2% in *TAP1*$^-$ RTOC to 12%–19% in *TAP1*$^+$ FTOC. Using these percentages and the total numbers of thymocytes recovered from FTOCs, we calculated the absolute numbers of CD8$^+$ P14 cells per thymic lobe. Thus, in the representative experiment shown in Figure 2-2, we observed a deficit in positive selection of these cells in *TAP1*$^-$ FTOC (5 x 10^3 CD8$^+$ P14 cells per thymic lobe) compared with *TAP1*$^+$ FTOC (37 x 10^3 CD8$^+$ P14 cells per thymic lobe). In all experiments performed, the number of CD8$^+$ P14 cells per thymic lobe ranged from 5 x 10^3 to 7 x 10^3 in *TAP1*$^-$ FTOC and 35 x 10^3 to 45 x 10^3 in *TAP1*$^+$ FTOC (n = 8).

Positive selection of CD8$^+$ transgenic P14 T cells is induced by addition of nominal antigen peptide

To understand the role of peptides in T cell development in greater detail, we examined the selection of CD8$^+$ transgenic P14 cells in *TAP1*$^-$ FTOC in the presence of various peptides. To this end we first examined the ability of the peptides to stabilize H-2D^b expression on the surface of *TAP2*-deficient RMA-S cells (24, 67). The data in Table 2-1 indicate that LCMV peptide stabilized H-2D^b on the surface these cells about 300 times less efficiently than the H-2D^b binding (51) influenza virus 1934 nucleoprotein peptide (366–374) (hereafter referred to as IF peptide). To estimate relative affinity of the two peptides for the P14 TCR, we examined the proliferation of P14 spleen cells *in vitro* in response to the LCMV and IF peptides. The IF peptide was at least 10^3 times less efficient than the LCMV peptide in inducing the proliferation of P14 spleen cells *in vitro* (Table 2-1). A third peptide, DB-S, retains asparigine and methionine at positions 5 and 9, respectively, which are thought to be required for binding with H-2D^b (68), but

Table 2-1. Relative abilities of peptides to stabilize surface H-2D^b and to stimulate P14 Spleen Cells *in vitro*

Peptide	Sequence	50_{max} H-2D^b (M)	50_{max} P14 (M)
LCMV	KAVYNFATM	2×10^{-4}	6×10^{-10}
LCMV-8.1	KAMYNFATM	3×10^{-4}	2×10^{-7}
LCMV-8.7	KALYNFATM	6×10^{-5}	$\sim 1 \times 10^{-4}$
LCMV-M	KAVYNFATM RGIF YGS	2×10^{-6}	2×10^{-7}
LCMV'	RGIFNYGSM	2×10^{-4}	$>1 \times 10^{-4}$
IF	ASNENMETM	6×10^{-7}	$>1 \times 10^{-4}$
DB-S	SSSSNSSSM	6×10^{-5}	$>1 \times 10^{-4}$

Compared with the LCMV peptide (69), the LCMV-8.1 and LCMV-8.7 variants have single amino acid changes at position 3 (underlined). The LCMV' peptide has every amino acid substituted with the most chemically related residue except for the H-2D^b anchor residues at positions 3 and 9 (68). The LCMV-M mix of peptides (128 peptides) was synthesized by incorporating the relevant amino acid from either the LCMV or LCMV' peptide at each position. The resulting mixture thus includes a spectrum of peptides differing in relatedness to LCMV or LCMV'. Both the IF (51) and DB-S peptides differ from the LCMV peptide at every position except the H-2D^b anchor residues. The relative abilities of peptides to stabilize H-2D^b molecules is expressed as the 50_{max} H-2D^b value. This is defined as the concentration of peptide required to rescue the surface expression of H-2D^b on RMA-S to a level that is 50% of maximum (maximum being about 35% of RMA) in an overnight *in vitro* experiment. Surface expression of H-2D^b was detected by staining with an anti-H-2D^b–biotin MAb (B22.249.R1, α1 specific) and then with streptavidin–PE and by FACS analysis. The relative ability of peptides to stimulate P14 spleen cells *in vitro* is expressed as the 50_{max} P14 value. This is defined as the concentration of peptide required to induce the proliferation of P14 *TAP1*$^+$ spleen cells to a level of 50% of the maximum value observed using the nominal antigen peptide. Proliferation was assessed by culturing P14 spleen cells (2×10^5) with peptide for 36 hr and then measuring the rate of cell division by pulsing with [^{3}H]thymidine for 12 hr and determining the amount of cell-associated label by counting the rate of β particle emission.

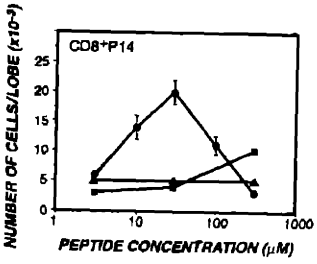
contains serine at all other positions. This peptide stabilized H-2D^b as efficiently as the LCMV peptide but failed to induce the proliferation of P14 spleen cells *in vitro* (Table 2-1).

The LCMV peptide supported the development of CD8$^+$ P14 precursor cells when added to *TAP1*$^-$ FTOC at concentrations as low as 10 μM, whereas at similar

concentrations neither the IF nor the DB-S peptide was effective (Figures 2-2 and 2-3). The LCMV peptide-mediated selection increased when we increased the concentration of the peptide, reaching a maximum level of selection at 30 μM. However, above this concentration, selection decreased with increasing peptide concentrations. When added at a high concentration (300 μM), the IF peptide also induced a moderate level of positive selection of $CD8^+$ P14 cells, whereas the DB-S peptide did not (Figure 2-3).

We attempted to determine the relative level of $H\text{-}2D^b$ expression induced by peptides on the surface of thymic stromal cells in P14 *TAP1*$^-$ FTOC (see Experimental Procedures). The IF peptide, which is a relatively potent $H\text{-}2D^b$ stabilizer (Table 2-1),

Figure 2-3 $CD8^+$ transgenic P14 cells generated in *TAP1*$^-$ FTOC in the presence of various $H\text{-}2D^b$-binding peptides



P14 *TAP1*$^+$ or *TAP1*$^-$ thymic lobes were cultured as described in the legend to Figure 2-2 with or without peptide at the concentrations shown. Thymocyte suspensions were prepared from FTOC treated with LCMV (closed circles), IF (closed squares), or DB-S (closed triangles) and analyzed for surface markers. The percentage of $CD8^+$ P14 cells was determined as described in the legend to Figure 2-2. The levels for the number of $CD8^+$ P14 cells per lobe is the mean (± SEM) of values determined from the analysis of between three and seven independently cultured thymic lobes (points without error bars had an SEM of $<0.25 \times 10^3$). *TAP1*$^+$ FTOC gave $38 \pm 5 \times 10^3$ $CD8^+$ P14 cells per lobe and *TAP1*$^-$ FTOC without peptide gave $5 \pm 1 \times 10^3$. Similar data was obtained from two other experiments performed at different times.

induced H-2D^b expression on the surface of I-A^b-positive thymic stromal cells at levels that were 5% (30 μM added IF peptide) and 35% (300 μM added IF peptide) of the level observed on the same type of cells from P14 *TAP1*$^+$ thymi. However, the LCMV peptide is a less potent H-2D^b stabilizer (Table 2-1) and at 30 or 300 μM did not induce the H-2D^b expression above the background level on the surface of thymic stromal cells. These data indicate that the positive selection by the LCMV peptide of CD8$^+$ P14 cells in *TAP1*$^-$ FTOC requires a very low density of peptide–H-2D^b complexes on the surface of thymic stromal cells, whereas the selection by the IF peptide requires a very high density of peptide–H-2D^b complexes.

We wanted to examine the possibility that the majority of CD8$^+$ P14 thymocytes that

Table 2-2. CD8$^+$ P14 T cells selected by LCMV peptide in *TAP1*$^-$ FTOC are not proliferating

Experiment	Concentration of LCMV Peptide (μM)	Duration of FTOC (Days)	Mean Forward Scatter	S Phase (%)
Experiment 1				
P14 *TAP1*$^+$ FTOC	—	9	373 ± 24	16 ± 6
P14 *TAP1*$^-$ FTOC	10	9	377 ± 24	13 ± 6
P14 *TAP1*$^+$ spleen cell culture	10	NA	525 ± 17	37 ± 5
Experiment 2				
P14 *TAP1*$^-$ FTOC	30	7	ND	20 ± 4
P14 *TAP1*$^-$ FTOC	30	8	ND	11 ± 4
P14 *TAP1*$^+$ spleen cell culture	10	NA	ND	51 ± 4

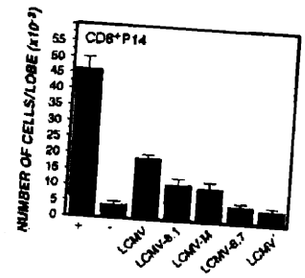
In experiment 1, P14 *TAP1*$^+$ (n = 56) or *TAP1*$^-$ (n = 49) thymic lobes were cultured with 10 μM LCMV peptide for 9 days as described previously (60), and then thymocyte suspensions were prepared. The suspensions were pooled before staining for cell surface markers and analyzed by FACS. Cells determined as Vα2$^+$ high Vβ8.1 ,8.2$^+$ high CD8$^+$ (CD8$^+$ P14) were sterilely sorted by FACS (*TAP1*$^+$ FTOC, 9 x 10^5 cells; *TAP1*$^-$ FTOC with LCMV 10 μM, 1.8 x 10^5 cells). We then estimated the size of the sorted cells by determining the mean value (± variance of histogram) of the forward light scatter from a histogram of forward scatter versus cell number. These cells were then permealized and chromosomal DNA stained overnight with RI. Quantitation of PI staining intensity was performed by FACS analysis. Statistical analysis (Modfit program) of the histogram of RI staining intensity versus cell number then allowed the determination of the percentage of cells in G0/G1 (*n* PI staining intensity), G2/M (*2n* PI staining intensity), and S phases of the cell cycle (intermediate staining intensity). P14-positive *TAP1*$^+$ spleen cell suspensions were prepared and incubated for 36 hr (1 x 10^6/ml) with the LCMV peptide at 10 μM. The sorted CD8$^+$ P14 cells (4 x 10^5) were then stained with PI, and the percentage of cells in the S phase of the cell cycle and the mean forward light scatter of the sorted population were determined as above. In experiment 2, P14 *TAP1*$^-$ thymi were cultured for 7 or 8 days with or without 30 μM LCMV peptide. As in experiment 1, at the end of culture, thymocyte suspensions were prepared and CD8$^+$ P14 cells purified by appropriate staining with MAbs and FACS sorting. In this experiment the number of CD8$^+$ P14 cells per P14 *TAP1*$^-$ lobe treated with 30 μM LCMV peptide after 7 days of FTOC was 7 ± 2 x 10^3 and after 8 days was 30 ± 4 x 10^3. P14 *TAP1*$^-$ lobes incubated without peptide gave 4 ± 0.7 x 10^3 after 7 days and 7 ± 2 x 10^3 after 8 days of FTOC. The level of CD8$^+$ P14 cells per thymic lobes is the mean (± SEM) of values determined from the analysis of between four and eight separately cultured lobes. Sorted CD8$^+$ P14 cells (P14 *TAP1*$^-$ FTOC with 30 μM LCMV peptide on day 7, 1 x 10^4 cells; on day 8, 8 x 10^4 cells) were stained with PI, and the percentage of cells in the S phase of the cell cycle was determined as in experiment 1. Proliferating P14 *TAP1*$^+$ spleen cells were prepared, and CD8$^+$ P14 cells were sorted (1 x 10^4 cells) and stained with PI as described in experiment 1. NA, data not applicable; ND, data not determined.

appeared in *TAP1*$^-$ FTOC supplemented with the LCMV peptide arose by proliferation of a small number of preexisting of CD8$^+$ P14 cells rather than by differentiation of CD4$^+$CD8$^+$ cells. To this end, we sorted CD8$^+$ P14 cells from *TAP1*$^-$ FTOC treated with 10 μM LCMV peptide and, as a control, from *TAP1*$^+$ FTOC. We then estimated the size of the sorted cells by determining the mean forward light scattering value of cells (after fluorescence-activated cell sorting [FACS] analysis) as well as the percentage of cells in S phase. For comparison we also analyzed the cell cycle status and size of CD8$^+$ P14 spleen cells that had been stimulated *in vitro* with the LCMV peptide. The data shown in Table 2-2 (experiment 1) indicate that the CD8$^+$ P14 cell population recovered from *TAP1*$^-$ FTOC supplemented by 10 μM LCMV peptide is similar to that recovered from *TAP1*$^+$ FTOC without added peptide in both cell size and percentage of dividing cells. In contrast, CD8$^+$ P14 cells recovered from a proliferating spleen cell culture exhibited a larger average cell size as well as a higher percentage of dividing cells. The data from Table 2-2 (experiment 2) show that at day 7 and day 8 in P14 *TAP1*$^-$ FTOC, the proportion of CD8$^+$ P14 cells in the S phase of the cell cycle is 20% and 11%, respectively. On the other hand, 51% of CD8$^+$ P14 spleen cells proliferating in response to the LCMV antigen are in S phase. Assuming that the proliferating cells in FTOC are equivalent to those in the spleen cell culture, we calculate that about 40% and 22% of the CD8$^+$ P14 cells are in the cell cycle at day 7 and day 8, respectively, in P14 *TAP1*$^-$ FTOC. We therefore conclude that the majority of the CD8$^+$ P14 cells that appeared in *TAP1*$^-$ FTOC upon an addition of the LCMV peptide arose by differentiation of CD4$^+$CD8$^+$ cells rather than by the proliferation of a small population of preexisting CD8$^+$ P14 cells.

Analogs of the nominal antigen peptide induce the positive selection of CD8⁺ transgenic P14 T cells in TAP1⁻ *FTOC*

From the data described in the previous section, it would appear that positive selection of $CD8^+$ P14 cells is peptide specific in that at input concentrations of between 10 and 100 μM, LCMV peptide is effective in inducing the positive selection while

Figure 2-4 Effects of LCMV peptide analogs added to *TAP1*⁺ FTOC on the positive selection of CD8⁺ transgenic P14 cells



P14 *TAP1*⁺ or *TAP1*⁻ thymic lobes were incubated with or without peptide as described in the legend to Figure 2. The plus sign designates the data obtained with *TAP1*⁺ FTOC with no added peptide, and the minus sign designates the data obtained with *TAP1*⁻ FTOC with no added peptide. All other data were obtained with *TAP1*⁻ FTOC supplemented with 30 μM of the indicated peptide. The numbers of selected $CD8^+$ P14 cells were determined by staining for the appropriate cell surface markers and by subsequent FACS analysis as described in the legend to Figure 2-2. The levels for the number of $CD8^+$ P14 cells per lobe is the mean (± SEM) of values determined from the analysis of between five and seven independently cultured lobes. Similar data was obtained from two other experiments performed at different times.

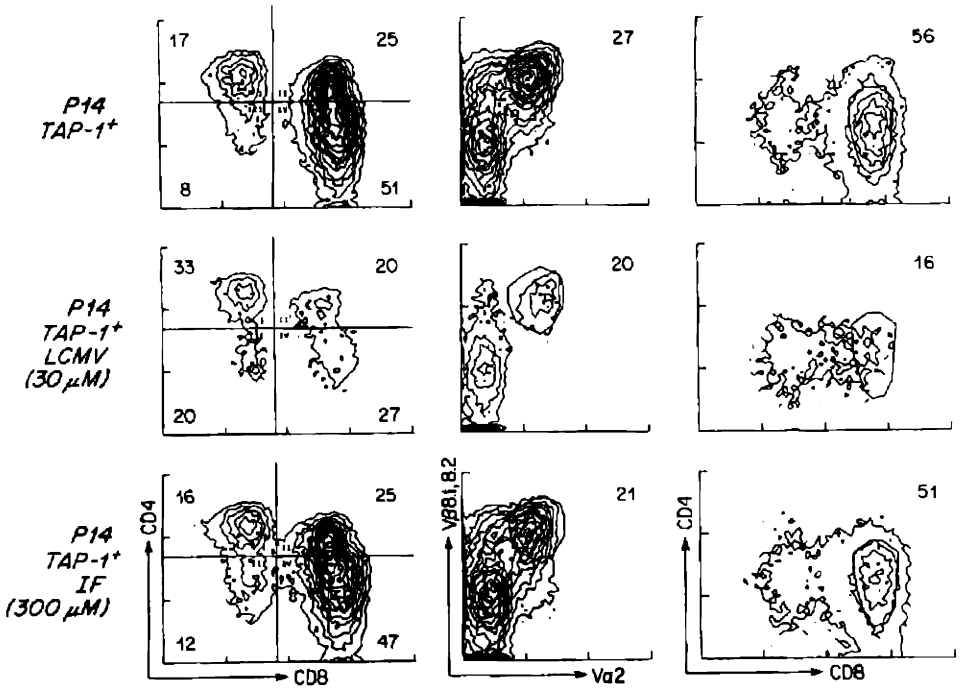
neither the IF nor the DB-S peptide is. To examine the peptide specificity of $CD8^+$ P14 cell selection in greater detail, we tested the ability of several LCMV-related peptides to drive the positive selection of $CD8^+$ P14 cells in *TAP1*⁻ FTOC. Two naturally occurring LCMV peptide variants, LCMV-8.7 and LCMV-8.1 (69), both stabilized $H\text{-}2D^b$ on the surface of RMA-S cells to similar extents (more precisely, LCMV-8.1 is 1.5-fold better

than LCMV, and LCMV-8.7 is 3-fold better than LCMV), but only LCMV-8.1 could induce the proliferation of $CD8^+$ P14 spleen cells *in vitro* to a detectable (albeit low) level. These peptides differ from the LCMV peptide by a single amino acid replacement at position 3 (see Table 2-1). We also tested a synthetic peptide, LCMV', which still has the anchor residues necessary to bind to H-$2D^b$ (68) but is replaced at every other position with the residue that is most chemically related to the corresponding residue of the LCMV peptide. The LCMV' peptide stabilized H-$2D^b$ as well as the LCMV peptide but failed to induce the proliferation of $CD8^+$ P14 spleen cells *in vitro* (see Table 2-1). We also synthesized a mixture of 128 peptides, called LCMV-M, by incorporating during the synthesis either the LCMV or LCMV' amino acid residue at each position. This resulted in a mixture including a spectrum of peptides differing in relatedness to LCMV or LCMV'. The LCMV-M peptides stabilized surface H-$2D^b$ on RMA-S cells 100-fold more efficiently than the LCMV peptide and could induce the proliferation of $CD8^+$ P14 spleen cells *in vitro* to a level distinctly lower than the level attained by the LCMV peptide but similar to the level attained by the LCMV-8.1 peptide. When added at 30 μM, the LCMV-8.1 peptide and LCMV-M peptides induced the positive selection of $CD8^+$ P14 cells, albeit at levels distinctly lower than those attained by the LCMV peptide (Figure 2-4). In contrast, neither LCMV-8.7 nor LCMV' at 30 μM induced the positive selection of $CD8^+$ P14 cells to a detectable level (Figure 2-4). At 300 μM the LCMV-8.1 peptide induced the positive selection of $CD8^+$ P14 cells to a level that is about 15% greater than the level attained at 30 μM (data not shown).

Negative selection of transgenic P14 cells by the nominal antigen peptide

As described above, when we increased the concentration of the LCMV peptide in *TAP1*$^-$ FTOC beyond 30 μM up to 300 μM, we observed a decrease in the number of CD8$^+$ P14 cells compared with the cultures supplemented with 30 μM of the same peptide (see Figure 2-3). We wanted to determine whether the decline in the number of CD8$^+$ P14 cells was due to negative selection. Negative selection of CD8$^+$ transgenic P14 cells by nominal antigen has previously been described both *in vivo* (65, 66) and *in vitro* (70). In these studies, negative selection led to a drastic reduction in the number of total

Figure 2-5 Flow cytometric data showing negative selection of CD8$^+$ transgenic P14 cells by the nominal antigen peptide in *TAP1*$^+$ FTOC

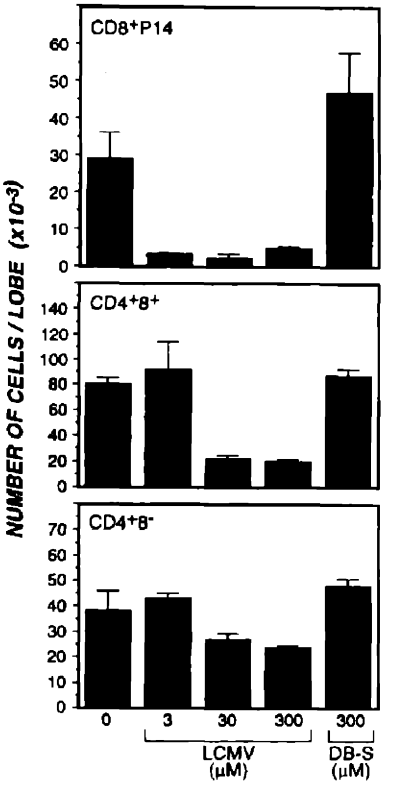


P14 *TAP1*$^+$ thymic lobes were incubated with or without the LCMV (30 μM) peptide or the IF peptide (300 μM) as described in the legend to Figure 2-2. Cell surface markers were stained and subsequently analyzed by FACS as described in the legend to Figure 2-2, except that all of the cells recovered from individual lobes were analyzed. Thus, the *TAP1*$^+$ lobe without peptide gave 8 x 10^4 stained cells, *TAP1*$^+$ lobes with the LCMV peptide (30 μM) gave 2 x 10^4 stained cells, and *TAP1*$^+$ lobes with the IF peptide (300 μM) gave 1 x 10^4 stained cells. The percentages of cells in each quadrant or window are indicated.

thymocyte numbers and in the percentages and absolute numbers of $CD8^+$ P14 cells and their $CD4^+CD8^+$ precursor cells.

We first examined whether we could induce negative selection of $CD8^+$ P14 cells by addition of the nominal antigen peptide to *TAP1*$^+$ FTOC. In one experiment the total numbers of thymocytes recovered from 9-day-old *TAP1*$^+$ FTOC supplemented with the LCMV peptide at 3, 30, and 300 μM were 100%, 42%, and 37%, respectively, of the control FTOC (no peptide). In two other experiments, the range of the total number of thymocytes recovered from 9-day-old *TAP1*$^+$ FTOC supplemented with the LCMV peptide at 3 μM was 100%–50%, at 30 μM was 47%–42%, and at 300 μM was 37%–18% of the number of thymocytes generated in FTOC without peptide. In *TAP1*$^+$ FTOC with 30 μM LCMV peptide, the percentages of $CD8^+$ P14 cells were clearly reduced compared with the

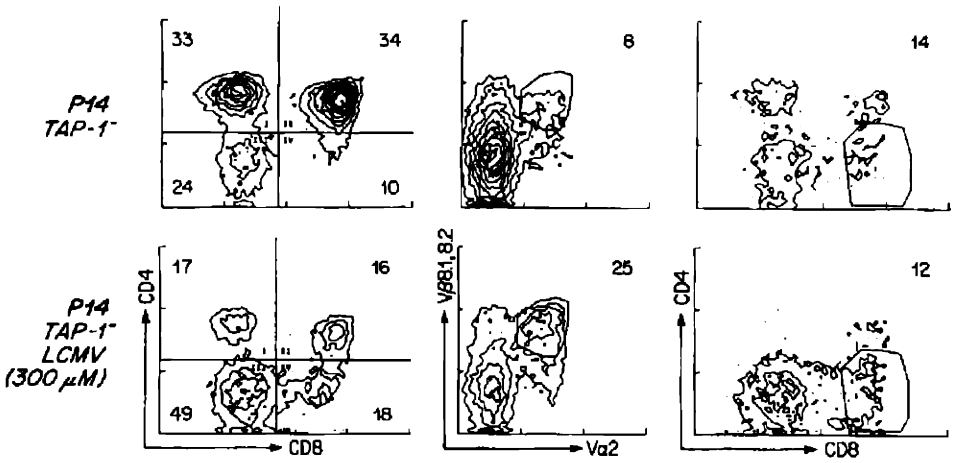
Figure 2-6 Histograms showing negative selection of $CD8^+$ transgenic P14 cells by the nominal antigen peptide in *TAP1*$^+$ FTOC



P14 *TAP1*$^+$ thymic lobes were cultured as described in the legend to Figure 2-2 with or without peptide at the concentrations indicated. Thymocyte suspensions were prepared, and the presence of the appropriate surface markers was detected by staining with the appropriate MAbs and subsequent analysis by FACS. The percentages of $CD8^+$ P14, $CD4^+CD8^+$, and $CD4^+$ cells were then determined as described in the legend to Figure 2-2. The level for the absolute number of a given cell type per lobe is the mean (± SEM) of values determined from the analysis of between three and five independently cultured thymic lobes. Similar data was obtained from two other experiments performed at different times.

control FTOC. In the representative experiment shown in Figure 2-5, the $CD8^+$ P14 cells were 5% (20% x 25%) with the peptide versus 20% (27% x 73%) without the peptide. The absolute number of $CD8^+$ P14 cells was also reduced by the addition of the LCMV peptide (Figure 2-6). The reduction was by 7- to 8-fold at the three input LCMV peptide concentrations tested (3, 30, and 300 μM) compared with the control FTOC with no added peptide. About a 4-fold reduction of the number per lobe of precursor $CD4^+CD8^+$ cells was also observed in *TAP1*$^+$ FTOC supplemented with 30 or 300 μM LCMV peptide, but no reduction was seen in the cultures supplemented with 3 μM LCMV peptide (Figure 2-6). In another experiment, similar results were obtained. However, in a third experiment, the number per lobe of $CD4^+CD8^+$ cells in the presence of 3 μM LCMV peptide was reduced by about 3-fold compared with the control FTOC without added peptide. We also quantitated the $CD4^+$ cell subset, most of which does not express the P14 TCR, and found that its number per lobe was reduced by up to 2-fold in *TAP1*$^+$ FTOC supplemented with 30 or 300 μM LCMV peptide, but not in the culture supplemented with 3 μM LCMV peptide (Figure 2-6). The reduction in the number of the $CD4^+$ cells is probably due to the fact that in the absence of the added peptide many of these cells arise from $CD4^+CD8^+$ cells whose TCRs have switched from the transgenic type to nontransgenic types by the lack of allelic exclusion in the TCR α locus. Depletion of $CD4^+CD8^+$ cells with P14 TCR by the added peptide will reduce the number of $CD4^+CD8^+$ cells with additional non-P14 TCRs and hence the number of $CD4^+$ cells.

Figure 2-7 Flow cytometric data showing negative selection of CD8$^+$ transgenic P14 cells by the nominal antigen peptide in *TAP1*$^-$ FTOC



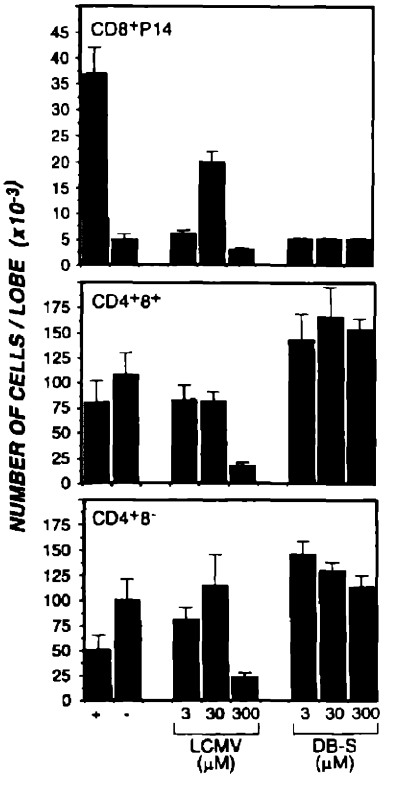
P14 *TAP1*$^-$ thymic lobes were cultured with or without LCMV peptide (300 μM) as described in the legend to Figure 2-2. Thymocyte preparations were prepared and analyzed (4 x 10^4 stained cells for lobes cultured without peptide and 3 x 10^4 for lobes cultured with 300 μM LCMV peptide) for the expression of surface markers as described in the legend to Figure 2-2. The percentages of cells in each quadrant or window are indicated.

The LCMV peptide-mediated reduction in the total number of thymocytes and in the numbers of cells in CD8$^+$ P14, CD4$^+$CD8$^+$, and CD4$^+$ subsets in P14 *TAP1*$^+$ FTOCs seemed to be peptide specific. Thus, neither the IF peptide (see Figure 2-5) nor the DB-S peptide (Figure 2-6), even at 300 μM, caused a reduction in the numbers of the total thymocytes or any of the three thymocyte subsets. The overall results demonstrate that the added LCMV peptide can induce negative selection of CD8$^+$ P14 cells in *TAP1*$^+$ FTOC. We suspect that not only the bulk TCR$^+$ low CD4$^+$CD8$^+$ cells but also a subset of cells that are derived from the CD4$^+$CD8$^+$ subset with somewhat up-regulated levels of TCR are susceptible to negative selection. In the presence of a low concentration (3 μM) of the LCMV peptide, only the latter subset will undergo negative selection, leading to a reduction in the number of CD8$^+$ P14 cells but not in the number of the bulk CD4$^+$CD8$^+$ subset (Figure 2-6). It should be noted that the concentrations of the LCMV peptides

required for negative selection of $CD8^+$ P14 precursor cells in *TAP1*$^+$ FTOC is considerably greater than those required for negative selection of these cells in thymocyte suspension cultures (70). This most probably reflects ineffective diffusion of peptides into thymic lobes during FTOC.

Having characterized the changes in the levels of total thymocytes and thymocyte subsets that accompany negative selection of $CD8^+$ P14 cells by the nominal antigen peptide in *TAP1*$^+$ FTOC, we then analyzed thymocytes from *TAP1*$^-$ FTOC supplemented with a relatively high concentration (300 μM) of the peptide. The total number of thymocytes per cultured lobe was reduced by about 2.5-fold in *TAP1*$^-$ FTOC with the LCMV peptide as compared with the cultures without

Figure 2-8 Histograms showing negative selection of $CD8^+$ transgenic P14 cells by the nominal antigen peptide in *TAP1*$^-$ FTOC



P14 *TAP*$^+$ or *TAP1*$^-$ thymic lobes were cultured as described in the legend to Figure 2-2 with or without peptide at the concentrations shown. The plus sign designates the data obtained from P14 *TAP1*$^+$ FTOC with no added peptide, and the minus sign designates the data obtained from P14 *TAP1*$^-$ FTOC with no added peptide. All other data were obtained from FTOC supplemented with the peptides indicated. Thymocyte suspensions were prepared and the presence of the appropriate surface markers was detected by staining with the appropriate MAbs and by subsequent analysis by FACS as described in the legend to Figure 2-2. The percentages of CDB^+ P14, $CD4^+CD8^+$, and $CD4^+$ cells were then determined as described in the legend to Figure 2-2. The level for the absolute number of a given cell type per lobe is the mean (± SEM) of values determined from the analysis of between three and seven independently cultured thymic lobes. Similar data was obtained from two other experiments performed at different times.

the peptide. Since the number of $CD8^+$ P14 cells is low in *TAP1*$^-$ FTOC without added peptide, we did not use reduction in the number of this thymocyte subset as a reliable measure for negative selection in *TAP1*$^-$ FTOC by added LCMV peptide. Indeed, the numbers of $CD8^+$ P14 cells in the peptide-supplemented FTOC fell within the background range (i.e., the numbers for peptide-negative FTOC) (see Figures 3 and 8). However, reduction in the numbers of $CD4^+CD8^+$ and $CD4^+$ subsets was clearly observed in *TAP1*$^-$ FTOC supplemented with 300 μM LCMV peptide (Figures 7 and 8). Such reduction was not observed in *TAP1*$^-$ FTOC supplemented with 3 or 30 μM LCMV peptide or in the similar cultures supplemented with 3, 30, or 300 μM DB-S peptide (Figure 8). We conclude that at a high concentration (300 μM) the LCMV peptide causes negative selection of $CD8^+$ P14 thymocytes in *TAP1*$^-$ FTOC. It is interesting to note that the negative selection was induced in *TAP1*$^+$ FTOC with distinctly lower concentrations of the LCMV peptide than in *TAP1*$^-$ FTOC (see below for a discussion on this point).

Discussion

In an earlier paper, we demonstrated that some, but not all, single H-$2D^b$- or K^b-binding peptides can induce positive selection of a diverse set of $CD8^+$ T cell clones when they are added at a relatively high input concentration (500 μM) to *TAP1*$^-$ (non-TCR-transgenic) FTOC (60). That study as well as another study performed using β2-microglobulin mutant mice (63) also showed that diverse mixtures of synthetic or natural H-$2D^b$- or K^b-binding peptides are effective positive selectors. Since the activity of a peptide (or a peptide mixture) to induce the positive selection and its activity to restore stable cell surface expression of class I H-2 molecules on thymic cells did not correlate, we concluded that the role of peptide in positive selection is not just to stabilize the class

I expression (the stability model); peptides also contribute to the specificity of positive selection of $CD8^+$ T cells in the thymus (the specificity model).

In the present paper, we extended our study on the role of peptides during thymic selection by examining the development of cells expressing a transgenic TCR. We draw two major conclusions. First, peptides shape the T cell repertoire, most probably by being directly recognized by the TCR during the positive selection. Second, the avidity of the interaction between TCRs and peptide–MHC complexes determines the fate of the immature ($CD4^+CD8^+$) thymocytes by inducing either positive or negative selection.

Peptides have a specificity-determining role in positive selection

The most striking finding made in the present study is that even the nominal antigen peptide, the LCMV glycoprotein (amino acids 33-41), can induce the positive selection of the P14 clone along the $CD8^+$ T cell pathway as long as the peptide is provided to the *TAP1*$^-$ FTOC at an appropriate concentration (10–100 μM). The selection is peptide specific in that its extent depends on the sequence of the peptide. The LCMV peptide and its three analogs possess similar H-$2D^b$-stabilizing activities. However, at 30 μM input concentration, the LCMV peptide induces the positive selection of $CD8^+$ P14 cells at a high level (about 40% of the level observed in *TAP1*$^+$ FTOC) and LCMV-8.1 peptide at an intermediate level (about 25%), whereas neither LCMV-8.7 nor LCMV' peptide induces the positive selection to a detectable level. Therefore, the substitution of just one amino acid has the potential either to abolish or to reduce the ability of a peptide to select. We also examined two other peptides, IF nucleoprotein (366-374) and DB-S, which have no sequence similarity to the LCMV peptide except for the two H-$2D^b$ anchor residues. Although these peptides are clearly either more effective (IF peptide) than or about as

effective (DB-S peptide) as the LCMV peptide in stabilizing the surface expression of H-2D^b molecules, they could not induce positive selection of CD8$^+$ P14 cells at 30 μM.

We have not directly measured the affinity of P14 TCR to any of the peptide-H-2D^b complexes. However, assuming a similar strength of binding to H-2D^b, the ability of a peptide to promote the proliferation of P14 spleen cells *in vitro* provides an indirect measure of the affinity. We found that among the LCMV peptide and its analogs with similar H-2D^b stabilizing activities, CD8$^+$ P14 cell selecting activities roughly correlate with P14 spleen cell stimulating activities. Thus, it is likely that the affinity between a TCR and a peptide–MHC complex is an important parameter in positive selection.

The second important parameter is the density of a peptide–MHC complex on the surface of selecting cells. This is suggested by the dose-response curve of positive selection of CD8$^+$ P14 cells by the LCMV peptide. At an input concentration below 3 μM or less, no CD8$^+$ P14 cells were generated in *TAP1*$^-$ FTOC above the background level. Between 3 μM and 30 μM, increasing levels of positive selection could be observed. It is reasonable to assume that with increasing concentrations of the LCMV peptide in *TAP1*$^-$ FTOC, the density of peptide–MHC complexes on thymic selecting cells increases since we have previously demonstrated a dose-response relationship for a peptide added to *TAP1*$^-$ FTOC and H-2D^b stabilization on thymic stromal cells (60).

It is interesting that the IF peptide can induce a detectable level of positive selection of CD8$^+$ P14 cells at a high input concentration (300 μM) (Figure 2-3). This peptide exhibited no detectable P14 spleen cell stimulatory activity but is highly effective in stabilizing the surface expression of H-2D^b molecules not only on RMA-S cells (Table 2-1) but also on thymic stromal cells in FTOC (see above). The observation that the IF

peptide can induce positive selection of $CD8^+$ P14 cells if it is given at a high concentration (Figure 2-3) suggests that even a low affinity peptide (so low that it cannot stimulate mature P14 T cells to a detectable level) has the capacity to promote positive selection of T cells as long as it is expressed at a very high density in association with an appropriate MHC molecule on selecting cells. These data thus illustrate the importance of peptide–MHC density as a parameter for positive selection.

It has been shown that the positive selection of a TCR-transgenic clone restricted to class II MHC is enhanced in mice expressing higher than normal levels of the selecting thymic MHC molecules (71). This observation also supports the view that peptide–MHC densities are a critical parameter in determining positive selection.

Specificity models of positive selection

It is now quite clear that peptide has a repertoire-shaping role during positive selection. A remaining question is whether peptide is directly recognized by TCR during this process as it is during the activation of mature T cells in the periphery. An alternative notion is that peptide plays a repertoire-shaping role by an indirect means without directly being recognized by TCR. Our finding that the same peptides that are recognized by mature T cells promote the positive selection of their precursor thymocytes argues for a direct role of peptide.

One way in which peptide can indirectly shape the T cell repertoire during positive selection is through steric hindrance of the interaction between TCR and MHC molecules (60). According to this model, a T cell clone is positively selected only when its TCR interacts with MHC molecules without interference from the MHC-bound peptide. However, our data with the DB-S peptide does not support this model. Except for the

anchor residues at positions 5 and 9, this peptide is composed of serines that carry less-bulky side chains than the corresponding amino acid residues of the LCMV peptide in 5 out of 7 positions. For each of the remaining two positions, the LCMV residue is alanine, whose side chain is only slightly less bulky than that of serine. Thus, it is very likely that the DB-S peptide when presented by H-2D^b molecules will sterically hinder the interaction between MHC and TCR less than the LCMV peptide. However, we found that the DB-S peptide did not select CD8$^+$ P14 cells, while the LCMV peptide did. These data do not support the steric hindrance model.

Another way in which peptide may shape T cell repertoire indirectly is by inducing conformational changes in MHC molecules, which might be preferentially recognized by TCR during positive selection. Our data do not argue against this possibility, and the occurrence of such conformational changes has been observed (72, 73). However, other studies found that these structural changes are relatively limited (74). In contrast, the structure of MHC-bound peptide was found to be a highly complex function of its entire sequence, potentially sensitive to even small sequence differences (74). It is difficult to imagine how TCR will ignore the structural variability of bound peptide and simply focus on relatively minor conformational changes induced in MHC molecules, either during positive selection or peripheral activation.

Differential avidity model of T cell selection

That apparently similar TCR–MHC interactions result either in the positive selection of MHC-restricted thymocytes or in the negative selection of autoreactive thymocytes has long intrigued many immunologists. While many hypotheses have been proposed to explain this thymic paradox (50), the parameters of the TCR–MHC interactions (or

subsequent signal transductions) that are crucial for the determination of different cell fate have remained largely unknown. Our present study begins to identify some of these parameters. Our results suggest that a single thymocyte clone can be either positively or negatively selected by the same peptide–MHC complexes depending on the avidity of the interaction between the TCRs and the peptide–MHC complexes. Here, we use the term avidity to indicate the product of the intrinsic affinity between a TCR and a peptide–MHC complex, the density of the TCR on the thymocyte, and the density of the peptide–MHC complex on the selecting cell. Avidity defined in this way is a measure of the number of engaged TCRs on the thymocyte. When the avidity is relatively low and within a certain range, the thymocyte undergoes positive selection. When the avidity exceeds this low range and reaches a certain threshold, negative selection occurs.

Several observations made in this study support this differential avidity model of T cell selection. We have already pointed out that the IF peptide that presumably has a very low affinity to P14 TCR can nevertheless positively select $CD8^+$ P14 cells in *TAP1*$^-$ FTOC when it is expressed at a very high density on thymic cells, presumably because a minimal avidity required for positive selection is reached. Conversely, the LCMV peptide that presumably has a high affinity for P14 TCR can also positively select $CD8^+$ cells in *TAP1*$^-$ FTOC when its expression on thymic cells is kept at a relatively low density so that the avidity is within the range for appropriate positive selection. However, when the same peptide is expressed at a higher density and hence the avidity exceeds a certain threshold, negative selection occurs. We have identified another peptide, LCMV-8.1, which can induce both positive (this work) and negative (69) selection. It is likely that the

different outcomes observed are due to differences in avidity under the two experimental conditions.

Another observation that supports the differential avidity model of T cell selection is that different fates are taken by the precursors of $CD8^+$ P14 in *TAP1*$^-$ FTOC and *TAP1*$^+$ FTOC supplemented by relatively low concentrations (~30 μM) of the LCMV peptide. In *TAP1*$^+$ FTOC without the exogenous peptide, an avidity sufficiently high for the positive selection of $CD8^+$ P14 cells is already reached by a variety of endogenous peptides. Hence, the system is very sensitive for negative selection when the high affinity LCMV peptide is added to the culture. By contrast, in *TAP1*$^-$ FTOC, the avidity is lower than the minimal level necessary for the positive selection in the absence of the exogenous peptides. When we add increasing concentrations of the LCMV peptide, the avidity necessary for positive selection is first reached, and then, upon a further increase in concentration, the avidity sufficient for negative selection is reached.

When transgenic mice that over express the CD8 coreceptor were crossed to class I-restricted TCR-transgenic mice, negative selection of the transgenic $CD8^+$ T cells was observed (75, 76). These observations are consistent with negative selection being determined by an increase in TCR–MHC avidity over that needed for positive selection. In those cases the increase in avidity was afforded by overexpression of the TCR–MHC class I coreceptor CD8.

In the past, a large amount of work on T cell selection was devoted to the identification of thymic stromal cell subsets that induce positive or negative selection. Initial studies seemed to indicate that radioresistant cortical epithelial cells and radiosensitive bone marrow-derived cells (macrophages and dendritic cells) specialize in

positive and negative selection, respectively (49). However, recent studies challenge these earlier notions and suggest that wider arrays of cell types, including fibroblasts, have the capacity to induce positive selection, negative selection, or both (50, 70, 77). Our differential avidity model, in its minimal form, does not depend on distinct thymic stromal cells specializing in each of the two types of selection. If distinct cell types are responsible for positive or negative selection and some adhesion molecules are differentially expressed on the positively and negatively selecting cells, they may modulate the avidity of interactions. However, the observation that a single peptide (the LCMV peptide) can induce either positive or negative selection depending on the concentration (Figure 2-3) indicates that the role of adhesion molecules in differentially influencing cellular interactions in positive versus negative selection is limited. This observation also argues against the notion that different sets of self-peptides must be produced by different selecting cells that, when recognized by thymocyte TCRs, lead to positive or negative selection (78).

As to the target of selection, thymocytes, the differential avidity model again does not depend on the occurrence of distinct subsets of cells for positive and negative selection. However, at least for the selection of thymocytes involving superantigens, it has been suggested that the thymocytes undergoing negative selection express TCRs at higher density than the thymocytes undergoing positive selection (79). When we supplemented P14 *TAP1*$^{-}$ FTOC with the LCMV peptide, we found that only a 10-fold difference (30 μM versus 300 μM) in the input peptide concentration resulted in distinct cell fates. Even if the TCR density is only slightly higher on thymocytes undergoing negative selection than those undergoing positive selection, that difference would amplify the discriminative

power of the avidity difference resulting from differences in affinity and peptide–MHC density.

A differential affinity model was previously formulated to explain the thymic paradox (80). In this model it is the affinity of TCR for a self-MHC or a peptide-self-MHC complex that is critical in the determination of the fate of thymocytes: low affinity results in positive selection and high affinity in negative selection. The differential avidity model (81) is distinct from the differential affinity model in that it incorporates as the critical parameters not only the intrinsic affinity of the TCR (perhaps including its coreceptor) for a peptide–MHC complex but also the cell surface density of the TCR as well as the density of the peptide–MHC complexes. In the thymus, TCRs of an immature thymocyte encounter MHCs loaded with a variety of peptides that have different binding affinities to the TCR and are expressed at different densities on the surface of selecting cells. In addition, it is possible that thymocytes with different levels of TCR density are involved in positive and negative selection. In the differential avidity model, it is the avidity or the summation of the interactions of the multiple but homogeneous TCRs with multiple and heterogeneous peptide–MHC complexes that determines the fate of an individual thymocyte. Thus, a relatively high affinity TCR–peptide/MHC interaction can lead to positive selection as long as the density of the TCR and that of the peptide–MHC complex are below certain levels. Conversely, a low affinity TCR–peptide/MHC interaction can theoretically lead to negative selection, although in practice the required high density of peptide–MHC is unlikely to be reached under physiological conditions. Of course, there is a minimal affinity below which no signal can be delivered through the

TCR. In this case, the density of neither TCR nor peptide–MHC complexes will be able to compensate the lack of sufficient affinity.

The nature of differential signals delivered to thymocytes during positive and negative selection remains unknown. Two possibilities can be considered. Owing to higher numbers of engaged TCRs, cells undergoing negative selection may receive more of the same signals than the cells undergoing positive selection. Alternatively, engaged TCRs at high densities may generate signals that are qualitatively different from the signals generated by engaged TCRs at low densities. For example, engaged TCRs at high densities may dimerize and thereby activate attached effector molecules (e.g., protein kinase) into a form that differs from the form generated by an engaged monomeric TCR.

After submission of this paper, Hogquist et al. (82) reported positive selection of another transgenic T cell clone by peptides in FTOC derived from β2-microglobulin mutant mice. Contrary to our findings, these authors reported that the nominal antigen peptides do not induce positive selection. We suggest that a range of peptide concentrations appropriate for positive selection was not tested in this study. Hogquist et al. (82) emphasize the correlation between the selecting peptides and so-called TCR antagonist peptides. However, we believe that this correlation is only apparent and is probably attributed to the fact that these peptides have a suitable range of affinity for promoting positive selection at the concentration (20 μM) the authors employed. Our view is that antagonicity is not a required property of positively selecting peptides. Rather, an appropriate range of the avidity as defined above is the critical parameter.

Experimental procedures

Mice

Control C57BL/6 mice (obtained from The Jackson Laboratory, Bar Harbor, Maine), *TAP1*-deficient mice ([129/SvJ x C57BL/6]F2) (62), and P14 transgenic mice (C57BL/6) (65) were maintained and bred under standard conditions. Mice homozygous for the *TAP1* mutated allele and for the *P14* transgene were crossed, and the progeny (Fl) were then intercrossed to produce F2 animals. $TAP1^{-/-}P14^{+/-}$ F2 animals were typed by screening peripheral blood for the absence of class I MHC expression by monoclonal anti-body (MAb) staining and FACS analysis (62) to detect $TAP1^{-}$ mice and by PCR analysis (*Vβ8.1* primer, 5'-CATGGAGGCTGCAGTCACCC-3'; *Cβ2* primer, 5'-GTTTGTTTGCGAGCTCTGTTTTGATGGCTC-3' of genomic DNA prepared from tail biopsies to detect P14 transgenes. These mice were then intercrossed to produce $TAP1^{-/-}$ $P14^{+/+}$ F3 mice, which were typed by backcrossing to normal C57BL/6 mice and screening the progeny for the presence of the transgene. F3 mice that gave 20 consecutive P14 progeny on back-crossing were considered to be homozygous for the transgene.

FTOCS

$TAP1^{-}P14^{+/+}$ males were mated with $TAP1^{-}$ females to produce P14 $TAP1^{-}$ fetal thymi, while $TAP1^{+/+}$ (C57BL/6) $P14^{+}$ males were mated with C57BL/6 females to produce $TAP1^{+}$ P14 fetal thymi. Fetal thymi (day 16 postcoitum) were cultured according to the method described previously (60) except that RPMI, 10% fetal calf serum (FCS) medium was used.

Flow cytometric analysis

The following MAbs were used: antiGD4 (allophycocyanin [APC] or R-phycoerythrin [PE] labeled), anti-CD8α (fluorescein isothiocyanate [FITC] labeled), anti-Vβ8.1, 8.2 (biotin-labeled), anti-Vα2 (R-PE-labeled), anti-H-2D^b (biotin-labeled B22-249R1, α1 specific, American Type Culture collection). and anti-l-A^b (PE-labeled). Thymocyte suspensions were prepared from cultured fetal thymi and analyzed as described previously (60). Thymocyte and mesenteric lymph node cell suspensions were prepared and analyzed as previously described (62). Chromosomal DNA of FACS-sorted cells was stained with propidium iodide (PI). In brief, cells were washed in phosphate-buffered saline (PBS) and then fixed in 70% ethanol (1 ml at -20°C) for 30 min. After washing in PBS, cells (1 x 10^6/ml) were permeabilized by incubation in 3 M HCI, 0.5% Tween 20 for 20 mi at 20°C and then neutralized by the addition of 3 ml of 0.1 M sodium borate. Washed cells (2 x PBS) were incubated with RNAase (150 μg/ml, 0.5 ml) for 20 min at 37°C, washed, and resuspended overnight with PI (50 μg/ml in PBS) at 4°C in the dark. Quantitation of the intensity of PI staining was performed by FACS analysis. The proportion of cells in G0/G1 (*n* PI staining), G2/M (*2n* RI staining), and S phases of the cell cycle (intermediate staining) was determined by statistical analysis (Modfit program) of the histogram of PI staining intensity versus cell number.

For the determination of H-2D^b expression on the surface of thymic stromal cells from FTOC, total thymic stromal cell suspensions from six whole fetal thymi were prepared exactly as described by Ashton-Rickardt et al. (60). Stromal cells were analyzed for surface expression of I-A^b and H-2D^b molecules. To quantitate the surface expression of H-2D^b on gated I-A^b cells, the log fluorescence intensity resulting from staining with

biotin-labeled anti-H-2D^b MAb (B22-249R1, α1-specific) and streptavidin-FITC was plotted against the log relative cell number. Analysis of the resulting histograms allowed the determination of the median level of fluorescence intensity resulting from anti-H-2D^b staining on I-A^b-positive cells. The relative level of H-2D^b was thus expressed as a percentage of the *TAP1*$^+$ level minus the level observed on cells from *TAP1*$^-$ thymi incubated without peptide.

Peptides

Amino acid sequences of synthetic peptides are as follows: LCMV (amino acids 33-41), KAVYNFATM: IF (amino acids 366-374), ASNENMETM; DB-S, SSSSNSSSM; LCMV-8.1 (amino acids 33-41), KAMYNFATM; LCMV-8.7 (amino acids 33-41), KALYNFATM; LCMV', RGIFNYGSM. For technical reasons (to prevent dimer formation), the original cysteines at anchor position 41 in LCMV, LCMV-8.1, and LCMV-8.7 were replaced by methionines. The LCMV, IF, and DB-S peptides were synthesized by the Biopolymers Laboratory (Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, Massachusetts), the LCMV-8.1 and LCMV-8.7 were synthesized by Neosystem Laboratoire (Strasbourg, France), and the LCMV' peptide and LCMV-M peptides were synthesized by Research Genetics (Huntsville, Alabama). All were purified by reverse phase high pressure liquid chromatography (HPLC) and the concentration determined by quantitative Ninhydrin colorimetric assay. All peptides used were over 99% pure as determined by HPLC analysis. Three separate preparations of LCMV (from three different syntheses) were used in FTOC experiments. All gave similar results.

H-2D^b stabilization assay for peptides

The relative abilities of peptides to stabilize H-2D^b surface expression was determined using the procedures described previously (52). After incubation for 16 hr, RMA-S (duplicate cultures with peptide dilutions 10^{-4} M to 10^{-9} M) cells (1 x 10^6/ml, 0.2 ml) were washed, and the surface expression of H-2D^b was determined by staining with B22-249R1-biotin (then streptavidin-PE) and FACS analysis. The relative level of surface H-2D^b expressed in arbitrary fluorescence units (RMA, 3251; RMA-S alone, 60) was determined after FACS analysis. The relative ability of peptide to stabilize surface H-2D^b was expressed as the concentration necessary to give 50% of maximum rescue (maximum rescue was 35% of RMA).

P14 spleen cell proliferation assay for peptides

Spleen cell suspensions (1 x 10^6 cells/ml) were prepared from P14 transgenic mice (C57BL/6) and incubated with peptide (triplicate wells, 2 x 10^5 cells) diluted from 10^{-4} M to 10^{-11} M for 36 hr in RPMI, 10% FCS medium. Cultures were pulsed with tritiated thymidine (1 μCi per well) for 16 hr, the cells in the wells were harvested, and the amount of radioactivity was determined by detection of β particle emission. The relative abilities of peptides to induce the proliferation of P14 spleen cells were expressed as the concentration of peptide necessary to give 50% of the maximum [^{3}H]thymidine incorporation (1.1 x 10^5 cpm) observed for the LCMV peptide. With the exception of the LCMV, LCMV-8.1, LCMV-8.7, and LCMV-M peptides, all other peptides tested failed to induce proliferation of P14 spleen cells at concentrations up to 10^{-4} M.

Acknowledgments

We wish to thank Juan Lafaille, Werner Haas, and Herman Eisen for useful comments on the manuscript; Carol P. Browne for skillful technical assistance; Glenn Paradis for flow cytometric analysis; and Jacqueline Collins and Eleanor Basel for excellent secretarial help. This work was supported by grants from the National Institutes of Health (AII7879 and CA53874) to S. T. and by grants from the Swiss National Foundation grants (31-3214541 and 31-0967.91) to R. M. Z. H.-P. P. is supported by the Stiftung Professor Clöetta. S. T. is an Investigator of Howard Hughes Medical Institute.

Received January 6, 1994; revised January 19,1994.

Contributions to work

The author assisted Dr. Philip Ashton-Rickardt in many aspects of the work presented here, including embryo dissection, thymic organ culture, FACS analysis, cell cycle analysis, class I stabilization assays, HPLC purification of peptides and even some editing of the manuscript. He also did some work using other TCR-transgenic mice (e.g., H-Y and F5) to complement Philips work with the P14 transgenics. This work, however, was not included in this publication.

Chapter 3 Positive and negative selection of 2C T cell receptor transgenic T cells

Introduction

In the thymus, T lymphocyte precursors follow a common developmental pathway of proliferation, TCR gene rearrangment and differentiation to the $CD4^+$, $CD8^+$ TCR^{low} stage (49). At this point, these double-positive cells undergo stringent selection ensuring that only cells that recognize self-MHC proteins mature into $CD4^+$ or $CD8^+$ T cells (positive selection) and that cells that are potentially autoreactive receive a signal to die (negative selection) (12, 15, 16, 44, 49, 50, 80). The signal for negative selection has long been know to be both peptide-dependent and MHC-allele dependent and mediated by the TCR (49, 50, 65). Negative selection is thought to involve bone marrow-derived stromal cells (macrophage and dendritic cells), while positive selection involves the thymic epithelium (49). Positive selection is MHC-allele dependent and TCR-mediated (10, 15, 45, 48).

In previous work, the Tonegawa group and others have shown that peptides contribute to positive selection of T cells in *TAP1* or β_2-microglobulin (β_2m)-deficient fetal thymic organ culture system (FTOC) (60, 63). In later studies, it was shown that peptide antigen and some variants can induce both positive and negative selection of transgenic P14 T cells in FTOC (42, 83) (see Chapter 2). Furthermore, it was shown that this selection is dependent on the concentration of peptide added to FTOC — high concentrations result in deletion, intermediate concentrations result in positive selection and low concentrations of peptide do nothing. These results were interpreted to mean that selection is dependent on the number of ligand-engaged TCR, (i.e., the avidity of

TCR–peptide–MHC interactions is a critical factor in determining the fate of immature T cells).

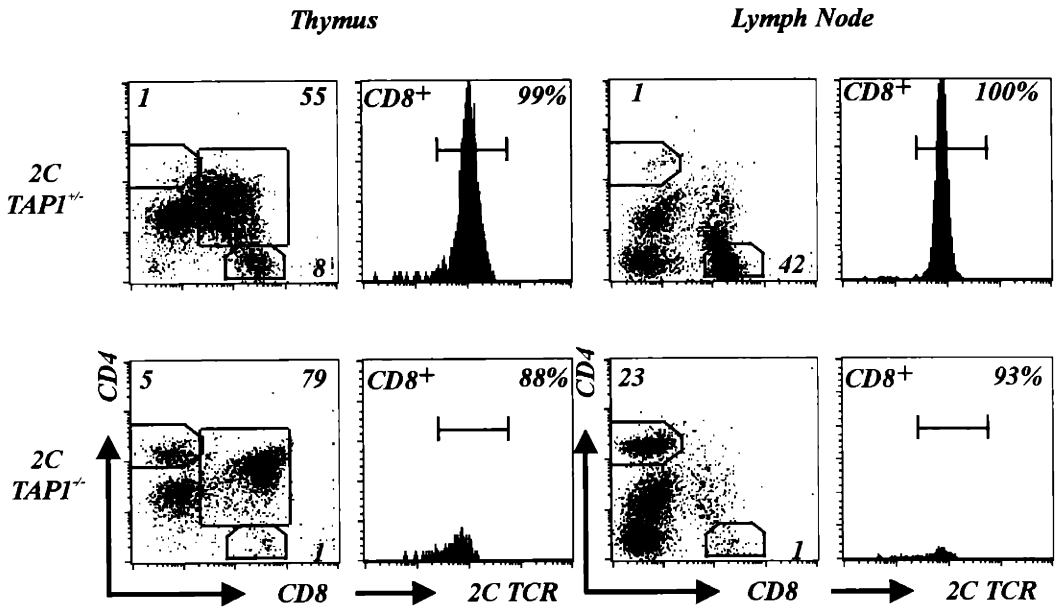
I expanded these findings by crossing *TAP1*-deficient mice with 2C TCR-transgenic mice. I employed reaggregate thymic organ culture (RTOC) techniques to test the activity of potential ligands on purified thymic epithelial cells without the potentially confounding effects that could result from a heterogeneous population of thymic APCs. Several conclusions can be drawn from the work presented here. First, positive and negative selection involve the recognition of peptide and MHC. Second, the number of TCR–peptide–MHC interactions determines the outcome of thymic selection. Third, peptide–MHC ligands with a broad range of affinities for the TCR ($K_a = 3 \times 10^3 M^{-1}$ to $1 \times 10^7 M^{-1}$) can both positively and negatively select thymocytes *in vitro*. Fourth, there is no correlation between a peptide's antagonist properties and its ability to induce the positive selection of cognate receptor-bearing thymocytes.

Results

Positive selection of 2C transgenic T cells is impaired in TAP1-deficient mice

It was shown previously that positive selection of $CD8^+$ T cells, but not CD4 T cells, is severely hampered in the thymus of *TAP1*-deficient mice (60, 62). Transgenic mice (2C) expressing an alloreactive TCR (V_αpHDS58, V_β8.2) that recognizes the naturally occuring peptide p2Ca (LSPFPFDL) presented by either H-$2K^b$ or H-$2L^d$ (36, 84, 85) were crossed to *TAP1*-deficient mice. I analyzed thymocytes and lymph node cells derived from 2C *TAP1*$^{-/-}$ and 2C *TAP1*$^{+/+}$ mice of the H-2^b background by flow cytometry. Typical results are shown in figure 3-1. The thymi of 2C *TAP1*$^{-/-}$ mice contained twice as many thymocytes as those of 2C *TAP1*$^+$ mice, the bulk of which are $CD4^+$ $CD8^+$ $2C^{low}$

Figure 3-1 Positive selection is impaired in 2C *TAP1*$^{-/-}$ mice



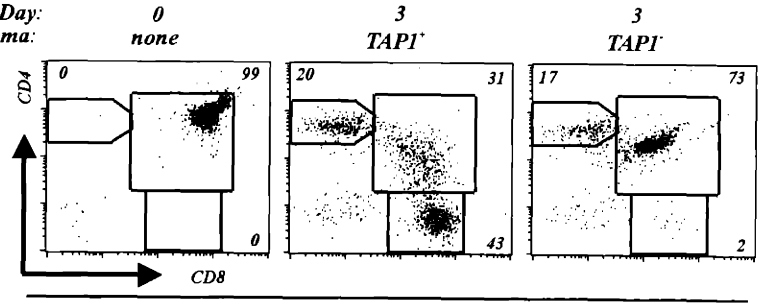
Thymocytes and mesenteric lymph node cells were prepared from 2C TCR transgenic *TAP1*$^{+/-}$ or *TAP1*$^{-/-}$ mice and stained with anti-CD8–FITC, anti-CD4–PE and anti-2C TCR–biotin–streptavidin–CyChrome.

cells. The lower expression of CD4 and CD8 on double-positive 2C cells was lower in *TAP1*$^{+/-}$ than on *TAP1*$^{-/-}$ mice (Figure 3-1). These findings suggest that some deletion may be occurring in *TAP1*$^{+/-}$ mice. This is consistent with the original analysis of 2C transgenic mouse, which has shown that the numbers of immature 2C thymocytes were higher in transgenic mice with a non-selecting MHC-haplotype than in mice with a positively selecting MHC-haplotype (i.e., H-2^{b}) (36). The percentage of CD8^{+} cells was reduced in 2C *TAP1*$^{-/-}$ mice (n = 4; range, 1%-1.5%) as compared to 2C *TAP1*$^{+/-}$ mice (n = 4; range, 3%-14%). In contrast, the percentage of CD4^{+} cells was augmented in 2C *TAP1*$^{-/-}$ mice (range, 3%-5%) by comparison with 2C *TAP1*$^{+/-}$ mice (range, 0.7%-1.7%).

Similar results were obtained with peripheral T cells. The mesenteric lymph nodes of 2C *TAP1*$^{-/-}$ mice contained approximately two-thirds of the number of cells as those of 2C *TAP1*$^{+/-}$ mice. A substantial reduction in the percentage of CD8^{+} cells was observed in 2C

TAP1$^{-/-}$ mice (range, 0.9%-1.9%) by comparison with 2C *TAP1*$^{+}$ mice (range, 25%-44%). Staining with the mAb 1B2, which recognizes the 2C clonotype, showed that the large majority of the CD8^{+} cells in 2C transgenic mice are 2C positive. These data indicate that the development of CD8^{+} transgenic 2C T cells is impaired in the *TAP1*$^{-/-}$ background.

Figure 3-2 Positive selection of 2C cells is impaired in *TAP1*$^{-/-}$ FTOC



After 3 days of culture, cell suspensions were prepared from RTOCs consisting of 2C^{+} CD4^{+} CD8^{+} thymocytes and epithelial cells from either *TAP1*$^{+/+}$ or *TAP1*$^{-/-}$ E14 embryos and analyzed as in Figure 3-1. Cell recovery was typically 5 x 10^{5} cells per lobe.

Stromal cells from TAP1$^{-/-}$ *thymus cannot positively select*

I performed RTOCs consisting of a prepurified population of DP 2C^{+} thymocytes and deoxyguanosine (dGuo)-treated (i.e., epithelial cell-enriched) *TAP1*$^{+/+}$ or *TAP1*$^{-/-}$ stromal cells. As observed *in vivo* in 2C transgenic mice, there were fewer CD8^{+} SP 2C thymocytes in cultures with *TAP1*$^{-/-}$ stroma than in cultures with *TAP1*$^{+/+}$ stroma. Figure 3-2 shows a representative result of several experiments (the left panel shows sorted thymocytes at day 0 of culture). In this experiment, the percentage of CD8^{+} cells ranged from 37-53% (n=3) in *TAP1*$^{+/+}$ RTOC as compared to 1-3% (n=4) in *TAP1*$^{-/-}$ RTOC. In other experiments, the frequency of CD8^{+} cells ranged from an average of 13% to as high as 40% in *TAP1*$^{+/+}$ RTOC compared with a variation of 1-10% in *TAP1*$^{-/-}$ RTOC and was consistently higher in *TAP1*$^{+/+}$ in comparison with *TAP1*$^{-/-}$ RTOC. The variation in different experiments could be due to differing numbers of haemopoietic dendritic cells

that may survive the dGuo treatment, the release of endogenous peptides from cells dying in culture or the presence of low numbers of class I complexes on the cell surface in the absence of a functional TAP transporter (see Chapter 4). Overall the results show that there is impaired positive selection of $CD8^+$ 2C cells in *TAP1*$^{-/-}$ RTOC relative to *TAP1*$^{+/+}$ RTOC.

Table 3-1 Peptides used in this study

peptide	amino acid sequence	K_a (M^{-1})	CTL activity	thymic selection	references
p2Ca	LSPFPFDL	3×10^3	+	+	(84-86)
SIY8	SIYRYYGL	1×10^7	+++	+	(87)
dEV8	EQYKFYSV	$\sim 10^6$	±	+	(88-90)
AYL	AAAAYAAL	ND	-	-	this study
VSV	RGYVYQGL	$<10^3$	-	-	(72)
pOV8	SIINFEKL	$<10^3$	-	-	(91)
p2Ca-Y5	LSPFYFDL	ND	-*	-	this study
E-VSV	RGYVYQEL	ND	-*	-	
GL8	GNYSFYAL	$\sim 10^5$	-*	-	

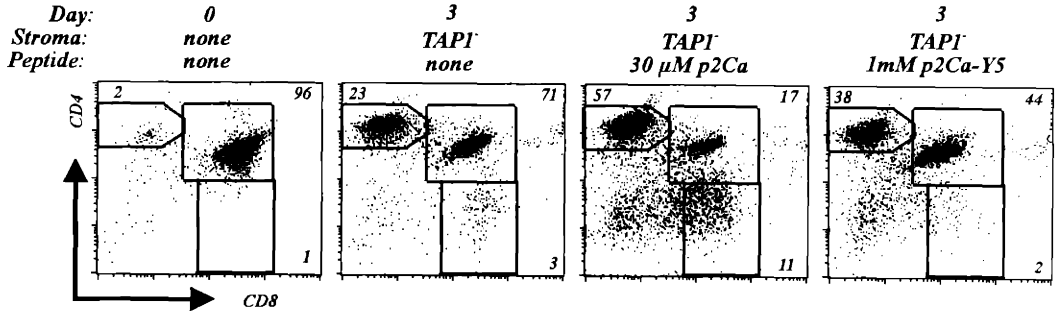
Each peptide was tested in at least 2 FTOC experiments over a range of concentrations. The maximum concentration tested was 1–3 x 10^{-3}M, unless the maximum solubility of the peptide was lower. The ability to induce positive selection is indicated by a "+". The K_a represents the measured affinity of the 2C TCR for the peptide–MHC complex indicated as determined by measurement on intact cells, except for the K_a of dEV8, which was measured by surface plasma resonance techniques (SPR; see references). The published K_a of the 2C TCR for dEV8–K^b is 1.2 x $10^4 M^{-1}$. The estimate shown here is based on the observation that when both values as determined by SPR and by measurement on intact cells are known, SPR values are typically 10-500 fold lower. The * indicates peptides that have antagonist activity in a CTL assay.

Positive selection of 2C transgenic CD8⁺ T cells can be induced in RTOC by the addition of cognate peptide ligand

Since several peptides, that are recognized by 2C T cells in association with K^b class I MHC proteins have been identified, I wanted to determine if they also could mediate positive selection of 2C cells in conjunction with *TAP1*$^{-/-}$ thymic epithelium in RTOC. The peptides p2Ca, dEV8 and SIY8 (Table 3-1) were tested over a broad range of concentrations.

These peptides induced the development of $CD8^+$ 2C cells at concentrations of 30 μM, 100 mM and 10 nM, respectively (Figure 3-3, see also Figure 5-4 and Table 5-2). At similar or higher concentrations the peptides p2Ca-Y5 (a synthetic p2Ca variant), pOV8 or a number of other H-2 K^b binding peptides had no such activity (Figure 3-3, Table 3-1, and data not shown).

Figure 3-3 Cognate peptides can restore positive selection of 2C cells in *TAP1*$^{-/-}$ RTOC



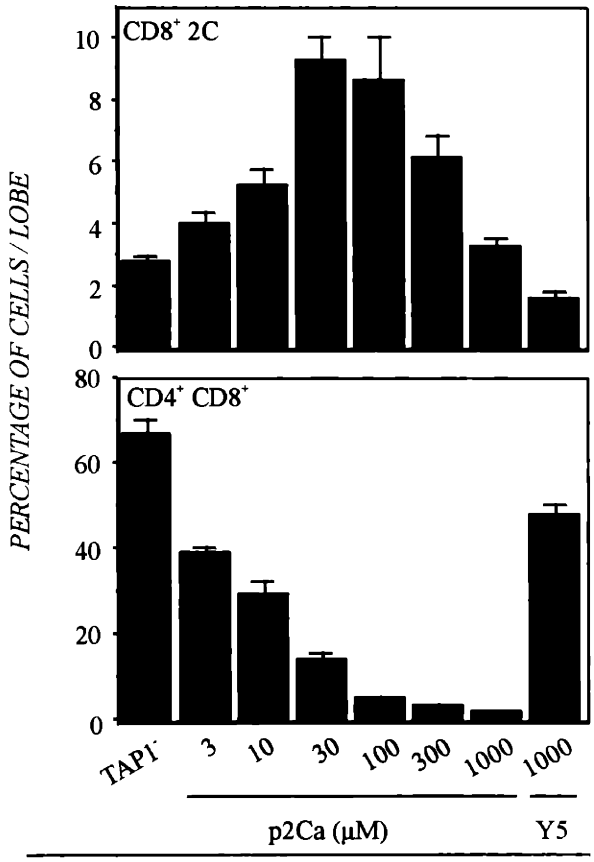
Reaggregate thymic organ cultures consisting of H-2^b *TAP1*$^{-/-}$ epithelial cells and $2C^+$ $CD4^+$ $CD8^+$ thymocytes were performed with or without peptide as indicated. After 3 days of culture, they were analyzed as in Figure 3-2, but with anti-CD8–TC, anti-CD4–PE and anti-2C TCR–FITC. Numbers indicate the percentage of cells in the respective gated areas.

Peptide-induced positive and negative selection of 2C transgenic cells

Peptide-driven positive selection as determined by FACS analysis peaked at 30μM for the peptide p2Ca and decreased at higher concentrations (Figure 3-4). This decrease

was not due to reduced conversion of DP cells to $CD8^+$ SP cells because the numbers of DP cells also decreased in a dose-dependent manner. These results are best interpreted to indicate that as the concentration of peptide ligand increases, negative, rather than positive, selection takes place.

Figure 3-4 Peptide-induced positive and negative selection of 2C cells in *TAP1*$^{-/-}$ RTOC



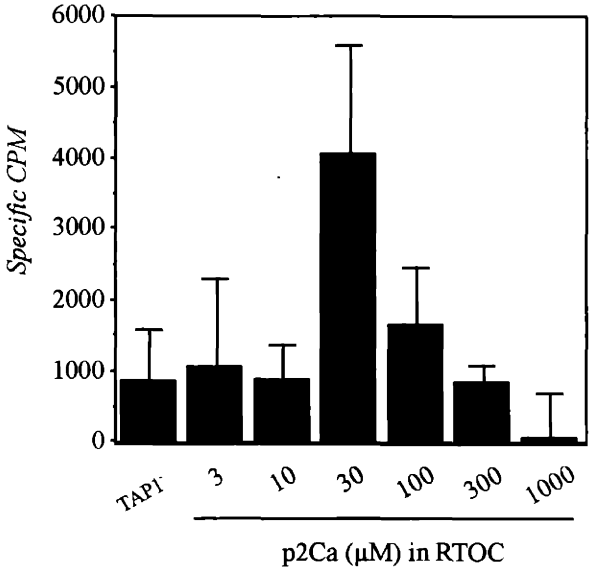
RTOCs were prepared as in Figure 3-3 and cultured with or without peptide as indicated. The value of the percentage of the given cells per lobe was taken from cytometric analysis as in Figure 3-3. The error bars indicate ± SD for 4 independent lobes.

Thymocytes selected on p2Ca are functional

It was important to determine if the $CD8^+$ 2C cells thymocytes produced as a result of positive selection in RTOC could respond to p2Ca-L^d-positive APC as cloned 2C cells

do. Figure 3-5 shows a comparison of the allo-proliferative response of thymocytes cultured with no peptide and that of thymocytes cultured with different concentrations of p2Ca. As p2Ca-presenting cells in the proliferation assay, T2 cells transfected with the gene encoding the L^d protein were used (92). Thymocytes derived from *TAP1*$^{-/-}$ RTOC not supplemented with peptide mounted a poor proliferative response to p2Ca plus T2-L^d APC, while thymocytes derived from *TAP1*$^{-/-}$ RTOC supplemented with 30μM p2Ca

Figure 3-5 Thymocytes positively selected by p2Ca are functional



Cells prepared from RTOCs were cultured as in Figure 3-3 with or without peptide as indicated above and then stimulated with T2-L^d target cells in the presence or absence of 100 μM p2Ca. The bars indicate ± SD (n=3) level of [^{3}H]thymidine incorporated during a 12-hour pulse as determined by the measure of β particle emission between samples cultured with and without peptide.

mounted a vigorous response. As expected, thymocytes cultured with higher concentrations of p2Ca showed a decreased response. The proliferative response of 2C cells generated in RTOC cultures was comparible to that of similar numbers of resting $CD8^+$ spleen cells from a 2C-transgenic mouse (data not shown). The proliferation was

proportional to the number of $CD8^+$ 2C cells assayed. One cannot rule out, however, that some cells become anergic at high peptide concentrations in RTOC. The results indicate that 2C cells selected by an agonist peptide in association with self-MHC (K^b) could respond to the same peptide in association with allogeneic MHC protein (L^d).

Discussion

Earlier work by the Tonegawa group and one other group has demonstrated that cognate peptide and variants thereof can positively and negatively select P14 transgenic cells in a dose-dependent manner in FTOC (42, 83). The Tonegawa group and others interpreted these findings to indicate that the number of receptors engaged (i.e., the avidity) is important in determining the fate of the immature T cells. Another group has reported that antagonist peptides, but not agonist peptides, can positively select OVA transgenic cells (82). They also stressed that strong agonists only delete in their TCR-transgenic system at any dose (93). They interpreted these results to indicate that if the intrinsic affinity of a peptide–MHC ligand for a TCR is above a certain threshold value, then only negative selection may occur. Thus, a ligand with high enough affinity for the TCR will always delete, regardless of the ligand-density and the number of TCRs engaged. The value of this threshold affinity was not determined. All of these studies employed foreign peptides and their variants. They offered no direct insight into the identity or nature of a possible natural thymic ligand responsible for positive selection *in vivo*. Furthermore in these studies, the exact affinities of the peptides for MHC and of the TCRs for their ligands were largely undetermined.

In this study, I demonstrated positive selection of T cells expressing a transgenic TCR by a thymic peptide with agonist activity. Furthermore, I demonstrated that a natural

peptide, that promotes the positive selection of immature T cells in combination with a particular MHC molecule, can also be a ligand for the resulting mature T cells in combination with another MHC molecule. Among the peptides tested for their ability to induce positive selection *in vitro*, the peptides p2Ca-Y5, E-VSV and GL8 can antagonize 2C CTL-mediated lysis of antigen-loaded APCs, but fail to induce selection of 2C thymocytes in RTOC. While the work of others suggests that some antagonist peptides are able to induce positive selection of cells expressing their cognate receptors, I have shown here there is no correlation between the ability to antagonize and to induce positive selection. One final point of interest is that negative selection can be observed in RTOC consisting of only thymocytes and thymic epithelial cells, provided that sufficient peptide-ligand is added. This could be due to the presence of contaminating dendritic cells in the epithelial cell preparations. Another possibility is that the costimulatory molecules implicated in negative selection may enhance TCR signaling quantitatively, rather than provide a qualitatively unique signal to die (94). Enhanced signalling could be accomplished either by favoring more TCR–peptide–MHC interactions (i.e., enhancing the overall avidity) or by increasing the sensitivity of the signal cascade response to TCR engagement.

Materials and methods

Mice.

Control C57BL/6 mice (The Jackson Laboratory, Bar Harbor, Maine), *TAP1*-deficient mice (62), and 2C transgenic mice (47) were maintained and bred under standard conditions. Mice homozygous for the *TAP1*-mutated allele and heterozygous for the 2C transgene were crossed and the progeny (F1) were typed by screening peripheral blood

for the predominance of the $CD8^+$ $V_\beta 8.2^+$ cells by monoclonal antibody (MAb) staining and FACS analysis. *TAP1*$^{+/-}$ $2C^{+/-}$ F1 animals were then intercrossed to produce F2 animals. TAP$^{-/-}$ $2C^{+/+}$ F2 animals were typed by three methods. For one, peripheral blood was screened for the predominance of $CD8^-$ $V_\beta 8.2^+$ cells and for the absence of expression of class I MHC expression by Mab staining. PCR analysis was also carried out ($2C_\alpha$ pHDS58 primer, 5'-TTCTGGTATGTCCAGTACCC-3'; $2C_\alpha$ junction primer, 5'-TCCAAATGTCAGCGCACTTG-3') on genomic DNA prepared from tail biopsies (95). Finally, *TAP1*$^{-/-}$ $2C^+$ mice were backcrossed to normal C57BL/6 mice and their progeny screened for the presence of the transgene. F2 mice that gave 20 consecutive $2C^+$ progeny were considered to be homozygous for the transgene.

Flow cytometric analysis.

The following mAbs were used: R-phycoerythrin (PE)-labeled RM4-5 (anti-CD4, Pharmingen), fluorescein isothiocyanate (FITC)-labeled 53-6.7 (anti-CD8a, Pharmingen), TRI-COLOR (TC)-labeled YTS 169.4 (anti-CD8a, Caltag), PE-labeled MR5-2 (anti-Vβ8.1,8.2, Pharmingen), biotin and FITC-labeled 1B2 [anti-2C TCR clonotypic, (96)], FITC-labeled HL3 (anti-CD11c, Pharmingen), PE-labeled AF6-120.1 (anti-I-A^b, Pharmingen), FITC-labeled 30-H12 (anti-Thy-1.2, Pharmingen), FITC-labeled M1/70 (anti-Mac-1, Pharmingen), MTS5 (anti-thymic epithelium, Pharmingen) and FITC-labeled G53-238 (anti-rat IgM, Pharmingen). Thymocyte and lymphocyte suspensions were prepared from cultured lobes or dissected tissue and analyzed as described previously (60, 62).

Cell isolation and RTOC.

Reaggregated thymic organ culture was performed as described previously (97). *TAP1*$^{-/-}$ and B6 thymic stromal cells were prepared by disaggregating deoxyguanosine-

(dGuo, Sigma) treated fetal thymic lobes using 0.05% trypsin (Gibco), 0.02%EDTA in Ca^{2+} and Mg^{2+}-free HBSS (GIBCO). The resulting cells were analyzed with anti I-A, anti-Mac-1 (macrophage specific), anti-CD11c (dendritic cell specific), anti-thymic epithelium (TE), and anti-Thy-1.2 antibodies. They were found to be approximately 80% $I\text{-}A^+$, 80% TE^+ 5% $MAC\text{-}1^+$, 10% $Thy\text{-}1.2^+$ and less than 1% $CD11c^+$, and are referred to as thymic stromal cells or thymic epithelial cells.

$CD4^+$ $CD8^+$ ("double-positive") $2C^+$ thymocytes were obtained by gently grinding freshly isolated newborn 2C transgenic *TAP1*$^{-/-}$ thymus lobes. The resulting suspensions were enriched for double-positive 2C cells using anti-CD4-biotin coupled to streptavidin bound magnetic microbeads (Miltenyi) and purity was checked by FACS analysis (usually > 95%). Reaggregates were formed by mixing together the desired stromal cells and double-positive thymocytes (at a ratio of 1:2) and pelleting by gentle centrifugation. After removal of the supernatant, the pellet was dispersed into a slurry, drawn into a fine glass pipette and placed as a standing drop on the surface of a nucleopore filter. Cultures were incubated for three days at 37°.

Peptides

All peptides were synthesized by the Biopolymers Laboratory (Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, Massachusetts). All were purified by reverse phase high pressure liquid chromatography (HPLC) and the concentration determined by quantitative Ninhydrin colorimetric assay or by submission of a sample to amino acid analysis. All peptides used were over 99% pure as determined by HPLC analysis. Table 3-1 lists the peptide sequences and the relevant references as appropriate.

Proliferation assay

Thymocyte suspensions were prepared from 2C RTOC that had been incubated with or without peptide as indicated in Figure 3-5. The cell suspension were divided into two sets of triplicate wells (1 x 10^4 cells/well) and incubated with or without 10^{-4}M p2Ca peptide and T2-L^d APC (1 x 10^4 cells/well) for 36 hr in RPMI, 10% FCS medium. Cultures were pulsed with tritiated thymidine (1 μCi per well) for 16 hr, the cells in the wells were harvested, and the amount of radioactivity was determined by detection of β particle emission. The net amount of reactivity is the difference between the cells from a given RTOC incubated with and without peptide during the three day proliferation experiment.

Contributions

All work presented in the work was performed by the author.

Chapter 4 Positive and negative selection are sensitive to TCR density and ligand affinities.

Introduction

In addressing the issues of thymic selection, much of the focus has been on the contribution of peptides and MHC proteins to the outcome of T cell development. This naturally followed from the development of uniquely amenable experimental systems made possible with transgenic and gene-knockout mice. TCR-transgenic mice allow for the study of homogeneous populations of T cells. Targeted disruptions in the β_2-microglobulin and *TAP1* genes have each created thymic environments in which class I MHC expression is not merely deficient, but is also restorable with exogenously added peptides. While this allows for a high degree of antigen manipulation, the T cell receptor itself has so far remained relatively unmolested by such experimentation.

To explore the contribution of cell-surface TCR to T cell development, we took advantage of 1B2, a monoclonal antibody that recognizes the 2C TCR clonotype. The target epitope of this antibody is the combined surface of the TCR α and β chains, which allows for its exquisite sensitivity and enables the antibody to block TCR engagement with peptide–MHC complexes. With this antibody, we determined the number of surface 2C TCR on immature 2C thymocytes, resting 2C splenocytes and activated 2C CTL. Through the addition of 1B2 to RTOC, we manipulated the number of 2C TCRs available for engagement by peptide–MHC. We found that just as the amount of ligand determines the fate of an immature thymocyte, so does the amount of TCR available for engagement by peptide–MHC.

Results

Determination of 2C TCR Density on the Cell Surface

To get an idea of the absolute number of TCRs involved in selection, ^{125}I-labeled 1B2 Fab′ was used to bind to 2C TCRs on the surface of immature DP 2C thymocytes, $CD8^+$ 2C thymocytes and resting $CD8^+$ 2C splenocytes. Extrapolation from the Scatchard plot of bound / free vs. free Fab′ revealed the number of 2C receptors per cell. Table 4-1 shows the results of several experiments. Immature thymocytes expressed approximately 5,000 receptors on their surface, whereas mature cells expressed a 10-fold greater number (40,000-60,000).

Table 4-1 Number of transgenic 2C TCR on immature ($CD4^+$ $CD8^+$) and mature ($CD4^-$ $CD8^+$) T lymphocytes

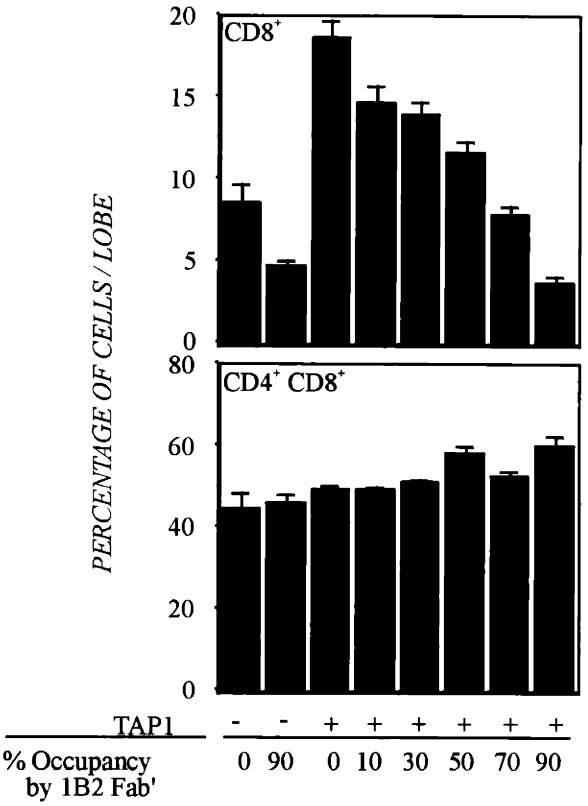
$CD4^+$ $CD8^+$ $2C^+$ thymocytes	$CD4^-$ $CD8^+$ $2C^+$ thymocytes	$CD4^-$ $CD8^+$ $2C^+$ splenocytes
5,385 ± 428	61,363 ± 1,000	38,500 ± 8,500

Average values and standard deviations derived from the results of at least two independent experiments are shown. See materials and methods for more details.

Blocking of TCR by clonotypic Fab′ reduces positive selection of $CD8^+$ 2C cells in H-2^b RTOC

To determine if positive selection is dependent on TCR density and to get an idea of the number of receptors required for positive selection, I performed *TAP1*$^+$ RTOCs in which we included different concentrations of Fab′ fragments from the antibody 1B2. From the total number of transgenic 2C TCRs on these thymocytes (n_t, Table 4-1), the equilibrium binding constant of Fab′ 1B2 for 2C TCR (K) and the free concentration (essentially equal to the total concentration) of the Fab′ fragments, I calculated the

Figure 4-1 Blocking of TCR by clonotypic Fab' reduces positive selection of CD8$^+$ 2C cells in H-2^b RTOC



RTOCs consisting of either H-2^b *TAP1*$^{+/+}$ or H-2^b *TAP1*$^{-/-}$ epithelial cells and 2C$^+$ CD4$^+$ CD8$^+$ thymocytes were carried out with or without added 1B2 Fab′ as indicated. Cell suspensions were prepared and analyzed with anti-CD8–FITC and anti-CD4–PE. Concentrations of added Fab′ were determined as indicated in the text. Bar levels indicate the percentage ± SD (n=3-5).

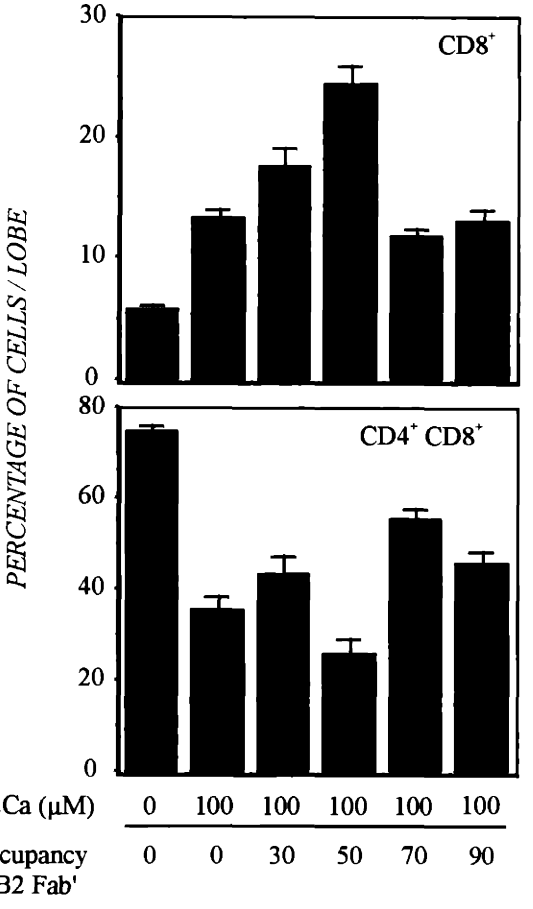
average number of occupied receptors per cell (n_{occ}) using the Karush form of the law of mass action (98):

$$n_{occ} = n_t \cdot K \cdot C / (1 + K \cdot C)$$

The number of accessible 2C receptors per cell is (n_t-n_{occ}). The blocking of only about 10% of the available receptors results in a slight decrease in positive selection while blocking of 90% abrogates positive selection. The reduction in positive selection was indicated by a decrease in the number and percentage of CD8$^+$ cells and a

complementary decrease in DP cells (Figure 4-1). These results show that positive selection is sensitive to the total number of available TCR molecules on the immature thymocyte.

Figure 4-2 Reducing the available TCR on double-positive cells changes the outcome of selection



RTOCs consisting of H-2^b *TAP1*$^{-/-}$ epithelial cells and 2C$^+$ CD4$^+$ CD8$^+$ thymocytes were carried out in the presence or absence of 100 μM p2Ca and with or without added 1B2 Fab′ as indicated. Analysis was as in Figure 4-1. Bar levels indicate the average percentage ± SD (n=3-5) of the given cell type.

Reducing the available TCR on double-positive cells changes the outcome of selection

Since the outcome of selection depends on ligand density on APCs, it is of interest to determine if it depends on receptor density as well. To show this, I carried out RTOCs

using *TAP1*$^{-/-}$ stromal cells to which p2Ca peptide was added at 100μM, a level at the boundary of positive and negative selection (Figure 3-4). In these cultures, I added increasing amounts of 1B2 Fab′ to block increasing numbers of 2C TCR, (Figure 4-2). While I observed a greater number of 2C CD8$^+$ cells in the presence of peptide than in its absence ("no peptide" control), I saw an even greater number with both peptide and 4nM Fab′, corresponding to an average occupancy of 50%. These data indicate that just as selection depends on ligand density, it also depends on TCR density. All of which implies a dependency on the number of ligations as a determinate factor in positive and negative selection.

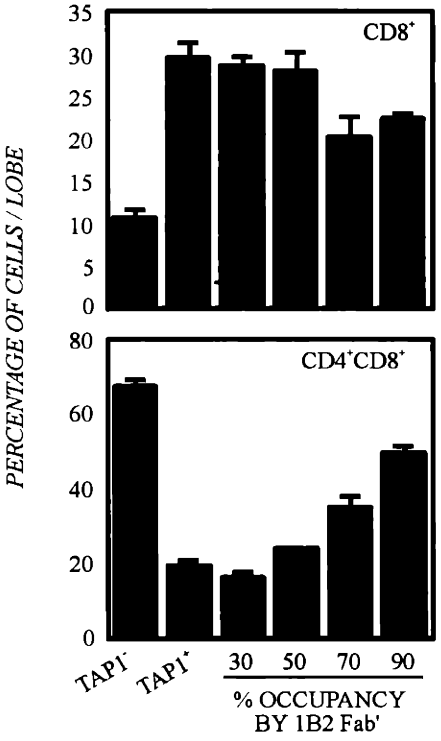
CD8$^+$ 2C cells are positively selected in H-2^d TAP1$^+$ RTOC and this selection is reduced by anti-clonotypic Fab′

As mentioned previously, 2C cells have been shown to be negatively selected in the thymus of H-2^d mice, presumably because of the expected high avidity interactions between the 2C thymocytes and the thymic stromal cells expressing p2Ca-L^d. However, in H-2^d RTOC in which dGuo-sensitive stromal cells are largely absent, positive selection of these thymocytes occurred.

Thus, more CD8$^+$ 2C cells were in H-2^d *TAP1*$^+$ RTOC than in H-2^b *TAP1*$^{-/-}$ controls (Figure 4-3). Again, positive selection was reducted with the addition of 1B2 Fab′, confirming that the CD8$^+$ 2C cells are indeed generated by positive selection via an interaction with the 2C TCR. However, only a modest decrease of CD8$^+$ cells with a complementary increase in DP cells was observed, even at the highest concentrations of 1B2 Fab′ tested (compare Figure 4-3 with Figure 4-2). Assuming that the high affinity ligand p2Ca-L^d, which is known to be present on H-2^d thymic stromal cells, is responsible for this selection, it is logical that a higher degree of occupancy of the TCRs was required

to modulate the selection in H-2^d RTOC than in H-2^b RTOC. These data again indicate that a high affinity ligand has the capacity to induce positive selection and that a lower density of TCR is required to attain a given level of positive selection with a higher affinity ligand.

Figure 4-3 CD8$^+$ 2C cells are positively selected in H-2^d *TAP1*$^+$ RTOC and this selection is reduced by anti-clonotypic Fab'



RTOCs consisting of either H-2^d *TAP1*$^{+/+}$ or H-2^b *TAP1*$^{-/-}$ epithelial cells and 2C$^+$ CD4$^+$ CD8$^+$ thymocytes were carried out with or without added 1B2 Fab′ as indicated. Cell suspensions were prepared and analyzed with anti-CD8–FITC and anti-CD4–PE. Concentrations of added Fab′ were determined as indicated in the text. Error bar levels indicate the percentage ± SD (n=3-5).

Discussion

The principal observation made here is that, just as altering the amounts of peptide ligand alters the outcome of thymic development (i.e., either positive or negative selection), altering the amount of available TCR has a similar effect. Blocking TCR does not merely reduce the efficiency of positive selection and negative selection, but can effectively alter the fate of a thymocyte and allow it to mature (positive selection) rather than die (negative selection). One implication of this is that thymocytes that somehow down-regulate the expression of a potentially autoreactive TCR could avoid negative selection and become positively selected instead.

As T cells mature, they up-regulate the surface expression of their TCR (15, 36). The rationale behind such developmental regulation is not clear. If we assume the law of mass action is applicable (99) to T cell–APC interactions, we can express them as follows:

$$[TCR] + [ligand] \rightleftarrows [TCR \cdot ligand]. \qquad \text{(Eq. 4-1)}$$

Logic suggests that the overall avidity of interactions resulting in positive selection is less than the avidity involving a peripheral response to antigen (e.g., positive selection < negative selection ≤ peripheral response) to avoid the survival of potentially autoreactive T cells. If the number of TCR engaged is the determining factor, than a lower TCR density would necessitate a higher ligand density and/or a higher affinity for the ligand. At first glance, this seems counterintuitive. A mature cell expressing a higher density of TCR might react to the same self-peptide–MHC complex in the periphery that positively selected it in the thymus. Fortunately specialized thymic APCs are present that help protect against this outcome [(49, 94); and chapter 5]. Thymic development allows only

an exquisitely small fraction of thymocytes to mature (49). The low levels of TCR on immature cells would encourage the development of T cells with a higher avidity for self-MHC. One advantage of this may be to prevent the development of T cells bearing TCR with affinities for MHC below a useful threshold.

It is quite interesting to note that blocking merely 10% (or 500 out of 5,000) of the available TCR results in a reduction of positive selection on H-2^b stromal cells. The peripheral response to antigen can involve very few ligands, fewer than 100 / APC, in contrast to the 50,000 to 100,000 TCRs (Table 4-1) on the T cell surface (100-102). If it only takes such an apparently small fraction of receptor ligations (maximum of 10^2 out of 10^5) to initiate a peripheral response, then we would expect that these T cells should be positively selected with an even smaller fraction of ligations. The finding that the blocking of only 500 out of 5,000 receptors results in significant blocking of selection of 2C T cells suggests that a large fraction of receptors must be available for positive selection to take place. A simple interpretation of this result is that in this situation the amount of available TCR is limiting in Equation 4-1. Thus any reduction in the amount of TCR available for useful engagement is detectable as a reduction in efficiency of positive selection. If one accepts the assumption that the signal for positive selection should be weaker than that for peripheral activation and combines that with the observations that peripheral activation involves fewer than 100 engagements (100-102), possibly fewer than 10 (103), and that the blocking of a small fraction of the available TCR reduces the efficiency of positive selection, then one can reasonably conclude that positive selection normally involves only one or critically few TCR engagements. Even though a thymocyte expresses a potentially useful TCR, not every encounter with thymic

epithelial cells may be productive and the thymocyte may naturally expire before maturation. However, this apparent inefficiency could be compensated by the ongoing generation of new T cells that might duplicate a useful TCR specificity and mature successfully. In a TCR-transgenic animal, many T cells of identical specificity arise simultaneously, but not every transgenic cell will receive the signal to mature. By interfering with the TCR–ligand interaction (e.g., by blocking with 2C TCR specific Fab′), we essentially reduced the percentage of transgenic cells that successfully matured.

Materials and methods

Mice

C57BL/6 (B6) mice were purchased from Taconic Farms. DBA/2 mice were purchased from Jackson Laboratories. *TAP1* knockout (62) and 2C TCR-transgenic mice (36) were bred in our colony (Center for Cancer Research, MIT).

Flow cytometric analysis

The following mAbs were used: R-phycoerythrin (PE)-labeled RM4-5 (anti-CD4, Pharmingen), fluorescein isothiocyanate (FITC)-labeled 53-6.7 (anti-CD8a, Pharmingen), PE-labeled MR5-2 (anti-Vβ8.1,8.2, Pharmingen), biotin-labeled 1B2 [anti-2C TCR clonotypic, (96)], FITC-labeled HL3 (anti-CD11c, Pharmingen), PE-labeled AF6-120.1 (anti-I-A^b, Pharmingen), PE-labeled AMS-32.1 (anti-I-A^d, Pharmingen), FITC-labeled 30-H12 (anti-Thy-1.2, Pharmingen), FITC-labeled M1/70 (anti-Mac-1, Pharmingen), MTS5 (anti-thymic epithelium, Pharmingen) and FITC-labeled G53-238 (anti-rat IgM, Pharmingen). Thymocyte and lymphocyte suspensions were prepared from cultured lobes or dissected tissue and analyzed as described previously (60, 62).

Cell isolation and RTOC

Reaggregated thymic organ culture experiments were performed as described

previously (97). *TAP1*$^{-/-}$, B6, and DBA/2 thymic epithelial cells (MHC haplotype H-2^b, H-2^b, and H-2^d accordingly) were prepared by disaggregating deoxyguanosine- (dGuo, Sigma) treated fetal thymic lobes using 0.05% trypsin (Gibco), 0.02%EDTA in Ca^{2+} and Mg^{2+}-free Hanks balanced salt solution (Gibco). The resulting cells were analyzed with anti-I-A, anti-Mac-1 (macrophage specific), anti-CD11c (dendritic cell specific), anti-thymic epithelium (TE), and anti-Thy-1.2 antibodies. They were found to be approximately 80% I-A$^+$, 80% TE$^+$ 5% Mac-1$^+$, 10% Thy-1.2$^+$ and less than 1% CD11c$^+$, and are referred to as thymic epithelial cell (TEC).

CD4$^+$ CD8$^+$ ("double-positive") 2C$^+$ thymocytes were obtained by gently grinding freshly isolated newborn 2C transgenic *TAP1*$^{-/-}$ thymus lobes. The resulting suspensions were enriched for double-positive 2C cells with biotinylated anti-CD4 antibody coupled to streptavidin-bound magnetic microbeads (Miltenyi); their purity, checked by FACS analysis was usually more than 95%. Reaggregates were formed by mixing together the desired epithelial cells and double-positive thymocytes (at a ratio of 1:2). After pelleting the cell mixture by gentle centrifugation, and removing the supernatant, the pellet was dispersed into a slurry, drawn into a fine glass pipette, and placed as a standing drop on the surface of a nucleopore filter suspended over complete medium (1640 RPMI supplemented with 10% fetal calf serum) with or without added peptide or 1B2 Fab′. Cultures were incubated for three days at 37°C.

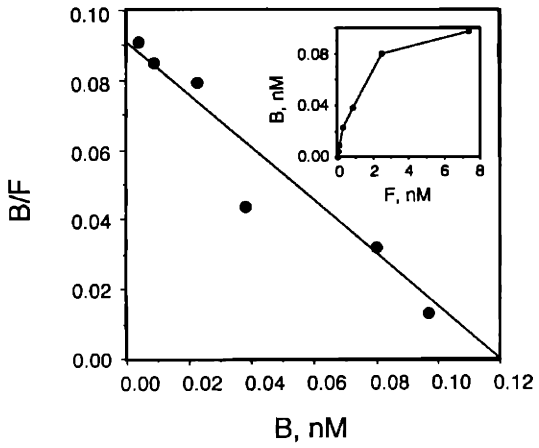
Isolation of cells from adult tissue was carried out as described previously (62). Cells were isolated from thymi of 2C transgenic and 2C transgenic, *TAP1*-deficient mice to obtain CD4$^-$ CD8$^+$ 2C$^+$ and CD4$^+$ CD8$^+$ 2C$^+$ thymocytes, respectively. Cells were isolated from spleens of 2C transgenic mice to obtain CD4$^-$ CD8$^+$ 2C$^+$ splenocytes. In each case, cells were labeled with anti-CD4 and anti-CD8 mAb and passed through a FACStar Plus™ (Becton Dickinson) and sorted for desired population of cells. An aliquot of

sorted cells was labeled with anti-CD4, anti-CD8 and anti-2C TCR mAb and analyzed again by FACS. In each case, at least 95% of the cells were of the desired type.

Peptides

All peptides were synthesized by the Biopolymers Laboratory (Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, Massachusetts). All were purified by reverse phase high pressure liquid chromatography (HPLC) and the concentration determined by quantitative Ninhydrin colorimetric assay or by submission of a sample to amino acid analysis. All peptides used were over 99% pure as determined by HPLC analysis. Table 3-1 lists the peptide sequences and the relevant references where appropriate.

Determination of 2C TCR density on cell surfaces



Various concentrations of ^{125}Fab′ 1B2 fragments and from 1 x 10^6 to 6 x 10^6 cells were combined in 100 µl of complete media and incubated in capped pre-siliconized tubes for an hour at room temperature (22-25°C). Cell-bound and unreacted ^{125}I-Fab′ were separated after rapid (10-30 sec.) centrifugation of the cell suspension through a layer of 84% silicone and 16% paraffin oil for radioactivity measurements (85). Titrations were carried out in duplicates. Non-specific binding of ^{125}I-FAB′ was estimated in the presence of 100-fold excess of unlabelled 1B2 antibody and did not exceed 5-10%. Concentrations of bound and free ^{125}I-Fab′ were used to produce Scatchard plots from which extrapolation to the x-intercept yielded numbers of 2C TCR per cell.

Contributions

The work presented here was performed in collaboration with Dr. Yuri K. Sykulev, a post-doctoral associate formally of Professor Herman N. Eisen's laboratory. Yuri generated the 1B2 FAB′ fragments used in these experiments, measured the TCR surface densities, and instructed the author in these techniques. All the other work was performed by the author.

Chapter 5 Differences in the level of expression of class I MHC proteins on thymic epithelial and dendritic cells influence the decision of immature thymocytes between positive and negative selection.

Introduction

Studies of T cell development indicate that T cell maturation in the thymus is largely driven by the extent to which antigen-specific receptors (T cell receptors or TCR) on immature thymocytes are engaged by peptide–MHC complexes on thymic antigen-presenting cells (APC) (42). When TCR interact with complexes that are present at relatively low levels, the corresponding thymocytes are stimulated to proceed along the maturation pathway (positive selection); if, however, the complexes are relatively abundant the thymocytes undergo cell death (negative selection). The thymic APC have also been the subject of many studies. Most reports indicate that positive selection is promoted by thymic epithelial cells while negative selection is mediated largely by haemopoietic dendritic cells (DC) (94). Studies of thymocyte maturation in the absence of thymic DC indicate that some T cells can be positively selected by the thymic epithelial cells even when they would otherwise be negatively selected by DC (104). In an attempt to understand the basis for the different outcomes mediated by thymic epithelial and dendritic cells, we used reaggregated thymic organ cultures to investigate cellular requirements for the selection of T cells that express the antigen specific receptor (TCR) of the $CD8^+$ CTL clone known as 2C.

The 2C clone was generated from an $H\text{-}2^b$ mouse (Balb.B) that had been immunized with $H\text{-}2^d$ bearing cells (96). It recognizes the allogeneic class I protein L^d in association

with a naturally processed peptide, LSPFPFDL (p2Ca) (84). The class I protein K^b has been identified as the positively selecting restriction element (47) and the 2C CTL line was also found to recognize the p2Ca peptide in association with K^b, albeit weakly (85, 105). More recently another naturally processed peptide, EQYKFYSV (dEV8), that can be recognized weakly in association with K^b by the 2C TCR has been identified (106).

In this study we followed the transition of double-positive ($CD4^+$, $CD8^+$) to single positive ($CD4^-$, $CD8^+$) 2C TCR^+ thymocytes from peptide-transport defective (*TAP1*$^{-/-}$) mice, in the presence of various concentrations of peptides, and APC with different MHC haplotypes. Our results encompass three principal findings. First, while the presence of the allogeneic MHC protein L^d in the thymus normally results in negative selection of 2C thymocytes, we show here that these cells are positively selected by L^d bearing thymic epithelial cells, if L^d bearing dendritic cells are absent. Second, we have identified a remarkably small range (three-fold or less) of cell surface peptide–MHC (epitope) densities on thymic epithelial cells that define the "epitope density threshold" amount of ligand above which thymocytes are negatively selected and below which they are positively selected. Finally, we found a 10-fold higher expression of class I MHC on thymic dendritic cells than on thymic epithelial cells and propose that this difference contributes to the disparate roles these cells play in thymocyte selection

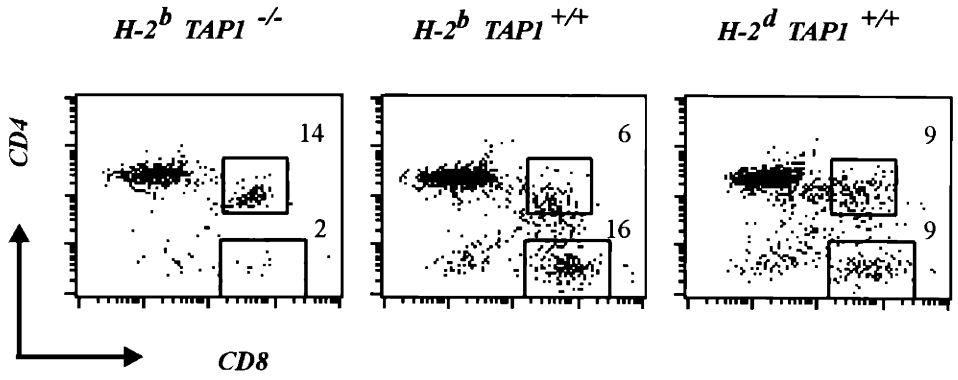
Results

Positive selection of 2C cells by alloantigen in reaggregation cultures in the absence of thymic dendritic cells

Thymocytes carrying the 2C TCR have been shown to be negatively selected in the thymus of H-2^d mice (36). To identify the thymic cells involved in 2C cell selection we

set up reaggregated thymic organ cultures (RTOC). These cultures consisted of two cell populations: (i) immature $CD4^+$ $CD8^+$ 2C thymocytes (>95% pure), and (ii) cells from deoxyguanosine treated thymic lobes, which consist of trypsinized thymic stromal cells (largely TEC) depleted of dendritic cells. The epithelial cells were obtained from H-2^d, H-2^b and *TAP1*$^{-/-}$, i.e., MHC class I deficient (62), H-2^b fetuses.

Figure 5-1 Flow cytometric analysis of positive selection of 2C cells in H-2^d *TAP1*$^{+/+}$ RTOC

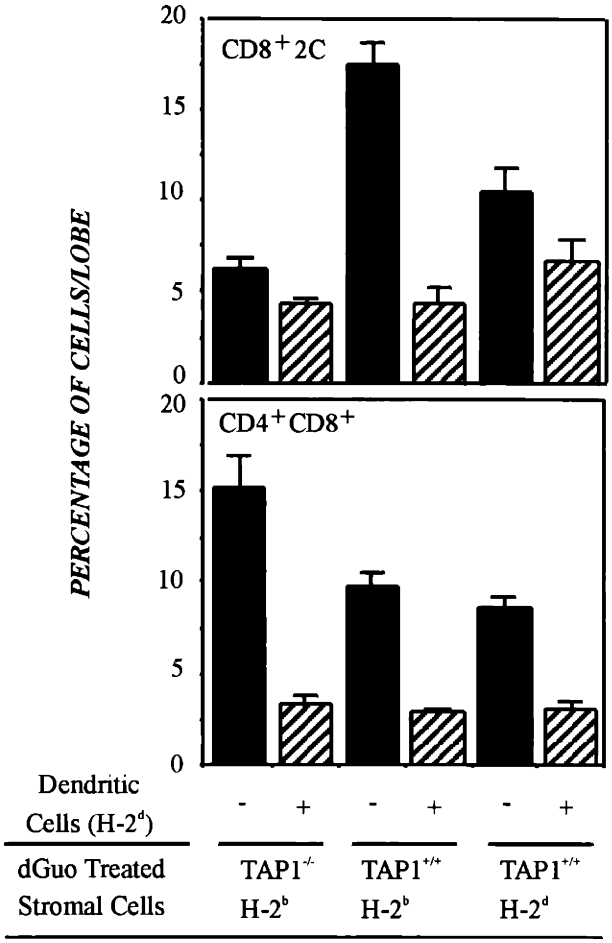


$CD8^+$ 2C cells are positively selected in H-2^d *TAP1*$^{+/+}$ RTOC. Reaggregate thymic organ cultures consisting of H-2^b *TAP1*$^{-/-}$, H-2^b *TAP1*$^{+/+}$ or H-2^d *TAP1*$^{+/+}$ epithelial cells and $2C^+$ $CD4^+$ $CD8^+$ thymocytes. Numbers indicate percentage of cells in the respective gated areas. Similar numbers of thymocytes were recovered from each lobe.

As one should expect, *TAP1*$^{-/-}$ TEC did not induce either positive or negative selection of 2C TCR^+ immature T cells. Interestingly, we found that mature $CD8^+$ 2C cells were not only generated in RTOC having H-2^b epithelial cells as expected, but also in those with H-2^d epithelial cells (Figure 5-1). Positive selection was less pronounced in H-2^d

RTOC than in H-2^b RTOC, but was clearly above the background level seen in H-2^b *TAP1*$^{-/-}$ RTOC (Figs. 5-1 and 5-2). The observed positive selection was mediated by the TCR, since clonotypic anti-TCR Fab′ fragments (96) blocked the appearance of mature $CD8^+$ 2C cells in the RTOC (data not shown). These findings suggest that the negative

Figure 5-2 Allogeneic dendritic cells mediate deletion of 2C cells in RTOC



Allogeneic dendritic cells mediate deletion of 2C cells in RTOC. Reaggregate thymic organ cultures consisting of dGuo-treated epithelial cells from H-2^b *TAP1*$^{-/-}$, H-2^b *TAP1*$^{+/+}$, or H-2^d *TAP1*$^{+/+}$ mice, and 2C$^+$ CD4$^+$ CD8$^+$ thymocytes, with or without the addition of H-2^d *TAP1*$^{+/+}$ spleen-derived dendritic cells, were cultured and analyzed as before. Where indicated, the equivalent of 1.5 x 10^4 dendritic cells per lobe was added to equal 5% of the total epithelial cell number. The total mean number of thymocytes recovered from each lobe (n=4) in Figure 2 reading from left to right was (x10^{-3}) 9.0, 10.9, 10.1, 9.4, 2.3 and 4.5, respectively. Error bars indicate the standard

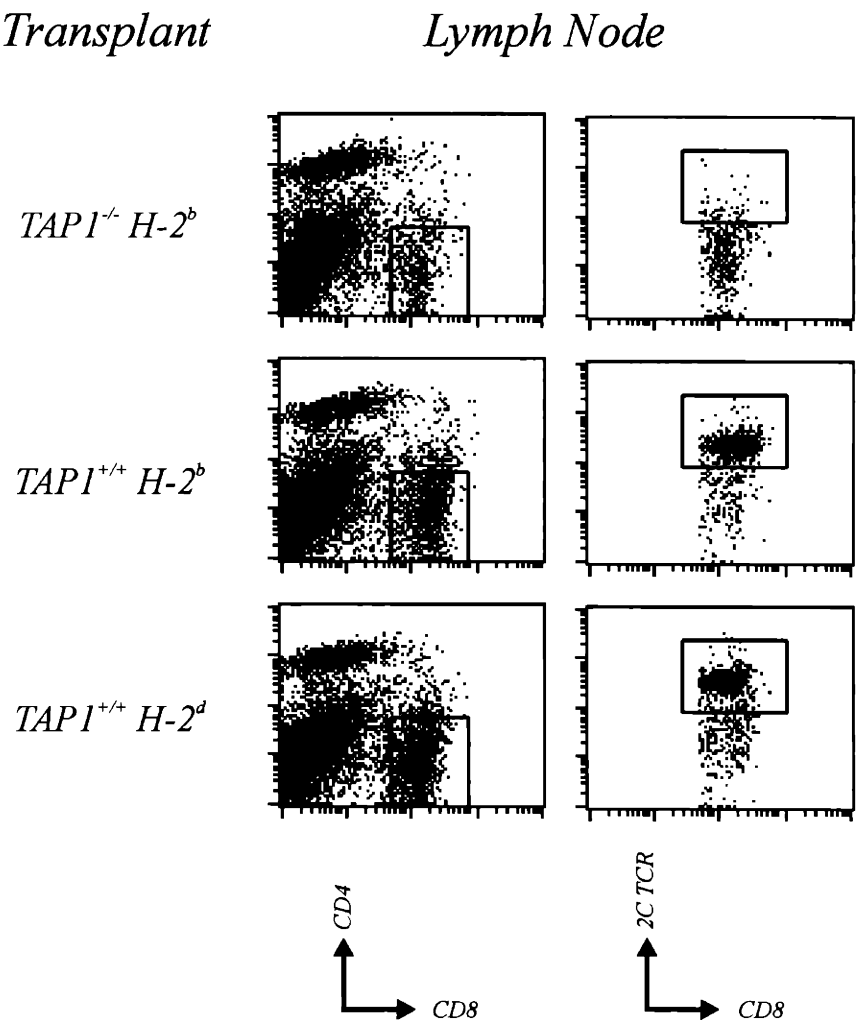
selection of 2C cells that is observed *in vivo* in H-2^d mice is largely mediated by deoxyguanosine sensitive cells, presumably thymic dendritic cells. To examine this

possibility, we set up the same RTOC as above and added dendritic cells from spleens of H-2^d mice. As shown in Figure 5-2, the dendritic cells promoted deletion of 2C cells in all cultures. We conclude that 2C cells are positively selected by thymic epithelial cells, which are deoxyguanosine insensitive, and negatively selected by H-2^d dendritic cells.

Positive selection of 2C cells in vivo by alloantigen in absence of class I$^+$ dendritic cells

To exclude the possibility that the positive selection of 2C cells by H-2^d epithelial cells was an artifact of the reaggregation cultures, we examined the selection of 2C thymocytes *in vivo* in thymectomized mice that were transplanted with intact deoxyguanosine treated lobes. The same types of deoxyguanosine-treated thymus lobes that had been used for the RTOCs (H-2^d, H-2^b, *TAP1*$^{-/-}$ H-2^b) were transplanted to thymectomized H-2^b mice that had been irradiated and reconstituted with 2C transgenic, *TAP1*$^{-/-}$ bone marrow cells. The bone marrow derived dendritic cells were therefore deficient in MHC class I expression and of the b haplotype, and thus should have no (or minimal) impact on selection. Three months after transplantation very few CD8$^+$ 2C cells were found in the lymph nodes of mice bearing H-2^b *TAP1*$^{-/-}$ thymus lobes (Figure 5-3) or mice that had not received any thymus lobes (data not shown). However, a considerable number of CD8$^+$ 2C$^+$ cells accumulated in the lymph nodes of mice receiving intact H-2^b or H-2^d thymus lobes. These CD8$^+$ 2C cells did not express the activation markers CD25 or CD44 (data not shown), indicating that they have been selected positively but have not been significantly expanded in the periphery by antigenic stimulation.

Figure 5-3 Allogeneic thymic epithelial cells positively select 2C cells *in vivo*



Lymph nodes were collected and analyzed (see methods) from animals having received either A. *TAP1*$^{-/-}$ H-2^{b}, B. *TAP1*$^{+/+}$ H-2^{b} or C. *TAP1*$^{+/+}$ H-2^{d} thymic transplants. The high proportion of CD4^{+} cells observed may be due either to the expansion of some mature CD4 cells present in the transplanted bone marrow or of cells that arose from some surviving host bone marrow. For each type of transplant two mice were studied with similar results.

Differences in the level of expression of class I MHC proteins on thymic epithelial and dendritic cells

Many endogenous peptides are likely to be generated at the same levels in different cells (107), and it is conceivable that differential expression of MHC, and therefore of cell surface densities of peptide–MHC complexes, may be responsible for the opposing

roles that epithelial and dendritic cells play in positive and negative selection, respectively. Dendritic cells are well known to express MHC proteins at high levels (104). However, a direct quantitative comparison of MHC expression on thymic epithelial and dendritic cells has not been reported. Using ^{125}I labeled antibodies specific for class I proteins, we determined the number of K^b and L^d MHC molecules on thymic dendritic cells and dGuo-resistant thymic epithelial cells. Both proteins were found at about 10-fold higher densities on thymic dendritic cells ($2.8x10^5$/cell) than on epithelial cells ($2.5x10^4$/cell) (Table 5-1). We also examined the number of K^b MHC molecules on splenic dendritic cells and found that these cells express class I MHC proteins at an intermediate level (Table 5-1), which is consistent with previous observations made with flow cytometry (108).

Table 5-1. dGuo-resistant thymic epithelial cells express fewer class I molecules than thymic and splenic dendritic cells.

Thymic Epithelial Cells		Thymic Dendritic Cells		Splenic Dendritic Cells
K^b	L^d	K^b	L^d	K^b
25,581 ± 8,907	26,513 ± 1,216	375,553 ± 140,195	212,600 ± 43,439	132,994 ± 30,064

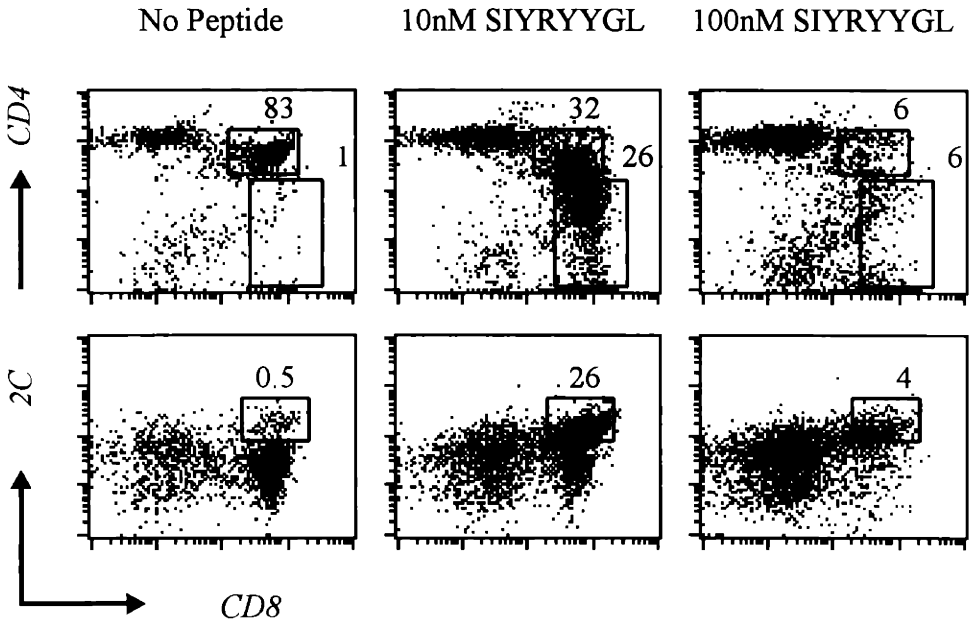
Average No. MHC class I molecules per cell and standard deviations shown are based on at least 2 independent measurements.

Peptide induced class I MHC expression required for positive and negative selection

Both positive and negative selection of 2C thymocytes can be achieved with the addition of appropriate amounts of peptide ligand. Figure 5-4 shows FACS analyses of RTOCs in which optimal positive and negative selection of 2C cells occurred at 10nM and 100nM of SIYRYYGL peptide (87), respectively, whereas no selection occurred without added peptide or with an irrelevant peptide (data not shown). The concentrations were determined from several peptide titration experiments. Peptide concentrations were

considered to be optimal for positive selection at the dose at which the most $CD8^+$ 2C T cells developed and optimal for negative selection at the lowest dose that eliminated nearly all double-positive cells and resulted in near background levels of $CD8^+$ 2C T cells. Two other peptides were also found to elicit positive and negative in RTOC although at very different concentrations. The peptide p2Ca (LSPFPFDL) could positively and negatively select at 30 and 500 μM, respectively, whereas dEV8

Figure 5-4 SIYRYYGL positively and negatively selects 2C cells in *TAP1*$^{-/-}$ RTOC



Reaggregate thymic organ cultures consisting of H-2^b *TAP1*$^{-/-}$ epithelial cells and $2C^+$ $CD4^+$ $CD8^+$ thymocytes with or without positively and negatively selecting concentrations peptide as indicated. Numbers indicate the percentage of cells in the respective gated areas.

(EQYKFYSV) did so at 100 and 3000 μM (data not shown). We compared the levels of peptide stabilized K^b on purified thymic epithelium at these concentrations, using ^{125}I-labelled antibody specific for K^b. The increase in the number of peptide-stabilized K^b complexes from positively to negatively selecting concentrations of peptide was only 3-

fold for p2Ca and even less for dEV8 (Table 5-2). We could not reliably detect SIYRYYGL-induced K^b expression above the background level with the peptide at a total concentration of either 10 or 100nM (Table 5-2). Thus as little as a three-fold difference in the density of a particular peptide–MHC ligand on the APC surface can determine whether a cell is positively or negatively selected.

Table 5-2 Level of K^b expression on *TAP1*$^{-/-}$ dGuo-resistant thymic epithelial cells in the presence and absence of exogenously added peptides

Peptide	Conc. (M)	Thymic selection[1]	No. peptide-induced K^b mol. per cell
none	none	none	none
LSPFPFDL	$3x10^{-5}$	positive	718 ± 428
LSPFPFDL	$5x10^{-4}$	negative	2,318 ± 1,243
EQYKFYSV	$1x10^{-4}$	positive	12,400 ± 1,500
EQYKFYSV	$3x10^{-3}$	negative	18,900 ± 4040
SIYRYYGL	$1x10^{-8}$	positive	ND
SIYRYYGL	$1x10^{-7}$	negative	ND

Average numbers and standard deviations shown are based on at least 3 independent measurements. ND = not detectable by assay. [1]As observed in independent RTOC experiments.

Discussion

Studies of thymocyte development have shown that the fate of double-positive thymocytes can be altered in peptide transport-deficient thymic organ cultures by varying concentrations of peptide, which presumably stabilize proportional amounts of MHC proteins on thymic APCs (42). At relatively low concentrations peptides promote positive selection and at high concentrations they lead to negative selection. These

effects can be accounted for by an "avidity" model which assumes that the cell's fate is determined by the extent to which its TCR are engaged by peptide–MHC complexes in the thymic environment (42). This view stresses the importance of the affinity of the developing cell's TCR for peptide–MHC complexes, and the abundance of these complexes in the thymic environment. At first glance, it does not account for the finding that different types of APC have divergent effects on thymocytes; i.e., thymic epithelial cells mediate positive selection and dendritic cells mediate negative selection (104). Indeed, thymic epithelium bearing H-2 L^d, an alloreactive restriction element for the 2C TCR, will positively select 2C thymocytes, whereas H-2 L^d bearing dendritic cells will induce negative selection both *in vitro* and *in vivo* (Figures 5-1 to 5-3). The disparate effects of epithelial and dendritic cells have been attributed to differences in various cell surface molecules involved in cell-cell interactions, such as LFA-1/ICAM-1 (109-111), CD28/B7.1 (112, 113), CD40/gp39 (114, 115), Fas/FasL (116) and CD30/CD30L (117). Experiments with knockout mice and specific inhibitors have suggested a role for these molecules in thymic selection, but also revealed that they are not always absolutely necessary (109-118).

The most important finding in this study is that the cell surface level of class I MHC molecules on dendritic cells is approximately 10-fold higher than on epithelial cells (Table 5-1). Taken in conjunction with the avidity model, this finding could account for the different roles of these APC in shaping the TCR repertoire of newly developed single positive $CD8^+$ T cells. Thus, if the pool of peptides produced in these APC were similar, those with the higher total MHC level would be expected to have a correspondingly higher level of any particular peptide–MHC complex and to be more likely to elicit

negative selection than APC with lower levels. The MHC class I level on thymic dendritic cells is not only higher that that on thymic epithelial cells, but also higher than that on splenic dendritic cells (Table 5-1). It is conceivable that the very high MHC class I expression in the thymus increases the likelihood that potentially autoreactive immature T cells are eliminated by negative selection in the thymus. There are other precedents for the natural exploitation of differential MHC expression. The down regulation of MHC levels by a variety of mechanisms helps many cancer cells and virus-infected cells to escape destruction by cytotoxic T cells (119-121). Large differences in the levels of MHC proteins between cells in the periphery may favor antigen presentation by some cells and minimize the risks of inadvertent immune injury of other cells (122).

Although thymic dendritic cells are largely responsible for negative selection, it is important to note that negative selection can occur in their absence (94). Thus, using thymic cell preparations consisting essentially of just double-positive thymocytes and epithelial cells, we observed both positive and negative selection, depending on the concentration of added peptide. With this simplified system, we were able to examine closely the effects of small changes in the abundance of peptide-K^b complexes ("epitope density') on the surface of thymic epithelial cells. The results are of particular interest with peptides p2Ca and dEV8, as both are naturally processed peptides derived from ubiquitous proteins (84, 106). The finding that approximately 700 p2Ca-K^b complexes per cell was associated with positive selection of 2C TCR^+ thymocytes (Table 5-2) may be compared with an earlier estimate of about 100-200 p2Ca peptide molecules per mouse thymus cell (84). The former value (700 complexes) is probably an overestimate (since it is based on incubation of cells with peptide for 2 hours while the RTOC assays

were carried out over three days, during which time peptide concentrations drop, perhaps due to proteases in the culture medium), and the latter value (100-200 peptide molecules) was probably too low, as it did not take into account losses incurred during peptide isolation. It is thus possible that the p2Ca peptide normally exists in the thymus at a level where it contributes to positive selection under natural conditions. The natural abundance of the dEV8 peptide has not been estimated (106), but it is doubtful that it is anywhere near the levels needed to approach the epitope density values shown in Table 5-2.

Surprisingly, the difference between an epitope level that led to optimal positive selection and one that led to near total negative selection was only 3-fold in the case of the peptide p2Ca and even less for the peptide dEV8. These remarkably small differences suggest that we have defined for each of these peptides a narrow epitope density zone, below which thymocytes are stimulated to make the transition from double-positive to single positive cells and above which they are triggered to undergo cell death. If we consider the midpoint between positively and negatively selecting values for a given peptide as a "threshold" value for the corresponding peptide-K^b complex, we see that the threshold is about 1500 complexes per cell for the p2Ca peptide and around 16,000 complexes per cell for the dEV8 peptide. For the SIYRYYGL peptide, the epitope density threshold could not be defined as the concentrations used to elicit positive and negative selection were too low. It is apparent, nevertheless, that in terms of required peptide concentrations, the order of effectiveness was SIYRYYGL>p2Ca>dEV8. This order matches the efficacy of these peptides in cytolytic assays, where the respective concentrations required for half maximal lysis of optimal K^{b+} target cells by 2C TCR^+

clones under standard assay conditions is about 10^{-13}M, 10^{-6}M, and indeterminate ($>10^{-4}$M) for SIYRYYGL, p2Ca, and dEV8, respectively (Sykulev, *et al.* in preparation) .

For the SIYRYYGL and p2Ca peptides the differences are in accord with the large differences in the affinity of the 2C TCR for these peptide–MHC complexes. Measured on intact $CD8^+$ 2C cells, the values are $1x10^7M^{-1}$ for SIYRYYGL-K^b (Sykulev, *et al.* in preparation) and $3x10^3M^{-1}$ for p2Ca-K^b (85). The corresponding affinity value for dEV8-K^b has not been measured under the same conditions, but has recently been reported to be $1.2x10^4M^{-1}$, as determined by surface plasma resonance using immobilized 2C TCR and soluble dEV8-K^b complexes (90). Equilibrium constants measured by the two methods have so far differed considerably, being 10 to 300-fold higher on intact cells (presumably due to the contribution of CD8 coreceptors which are absent in the cell free system). Thus, the affinity of the 2C TCR on intact cells is likely to be much greater for dEV8-K^b than for p2Ca-K^b. Given this difference, it is notable that dEV8-K^b is far less effective than p2Ca-K^b in promoting the maturation of 2C thymocytes. A possible explanation is suggested by the relative instability of the complex formed by the 2C TCR with dEV8-K^b (37). If the corresponding TCR bond with p2Ca-K^b were more stable, the disparity between the epitope density thresholds for p2Ca-K^b and dEV8K^b might be accounted for. Alternatively, the explanation may be structural rather than kinetic: it is possible that different conformations of the TCR, resulting from ligation with different peptide–MHC ligands, could have confounding effects that are responsible for the dEV8/p2Ca disparity.

Materials and methods

Mice

C57BL/6 (B6) mice were purchased from Taconic Farms. DBA/2 mice were purchased from Jackson Laboratories. *TAP1* knockout (62) and 2C TCR-transgenic mice (36) were bred in our colony (Center for Cancer Research, MIT)

Flow cytometric analysis

The following mAbs were used: R-phycoerythrin (PE)-labeled RM4-5 (anti-CD4, Pharmingen), fluorescein isothiocyanate (FITC)-labeled H53-6.7(anti-CD8a, Pharmingen), PE-labeled MR5-2 (anti-Vβ8.1,8.2, Pharmingen), biotin-labeled 1B2 (anti-2C TCR clonotypic (96), FITC-labeled HL3 (anti-CD11c, Pharmingen), PE-labeled AF6-120.1 (anti-I-A^b, Pharmingen), PE-labeled AMS-32.1 (anti-I-A^d, Pharmingen), FITC-labeled 30-H12 (anti-Thy-1.2, Pharmingen), FITC-labeled M1/70 (anti-Mac-1, Pharmingen), MTS5 (anti-thymic epithelium, Pharmingen) and FITC-labeled G53-238 (anti-rat IgM, Pharmingen). Thymocyte and lymphocyte suspensions were prepared from cultured lobes or dissected tissue and analyzed as described previously (60, 62).

Cell isolation and RTOC

Reaggregated thymic organ culture experiments were performed as described previously (97). *TAP1*$^{-/-}$, B6, and DBA/2 thymic epithelial cells (MHC haplotype H-2^b, H-2^b, and H-2^d accordingly) were prepared by disaggregating deoxyguanosine- (dGuo, Sigma) treated fetal thymic lobes using 0.05% trypsin (Gibco), 0.02%EDTA in Ca^{2+} and Mg^{2+} -free Hanks balanced salt solution (Gibco). The resulting cells were analyzed with anti-I-A, anti-Mac-1 (macrophage specific), anti-CD11c (dendritic cell specific), anti-thymic epithelium (TE), and anti-Thy-1.2 antibodies. They were found to be approximately 80% I-A^+, 80% TE^+ 5% Mac-1^+, 10% Thy-1.2^+ and less than 1% CD$11c^+$, and are referred to as thymic epithelial cell (TEC).

$CD4^+CD8^+$("double-positive") $2C^+$ thymocytes were obtained by gently grinding freshly isolated newborn 2C transgenic *TAP1*$^{-/-}$ thymus lobes. The resulting suspensions were enriched for double-positive 2C cells with biotinylated anti-CD4 antibody coupled to streptavidin-bound magnetic microbeads (Miltenyi); their purity, checked by FACS analysis was usually more than 95%. Reaggregates were formed by mixing together the desired epithelial cells and double-positive thymocytes (at a ratio of 1:2). After pelleting the cell mixture by gentle centrifugation, and removing the supernatant, the pellet was dispersed into a slurry, drawn into a fine glass pipette, and placed as a standing drop on the surface of a nucleopore filter suspended over complete medium (1640 RPMI supplemented with 10% fetal calf serum) with or without added peptide. Cultures were incubated for three days at 37°.

Thymus- and spleen-derived dendritic cells were purified from B6 and DBA/2 tissues as described previously (108). Purity was checked by staining with anti-CD11c, anti-I-A^b and anti-I-A^d antibodies and analyzing by FACS. Splenic preparations were typically 90% dendritic cells, while thymic preparations usually were 70% dendritic cells, the primary contaminants being T cells.

Thymectomized, irradiated and bone marrow reconstituted (TIR) mice and thymus transplants

B6 mice were thymectomized as described previously (123). They were then lethally irradiated (1100 rad) and reconstituted with 2C transgenic *TAP1*$^{-/-}$ bone marrow as described (124). In brief, bone marrow cells were isolated by flushing the tibia and femur of adult mice with ice cold phosphate buffered saline (PBS). After subjecting the cells to two rounds of complement lysis using anti-CD4, anti-CD8 and anti-Thy1 antibodies and rabbit complement, approximately $5x10^6$ cells in 0.5ml PBS were injected intravenously into the TIR mice. Thymic transplants were performed by placing 2 or 3 dGuo treated

fetal thymic lobes under the left kidney capsule of these TIR animals as described previously (125). Donor thymi came from E14 embryos of either B6, DBA/2 or *TAP1*$^{-/-}$ strains.

Quantitation of K^b and L^d complexes

To measure the number of K^b and L^d molecules on cells from B6 and DBA/2 mice, we used a monoclonal anti-K^b antibody, Y3 (17), and a monoclonal anti-L^d antibody, 30-5-7 (126), respectively. We used bivalent antibody molecules, whose apparent affinity for the multivalent antigenic determinants on a cell surface is higher than the intrinsic affinity of monovalent Fab fragments (127), to overcome problems of sensitivity and background. We determined apparent equilibrium binding constants (K_a) for the Y3 antibody to K^b and the 30-5-7 antibody to L^d using K^{b+} EL4 and L^{d+} P815 cells respectively. Various concentrations of ^{125}I-labeled antibody were incubated with 2-4x10^5 cells in a total volume of 50 µl of complete medium for 30 min. at room temperature (22-25°C). All reactions were carried out in duplicate. Cell-bound and unbound ^{125}I-antibody were separated by centrifugation on fetal calf serum and measured. The concentration of specifically bound ^{125}I-antibody was determined as the difference between cell-bound radiolabelled antibody in the presence and absence of a 100-fold molar excess of unlabelled antibody. The binding data were analyzed according to Klotz (128). The concentration of specifically bound antibody (B) was plotted as a function of the logarithm of the unbound (free) concentration (F), and experimental points were fitted to the equation:

$$B = K_a \bullet B_{max} / (K_a + 10^{-logF}), \qquad \text{(Eq.1)}$$

where B_{max} is the concentration of class I bound antibody at saturation, i.e., as F approaches infinity. Values of B_{max} and K_a were derived from the best fit. The K_a values were found to be $3x10^8$ M^{-1} for the Y3 antibody and $2x10^8$ M^{-1} for the 30-5-7 antibody (the Fab′ fragment of the Y3 antibody to cell surface K^b on the same cells had an approximately 30-fold lower K_a than the bivalent antibody, unpublished observation).

The fractional occupancy (α) of class I for any given free antibody concentration was determined from:

$$\alpha = K_a \bullet F / (1 + K_a \bullet F). \qquad \text{(Eq. 2)}$$

From the specific radioactivity of the radioiodinated antibodies and the number of cells in the analyzed samples we determined the number (N_f) of antibody molecules specifically bound to class I MHC molecules per cell at a given free antibody concentration (F). The maximal number of class I MHC bound antibody molecules per cell was taken to be N_f / α and is reported as a measure of the total number of class I molecules per cell (Table 5-1); the actual number of the corresponding MHC molecules per cell lies between N_f / α and twice this value.

To measure the number of peptide-K^b complexes formed on thymic epithelial cells at positively and negatively selecting peptide concentrations, cells from *TAP1*$^{-/-}$ thymi were incubated for two hours at 37° in complete medium with or without peptide followed by analysis with ^{125}I-Y3 as described (Table 5-2).

Acknowledgements

We thank Dennis Y. Loh for contribution of the 2C transgenic mice, Chanel Lovett and Carol McKinley for technical assistance, Christiaan Levelt and Suzana Marusic-Galesic for advice and discussion, Emily Rossie for secretarial assistance, and Werner

Haas for discussion and critical reading of the manuscript. This work was supported in part by grants from the National Institutes of Health/National Cancer Institute (R35-CA53874, CA-60686, AI-34247) and a training grant in immunology (R37-AI17879).

Contributions

The work presented here was performed in collaboration with Dr. Yuri K. Sykulev, a post-doctoral associate formally of Professor Herman N. Eisen's laboratory. Yuri determined the binding constants of the antibodies used in these studies and performed most of the direct measurements of surface levels of MHC proteins. The author performed the remainder of the antibody-labeling and surface MHC-counting experiments. All cell preparation, organ culture, transplantation and analysis was performed by the author with some technical assistance from Chanel Lovett.

Chapter 6 Autoimmunity

Introduction

Much about the phenomenon of autoimmunity remains to be understood. Autoimmune diseases can be categorized according to their immunopathogenic mechanisms. Many involve the binding of antibodies (usually of the IgG type) to cell-surface or matrix-associated antigens. This can result in destruction of tissue by complement-mediated lysis or phagocytosis by FcR^+ cells. This happens, for example, in autoimmune hemolytic anemia, where antibody binding to cell surface antigens on red blood cells results in red blood cell destruction. If the cell surface antigen happens to be a receptor important for some signal pathway, antibodies might act as either agonists or antagonists of that receptor. In Graves disease, autoantibodies bind to the thyroid-stimulating hormone receptor on thyroid cells and thereby stimulate the production of thyroid hormone, resulting in hyperthyroidism. Patients suffering from myesthenia gravis produce autoreactive antibodies that bind to the acetylcholine receptor present at neuromuscular junctions, thereby blocking neuromuscular transmission.

Alternatively, autoantibodies may develop that bind a soluble antigen. The antigen–antibody complexes could form deposits in tissues or small blood vessels and result in inadvertent complement-fixation or even simple physical blockage. In systemic lupus erythematosis, production of antibodies recognizing common cellular components, such as DNA, histones, and other nuclear proteins can result in a variety of symptoms including glomerulonephritis, vasculitis and arthritis.

A large number of chronic inflammatory autoimmune diseases are mediated primarily by T cells. Infiltration of pancreatic islet-cells by by autoreactive T cells results in insulin-dependent diabetes mellitus (IDDM). Experimental autoimmune encephalomyelitus (EAE), and a similar human disease, multiple sclerosis, result from the invasion of the central nervous system by $CD4^+$ T cells. Immunization of certain mouse strains with collagen in complete Freund's adjuvant leads to joint inflammation which resembles in many respects the human disease rheumatoid arthritis.

While experiments in animals have shown that $CD4^+$ T cells are sufficient to cause these diseases, antibodies are thought to contribute to the pathogenesis of autoimmine diseases which arise spontaneously in humans or certain mouse strains. In most autoimmune diseases, multiple antigens are involved. Indeed, over a dozen antigens have been implicated in IDDM in both human patients and mouse models including epitopes from insulin itself, 65 kDa heat-shock protein (Hsp65), carboxypeptidase H, and a β-granule antigen.

The existence of autoimmune disorders indicate that the mechanisms of central tolerance (i.e., clonal deletion of autoreactive T cells in the thymus and B cells in the marrow) are obviously not perfect. However, autoimmunity is the exception rather than the rule. Obviously there are safeguards in place that prevent or at least attenuate autoimmune responses should they arise. Three general mechanisms of this peripheral tolerance suggest themselves.

First, self-antigens may be present in the organism, but are normally sequestered and thus hidden from immune surveillance. While the lymphocytes do not have the opportunity to be deleted by this antigen, they also do not normally encounter it during

their mature stage. Mice of inbred strains that are susceptible to EAE are normally healthy and free of disease unless an investigator inoculates the mice with antigen in the form of myelin basic proteins or its antigenic determinants and adjuvant. Such a procedure will activate the $CD4^+$ T cells that cause the disease.

Second, some self-antigens may be present and accessible to recognition by lymphocytes but are not presented in immunogenic form at the surface of professional APC. Furthermore, self-antigen specific lymphocytes may not develop into large pathogenic clones unless they are helped by other lymphocytes that require independent activation.

A third possible mechanism of peripheral tolerance, and possibly the most elusive, is that of active suppression of immune responses to self-antigens. Recent evidence for suppresser T cells comes from work involving mice transgenic for genes encoding TCR α and β chains that together recognize a peptide antigen from myelin basic protein. Mice that are transgenic for this TCR display a very low incidence of spontaneous EAE. However, mice that carry both the TCR transgenes and homozygous knockout-alleles for the *RAG1* recombinase gene suffer 100% incidence of EAE (40). Due to the *RAG1* deficiency, these mice have no B or T cells except for those $CD4^+$ T cells expressing the transgenic receptor. One interpretation of this finding is that T or B cells that develop in the $RAG1^+$ background are responsible for maintaining tolerance to the EAE antigen and that, in their absence, nothing prevents the onset of disease. Addition evidence comes from studies in which the transfer of purified populations of $CD4^+$ T cells could protect rodents against experimentally induced autoimmune diseases, such as diabetes or thyroiditis (129).

Most incidents of autoimmune disease of the skin fall into two broad categories: pemphigoid blistering disease and chronic psoriasis. The pemphigoid diseases are perhaps the only true autoimmune diseases of the skin that have a well characterized etiology. Pathogenesis is mediated by autoantibodies specific for inter-epidermal (e.g. adherins junctions and desmosomes) or dermal-epidermal structures (e.g. hemidesmosomes and collagen). Antibody binding alone is often sufficient to disrupt cell–cell interactions and cause detachment of epidermis from dermis, and thus blistering, or acute dermatitis. Complement fixation is often a component of the more severe cases of pemphigoid diseases. For reasons that are not entirely clear, the epidermis and mucous membranes of the eyes, mouth and throat are the principal sites affected by these diseases (130).

Patients who suffer from psoriasis develop thickened, roughened layers ofepidermis that become flaky and scaly. These symptoms can occur in broad patches of skin or in smaller disc-like areas known as psioratic plaques. In either case, the rash can occur anywhere on the body but common sites include the scalp and regions of the skin covering joints (e.g. elbows, knees, knuckles, etc.). The disease can be triggered by a variety of stimuli: infection, wounding, UV-irradiation, contact hypersensitivity, etc. Once triggered, though, symptoms can last for years. Patients who recover are often prone to chronic relapses. Chronic psoriasis is associated with lymphoid and myeloid inflammatory responses and is linked to certain HLA alleles, but its exact etiology remains undefined (130). Development of an animal model for this disease could provide a valuable tool for understanding the etiology of the disease and developing new treatments.

In this study, I crossed the 2C TCR transgenes with DBA/2 mice that express the allo-antigenic H-2L^d MHC protein. In accordance with central tolerance theory, the presence of antigen results in the deletion of transgenic CD4 CD8 double-positive cells and prevents the maturation of CD8$^+$ 2C$^+$ T cells. Surprisingly, when these mice were intercrossed, some of the offspring developed a peculiar pathology consisting of loss of hair and swelling of the skin that was restricted to the region surrounding the eyes and covering the snout. This pathology was associated always with expression of the 2C TCR and H-2^d MHC proteins. Closer examination of the lymphocytes of these animals revealed a population of unusual transgenic T cells that escaped thymic deletion. These animals may provide an interesting model system for the study of peripheral tolerance by comparing affected and unaffected (i.e. tolerant) animals. More importantly, these mice may provide the first animal model for the study of spontaneous autoimmune skin diseases.

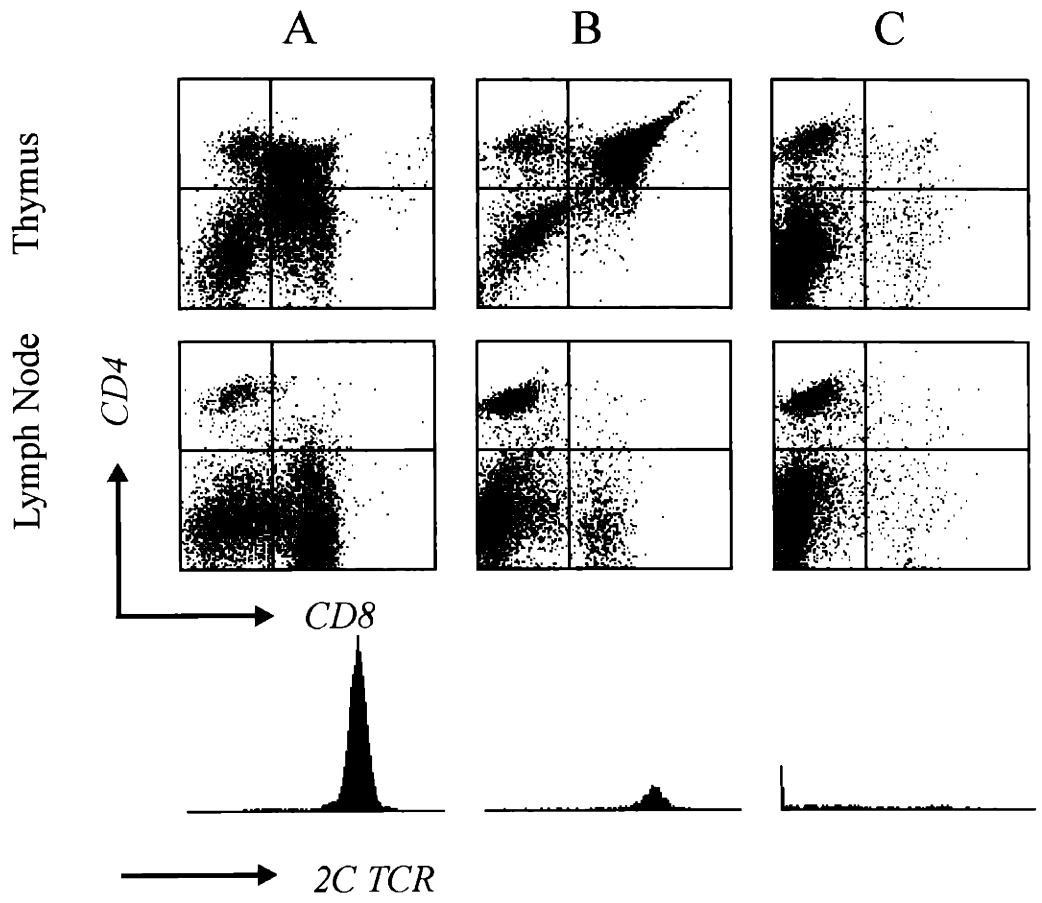
Results

Negative selection of 2C thymocytes in 2C TCR-transgenic H-2$^{b/d}$ mice

In order to study mechanisms of self-tolerance, mice transgenic for the genes encoding the 2C TCR α and β chains were crossed with DBA/2 that express the allo-antigenic class I MHC H-2 L^d. The offspring of this cross therefore expressed both the H-2^b and H-2^d MHC proteins. I analyzed thymocytes and lymph node cells from transgenic F1 mice by flow cytometry. The thymi from these mice were greatly atrophied and contained only a tenth of the cells found in 2C transgenic H-2^b thymi. The majority of cells were CD4$^-$ and CD8$^-$ (n=4; range, 80-90%) and the remainder were CD4$^+$ SP cells (Figure 6-1c). The failure of CD8$^+$ SP cells to develop and the elimination

of DP thymocytes indicate that negative selection is occurring in 2C transgenic H-2^{d+} animals (compare with $2C^+$ *TAP1*$^+$ and $2C^+$ *TAP1*$^{-/-}$, Figure 6-1). This is consistent with the original analysis by Sha et al. in which 2C TCR-transgenic mice were crossed with H-2^d-expressing BALB/C mice (36).

Figure 6-1 Thymic deletion of 2C cells in 2Cd mice



Flow cytometric analyses of thymocytes and lymph node cells from (A) $2C^+$ *TAP1*$^+$ H-$2^{b/b}$, (B) $2C^+$ *TAP1*$^{-/-}$ H-$2^{b/b}$ and (C) $2C^+$ *TAP1*$^+$ H-$2^{d/b}$ mice. Cells were stained with anti-CD4–PE, anti-CD8–TC and anti-2C–FITC. Histograms show the 2C TCR profile of the $CD4^-$ $CD8^+$ cells from the lymph nodes of the respective mice.

Table 6-1 Summary of observations made of the 2Cd mice

	pathology			
genotype	healthy	moderate	severe	total
$2C^+$ H-$2^{d/b}$	5	5	10	20
$2C^+$ H-$2^{d/d}$	74	58 (12)	15 (1)	147
$2C^-$ H-$2^{d/d}$	40			40

Phenotype of mice derived from intercrossing F2 progeny from a 2C TCR-transgenic (H-$2^{b/b}$) and DBA/2 wild type (H-$2^{d/d}$). F3 mice that were $2C^+$ and H-$2^{d/d}$ were then crossed to produce more mice of the $2C^+$ H-$2^{d/d}$ genotype. See Figure 6-2 for an evaluation of the symptoms. Numbers in parentheses indicate the number of animals that largely, if not totally, recovered.

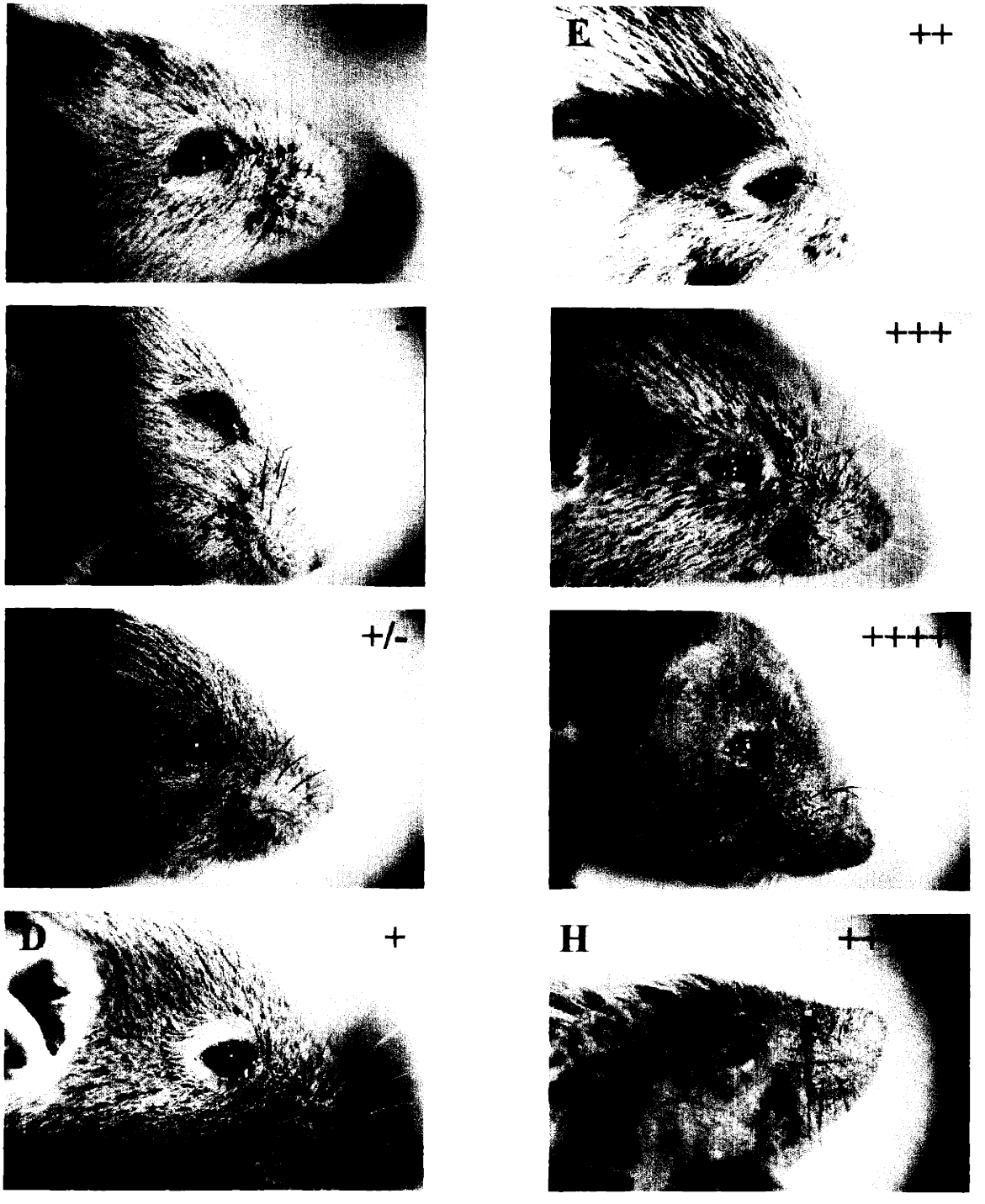
The $2C^+$ H-$2^{b/d}$ F1 animals were normal and seemed as healthy as $2C^+$ H-2^b parental type mice. However, when the F1 animals were subsequently intercrossed (these referred to hereafter as 2Cd mice), some interesting observations were made. Approximately 1/3 of these animals developed a pathology of inflammation that was first noticeable when the eyelids became thickened and roughened often accompanied by hair loss around the eyes (Figure 6-2). Sometimes the swelling was so pronounced that the eyelids were swollen shut. Many mice remained with this level of pathology and even a few recovered at this point (Table 6-1). However, a certain proportion of animals developed more severe symptoms. The inflammation and loss of hair proceeded down the face to the snout. The skin sometimes became flaky and obviously irritated, resembling dermatitis (Figure 6-2). Development of these symptoms was always symmetrical about the face, but not always uniform from mouse to mouse. Some mice suffered acute swelling of the eyelids without any loss of hair. Others showed acute dermatitis and hair loss across the face, yet the eyelids were not nearly as swollen as others that had no loss of hair. The onset of pathology was usually within 6-10 weeks of age. 2Cd mice that did not develop the pathology within 16 weeks usually remained asymptomatic to 12 months of age. All mice that developed any sort of pathology were transgenic for the 2C TCR, while all non-

transgenic littermates remained asymptomatic. Table 6-1 shows a summary of the observations of the mice generated during the course of this study.

Histological examination of skin from the face of affected and unaffected 2Cd littermates showed that the severely affected mice suffered from a massive infiltration of multiple types of white blood cells. Large numbers of neutrophils, eosinophils, macrophage and lymphocytes can be seen throughout the dermal layer of the skin (Figure 6-3). The epidermis itself has become several cell layers thick rather than the one to two cell thickness seen in normal tissue (Figure 6-3). Tissue samples from less severely affected 2Cd mice show again a thickening of the epidermis in parts, but not the massive infiltration of white blood cells that is seen in the most severe cases. Inflammation and infiltration by neutrophils and eosinophils could often be seen in and around the hair follicles in these less symptomatic mice, suggesting that some follicular antigen may be associated with the disease.

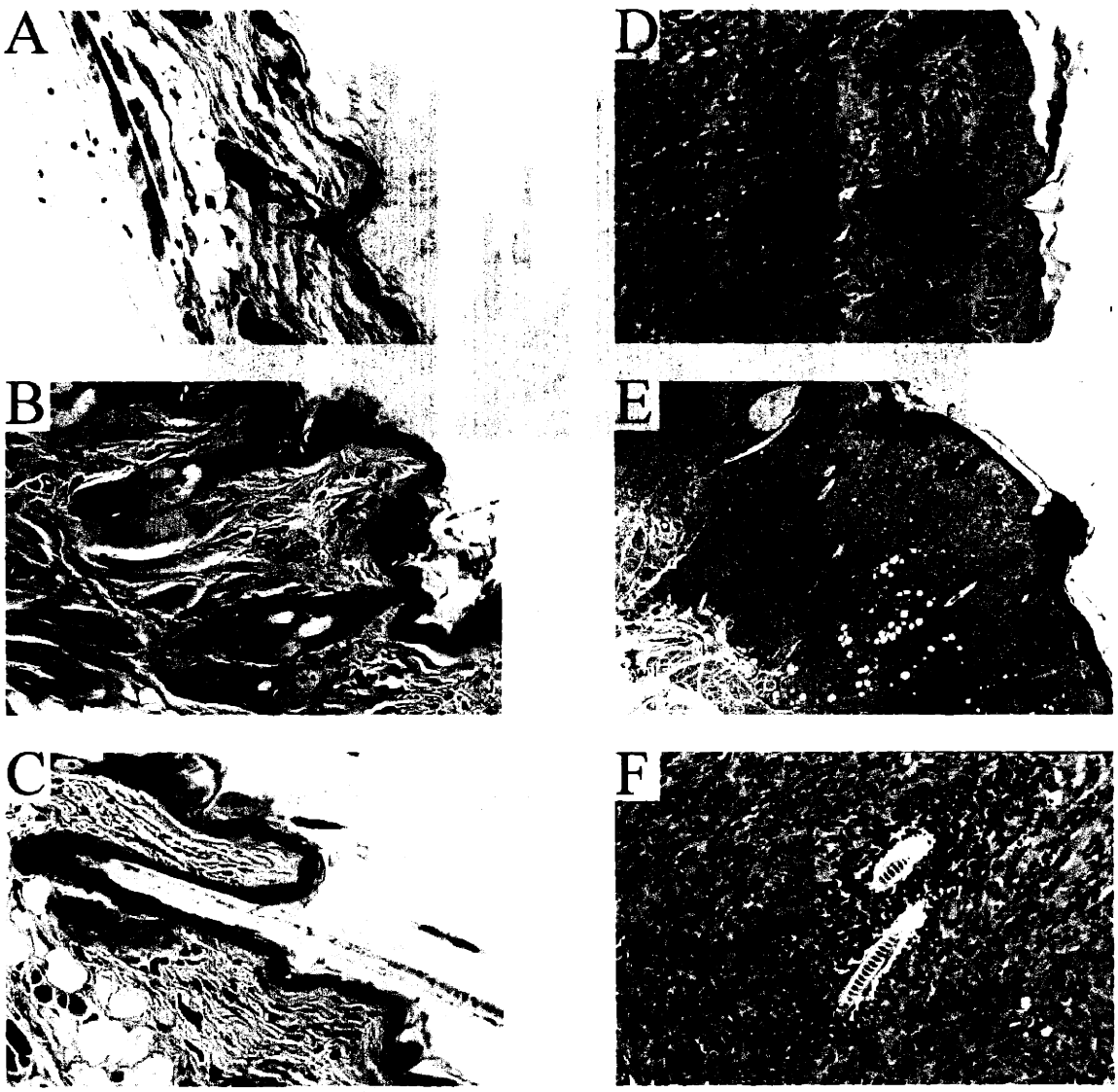
Since autoimmune diseases of the skin often involve self-reactive antibodies, I examined blood serum levels in 2Cd mice and compared them with non-transgenic littermates. 2Cd mice had elevated titers of multiple isotypes of antibodies, including IgG_1, IgG_{2a}, IgG_{2b}, IgM, IgE and marginally IgA (Fig 6-4). Histological analysis of the spleen of an affected 2Cd mouse, showed germinal centers and many plasma cells indicative of a B cell response (Figure 6-5). Suspecting that autoreactive antibodies may therefore be involved in the pathology of the 2Cd mice, I stained for mouse Ig in frozen skin sections of 2Cd and control mice. No specific immunohistochemical staining of endogenous antibody deposits could be observed in sections from 2Cd mice (data not shown).

Figure 6-2 Facial pathology of 2Cd mice



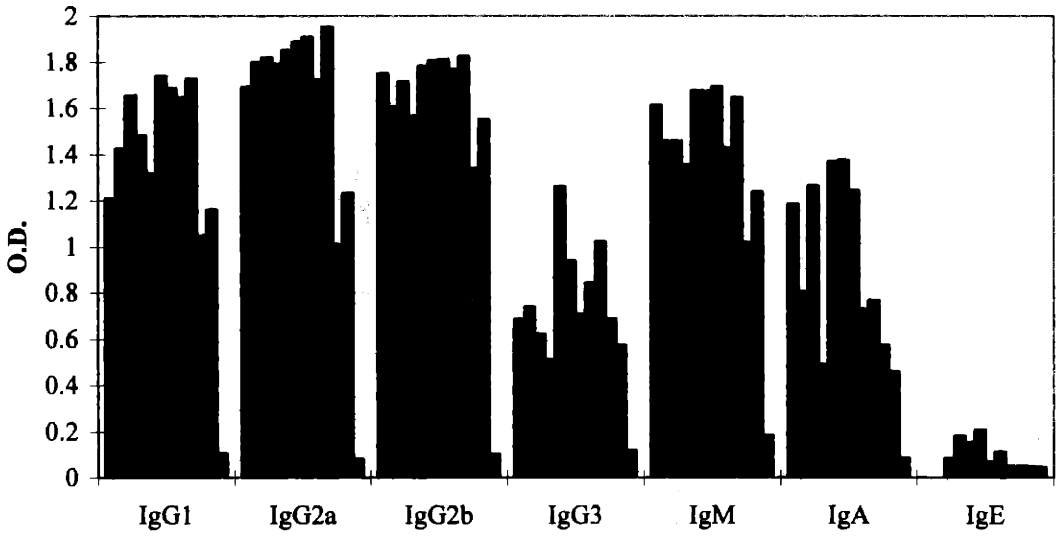
Panel A shows a non-transgenic eight-week-old littermate. Panels B-H show 2C TCR-transgenic littermates that exhibit increasingly more severe symptoms. In the top, right corner of each panel, an arbitrary rating of severity is offered on a scale of "-" to "+++++".

Figure 6-3 Histological analysis of skin from 2Cd mice



Comparison of hematoxilin- and eosin-stained paraffin sections (10 μm) of skin from a healthy 2Cd mouse (Panels A-C) and a severely symptomatic littermate (Panels D-F). Panels A and D compare skin from the eyelids. Panels B and E compare skin from the snouts. The arrow in panel E points to the hair follicle that is enlarged in panel F. Note the thickened epidermis in panels D and E compared with A and B, respectively. Also note the lack of intact, open hair follicles in the severely affected animal as compared with the healthy animal. Myeloid infiltrates are marked by arrows in panels D and F: E, eosinophil; M, macrophage; N, neutrophil. The remainder of the infiltrating cells are mostly lymphocytes.

Figure 6-4 Elevated levels of serum Ig in 2Cd mice

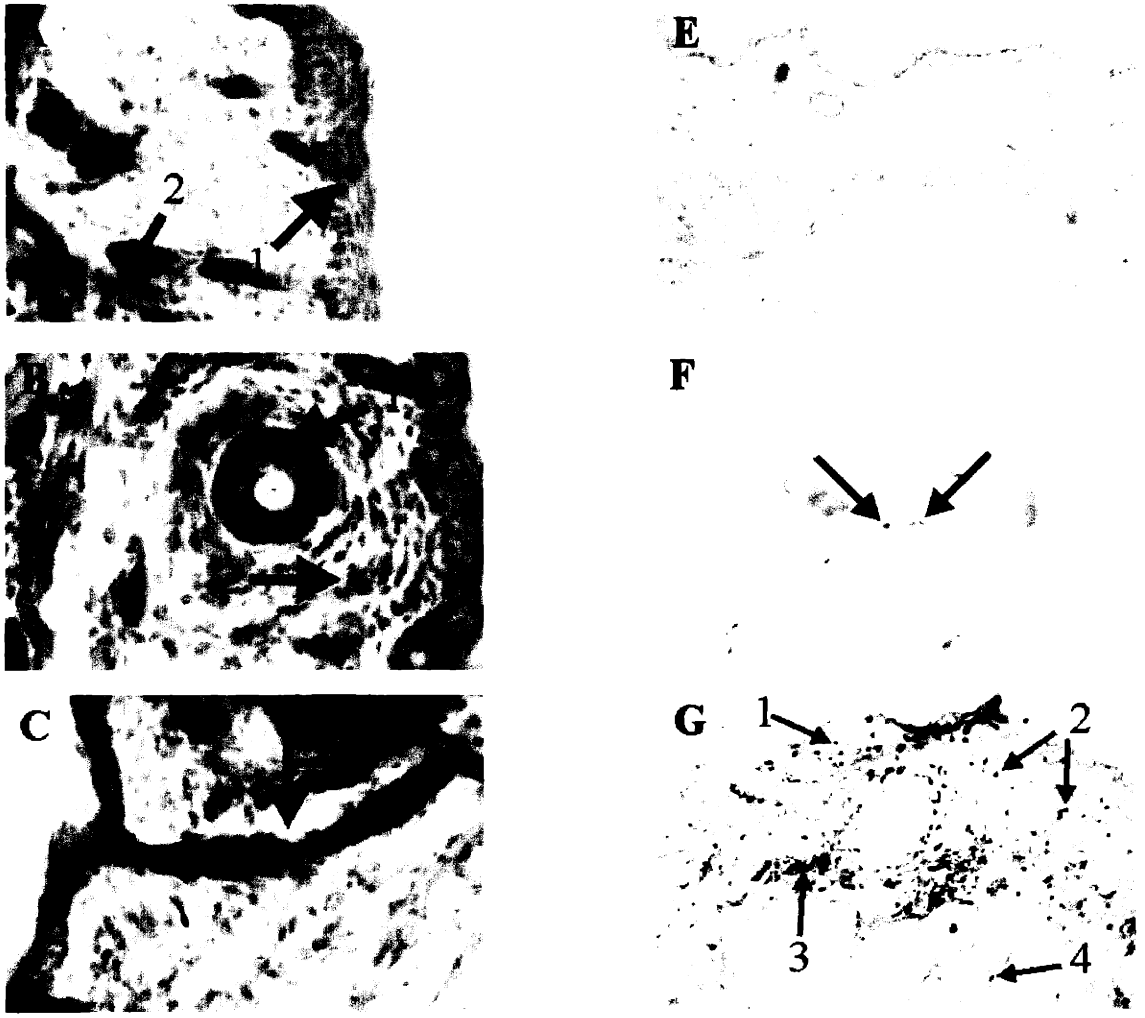


Comparison of serum Ig levels for a variety of isotypes between H-2^{d+} 2C TCR-transgenic (blue) and non-transgenic (red). Black bars indicate no serum (PBS) controls.

Figure 6-5 Germinal center in spleen of 2Cd mouse

Hemotoxilin and eosin staining of a section (10 μm) of a spleen from a severely affected 2Cd mouse. The disproportionate amount of white pulp (blue) versus red pulp (red) is indicative of extensive myelo- and lymphopoiesis. Arrow indicates a germinal center that is the site of B cell maturation and proliferation.

Figure 6-6 2C TCR$^+$ cells infiltrate the epidermis and follicular sheaths



Immunohistochemistry was performed on frozen, acetone fixed 10 micron sections of skin from around the eye of a moderately affected (+++, see legend Figure 6-2) 2Cd mouse (Panels A-D, F, G) and a normal DBA/2 mouse. After immunohistostaining, sections were stained with hemotoxalin. Panels A-D show immunohistostaining with 1B2–biotin followed by ABC-peroxidase and AEC colorimetric reagents. Arrows indicate 1B2$^+$ cells: A1) within the epidermis, A2) in deep dermis, B1) within the epithelial sheath surrounding a hair follicle, B2) in dermis, C) in follicular shaft, and D) at the junction of hair follicle and epidermis. Panels E and F show immunohistostaining with 1B2–biotin followed by streptavidin–peroxidase and DAB colorimetric reagents. Panel E shows no specific staining by 1B2 in a TCR non-transgenic DBA/2 skin section. Panel F shows 1B2 positive cells in the epithelial sheath surrounding a hair follicle. Panel G shows immunohistostaining with anti-CD3 mAb visualized with anti-rat-peroxidase mAb and DAB colorimetric reagents. Arrows indicate CD3$^+$ cells: 1) in the epidermis, 2) along the basal membrane, 3) in association with hair follicles, and 4) in deep dermis.

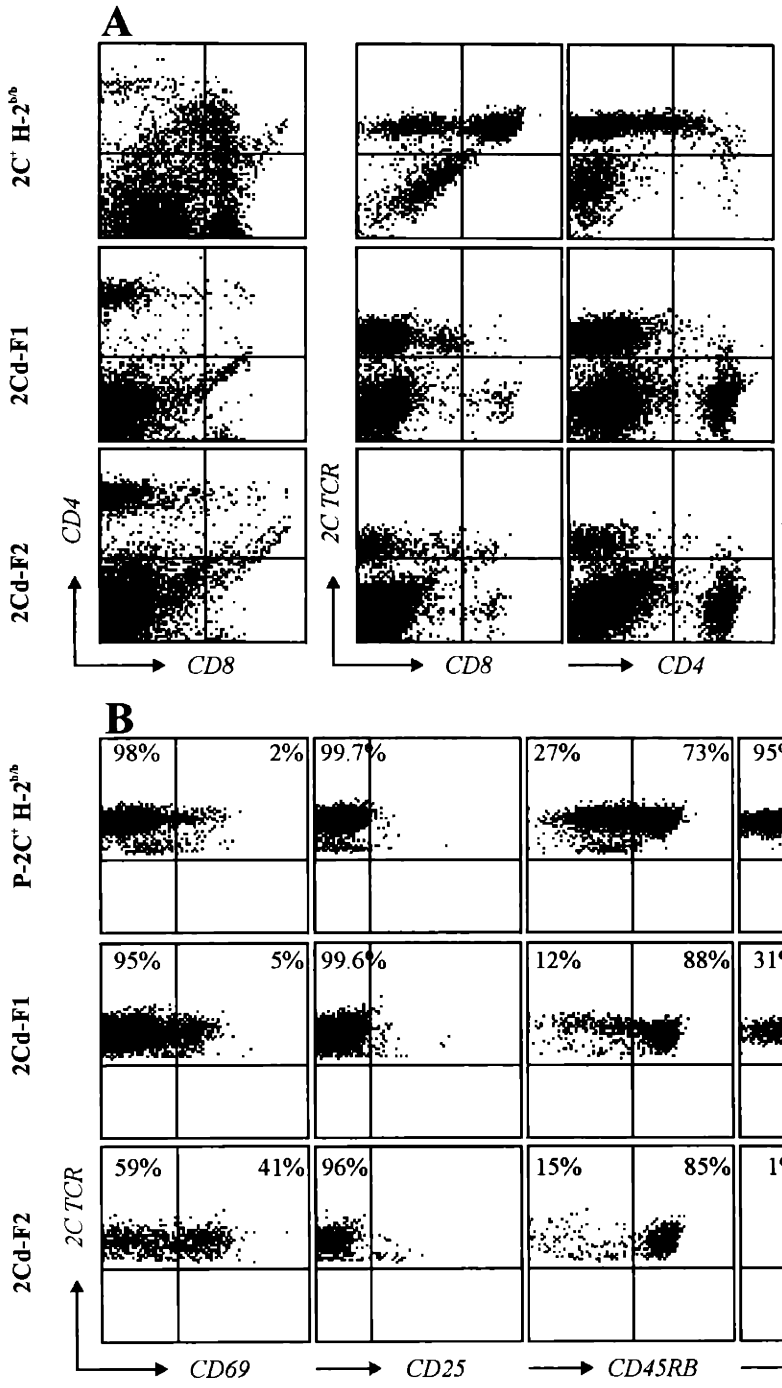
Tissue-specific infiltration by 2C transgenic cells

Since only those mice that were 2C TCR-transgenic as well as H-2^{d+} became sick, skin from around the eyes and face of 2Cd mice was examined for infiltrating 2C TCR-transgenic cells. Mice exhibiting mild to moderate symptoms (++ to +++, see Figure 6-2 legend) were selected for examination so that tissue-specific localization might be revealed more easily. Figure 6-6 shows immunohistochemical staining for 2C TCR^+ cells (Panels A-F) and for $CD3^+$ cells (Panel G). Relatively few 2C TCR^+ cells were present in the skin of sick animals compared with total $CD3^+$ T cells. However, the few 2C TCR^+ cells present were found primarily infiltrating the epidermis or the epithelial sheath surrounding hair follicles (Figure 6-6, Panels A-D, F). Large numbers of $CD3^+$ T cells were also present, but not associated with any particular dermal or epidermal structure. $CD3^+$ T cells could be observed in association with the epidermis, along the dermal-epidermal junction, surrounding follicles and in the deep dermal layer (Figure 6-6, Panel G). The specific association of 2C TCR-transgenic cells with the epidermis and follicular sheath suggests their influence on the hyperproliferation of the epidermis and the loss of hair suffered by these animals.

Examination of $2C^+$ cells from 2Cd mice

I examined more closely the lymphocytes found in the periphery of these animals. Cells from mandibular lymph nodes were examined since these are the relevant draining nodes for the head along with cells from mesenteric lymph nodes and splenocytes. Figure 6-7 shows FACS analysis with antibodies specific for a variety of markers that have expression patterns indicative of the activation state of the cell (i.e., naïve, activated, or memory phenotype).

Figure 6-7 Analysis of activation markers on transgenic lymphocytes from 2Cd mice

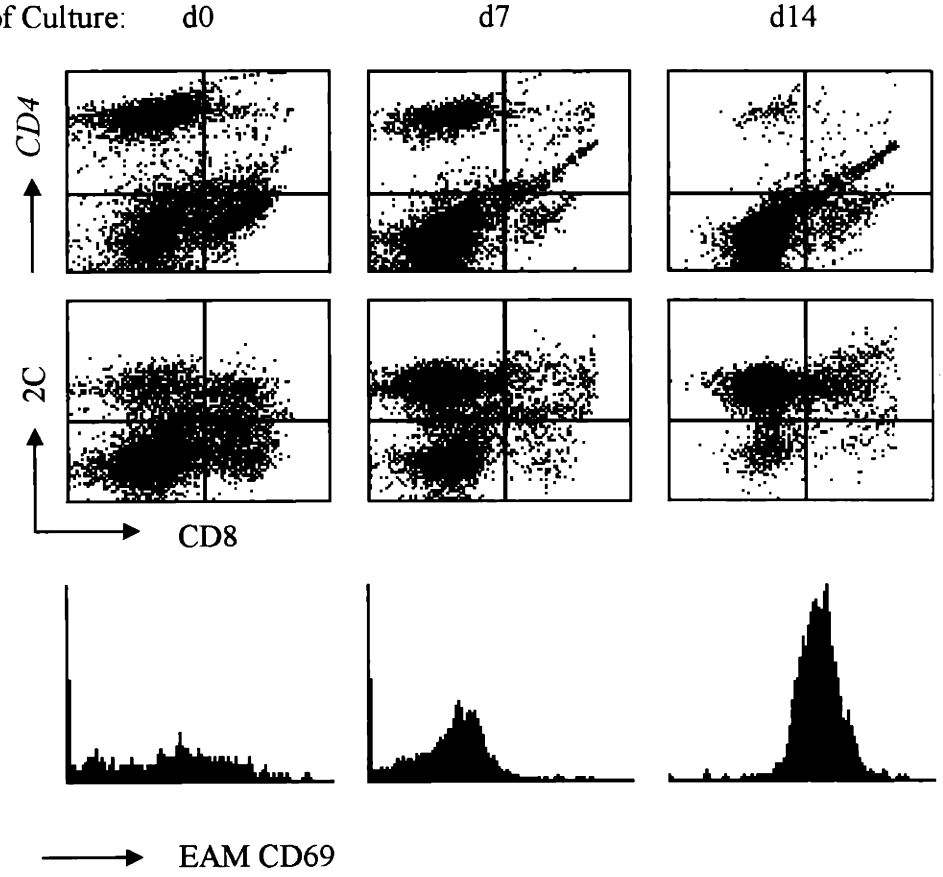


Flow cytometric analysis of mandibular lymph node cells from 2C TCR transgenic mice of three different genetic backgrounds: C57BL/6 (H-$2^{b/b}$), C57BL/6 x DBA/2 (F1 progeny, H-$2^{d/b}$), and the intercross of the F1 mice (F2 progeny selected for expression of the 2C TCR and H-2^{d} MHC proteins). (A) Lymphocytes were stained with anti-2C TCR–FITC, anti-CD4–PE and anti-CD8–TC. (B) Lymphocytes were stained with anti-2C TCR–FITC and either anti-CD69–PE, anti-CD25–PE, anti-45RB–PE, anti-44–PE or anti-L-selectin–PE. The cells were gated on 2C+ cells and displayed as a 2D plot to better visualize the differences between the populations.

While 2C transgenic cells were found in the periphery of both 2Cd F1 and F2 mice, some marked differences were noticed. A higher percentage of $2C^{+}$ cells were observed in the mandibular lymph node, the mesenteric lymph nodes and the spleen of F1 2Cd

mice than in the same tissues from F2 mice. The $2C^+$ cells from the F1 mice were largely $CD4^-$, $CD8^-$, $IL2R\alpha^-$(CD25), $CD45RB^+$, $CD69^-$ and $CD44^+$. The $2C^+$ cells from F2 mice differed in that they were $CD69^+$ and $CD44^{low}$, indicating that these cells are largely activated in F2 but not F1 mice.

Figure 6-8 Flow cytometric analysis of cultured 2C transgenic cells from 2Cd mice



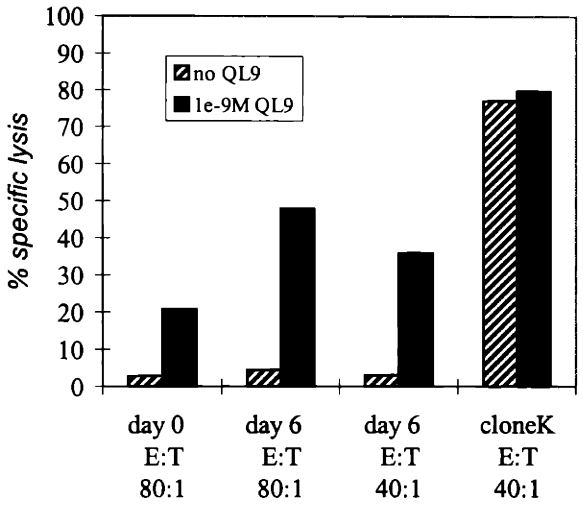
Mandibular lymph node cells were isolated and analyzed by flow cytometry after zero, seven or 14 days of culture with T2-L^d cells and added QL9 peptide. Aliquots of lymphocytes were stained with anti-CD4–PE, anti-CD8–TC and anti-2C–FITC or with anti-CD69–PE, anti-CD8–TC and anti-2C–FITC. Histograms show CD69 staining of $2C^+$ lymphocytes.

In vitro *responses of cells from 2Cd mice*

I tested the ability of 2C transgenic cells from 2Cd mice (referred to hereafter as 2Cd-cells) to proliferate in response to antigen and to lyse target cells. Cells from the

mandibular lymph nodes of 2Cd F2 mice were isolated and cultured *in vitro* with T2-L^d (a *TAP1*-deficient human cell line transfected with the H-$2L^d$ gene) cells in the presence or absence of the peptide QL9 (91). The $2C^+$ cells did not proliferate in response to stimulation but showed better survival compared with other cell types in the cultures (Figure 6-8) and with cells cultured without peptide, which did not survive a week's culture at all (data not shown). This inability to proliferate was confirmed by the lack of [^{3}H]thymidine incorporation in a standard three-day proliferation assay (data not shown).

Figure 6-9 $2C^+$ DN cells can lyse target cells



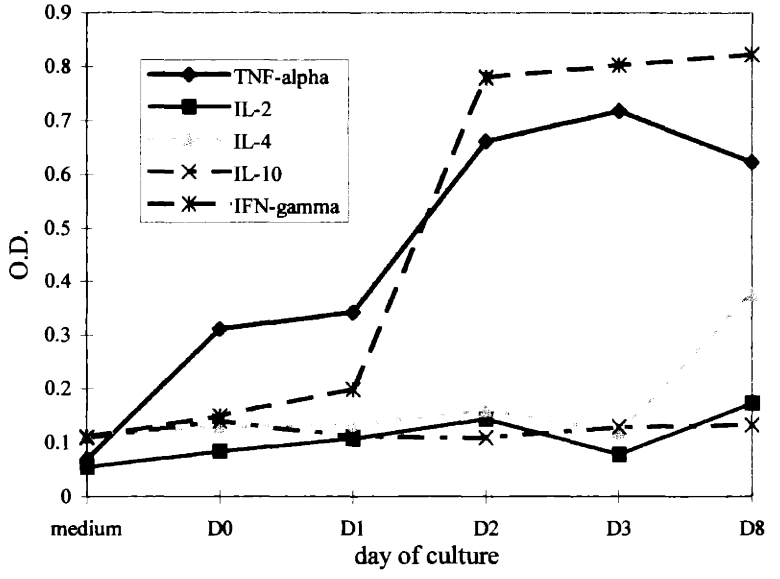
Specific lysis of ^{51}Cr-labeled P815 cells in the presence and absence of the peptide QL9 (QLSPFPFDL). Day 0 and day 6 indicate how long the 2Cd lymphocytes were cultured prior to the assay. Clone K is a normal $CD8^+$ 2C clone that recognizes H-$2L^{d+}$ P815 cells in the absence of added cognate ligand. E:T indicates the effector-to-target ratio in each case.

These cells were also tested for CTL activity. The cell lines P815 and T2-L^d were used as APCs. P815 is a murine-derived cell line that expresses H-2^d MHC proteins and is lysed by normal 2C CTL. 2Cd-cells could not lyse P815 cells in the absence of exogenously added peptide antigen, but could lyse both P815 and T2-L^d APCs when they

were loaded with QL9 peptide (Figure 6-9 and data not shown). The CTL activity was blocked by the addition of 1B2 antibody, confirming that it was 2C TCR-mediated lysis (data not shown). The CTL activity of 2Cd-cells was not inhibited by the addition of anti-CD8 antibody, whereas normal 2C CTL activity largely was (data not shown). This is not surprising since the 2Cd-cells do not express CD8. Interestingly, 2Cd-cells could lyse targets *ex vivo* without any prior *in vitro* stimulation, likely reflecting prior *in vivo* activation.

I then examined the profile of cytokines secreted by these cells. ELISA analysis detected high levels of TNFα and IFNγ, possibly low levels of IL-4, and no IL-2 or IL-10 (Figure 6-10). The mRNA expression of IL-4, TNFα, IFNγ and TGFβ but not IL-2 or

Figure 6-10 *In vitro* cytokine response of 2Cd cells



Lymph node cells from 2Cd mice were isolated and cultured in the presence of $1x10^{-9}$M QL9 peptide. Aliquots of culture medium were taken and tested in a standard ELISA assay (see methods). Each point is the average of duplicate experiments.

IL-10 was detected by rtPCR (data not shown). This reflects a largely inflammatory (T_H1) profile of cytokines.

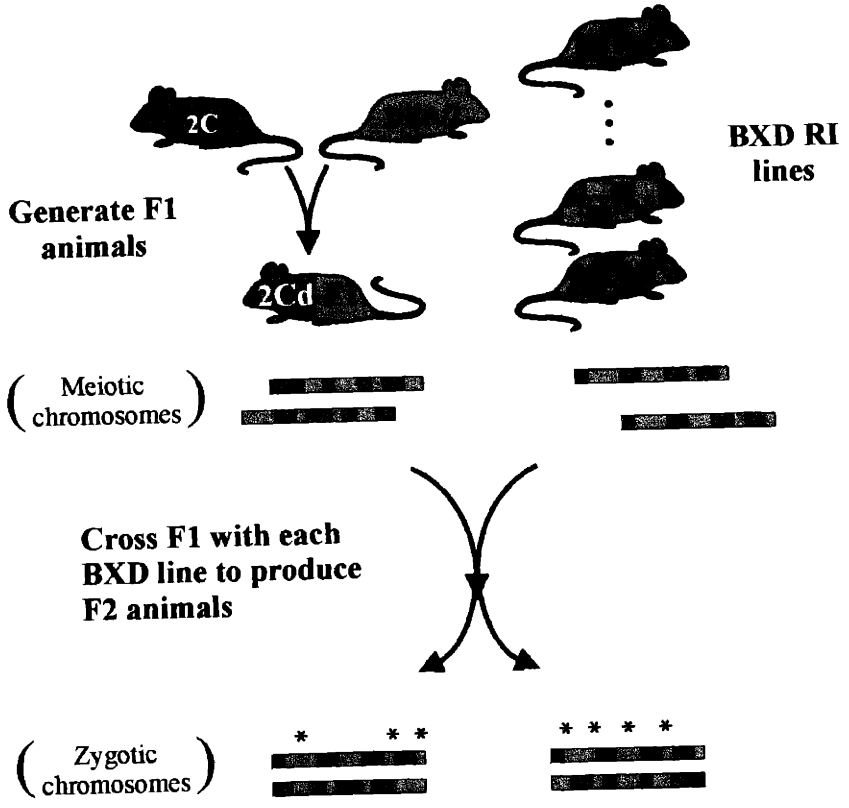
Recombinant inbred crosses

One of the interesting features of the disease in the 2Cd mice is that the symptoms have only been observed in a certain proportion of F2 animals. Other studies have examined 2C transgenic animals crossed with BALB/c mice, another H-2^{d+} strain, and reported no such symptoms (36, 131). I crossed 2C transgenic mice with BALB/c mice to generate $2C^{+/-}$ H-$2^{b/d}$ F1 animals. Thirty of these animals were observed for five months, and none developed symptoms like those of the 2Cd mice. These F1 animals also were intercrossed and three litters of F2 animals were monitored for symptoms. None of the transgenic animals (n=12) developed symptoms like those of the 2Cd mice. Taken together, these data strongly suggest that genetic contributions, other than *MHC* alleles, from the DBA/2 strain determine the etiology of the disease.

To dissect the genetic component or components responsible for the disease phenotype, I took advantage of existing recombinant inbred (RI) mice derived from C57BL/6 and DBA/2 mice (designated BXD mice; the Jackson laboratory, Bar Harbor, Maine). Each of the 32 BXD RI lines has a unique distribution of C57BL/6 and DBA/2 genetic material. Each line has been genotyped at hundreds of polymorphic loci across all of the chromosomes, thereby generating a map of the C57BL/6 and DBA/2 genetic contributions. In some cases, sufficient polymorphic markers were examined to define regions as small as 1 centi-Morgan (cM). The experimental plan involved crossing 2Cd-F1 mice with animals from each of the RI lines and scoring the progeny for the development of symptoms likes those in 2Cd-F2 mice (Figure 6-11). To limit the size of

the experiment and to simplify genotyping, only 15 BXD strains that carry the *MHC* locus of DBA/2 origin (i.e., H-$2^{d/d}$) were selected.

Figure 6-11 Recombinant inbred cross strategy



Although the genome of the animals resulting from the crossing of 2C transgenic mice with DBA/2 inbred mice is made up of half of each parental genotype, the gametes produced by these animals with be genetically heterogeneous due not only to random segregation of sister chromosomes, but to meiotic recombinatorial events as well. Each BXD RI mouse is genetically chimeric, having a unique composition of B6 and DBA/2 loci that have been bred to uniform homozygosity. On average, half of the offspring from matings between 2Cd-F1 mice and BXD RI mice will be homozygous for any particular DBA/2 locus that the particular BXD line carries, as noted by an * next to homozygous DBA/2 (blue) loci on the F2 chromosomes. Assuming that a limited number of loci are responsible for the onset of disease, only certain BXD lines will produce susceptible progeny. By a comparing the distribution of DBA/2 and B6 chromosomal regions among the susceptible and asymptomatic BXD lines, it may be possible to identify chromosomal regions and candidate loci that influence the onset of disease.

Table 6-2 summarizes the data obtained from these crosses. As was the case in the original generation of the 2Cd animals (Table 6-1), only animals that were 2C TCR-

transgenic developed any signs of disease. Only three lines, BXD-12, -27 and -32, produced progeny that did not develop symptoms of the disease. Each line was evaluated for severity of symptoms and rated as explained in Figure 6-2. Four lines, BXD-11, -16, -25 and -31, yielded progeny that developed only mild symptoms. BXD-9 was

Table 6-2 Result of BXD crosses

BXD strain	$2C^-$	$2C^+$ H-$2^{b/d}$	$2C^+$ H-$2^{d/d}$	total $2C^+$	Severity
1	0/3	4/4 (1)	1/2	5/6	+++++
5	0/7	0/4	0/1	0/5	++
6	0/8	0/7	0/4	0/11	++
9	0/8	4/11 (1*)	1/4	5/15	++
11	0/16	3/6	2/4	5/10	+
12	0/4	0/1	0/2	0/3	-
16	0/3	0/2	1/2	1/4	+
18	0/6	2/4 (2)	2/4	4/8	++++
22	0/11	2/4	1/8	3/12	+++
24	0/12	0/2	0/2	0/4	+++
25	0/11	2/7 (1)	1/8	3/15	+
27	0/3	0/4	0/1	0/5	-
30	0/4	0/1	2/2 (1)	2/3	+++
31	0/10	1/9 (1)	1/8 (1)	2/17	+
32	0/12	0/7	0/3	0/10	-

Phenotype of the mice resulting from the crossing of 2Cd F1 mice ($2C^{+/-}$ H-$2^{d/b}$) with the indicated BXD RI lines, all of which were H-$2^{d/d}$. The possible genotypes were $2C^+$ H-$2^{d/b}$, $2C^+$ H-$2^{d/d}$, $2C^-$ H-$2^{d/b}$ and $2C^-$ H-$2^{d/d}$. The progeny were typed for expression of the 2C TCR transgene and the expression of I-A^b MHC. The data for the $2C^-$ H-$2^{d/b}$ and $2C^-$ H-$2^{d/d}$ mice were pooled for the purpose of this table. The $2C^+$ total column contains the pooled data for the $2C^+$ H-$2^{d/b}$ and $2C^+$ H-$2^{d/d}$ mice. The ratios indicate the fraction of mice that exhibited some signs of the pathology (i.e., swelling, inflammation, and, or loss of hair around the eyes and snout). The number in parentheses indicates the number of affected mice that recovered, if any. The asterisk notes a mouse that suffered an unusually acute case of dermatitis and loss of hair that extended over the entire head down the shoulders. This animal then recovered a near totally healthy appearance. Severity was judged as in Figure 6-2 and described as follows: +++++ severe, severe dermatitis and loss of hair around eyes and snout; +++ moderate, substantial swelling and loss of hair restricted to eyelids, possibly some inflammation on snout; + mild, limited loss of hair or swelling usually restricted to a small (≤ 1mm) band around the eyes.

designated as mild-moderate in severity since crosses with this line yielded mostly mildly symptomatic progeny with one exception that exhibited severe symptoms but then completely recovered. Two other lines, BXD-5 and -6, yielded progeny that showed symptoms that were mild-moderate in severity. Three lines, BXD-22, -24 and -30, generated offspring with moderate symptoms. The transgenic progeny from the cross with BXD-18 mice all had at least moderate symptoms. The BXD-1 line was unique in that it produced the largest proportion (5/6) of severely affected animals.

Discussion

The crossing of 2C TCR-transgenic mice with mice of the DBA/2 strain has led to several serendipitous and interesting observations. Immunological dogma would suggest that mice that express both a TCR and its cognate alloantigen should be largely healthy since thymic deletion should eliminate the potentially auto-reactive T cells. However, this was not the case. Mice that express the 2C TCR transgenes and the alloantigen H-$2L^d$ are much more prone than non-transgenic animals to develop a variety of ailments including perivascular inflammation, colitis, cachexia, dermatitis and alopecia.

The dermatitis and alopecia observed in these animals are particularly interesting for several reasons. Incidents of colitis and cachexia could be observed in any 2C TCR-transgenic mouse, although at a higher incidence in H-2^d backgrounds. Both these syndromes are observed in several other strains of immunologically perturbed mice housed in our colony (e.g., $RAG^{-/-}$, TCR-$\alpha^{-/-}$, TCR-$\delta^{-/-}$, etc.), suggesting that they may arise from an imbalanced immune system, rather than from a specific autoimmune response. On the other hand, the dermatitis and alopecia were only observed in F2 animals originating from the cross of a 2C TCR-transgenic mouse with a DBA/2 mouse.

The same was not true in F2 animals derived from the cross of a 2C TCR-transgenic mouse with a BALB/c mouse. For these reasons, the etiology of the dermatitis and alopecia in these mice was the focus of this study.

The correlation between the likelihood of developing the facial skin pathology and the inheritance of the 2C TCR transgenes is a strong indication that transgenic cells are in some way involved in the course of the syndrome. Localization of 2C TCR^+ cells to the epidermis and follicular sheaths and analysis of the transgenic cells from the affected animals support this hypothesis. The cells are quite unusual based on the expression pattern of a variety of T cell surface proteins. To start, they do not express the CD8 coreceptor that is normally associated with class I MHC restricted α/β T cells. Perhaps it is by virtue of this down-regulation of CD8 surface expression that these transgenic cells were able to escape thymic deletion. When I compared $2C^+$ $CD8^-$ cells from 2Cd-F2 mice with those from a 2Cd-F1 and with $2C^+$ $CD8^+$ cells from a 2C TCR-transgenic mouse, some interesting differences were revealed. While both F1 and F2 mice accumulated $2C^+$ $CD8^-$ cells in the periphery, F2-derived cells show signs of activation as determined by up-regulation of the markers CD69 and CD44 and to a small extent L-selectin as compared with F1-derived $2C^+$ $CD8^-$ and normal resting $2C^+$ $CD8^+$ cells. Interestingly, though the cells seemed largely activated by virtue of these markers, they remained absolutely negative for surface expression of the Il-2 receptor (CD25). This marker is normally expressed upon activation of T cells, but the transgenic cells from 2Cd-F2 mice remained negative for CD25 expression even when stimulated with peptide and APCs or with a mitogen, such as concavalin A. Taken together with the inability of the cells to proliferate upon *in vitro* stimulation (Figure 6-8), the lack of CD25 expression

suggests that cells are in some sense anergic. However, the cells are active enough to potentially cause damage directly by cytolysis (Figure 6-9), or indirectly by cytokine secretion and activation of other cells.

One possibility that must be considered is that these mice might be more susceptible to chronic infection due to the "imbalance" of their immune systems. Several facts indicate that this is unlikely. Known immune-deficient mice were housed under the same conditions and were not affected. A variety of antibody- and PCR-based tests failed to detect any pathogens aside from the normal flora in sick animals. Ongoing treatment of the mice from birth with Septra (Trimethoprim sulfa, Teva Pharmaceuticals USA, Sellersville, PA) did not prevent onset of the symptoms. Finally, the strict segregation of symptoms with the presence of 2C transgenic cells strongly indicates that this is an autoimmune disorder.

Cytokines are potential mediators of this autoimmune disorder. The transgenic 2C cells from 2Cd mice secreted TNF-α, IFN-γ, TGF-β and IL-4. TNF-α is a particularly good candidate for several reasons. Transgenic mice that systemically overexpress TNF-α suffer from cachexia, dermatitis and arthritis (132). Transgenic mice that overexpress TNF-α specifically in T cells show signs of cachexia, ischemia, and lymphoid and myeloid hyperplasia (133). Tristetraprolin- (TTP) deficient mice also develop an autoimmune syndrome that includes cachexia, arthritis, myeloid hyperplasia, dermatitis and "patchy" alopecia (134). In both TNF-α transgenic mice and TTP-deficient mice, virtually all symptoms could be ameliorated by the administration of neutralizing antibody specific for TNF-α, confirming that TNF-α is the primary mediator of these symptoms (132-134).

In many other instances, TNF-α has been implicated in the pathology of the skin. In addition to lymphocytes, other cells, such as macrophage and epithelial cells, also secrete TNF-α. Furthermore, epithelial cells also express the 55-kDa form of the TNF receptor providing for autocrine activation of epithelial cells and TNF-α production. There also is a correlation between TNF-α production and inflammatory skin diseases, such as cutaneous hypersensitivity reactions, psoriasis or graft vs. host disease (135).

Model for autoimmune disease of the skin

The pathology observed in the 2Cd mice most closely resembles the human disease psoriasis [Dermatology in General Medicine (130) provides a thorough overview of human psoriasis]. The microscopic pathology of psoriasis includes a greatly thickened epidermis (three to five times the normal cell number), increased epidermal proliferation rates and inflammatory infiltrates within the dermis consisting of lymphocytes, macrophages, neutrophils and mast cells. There is an association of psoriasis with inflammatory bowel disease, occlusive vascular disease and exfoliative erythroderma. Serum IgA levels and IgA immune complexes are elevated in patients with psoriasis and are thought to be the result of a compensatory response to disease progression. Evidence that psoriasis is an autoimmune disorder includes the presence of large numbers of T cells in psoriatic lesions, strong linkage to certain class I *HLA* loci, association of TNF-α production (among other cytokines, including IL-1, -6, -8, -12 and TGF-α) with psoriatic lesions and a responsiveness to treatment with immunosuppressive reagents, such as cyclosporin A. Arguing against the validity of these mice as a model for psoriasis is the lack of a systemic hyperplasia of lymphoid and myeloid cells, and the lack of a broad hyper-Ig response in human patients. Although the scalp is one of the principal sites

prone to developing psoriasis, the disease often affects many other areas of the body, especially the areas of the skin covering joints. The tight regional restriction to the skin around the eyes and face in mice may reflect an influence peculiar to the 2C TCR-transgenic cells, or some physiologic difference in the skin of the head and face as compared with that of the rest of the body.

Genetic contributions to skin disease

As noted above, there is a strong association between psoriasis and certain *HLA* alleles (130). One interpretation of this finding is that these HLA proteins present more efficiently an antigen involved in the pathogenesis of psoriasis. The *TNF* locus also is located within the *MHC* locus of both humans and mice (2, 135). Mice can be distinguished in terms of epithelial TNF-α production in response to skin irritants. For example, BALB/c are low responders whereas CBA and C57BL10 are high responders (135). In congenic mice, the response patterns co-segregated with the *MHC* locus. This suggests that strain variations in TNF-α production may reflect polymorphisms at the *TNF* locus itself. Such a genetic difference between DBA/2 and BALB/c mice could explain some of the differences observed between these two strains. However, this could not explain why the disease does not occur in 2Cd F1 mice, yet it does in 2Cd-F2 mice that are heterozygous at the *MHC* locus.

The purpose of crossing the 2Cd-F1 mice with the various BXD-RI lines was to approach the identification of genes that contribute to the etiology of the disease. For instance, if the pathology were controlled by a single DBA/2 recessive locus, then one would predict that roughly half of the lines tested should give TCR-transgenic offspring that are susceptible to disease, and that half of such transgenic offspring should actually

develop the disease. If the pathology were controlled by two such loci, then the ratios expected would be one in four. Instead, a broad range of phenotypes developed among all of the RI lines tested. Some lines were prone to severe cases of disease, while others were prone to milder forms, and still others were asymptomatic. The data are difficult to interpret because of the small sample size. What the data may suggest is that multiple loci influence the pathogenesis of the disease. The inheritance of any one locus could result in mild symptoms, while the inheritance of multiple loci could result in progressively more severe symptoms. Progeny of an RI line that carried a negative regulator of the disease might only suffer mild symptoms, whereas progeny of a line without such a locus might be prone to more severe symptoms.

Based on the data shown here, it would be worthwhile to screen for chromosomal polymorphisms that segregate with the disease phenotype. By examining a large number of individual meiotic events, one should be able to generate a more statistically reliable assessment of the genetic components of the disease. Such screening should be initially guided by the results obtained with the RI crosses. Due to the small number of lines tested, these data cannot be considered highly significant, but the trends observed in the distribution of previously mapped C57BL/6 and DBA/2 alleles among the susceptible and asymptomatic RI lines may provide a useful place to start genetic mapping (see Appendix B). In the end, such analysis may provide answers to two important questions: why do some animals that seemingly have all of the components of an autoimmune disease do not develop the disease and why do those animals that do develop disease suffer such anatomically restricted symptoms? Hopefully, such answers will provide a better understanding of the regulatory mechanisms of the immune system and insight into

the unique qualities of the facial epidermis that make it particularly susceptible to autoimmune disease.

Materials and methods

Mice

All mice were bred and maintained under standard conditions. The 2C transgenic mice were maintained in our colony at the Center for Cancer Research, MIT, Cambridge, Massachusetts. DBA/2 and BXD mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. BXD lines 1, 5, 6, 9, 11, 12, 16, 18, 21, 22, 24, 25, 27, 30, 31 and 32 were selected for their expression of H-2^{d} MHC proteins. Homozygous 2C transgenic mice were crossed with DBA/2 mice to produce H-$2^{d/b}$ 2C $^{+/-}$ F1 animals. F1 animals were then intercrossed to produce F2 animals. H-$2^{d/d}$ $2C^{+}$ F2 were typed by peripheral blood staining the $2C^{+}$, H-2^{d+} and H-2^{b-} phenotype. These F2 mice were then bred to continue production of H-2^{d} $2C^{+}$ mice. F1 animals also were crossed with each of the BXD lines. The offspring were typed by peripheral blood staining for 2C TCR and IA^{b} expression.

Septra treatment was performed 3 days a week and administered in the drinking water. Working solutions were prepared mixing 6.25ml concentrated Septra suspension (40 mg/ml sulfamethoxazole and 8 mg/ml trimethoprim, Teva Pharmaceuticals USA, Sellersville, PA) into 200 ml of drinking water.

Flow cytometric analysis

The following mAbs were used: R-phycoerythrin (PE)-labeled RM4-5 (anti-CD4, Pharmingen), fluorescein isothiocyanate (FITC)-labeled 53-6.7 (anti-CD8a, Pharmingen), TRI-COLOR (TC)-labeled YTS 169.4 (anti-CD8a, Caltag), biotin- and

FITC-labeled 1B2 [anti-2C TCR clonotypic (96)], PE-labeled AF6-120.1 (anti-I-A^b, Pharmingen), PE-labeled 3C7 (anti-CD25, Pharmingen), PE-labeled IM7 (anti-CD44, Pharmingen), PE-labeled 23G2 (anti-CD45RB, Pharmingen), PE-labeled MEL-14 (anti-CD61L, Pharmingen), PE-labeled H1.2F3 (anti-CD69, Pharmingen) Splenocyte and lymphocyte suspensions were prepared from dissected tissue and analyzed as described previously (60, 62).

CTL assay

^{51}Cr-labeled T2-L^d target cells (92) and 2C CTL (136) were combined in 150 µl of K medium (RPMI 1640 supplemented with 10% FCS, 10 mM HEPES, 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM β-mercaptoethanol) in round bottom wells of microtiter plates. To each well, 50 µl of peptide at various concentrations in PBS were added. The plates were incubated in a CO_2 incubator for 4 hours at 37°C. Percent specific lysis was calculated from the average of duplicate wells as:

$$100 \times \frac{^{51}\text{Cr experimentally released - spontaneous release}}{\text{total release in 0.1\% NP40 - spontaneous release}}.$$

Cytokine measurements

The presence of cytokines in culture supernatants was determined by ELISA using the antibodies JES6-1A12 and JES6-5H4 (Pharmingen) for the detection of IL-2, antibodies BVD4-1D11 and BVD6-24G2 (Pharmingen) for the detection of IL-4, antibodies JES5-2A5 and SXC-1 (Pharmingen) for the detection of IL-10, antibodies R4-6A2 and XMG1.2 (Pharmingen) for the detection of IFN-γ, and the Factor-test-X kit (Genzyme) for the detection of TNF-α.

Histology and immunohistochemistry

Tissue samples were fixed in 10% buffered formalin phosphate (Fischer) over night, washed in 70% ethanol, processed and embedded in paraffin blocks. Sections (6 μm) were cut and stained with hematoxilin and eosin and then mounted with Permount (Fischer).

Alternatively, tissue samples were placed in Tissue-Tek O.C.T. compound (Miles), snap frozen in a dry ice/ethanol bath and stored at -20 ° C. Sections (6-10 μm) were cut and stored at -20 ° C. Slides were briefly fixed in acetone and blocked with normal serum. Biotinylated antibodies were added (0.1–0.5 μg/ml) and incubation was carried out for 30 minutes at room temperature. Visualization was carried out using the Vectastain ABC kit and either the DAB or AEC substrate kits (Vector Laboratories). Color development was allowed to continue for 5 to 10 minutes. Slides were then rinse in water, counterstained with hematoxilin and mounted with DAKO Glycergel.

Antibodies used were either biotin-labeled 1B2 [anti-2C TCR clonotypic (96)] or biotin-labeled 145-2C11 (anti-CD3ε, Pharmingen).

Contributions

CTL assays were performed with the assistance of Carol McKinley, a research assistant to Professor Herman Eisen. Dr. Mark Schrenzel of the Department of Comparative Medicine at MIT, provided a clinical evaluation and independent verification of the histology results. All other work was performed by the author.

Chapter 7 Discussion overview

The body of work presented here has largely focused on the interactions between TCR and peptide–MHC complexes and their influence on the development of $CD8^+$ T cells in the thymus. Several major issues concerning the nature of these interactions were studied.

In Chapter 2, three important insights into the positive selection of $CD8^+$ T cells were discussed. First, it was found that the nominal LCMV-derived antigen that is recognized by P14 TCR-transgenic T cells could also positively select P14 thymocytes in FTOC. Second, the efficacy of different peptides to positively select P14 thymocytes correlated with their efficacy to elicit immune responses from mature P14 lymphocytes. Third, the same peptide that induced positive selection of P14 T cells also induced negative selection of P14 cells, when at higher peptide concentrations. These data were interpreted to indicate that, the outcome of thymic selection is determined by three criteria: (i) intrinsic affinity between a TCR and peptide–MHC complex, (ii) the density of TCR on immature thymocytes, and (iii) the density of peptide–MHC complexes on thymic APC. The product of these three factors is defined as the avidity of interaction between the TCRs of thymocytes and peptide–MHC complexes on presenting cells. This model suggests that there is no qualitative difference between peptides that are responsible for the positive selection of mature T cells and those that are responsible for the negative selection of potentially autoimmune thymocytes. Implicit in this model is the notion that the peptides presented by the MHC complexes not only stabilize the MHC proteins, but also contribute to the specificity of positive selection. Furthermore, this model downplays the significance of costimulatory signals in determining thymocyte fate since

the outcome of thymic selection could be controlled by simply manipulating the amount of peptide added to the system.

Chapter 3 introduces the 2C TCR-transgenic mouse line and the technique of RTOC as an experimental system to study thymic development. The 2C line offered distinct advantages over the other transgenic model systems. The 2C TCR was discovered by virtue of its cross-reactivity with an allogeneic MHC allele. As a result, several cognate peptides of host origin (rather than viral origin, for example) were identified. Many peptides, both of natural and synthetic origin, have been biochemically characterized. Thus, the experiments could be interpreted with respect to detailed binding data. It was found that a peptide with an affinity as low as $3 \times 10^3\ M^{-1}$, or as high as $1 \times 10^7\ M^{-1}$, could both positively and negatively select at relatively low and high concentrations. This was a direct demonstration of the first of the key tenets of the differential avidity model, as outlined above. Interestingly, the peptide p2Ca, which is recognized by 2C cells when bound to H-2L^d, could both positively and negatively select 2C thymocytes in association with the appropriate H-2K^b self-restriction element. This was the first demonstration of a host-derived peptide that could elicit the positive selection of CD8$^+$ T cells. In addition to antigenic and unrelated peptides, peptides that could antagonize mature 2C T cell responses were also tested for their ability to induce positive or negative selection. In this experimental system, antagonist peptides elicited no responses in RTOC. Again, the issue of costimulation was raised since, in this instance, reaggregate thymic cultures consisting of purified thymic epithelial cells in the absence of dendritic cells could mediate both positive and negative selection. This is significant, since dendritic cells

have long been known to play a role in the thymic deletion of T cells and were thought to provide important costimulatory signals.

Chapter 4 focused on the contribution of the surface density of TCR to thymic selection. Through antibody blocking experiments, it was found that, just as altering the amount of available ligand alters the outcome of thymic selection, so does altering the amount of available TCR. By blocking the TCRs on the surface of thymocytes, the efficiency of positive selection was reduced, or the outcome of selection was changed from negative to positive. To put these findings in a more quantitative context, the absolute number of TCR on immature and mature 2C cells was measured and found to be approximately 5,000 and 50,000 TCR / cell, respectively. It was found that blocking less than 50% of the surface TCR could have a dramatic effect on selection.

There were three principal observations in Chapter 5. First, thymic epithelial cells that express an alloantigen, H-2L^{d}, could positively select 2C TCR-transgenic thymocytes in the absence of H-2L^{d+} dendritic cells. Second, the level of expression of class I MHC proteins was measured and found to be ten-fold higher on thymic dendritic cells than on thymic epithelial cells. Finally, the level of peptide-stabilized MHC proteins on the surface of thymic epithelial cells was measured and found to be less than three-fold higher at negatively than at positively selecting doses of added peptide. Together, these data suggest that differential expression of class I MHC proteins on thymic dendritic and epithelial cells contributes to the physiologically disparate roles these cells play in the positive and negative selection of thymocytes.

Several molecules involved in cell–cell interactions have been implicated in the negative selection of thymocytes. B7 expression on APC or crosslinking of CD28 with

specific antibodies promotes the deletion of thymocytes *in vitro*. Co-expression of ICAM-1 with B7 on APC resulted in enhanced deletion of thymocytes *in vitro*. *In vivo* administration of antibody specific for gp39, the ligand for CD40, blocked the deletion of superantigen-reactive T cells and the deletion of TCR-transgenic T cells in response to low, but not high, doses of antigen (114). However, thymic development seems largely normal in CD40-, and gp39-deficient mice (137). Mice deficient for the genes encoding B7 or CD28 could generate largely normal primary T cell responses as well. The exact roles of these costimulatory molecules may become clear once their associated intracellular signaling responses are fully understood.

The function of the TCR is primarily that of antigen recognition carried out by its large extracellular domain. The TCR is linked to the intracellular signaling process by virtue of the non-covalent association of its small intracellular domain with CD3εγ, CD3εδ and CD3ζζ or CD3ζη dimers (138). Two different, though not mutually exclusive, mechanisms for variable signaling through the TCR are possible. The TCR complex itself could act as a switch, transmitting qualitatively different signals based on the quantity of receptor engagements. Cross-linking experiments have shown that oligomerization, rather than dimerization, of the TCR complex was required for signaling in mature T cells (139, 140). Such clustering might facilitate intermolecular phosphorylation of the CD3 chains and their associated enzymes/substrates (e.g. $p59^{fyn}$ tyrosine kinase, phospholipase C). Non-clustering doses of ligand may only activate a subset of target enzymes or signal cascades (e.g., $p59^{fyn}$ and CD4- or CD8-associated $p56^{lck}$) and may result in positive selection. At full clustering concentrations the full

repertoire of target pathways could be activated (e.g., $p59^{fyn}$, $p56^{lck}$, PLC, PKC, etc.) and may result in negative selection.

Alternatively, the TCR may simply convey the intensity of signal, and the interpretation of this signal may lie farther downstream. Differences in intracellular calcium responses to antigen exposure have been detected between naïve and tolerant lymphocytes. Naïve cells exhibit a strong calcium response, including the nuclear translocation of the transcription factors NFAT and NFκB and the activation of the JNK and ERK kinases. Tolerant or anergic lymphocytes produce a weak calcium response and NFAT activation, but not NFκB, JNK or ERK activation. In mature T cells, phosphotidyl inositol (PI) turnover is associated with calcium signaling. Evidence suggests that PI turnover does not occur in immature thymocytes, although low-levels of calcium flux are detectable in thymocytes undergoing negative selection (141). Enzymes that are activated in response to different levels of calcium could then be responsible for the divergent signals. A low level calcium response occur by the release of calcium stores from the ER. An increased response could be obtained by the opening of channels allowing the influx of extracellular calcium. If calcium release is proportional to TCR ligation, then positive and negative selection could be the result of low and high levels of intracellular calcium, respectively. However, it also is conceivable that differential signaling could be achieved through the depletion of an important resource, rather the accumulation of one. Negative selection could be the result of the inactivation of an enzyme or exhaustion of a substrate necessary for the survival of the thymocyte. Such a system might be advantageous since it would not only promote negative selection in

response to acute engagement of TCR, but also in response to a sustained low level of TCR engagement.

Several components of the intracellular signal pathways responsible for positive and negative selection have been identified. The immunosuppressive drug cyclosporin A (CsA) inhibits the activity of the calcium-dependent phophatase calcineurin. Treatment of mice with CsA disrupts both positive and negative selection of thymocytes. However, a few self-reactive thymocytes often escape to the periphery of these animals, resulting in a high incidence of autoimmune disease. This could be interpreted to indicate that CsA-treatment disrupts negative selection more severely than it does positive selection *in vivo* (i.e., a few cells are successfully positively selected, but self-reactive cells are not subsequently negatively selected). *In vitro* experiments with CsA have had conflicting results, some suggesting that CsA partially inhibits deletion, others suggesting that it has no effect (142-145). Another inhibitor of calcineurin, FK506 differs from CsA in that it clearly disrupts positive selection, but not negative selection, *in vivo* (145). Mice that express a dominant negative form of $p21^{ras}$ show disrupted positive, but not negative, selection of T cells *in vivo* (146). Similarly, positive but not negative selection is inhibited in mice that express MEK-1, an inactivated form of MAP kinase (147). Mice deficient for the protein tyrosine kinase ZAP-70 show neither positive nor negative selection of thymocytes (148).

From these data it seems clear that positive and negative selection involve shared signaling pathways. However, several components that are necessary for positive selection, are not strictly required for negative selection. Calcineurin-dependent pathways seem to be required for positive selection. Negative selection, however, seems

to involve additional redundant pathways that are calcineurin-independent. The contribution of secondary signals could explain the conflicting results with calcineurin inhibitors. *In vitro* deletion experiments are often carried out in the presence of professional APCs. The variable influence of CsA could reflect the variable ability of the APCs used to provide secondary signals necessary for calcineurin-independent negative selection.

Of particular interest in the study of thymic development is the nature of the peptides involved in the positive selection of developing thymocytes *in vivo*. In this study, different peptides were studied for their effect on thymocytes expressing transgenic 2C TCR. Different peptides of limited sequence homology to each other and of greatly different affinities for the 2C TCR could all positively and negatively select 2C thymocytes. Peptides that did not elicit any sort of response from mature 2C cells had no effect on thymic development. Peptides that could antagonize mature 2C T cell responses also had no selection activity. It was previously shown that antagonist, but not agonist, peptides for the ovalbumin-specific TCR could induce the positive selection of anti-ovalbumin TCR transgenic thymocytes (82, 93). By contrast, agonist peptides could positively select P14 TCR-transgenic thymocytes (42, 83). The discrepancy between the anti-ovalbumin specific TCR transgenic system and the other two systems may be due to unknown differences between the different TCRs or the class I-deficient mice used in these experiments (β2m- vs. TAP1-deficient).

Recently, peptides that could promote positive selection *in vitro* have been purified from cell lines of either lymphoid or thymic epithelial cell origin. Using a lymphoid tumor cell line, Hogquist, *et al.* identified eight HPLC peaks that contained peptides that

could elicit responses from anti-ovalbumin specific thymocytes (149). One of these peptides was purified and found to have positively selecting activity *in vitro*, albeit at extremely high concentrations (500 μM). Based on its sequence, this peptide appears to be derived from the mouse F-actin capping protein, which is expressed in thymic epithelial cells. The only common amino acid residues it shares with the nominal antigen peptide are the two H-2D^b binding residues at positions five and eight. Interestingly, this peptide could not sensitize target APC for lysis even at these extremely high concentrations.

From a transformed thymic epithelial cell line, Hu *et al.* purified and sequenced three peptides that had varying abilities to positively select either P14 (LCMV-specific) or F5 (influenza-specific) TCR-transgenic thymocytes (150). This activity was only evident at high doses of peptide (300 μM). Interestingly, these peptides also had varying abilities to positively select polyclonal (i.e., non-transgenic) thymocytes. In contrast with Hogquist *et al.*, these peptides could sensitize APC for lysis by the appropriate T cell line, albeit very weakly.

These data suggest that positive selection involves a substantial amount of cross-reactivity between particular peptides and different TCRs, and vice versa. In such a model, several different thymic peptide–MHC complexes could contribute to the overall avidity with the TCRs of a particular thymocyte. Conversely, any particular peptide may participate in the positive selection of different thymocytes with several different antigen-specificities. The surface density of peptide–MHC proteins that results in positive selection was not directly determined in previous studies. However, the high concentrations required for positive selection and the half-maximal class I stabilization

activity of each of the peptides studied (range, 2 x 10^{-8} to 2 x 10^{-5} M) suggest that the concentrations of peptides used do not reflect physiological conditions (149, 150). This also suggests that the peptide–MHC complexes formed with these peptides have very low affinities for their respective TCRs. This is only speculation until such measurements are taken directly. In any case, it is likely that these peptides would need to work in concert with others to provide sufficient signaling for positive selection.

The ideal experiment to prove that a peptide is responsible for the positive selection of a particular T cell clone would be to remove that peptide from an animal's repertoire and show that this abrogates the positive selection of that T cell clone *in vivo*. A potential problem with this approach is that several peptides may be involved in the selection of any particular T cell clone. In this case the approach would only be useful if each peptide were present at a critical level so that the removal of any one would result in a significant defect in positive selection.

These recent advances in our understanding of the development of $CD8^+$ T cells come from studies in which T cell repertoire was restricted to a single TCR and the peptide ligand was varied in quantity and identity. Class II MHC proteins undergo different metabolic processing than class I MHC proteins and are not as amenable to the loading of pure species of exogenously added peptide. Instead, researchers examined the selection of diverse populations of $CD4^+$ T cells in transgenic and knockout mouse systems that express single peptide–MHC class II protein combinations.

Ignatowicz *et al.* generated mice that expressed a transgene encoding the class II MHC $IA^b\beta$ chain covalently linked to an IA^b-restricted peptide. These mice were then crossed into a $IA^b\beta$ chain- and Ii chain-knockout background resulting in mice that

predominantly express a single peptide–MHC class II species. The total amount of surface class II MHC proteins was significantly lower in the mutant animals than in normal mice and the mutant animals generated only approximately 20% of the number of $CD4^+$ T cells that develop in normal mice. The TCRs of these cells used diverse Vβ chains. However, the Vβ chain distribution differed slightly between mutant and normal animals (151).

Other groups generated mice that were deficient for the gene encoding the H2-M protein. H2-M is a MHC class II-like protein that facilitates the release of the fragments of the class II-associated invariant chain peptides (CLIP) that occupy the peptide-binding groove of MHC class II proteins (152, 153). In the absence of H2-M, CLIP remains bound to class II proteins and prevents the binding of other peptides in the lysosomal compartments. H2-M-deficient mice expressed normal levels of IA^b at the cell surface, but only in association with CLIP. Approximately 50% of the number of $CD4^+$ T cells were present in mutant mice compared with normal littermates. Again, $CD4^+$ cells from mutant mice displayed a diverse selection of Vβ chains with only slight variations from normal animals (154, 155).

Interestingly, it was found that $CD4^+$ T cells from both the H2-M-deficient and $IA^b\beta$-peptide-transgenic mice cross-reacted strongly with syngeneic and allogeneic MHC class II proteins presenting a naturally diverse repertoire of peptides (151, 154, 155). Furthermore, Ignatowicz *et al.* estimated that T cells selected on the single peptide–MHC combination reacted with allogeneic MHC three to five times more frequently than did T cells from normal mice. Surprisingly, T cells from the peptide–MHC-homogeneous mice

cross-reacted with cells expressing naturally diverse peptides bound to syngenic IA^b proteins twice as frequently as they did with allogeneic cells (151).

Further evaluation of the H2-M-deficient mice yielded very interesting results. These animals could mount a $CD4^+$ T cell response comparable to that of normal animals when immunized with foreign peptides (156). Interestingly, the mutant mice mounted a poor response to immunization with intact protein, suggesting that peptide-immunization might by-pass critical processing pathways (156, 157). The mutant animals could also mount a competent IgG response to viral infection, indicating that an intact T helper cell-response was present in these animals (156). In spite of the ability to mount diverse $CD4^+$ T cell responses, H2-M-deficient background mice nevertheless failed to positively select five different transgenic TCR specificities (156-158). The cross-reactivity of cells from these mice was also examined in more detail. About twice as many precursors were found that reacted with normal syngeneic APC than with allogeneic MHC proteins. In bone marrow chimeras, the presence of normal syngeneic (in this case C57BL/6 or B6) donor-derived APC reduced the number of $CD4^+$ T cells by two to three fold in H2-M-deficient mice and eliminated the cross-reactivity with B6 APC. Depletion of B6-reactive cells resulted in a substantial increase in the frequency of alloreactive cells among the remaining $CD4^+$ T cell population.

In a different approach, Nakano *et al.* used an adenovirus-derived expression system that could target vector-encoded peptides to endosomal-lysosomal compartments for loading onto class II MHC proteins (159). The adenovirus vector was injected intrathymically in Ii-deficient mice. This system had the advantages that it did not require the generation of transgenic animals or the crossing of multiple mutant lines. It

was found that the nominal antigen peptide, variants of that peptide and unrelated peptides could positively select endogenous (i.e. non-transgenic) T cells specific for a moth cytochrome C (MCC)-derived peptide in conjunction with IE^k proteins. The MCC-reactive T cells bore TCRs of limited Vα and Vβ chain diversity. MCC-reactive T cells selected by one peptide usually had very limited cross-reactivity with the other peptides used in that study.

Liu *et al.* also studied MCC-reactive T cells and created a variety of animals that expressed transgenes encoding $IE^k\beta$ chains linked to a variety of peptides (160). The resulting mice expressed IE^k only in association with the transgene-encoded peptide and IA^f in association with endogenously derived peptides. Liu *et al.* largely recapitulated what Nakano *et al.* reported with one notable exception. In this experimental system, the same unrelated peptide as that used in the adenovirus system could not select MCC-specific T cells. The authors offer two main explanations for this discrepancy. First, MCC-reactive T cells selected by the unrelated peptide coincidentally may have been eliminated by endogenous peptides bound to IA^f. Second, differences in the level of expression between transgene-encoded peptide–IE^k and adenovirally introduced peptide complexes may be responsible for the disparate results. Another possibility is that the expression of the adenovirus vector was restricted to the cortical and medullar epithelium of the thymus (159). The expression of peptide–IE^k proteins on thymic dendritic cells in transgenic mice may be responsible for the deletion of MCC-reactive cells in these animals.

The issue of what makes an APC particularly appropriate for mediating the negative selection of T cells was not addressed in these studies. When presenting a diverse array

of peptides, bone marrow-derived APC mediated the deletion of many self-reactive T cells that otherwise would have matured in H2-M mice. This result suggests that self-reactive T cells are mostly eliminated by MHC proteins presenting peptides different from those that impart the positively selecting signal. To gauge the amount of negative selection mediated by a single CLIP peptide in these animals, an interesting experiment might have been to create chimeric mice by transfer of class II-deficient bone marrow cells into H2-M-deficient recipients ($II^{-/-} \rightarrow H2\text{-}M^{-/-}$). If a greater number of T cells develop than in an $H2\text{-}M^{-/-} \rightarrow H2\text{-}M^{-/-}$ transfer, or, more profoundly, if the $II^{-/-} \rightarrow H2\text{-}M^{-/-}$ animals developed autoreactive T cells, this would indicate that the same peptide that induces positive selection of T cells can also induce negative selection *in vivo*. The caveat to this experiment is that cells other than those haemopoietically derived could mediate thymic deletion. However, the natural recombination of receptor genes may generate novel TCR rearrangements that result in T cells selected by a peptide–MHC combination that recognize that same combination as mature cells. Regardless of the outcome of these experiments, it would seem that whatever specialized features (e.g. costimulatory signals, controlled expression of MHC proteins, etc.) the immune system has adopted to ensure the elimination of self-reactive cells, the most important factor is the presentation of a comprehensive set of self-derived antigens during development.

Due to differences between the MHC class I and class II experimental systems, the questions asked regarding each system were different. The transgenic TCRs examined in the class I system had already undergone the natural selection process and thus already exhibited three of the cardinal features of an antigen receptor: self-restriction, antigen specificity and self-tolerance. The class II experiments highlighted the resourcefulness of

the recombinatorial mechanism for TCR diversity. In spite of being restricted to encountering a single peptide-MHC class II ligand during maturation, the $CD4^+$ T cells from these animals competently responded to antigenic and pathogenic challenge. This would suggest that the purpose of the presentation of a diverse set of peptides by MHC proteins during thymic development is primarily to eliminate self-reactive T cells. The contribution of diverse peptides might seem trivial in light of these results, but a diverse repertoire obviously is responsible for the larger repertoire of T cells observed in normal mice and for the selection of the few specific TCR combinations that have been tested in these systems. Survival of an organism, and of a species, does not entail responding to a challenge in a sufficient or adequate manner. Rather, it involves meeting each challenge with the most vigorous response possible. To combat infection as effectively, vertebrate immune systems developed the ability to process and present as diverse a population of antigens as possible. This came with the cost of a greater chance of autoimmune disease. Fortunately, the processing machinery that allows for the careful surveillance of foreign pathogens also helps insure that harmful self-reactive lymphocytes never develop.

Future advances in our understanding of the interactions between TCR and peptide–MHC proteins are likely to come from more detailed x-ray crystallographic and other structural analyses. Although natural, positively selecting peptides identified thus far have had very weak affinities for their cognate TCRs, no distinguishing characteristics have been identified. It seems that the features required for TCR engagement during thymic development are essentially those necessary for TCR engagement by mature lymphocytes.

One interesting issue raised in this study is the incongruity of the data regarding the peptides dEV8 and p2Ca. Even though the dEV8 peptide had a higher affinity than p2Ca for H-2K^b and the dEV8–K^b complex had a higher affinity than the p2Ca-K^b complex for the 2C TCR, dEV8 was still less efficient in promoting the positive selection of 2C cells (Chapter 5). This correlated, however, with the relatively weaker ability of dEV8 to sensitize T2-K^b target cells for lysis by 2C CTL. Interestingly, the relative efficacy of dEV8 to sensitize APC for lysis was dependent on the APC used. The order of increasing efficacy to sensitize T2-K^b APC for lysis by 2C CTL was dEV8 < p2Ca < SIY8, while the order to sensitize concavalin A-stimulated splenocytes was usually p2Ca < dEV8 < SIY8 (H.N. Eisen, personal communication). Thymic epithelial and dendritic cells were also tested as targets in CTL assays and found to behave similarly to T2-K^b cells (data not shown). These data suggest that some additional factor may be present on certain APC that participates in or influences the TCR–peptide/MHC interaction. These data also suggest that there may be some unique quality of either the p2Ca or dEV8 peptide that is responsible for enhanced or diminished activity, respectively. For example, peptide binding could result in subtle conformational changes in the MHC protein that discourage dimer or oligomer formation. Likewise, particular peptide–MHC combinations might have a similar influence on the TCR. Thus, dEV8–K^b complexes might be less potent activators than p2Ca–K^b complexes despite their higher intrinsic affinity for the 2C TCR. A comparative examination of the oligomerization of these two complexes might be revealing in this regard. The crystal structure of the 2C TCR engaged by dEV8–K^b complexes has been determined (89). A comparison of crystal structure data of the 2C TCR engaged by p2Ca–K^b with the previously published data involving dEV8 could

resolve this issue and reveal novel aspects of TCR engagement important for proper activation of T cells.

Chapter 6 is primarily a clinical evaluation of an autoimmune disease of the skin that develops in certain strains of 2C TCR-transgenic mice that express the H-2L^d protein. The most interesting feature of this syndrome is that it occurred in a subset of 2C-positive, H-2^d-positive F2 mice resulting from the cross of a 2C TCR-transgenic mouse with a DBA/2 inbred mouse (2Cd-F2 mice) and not in any F2 mice resulting from the cross of a 2C transgenic mouse with a BALB/c inbred mouse. The disease was observed both in mice that were H-2^{dxd} or H-2^{bxd}. This suggests that a locus, other than the *MHC*, that is present in DBA/2 and not in BALB/c mice contributes to the etiology of the disease. This hypothesis is supported by the result that only certain BXD-RI lines were also susceptible to the disease.

The disease seems to be the result of 2C TCR transgenic cells that are present in the periphery of the 2Cd-F2 animals. These cells escape thymic deletion presumably through the down regulation of the CD8 coreceptor, as has been observed in other experimental systems (161, 162). The mere presence of the 2C$^+$ DN cells is not sufficient to cause disease, since they also are present in both 2Cd-F1 mice and 2C TCR-transgenic/BALB/c mice which do not develop disease. It remains to be seen if factors intrinsic or extrinsic to the 2C$^+$ DN cells are responsible for the onset of pathology. When suitably inbred lines of mice become available, this issue may be examined through adoptive transfer experiments. TNF-α may play a critical role, since it is secreted by the 2C$^+$ DN cells and is known to result in symptoms similar to those seen here. Treatment of animals with antibody specific for TNF-α might protect them from the onset of disease.

A more detailed genetic analysis of the disease may lead to the identification of a novel genetic locus that influences T cell behavior. Alternatively, it may lead to the identification of a new property of a previously identified gene. One possibility is that certain 2Cd mice express a novel antigen in a tissue specific manner, resulting in activation and infiltration of 2C T cells into the epidermis of the face. However, such an antigen would have to display a recessive expression pattern or be influenced by some recessive trait that would only be present in a subset of 2Cd mice. Another possibility is that the disease results from some influence on the production of TNF-α.

Differences in immune responses between inbred strains of mice have long been known. Oddly enough, BALB/c inbred mice are highly susceptible to autoimmune diseases, whereas DBA/2 and C57BL/6 inbred mice are resistant (163). The study of a disease model that develops in a DBA/2, but not BALB/c mouse strain could reveal the underlying causes for these differences.

It also will be interesting to evaluate these mice further as a model for human autoimmune skin disease. While the dermal and epidermal pathology is similar to that seen in human psoriasis, the tissue restriction and some of the associated disorders observed in these mice are not seen in human patients. If these mice respond to the clinical treatments for psoriasis, and if lines of mice can be established that more reliably and uniformly develop symptoms, then these animals could prove to be an extremely useful tool, offering the chance to better understand the etiology of autoimmune skin disease and to develop new and better treatments for use in human patients.

Future experimental plans

Several aspects of the autoimmune skin disease observed in the 2Cd animals merit further investigation. Previous studies have shown that the administration of TNF-α produced dermatitis, inflammation and cachexia similar to that seen in the 2Cd mice (135). It has also been shown that the administration of neutralizing antibody specific for TNF-α protected animals against these symptoms (133, 134). Since the 2C TCR^+ DN cells secreted TNF-α, *in vitro*, the role of both TNF-α and $2C^+$ cells were tested by the *intra peritoneal* administration of neutralizing antibody specific for either TNF-α (TN3-19.12) (164) or the 2C clonotype (1B2), *in vivo*. The preliminary results indicate that the neutralization of TNF-α resulted in a reduced activation of the $2C^+$ DN cells and protection against skin disease. The elimination of the $2C^+$ cells with 1B2 antibody also protected against the development of the skin disease. Histological and immunohistochemical analysis needs to been done to determine whether TN3-19-12 administration prevented the activation and infiltration of the skin by 2Cd cells or if the cells still targeted the skin, but were prevented from causing damage.

For future experiments it will be important to establish lines as genetically pure as possible. Back crossing transgenic animals to a purer DBA/2 background may yield animals that more consistently develop disease. Also with a purer background, 2Cd cells could be adoptively transferred to non-transgenic littermates. This would confirm that the 2Cd cells are sufficient for disease in the appropriate background. It will also be possible to track the pathology from the moment of introduction of the transgenic cells. The collected RI crosses suggest that the genetics of the disease may not be simple. More lines developed disease than not (Table 6-2). This could mean that more than one locus

is sufficient to result in the onset of the disease. This, however, is not consistent with the relative low incidence of disease observed in the F2 animals. The severity of symptoms also varied from RI line to RI line. Instead of multiple loci contributing to the onset and severity of disease, one or more loci might actually ameliorate symptoms. An RI line that developed mild symptoms may carry the disease promoting alleles at one locus and protective alleles at another. RI lines that developed more severe symptoms might have the disease causing alleles, but lack any protecting ones. The protective alleles could be either of C57BL/6 or DBA/2 origin. If extensive backcrossing to the DBA/2 background results in a more severe pathology, then the protective alleles are likely to be C57BL/6 in origin.

Although the 2Cd exhibited characteristics of α/β T cells, α/β DN cells have been identified in other systems that may have been either of the γ/δ lineage, B cell lineage or had otherwise developed extrathymically (e.g., IELs) (131, 165, 166). The 2Cd cells did not express γ/δ receptors or B cell markers (data not shown). To determine if the cells are truly thymus-derived, 2Cd neonates should be thymectomized and observed for the development of disease. Alternatively, young thymectomized animals can be lethally irradiated and reconstituted with bone marrow from affected 2Cd mice. If disease fails to develop in these animals, this would suggest that the 2Cd cells developed extrathymically and are not of the normal α/β T cell lineage. In this case, the cells should be examined for rearranged γ/δ receptor genes. The animals should also be examined to determine the true site of development of the cells.

There is clearly a difference, other than MHC, between 2Cd-F1 animals and 2Cd-F2 animals that results in some F2 animals becoming sick and none of the F1 animals doing

so. The reconstitution of 2Cd-F1 and -F2 (or otherwise inbred) animals with bone marrow from symptomatic animals would establish whether the genetic contribution is intrinsic to the 2Cd cells themselves. If the genetic influence is extrinsic to the 2Cd cells, then only the expected proportion of reconstituted F2 animals should become sick. If reconstituted F1 animals developed symptoms, then the background contribution is intrinsic to the cells. Furthermore, if the bone marrow comes from individuals which are, themselves, symptomatic, then we might expect all reconstituted animals to become ill.

The possibility exists that a unique antigen is present in DBA/2 animals that is responsible for the peculiar behavior of the 2Cd cells. The immediate drawback to this theory is that such an antigen must have a recessive expression pattern, since F1 animals do not develop disease. Such an antigen would be compatible with the theory that the genetic factor is extrinsic to the 2Cd cells themselves. This hypothesis can be directly tested by the fractionation of acid extracts of peptides from DBA/2 tissue. These extracts can be HPLC fractionated and each fraction tested for its ability to sensitize H-2^{d+} targets for lysis by 2C CTLs. The profile of active fractions can be compared with a profile of fractions from BALB/c tissue to determine if any active fractions are unique to the DBA/2 background. Unique active fractions should be further fractionated by HPLC and retested. Samples can be analyzed by mass spectrometry to determine purity and the peptides sequenced. The sequence can then be checked against known sequences to determine if the source protein has been identified and, if so, if anything can be deduced from its function or pattern of expression.

Possibly the most interesting task will be to map the gene or genes involved in the disease. Over 200 genome-wide polymorphisms between the DBA/2 and C57BL/6

strains have been mapped and can be detected by PCR. To generate the offspring one will look at, 2Cd-F1 mice should be crossed to DBA/2 mice and the offspring scored for disease. Symptomatic and asymptomatic mice will then be compared for the inheritance of the C57BL/6 (B) vs. DBA/2 (D) markers. The expected inheritance ratio is 50:50 BxD:DxD at any random locus. If a marker is linked to a disease-causing gene, then the observed frequency will deviate from the expected 50:50. Symptomatic animals should carry a higher frequency of the D-type polymorphism, while healthy animals should be more frequently B-type. A gene that confers resistance to disease can also be detected in this manner. However, one does not know apriori if such a gene will be B- or D-derived. However, careful observations of backcrosses should reveal the existence of such a locus and its strain of origin. The initial choice of chromosomes to analyze in this manner can be guided by information inferred from the RI crosses already performed, notably chromosomes 5, 7, 12, and 13 (see Appendix B).

Appendix A Some mathematical speculations

The key point of the differential avidity model is that engagement of the TCR by relatively few peptide–MHC ligands results in positive selection, whereas engagement of a relatively greater number of TCR results in negative selection. An ideal experiment would directly determine the number of TCR engagements required for positive and negative selection. Experimental limitations prevent such direct measurements from being taken and allow only indirect approximations. Thus we can measure the number of TCR available on the surface of the thymocyte and how many peptide–MHC complexes may be stabilized on the thymic epithelial cell surface. Based on these observations and the published binding data of the 2C TCR for a variety of its ligands, one might be able make some "back of the envelope" calculations to approximate how many engagements might occur under the conditions that promote positive or negative selection.

The key to this approach is to assume that the Law of Mass Action applies and that the interaction between TCR and peptide–MHC (from here on referred to as L in the context of equations) can be expressed in the form:

$$[TCR]+[L]\rightleftarrows[TCR\cdot L]. \qquad \text{(Eq. A-1)}$$

The equilibrium binding constant (K) is therefore defined by the expression:

$$K=\frac{[TCR\cdot L]}{[TCR][L]}. \qquad \text{(Eq. A-2)}$$

Adherence to the Law of Mass Action is demonstrably true in experiments where at least one of the reactants is available in a freely diffusing and soluble form (e.g., soluble peptide–MHC complexes), and arguably true in the case of CTL–APC interactions (99). Factors other than the TCR and MHC, including many intercellular adhesion molecules,

influence the interactions between thymocytes and APC. The TCR–peptide–MHC interaction probably does not add significantly to the overall energy of interaction between the two cells and only comes about after the two cells have embraced by other means. If we assume that TCR and MHC proteins freely diffuse across the cell surface and within the micro-environment of the thymocyte–APC interface, then we can proceed with our analysis.

The volume of interaction can be imagined as the product of the surface area of the cell–cell interface and the distance (i) between the two cells. For simplicity, let us assume that the area of interaction is equivalent to half of the surface area of the thymocyte, that r is the radius of a thymocyte in μm and that a thymocyte approximates a sphere. Using the formula for the surface area of a sphere:

$$S = 4\pi r^2,$$

and taking the value of 5,000 2C TCR / thymocyte from Table 4-1, we calculate:

$$[TCR] = \frac{5{,}000}{4\pi r^2} \cdot \frac{2\pi r^2}{2\pi r^2 \cdot i} \cdot \frac{mole}{N_a} \cdot \frac{10^{15}\,\mu l^3}{l} = 6.6 \times 10^{-7}\, mol/r^2 \cdot i. \quad \text{(Eq. A-3)}$$

Taking the value of 700 p2Ca–K^b complexes / thymic epithelial cell (Table 5-2) and letting n be the ratio of the radius of a thymic epithelial cell to that of a thymocyte, we can determine the concentration of ligand (L):

$$[L] = \frac{700}{4\pi(nr)^2} \cdot \frac{2\pi r^2}{2\pi r^2 \cdot i} \cdot \frac{mole}{N_a} \cdot \frac{10^{15}\,\mu l^3}{l} = 9.25 \times 10^{-7}\, mol/n^2 r^2 \cdot i. \quad \text{(Eq. A-4)}$$

If we assume that [TCR] and [L] are initial values and x is [TCR·L] at equilibrium, then we can rewrite equation A-1:

$$K = \frac{[TCR \cdot L]}{[TCR][L]} = \frac{x}{([TCR]-x)([L]-x)}, \qquad \text{(Eq. A-5)}$$

which can be written in the form of the quadratic equation:

$$Kx^2 - (K[TCR] + K[L] + 1)x + [TCR][L]K = 0. \qquad \text{(Eq. A-6)}$$

If r=5μm, n=2, and i=0.05μm, then substituting these values into equations A-3 and A-4 will determine the values of $[TCR]$ and $[L]$, respectively. Entering these values and $K=3\times10^3 M^{-1}$ into equation A-6 and solving the quadratic yields:

$$3\times10^3 x^2 - x + 2.93\times10^{-11} = 0,\ x = 2.93\times10^{-11} M,$$

and converting M into absolute numbers yields:

$$\frac{2.93\times10^{-11} mol}{l} \times \frac{N_a}{mol} \times \frac{l}{10^{15}\,\mu l^3} \times 2\pi(5\mu m)^2 (0.05\mu m) = 0.138 \text{ TCR engagements}.$$

This is an equilibrium value that is based on the assumptions we made about the environment that exists between two cells. One must remember, however, that equilibrium is dynamic. As new bonds form, old bonds break apart in accordance with concentration and the on/off rate constants. If we had calculated that exactly one bond formed during a thymocyte's encounter with an APC, we could not conclude that one bond was stable at all times. At any given moment, one, none, or 2 bonds might exist. In essence, what one observes is the peak value of a normal distribution. A value of 0.138 suggests that TCR is rarely engaged under these conditions. Another interpretation is that given 100 transgenic thymocytes, on average 13 or 14 will experience a successful TCR engagement by MHC proteins on thymic epithelial cells. Perhaps one or two will have two successful engagements. When the transgenic thymocytes encounter MHC proteins on a thymic dendritic cell surface, where the MHC density is 10 fold higher, nearly all of them will experience the engagement of one or two TCRs, and some possibly will

experience a sufficient number of engagements to receive the negative selection signal. This is consistent with the observation that in 2C transgenic mice expressing only syngeneic MHC proteins, some negative selection seems to occur [(36); and Chapter 3]. It is also consistent with the broad observation that so few thymocytes survive thymic development. Such mechanics clarify how positive and negative selection produce an effective and self-tolerant repertoire of T cells that is nevertheless often on the brink of autoimmunity.

One factor that this analysis largely ignores is the possibility of mechanisms that actively encourage TCR engagement. Adhesion molecules that secure the thymocyte and APC together give more opportunity for a positive (as well as negative) signal to occur. Clustering or "capping" mechanisms that increase the local concentration of TCR and MHC proteins would favor more interactions. TCR clustering could explain how a single receptor can transmit different messages. A lone TCR might transmit one quality of signal, while simultaneous engagement and clustering might create a higher order structure that could transmit a qualitatively different signal. Otherwise, a cell needs an accounting system that keeps track of the amount of signal received. For example, some factor that is altered or consumed during TCR signaling may be necessary for survival. Thus a modest degree of signaling results in positive selection but does not deplete this factor, and signaling beyond a certain degree depletes this factor, resulting in cell death.

Appendix B Limiting mapping with RI crosses

Normally, genetic mapping strategies involve examining a large number of individuals and correlating inheritance of a certain genotype with phenotype. Even in the simplest scenario, this is no small task. The participation of multiple genetic loci and the possibility of partial penetrance make the task even harder. A simplified form of genetic analysis can be performed by taking advantage of recombinant inbred lines of mice.

The BXD-RI lines have been well characterized at thousands of loci across all chromosomes. An example of such data is shown in Table B-1, under BXD-line genotypes. Each line has inherited one of the two founder alleles (B or D) for each chromosomal marker indicated. The original experimental design of crossing a 2Cd-F1 mouse with each of the BXD-RI lines (Figure 6-11) allowed us to score for lines with progeny that developed the autoimmune skin disease. RI lines with susceptible progeny should carry the allele(s) responsible for disease, whereas RI lines with immune progeny should not. A comparison of the genotypes between these two groups should reveal markers that segregate accordingly.

Unfortunately, the pattern of disease was not uniform among the susceptible RI lines (see Table 6-2). In some cases, although present, symptoms never progressed beyond the mildest of stages. In others, the symptoms were consistently severe. In some cases, fully half of the transgenic offspring developed symptoms, suggestive of the segregation of a single locus. In others, only a small fraction of transgenic offspring developed disease. In light of these findings, it is necessary to examine the data from more than one perspective. Although the animals are not easily categorized as affected or unaffected, they can be grouped according to severity. By comparing asymptomatic with severe, or

asymptomatic and mildly symptomatic with moderately to severely symptomatic, or other permutations thereof, some interesting patterns do emerge.

In order to ascribe a statistical framework to this analysis, a simple chi-square test was performed. The basis of this analysis was the assumption that should a marker have no relevance to pathology, then its frequency among the lines grouped according to phenotype should not differ significantly from its frequency among all lines. The formula for the chi-square is

$$\chi^2 = \frac{(n_o - n_t f_e)^2}{n_t f_e},$$

where n_o is the number of lines that carry the D allele, n_t is the total number of lines in that group and f_e is the expected frequency of D alleles among all 15 lines. This analysis is performed for each of the two groups, compared and totaled. P values for that locus can then be obtained from a chi-square distribution table (167).

For example, among the 15 lines examined, seven carry the B allele and seven carry the D allele of the *Bcl2* locus on chromosome 1 (Table B-1). The expected frequency of the D allele is $f_e = 8/15$. A comparison of groups II and V yields:

$$\chi^2 = \frac{(2 - 8 \cdot 8/15)^2}{8 \cdot 8/15} + \frac{(5 - 7 \cdot 8/15)^2}{7 \cdot 8/15} = 0.869,$$

indicating that there is no statistically significant linkage of this locus with disease. On the other hand, the same comparison of the D5Rik81 marker on chromosome 5 yields:

$$\chi^2 = \frac{(7 - 8 \cdot 10/15)^2}{8 \cdot 10/15} + \frac{(3 - 7 \cdot 10/15)^2}{7 \cdot 10/15} = 3.923,$$

which in turn yields a p-value of 0.05, suggesting that there may be significant skewing of this locus between these two populations. Several such loci can be identified among several chromosomes (Table B-1). A closer examination of these regions both in terms of testing individual animals for the inheritance of these markers and in terms of identifying previously characterized genes that may influence immune responses could lead to the identification of loci that influence the autoimmune events in the 2Cd mice.

Table B-1 Collected data on BXD RI lines and crosses with 2Cd mice.

Chromosomal marker data is available from The Jackson Laboratory at http://www.informatics.jax.org.

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		BXD-line															Grouping of BXD lines by symptoms (line nos.)																	
																	I. None (12,27,32)		II. None to Mild (9,11,12,16,25,27,31,32		III. Mild (9,11,16,25,31)		IV. Mild to Moderate (5,6,9,11,16,25,31)		V. Mild to Severe (1,5,6,18,22,24,30)		VI. Moderate to Severe (1,18,22,24,30)		All lines							
Chr	Marker	1	5	6	9	11	12	16	18	22	24	25	27	30	31	32	B alleles	D alleles	B alleles	D alleles	B alleles	D alleles	B alleles	D alleles	B alleles	D alleles	B alleles	D alleles	B alleles	D alleles	I vs.VI χ^2	p-value	II vs.VI χ^2	p-value	I vs.V χ^2	p-value	II vs.V χ^2	p-value
1	Acrg	B	B	B	B	B	B	B	.	D	D	.	.	D	B	D	1	1	5	1	4	0	6	0	3	3	1	3	8	4	2.167	ns	2.333	ns	0.583	ns	0.750	ns
1	Nppc	B	B	?	B	B	B	B	B	.	D	D	B	D	B	D	2	1	6	2	4	1	5	1	3	2	2	2	9	4	0.484	ns	0.519	ns	0.141	ns	0.177	ns
1	D1Mit11	.	D	D	B	D	B	B	D	D	D	D	B	.	.	.	2	0	4	2	2	2	2	4	0	5	0	3	4	7	2.851	0.1	2.139	ns	2.238	ns	1.526	ns
1	D1Mit135	.	D	D	B	D	B	B	D	D	D	B	B	D	B	D	2	1	6	2	4	1	4	3	0	6	0	4	6	8	1.683	ns	3.214	0.1	0.492	ns	2.024	ns
1	Bcl2	B	D	B	B	D	B	B	D	D	D	B	B	D	B	D	2	1	6	2	4	1	5	2	2	5	1	4	8	7	1.290	ns	1.895	ns	0.265	ns	0.869	ns
1	Psmb6-rs1	B	D	D	B	D	B	D	D	D	D	B	B	D	B	D	2	1	5	3	3	2	3	4	1	6	1	4	6	9	0.867	ns	1.346	ns	0.543	ns	1.022	ns
3	D3Jkn1	B	B	B	B	D	D	B	D	B	B	D	B	B	B	B	2	1	5	3	3	2	5	2	6	1	4	1	11	4	0.102	ns	0.211	ns	0.421	ns	0.530	ns
3	Pmv26	B	B	B	D	D	D	B	D	B	B	D	B	B	B	D	1	2	3	5	2	3	4	3	6	1	4	1	9	6	0.856	ns	1.175	ns	1.513	ns	1.832	ns
3	Iapls2-14	B	B	B	D	D	D	B	D	B	B	D	D	B	B	D	0	3	2	6	2	3	4	3	6	1	4	1	8	7	2.362	ns	1.966	ns	3.173	0.1	2.777	0.1
3	P40-rs4	D	B	B	D	D	D	B	D	D	B	D	D	B	B	D	0	3	2	6	2	3	4	3	4	3	2	3	6	9	1.200	ns	0.450	ns	1.543	ns	0.793	ns
3	Rnu1-ps2	B	.	B	D	D	D	B	D	B	B	.	D	D	B	B	1	2	3	4	2	2	3	2	4	2	3	2	7	6	0.275	ns	0.198	ns	0.448	ns	0.371	ns
4	Cfp1	D	B	D	D	B	B	B	D	.	D	B	B	.	.	.	2	0	5	1	3	1	4	2	1	4	0	3	6	5	2.721	0.1	2.875	0.1	0.990	ns	1.144	ns
4	Cxv2	D	B	B	B	B	D	D	D	B	B	D	D	D	.	.	0	2	2	4	2	2	4	2	4	3	2	3	6	7	0.958	ns	0.249	ns	1.080	ns	0.371	ns
4	D4Rik102	D	B	B	B	B	B	B	B	D	D	D	B	D	B	B	3	0	7	1	4	1	6	1	3	4	1	4	10	5	3.767	0.1	3.788	0.1	1.690	ns	1.711	ns
4	D4Rik103	D	B	B	B	B	B	B	B	D	D	D	B	D	B	B	3	0	7	1	4	1	6	1	3	4	1	4	10	5	3.767	0.1	3.788	0.1	1.690	ns	1.711	ns
4	D4Rik104	D	B	.	.	B	B	B	B	D	D	D	B	D	B	.	2	0	5	1	3	1	4	1	2	4	1	4	7	5	2.359	ns	2.406	ns	1.495	ns	1.543	ns
4	D4Rik105	D	B	B	B	B	B	B	B	D	D	D	B	D	B	.	2	0	6	1	4	1	6	1	3	4	1	4	9	5	3.143	0.1	3.246	0.1	1.297	ns	1.400	ns
4	D4Rik106	D	B	B	D	B	B	B	B	B	D	D	D	D	B	B	2	1	5	3	3	2	5	2	4	3	2	3	9	6	0.522	ns	0.508	ns	0.037	ns	0.023	ns
4	D4Rik107	D	B	B	.	B	B	B	B	B	D	D	D	D	B	B	2	1	5	2	3	1	5	1	4	3	2	3	9	5	0.828	ns	0.881	ns	0.103	ns	0.156	ns
4	D4Rik125	B	B	B	D	D	B	D	D	B	D	D	B	D	B	D	2	1	3	5	1	4	3	4	4	3	2	3	7	8	0.299	ns	0.186	ns	0.401	ns	0.288	ns
4	D4Rik127	D	B	D	B	D	B	B	B	D	D	D	D	D	B	D	1	2	4	4	3	2	4	3	2	5	1	4	6	9	0.367	ns	0.533	ns	0.043	ns	0.210	ns
4	D4Rik128	D	B	B	B	B	B	B	B	D	D	D	B	D	B	B	3	0	7	1	4	1	6	1	3	4	1	4	10	5	3.767	0.1	3.788	0.1	1.690	ns	1.711	ns
4	D4Rik129	D	B	B	B	B	D	B	D	B	B	D	B	D	B	D	1	2	5	3	4	1	6	1	4	3	2	3	9	6	0.856	ns	0.508	ns	0.370	ns	0.023	ns
4	D4Rik130	D	B	B	D	B	D	D	D	B	B	B	D	D	B	D	0	3	3	5	3	2	5	2	4	3	2	3	7	8	1.442	ns	0.186	ns	1.544	ns	0.288	ns
4	Mtv14	D	B	D	B	D	B	B	D	D	D	D	D	D	B	D	1	2	4	4	3	2	4	3	1	6	0	5	5	10	0.833	ns	1.500	ns	0.024	ns	0.690	ns
4	D4Bir4	D	B	D	B	D	B	B	B	D	D	D	D	D	B	D	1	2	4	4	3	2	4	3	2	5	1	4	6	9	0.367	ns	0.533	ns	0.043	ns	0.210	ns
4	D4Bir2	D	B	B	B	B	B	B	B	D	D	D	B	D	B	B	3	0	7	1	4	1	6	1	3	4	1	4	10	5	3.767	0.1	3.788	0.1	1.690	ns	1.711	ns
4	D4Nds3	D	B	B	B	B	B	B	B	D	D	D	B	D	B	B	3	0	7	1	4	1	6	1	3	4	1	4	10	5	3.767	0.1	3.788	0.1	1.690	ns	1.711	ns
4	Pmv30	D	B	B	B	B	B	B	B	D	D	D	B	D	D	D	2	1	5	3	3	2	5	2	3	4	1	4	8	7	1.290	ns	1.317	ns	0.265	ns	0.291	ns
4	Mltr3	D	B	B	B	B	B	B	B	D	D	D	B	D	B	D	2	1	6	2	4	1	6	1	3	4	1	4	9	6	2.022	ns	2.300	ns	0.537	ns	0.814	ns
4	Scl2	D	B	B	.	B	B	.	.	.	D	D	B	.	.	D	2	1	3	2	1	1	3	1	2	2	0	2	5	4	1.456	ns	1.407	ns	0.094	ns	0.046	ns
4	Xmmv8	D	B	B	B	B	B	B	B	D	D	D	B	D	B	D	2	1	6	2	4	1	6	1	3	4	1	4	9	6	2.022	ns	2.300	ns	0.537	ns	0.814	ns
4	D4Nds8	D	B	B	B	B	B	B	B	D	D	D	B	D	B	B	3	0	7	1	4	1	6	1	3	4	1	4	10	5	3.767	0.1	3.788	0.1	1.690	ns	1.711	ns
4	Tmod	D	B	B	B	B	B	B	B	D	D	D	B	D	B	B	3	0	7	1	4	1	6	1	3	4	1	4	10	5	3.767	0.1	3.788	0.1	1.690	ns	1.711	ns
4	Cd72	D	B	B	B	B	B	B	B	D	D	D	B	D	B	B	3	0	7	1	4	1	6	1	3	4	1	4	10	5	3.767	0.1	3.788	0.1	1.690	ns	1.711	ns
4	Lyb4	D	B	B	B	B	B	B	B	D	D	D	B	D	.	.	2	0	5	1	3	1	5	1	3	4	1	4	8	5	2.724	0.1	2.706	0.1	1.116	ns	1.098	ns
4	D4Nds10	D	B	D	D	D	B	D	D	B	D	B	D	B	D	D	1	2	2	6	1	4	2	5	3	4	2	3	5	10	0.033	ns	0.200	ns	0.595	ns	0.762	ns
4	Nsk1	D	B	B	B	B	B	B	B	D	D	D	B	B	.	B	3	0	6	1	3	1	5	1	4	3	2	3	10	4	2.071	ns	1.929	ns	0.843	ns	0.700	ns
4	D4Nds6	D	B	B	B	B	B	B	B	D	D	D	B	B	B	B	3	0	7	1	4	1	6	1	4	3	2	3	11	4	2.374	ns	2.302	ns	0.979	ns	0.907	ns
5	D5Rik81	D	D	D	D	D	D	D	B	B	B	B	D	B	D	D	0	3	1	7	1	4	1	6	4	3	4	1	5	10	2.633	ns	2.675	ns	3.881	0.05	3.923	0.05
5	D5Rik82	D	D	D	D	D	D	D	B	B	B	B	D	B	D	D	0	3	1	7	1	4	1	6	4	3	4	1	5	10	2.633	ns	2.675	ns	3.881	0.05	3.923	0.05
5	Pmv40	D	D	D	D	D	D	D	B	B	B	B	D	B	D	D	0	3	1	7	1	4	1	6	4	3	4	1	5	10	2.633	ns	2.675	ns	3.881	0.05	3.923	0.05
5	D5Mit1	.	D	D	D	.	D	D	B	B	B	B	D	.	.	.	0	2	1	4	1	2	1	4	3	2	3	0	4	6	2.600	ns	2.300	ns	3.800	0.1	3.500	0.1
5	Fgl2	D	D	D	B	D	D	D	B	B	B	B	D	B	D	D	0	3	2	6	2	3	2	5	4	3	4	1	6	9	2.533	ns	1.783	ns	3.638	0.1	2.888	0.1
5	D5J3	D	D	B	B	D	D	D	B	B	B	B	D	B	D	D	0	3	2	6	2	3	3	4	5	2	4	1	7	8	2.442	ns	1.846	ns	3.401	0.1	2.806	0.1
5	D5Mit61	D	D	B	B	D	D	D	B	B	B	B	.	B	D	D	0	2	2	5	2	3	3	4	5	2	4	1	7	7	1.900	ns	1.543	ns	2.786	0.1	2.429	ns
5	D5Mit72	D	D	B	B	D	D	D	B	B	B	B	.	B	D	D	0	2	2	5	2	3	3	4	5	2	4	1	7	7	1.900	ns	1.543	ns	2.786	0.1	2.429	ns
5	Xmv17	D	D	B	B	D	D	D	B	B	B	B	D	B	D	D	0	3	2	6	2	3	3	4	5	2	4	1	7	8	2.442	ns	1.846	ns	3.401	0.1	2.806	0.1
5	Xmv45	D	D	B	B	D	D	D	B	B	B	B	D	B	D	D	0	3	2	6	2	3	3	4	5	2	4	1	7	8	2.442	ns	1.846	ns	3.401	0.1	2.806	0.1
5	Zrf1	D	D	B	B	D	D	D	B	B	B	B	D	B	D	D	0	3	2	6	2	3	3	4	5	2	4	1	7	8	2.442	ns	1.846	ns	3.401	0.1	2.806	0.1
5	Mpmv23	D	D	B	B	D	D	D	B	B	B	B	D	B	D	D	0	3	2	6	2	3	3	4	5	2	4	1	7	8	2.442	ns	1.846	ns	3.401	0.1	2.806	0.1
5	Xlr8	D	D	B	B	D	D	D	B	B	B	.	D	B	D	D	0	3	1	6	1	3	2	4	5	2	4	1	6	8	2.493	ns	2.540	ns	3.536	0.1	3.583	0.1
5	D5Rik83	D	D	B	B	D	D	D	B	D	B	B	D	B	D	D	0	3	2	6	2	3	3	4	4	3	3	2	6	9	1.533	ns	0.783	ns	2.352	ns	1.602	ns

Legend: BXD origin = C57BL/6J x DBA/2J; B = C57BL/6J; D = DBA/2J; . = untyped;
Z = heterozygous; X = new allele; ? = ambiguous typing; * = inconsistent typing

number of lines within group that carry alleles of B or D origin

ns=not significant

		BXD-line															Grouping of BXD lines by symptoms (line nos.) I. None (12,27,32)		II. None to Mild (9,11,12,16,25,27,31,32		III. Mild (9,11,16,25,31)		IV. Mild to Moderate (5,6,9,11,16,25,31)		V. Mild to Severe (1,5,6,18,22,24,30)		VI. Moderate to Severe (1,18,22,24,30)		All lines		I vs.VI		II vs.VI		I vs.V		II vs.V	
Chr	Marker	1	5	6	9	11	12	16	18	22	24	25	27	30	31	32	B alleles	D alleles	B alleles	D alleles	B alleles	D alleles	B alleles	D alleles	B alleles	D alleles	B alleles	D alleles	B alleles	D alleles	χ^2	ρ-value	χ^2	ρ-value	χ^2	ρ-value	χ^2	ρ-value
5	D5Rik84	D	D	B	B	D	D	D	B	D	B	B	D	B	D	D	0	3	2	6	2	3	3	4	4	3	3	2	6	9	1.533	ns	0.783	ns	2.352	ns	1.602	ns
5	Slc4a2	D	D	B	B	D	D	D	B	D	B	B	D	B	D	D	0	3	2	6	2	3	3	4	4	3	3	2	6	9	1.533	ns	0.783	ns	2.352	ns	1.602	ns
5	Xmv34	D	D	B	B	D	D	D	B	D	B	B	D	B	D	D	0	3	2	6	2	3	3	4	4	3	3	2	6	9	1.533	ns	0.783	ns	2.352	ns	1.602	ns
5	Mpmv13	B	D	B	B	D	D	D	B	D	D	B	D	B	D	D	0	3	2	6	2	3	3	4	4	3	3	2	6	9	1.533	ns	0.783	ns	2.352	ns	1.602	ns
5	D5H4S43E	B	D	B	B	D	B	D	B	D	D	B	B	D	D	D	2	1	4	4	2	3	3	4	3	4	2	3	7	8	0.299	ns	0.061	ns	0.401	ns	0.163	ns
5	D5J4	B	D	B	B	D	B	D	B	D	D	B	D	D	D	D	1	2	3	5	2	3	3	4	3	4	2	3	6	9	0.033	ns	0.013	ns	0.376	ns	0.355	ns
5	D5Nds1	.	D	B	B	D	B	D	B	D	D	B	D	D	D	D	1	2	3	5	2	3	3	4	2	4	1	3	5	9	0.076	ns	0.079	ns	0.195	ns	0.198	ns
5	D5Rik66	B	D	B	B	D	B	D	B	D	D	B	D	D	D	D	1	2	3	5	2	3	3	4	3	4	2	3	6	9	0.033	ns	0.013	ns	0.376	ns	0.355	ns
7	Zfp30	B	B	D	B	B	D	B	B	B	B	B	B	B	D	D	1	2	5	3	4	1	5	2	6	1	5	0	11	4	1.988	ns	1.461	ns	2.521	ns	1.995	ns
7	Pmv18	B	B	D	B	B	D	B	B	B	B	B	B	B	D	D	1	2	5	3	4	1	5	2	6	1	5	0	11	4	1.988	ns	1.461	ns	2.521	ns	1.995	ns
7	Cd22	B	B	D	B	B	D	B	B	B	B	.	D	B	B	D	0	3	4	3	4	0	5	1	6	1	5	0	10	4	3.571	0.1	1.629	ns	4.143	0.05	2.200	ns
7	Atp4a	B	B	D	B	B	D	B	B	B	B	B	D	B	D	D	0	3	4	4	4	1	5	2	6	1	5	0	10	5	3.667	0.1	2.000	ns	4.333	0.05	2.667	ns
7	Abpa	B	B	D	B	B	D	B	B	B	B	B	D	B	D	D	0	3	4	4	4	1	5	2	6	1	5	0	10	5	3.667	0.1	2.000	ns	4.333	0.05	2.667	ns
7	Gpi1	B	B	D	B	B	D	B	B	B	B	B	D	B	D	D	0	3	4	4	4	1	5	2	6	1	5	0	10	5	3.667	0.1	2.000	ns	4.333	0.05	2.667	ns
7	Gpi1-s	B	B	D	B	B	D	B	B	B	B	B	D	B	D	D	0	3	4	4	4	1	5	2	6	1	5	0	10	5	3.667	0.1	2.000	ns	4.333	0.05	2.667	ns
7	Mag	B	B	D	B	B	D	B	B	B	B	B	D	B	D	D	0	3	4	4	4	1	5	2	6	1	5	0	10	5	3.667	0.1	2.000	ns	4.333	0.05	2.667	ns
7	Cebpa	B	B	D	B	B	D	B	B	B	B	B	D	B	D	D	0	3	4	4	4	1	5	2	6	1	5	0	10	5	3.667	0.1	2.000	ns	4.333	0.05	2.667	ns
7	D7Rp2e	B	B	D	B	B	D	B	B	B	B	B	D	B	D	B	1	2	5	3	4	1	5	2	6	1	5	0	11	4	1.988	ns	1.461	ns	2.521	ns	1.995	ns
7	Pmv15	B	B	D	B	B	D	B	B	B	B	B	D	B	D	D	0	3	4	4	4	1	5	2	6	1	5	0	10	5	3.667	0.1	2.000	ns	4.333	0.05	2.667	ns
7	Odc-rs6	B	D	D	B	B	D	B	B	D	B	D	B	B	D	B	2	1	5	3	3	2	3	4	4	3	4	1	9	6	0.522	ns	0.508	ns	1.179	ns	1.165	ns
7	Tam1	B	D	D	B	B	D	B	B	D	B	D	B	B	D	D	1	2	4	4	3	2	3	4	4	3	4	1	8	7	0.987	ns	0.779	ns	1.798	ns	1.589	ns
8	D8Bir2	B	B	B	B	D	B	B	B	D	D	B	B	D	B	D	2	1	6	2	4	1	6	1	4	3	2	3	10	5	1.067	ns	1.150	ns	0.190	ns	0.274	ns
8	D8Byu2	B	B	B	B	D	B	D	D	D	D	B	B	D	D	D	2	1	4	4	2	3	4	3	3	4	1	4	7	8	0.924	ns	0.686	ns	0.276	ns	0.038	ns
8	D8Mit4	.	B	B	B	D	B	D	D	D	D	B	B	.	.	.	2	0	4	2	2	2	4	2	2	3	0	3	6	5	2.721	0.1	2.125	ns	0.990	ns	0.394	ns
8	D8Rik81	D	B	B	B	D	B	D	D	D	D	B	B	D	D	D	2	1	4	4	2	3	4	3	2	5	0	5	6	9	1.867	ns	1.533	ns	0.686	ns	0.352	ns
8	Rasl5-2	B	B	B	B	D	B	D	D	D	D	B	B	D	D	D	2	1	4	4	2	3	4	3	3	4	1	4	7	8	0.924	ns	0.686	ns	0.276	ns	0.038	ns
9	D9Mit10	D	B	D	B	B	D	D	D	D	B	B	B	D	B	B	2	1	6	2	4	1	5	2	2	5	1	4	8	7	1.290	ns	1.895	ns	0.265	ns	0.869	ns
9	D9Ncvs53	D	B	D	B	B	D	D	D	D	B	D	B	D	B	B	2	1	5	3	3	2	4	3	2	5	1	4	7	8	0.924	ns	1.096	ns	0.276	ns	0.449	ns
9	Rbp1	D	B	D	B	B	D	B	.	.	.	.	B	D	B	B	2	1	6	1	4	0	5	1	1	3	0	2	7	4	2.232	ns	2.763	0.1	0.209	ns	0.741	ns
9	D9Rik65	D	B	D	B	B	B	B	D	D	B	D	B	B	B	D	2	1	6	2	4	1	5	2	3	4	2	3	9	6	0.522	ns	0.800	ns	0.037	ns	0.314	ns
9	P9Ehs1	D	B	D	B	B	B	B	D	D	B	D	B	B	.	.	2	0	5	1	3	1	4	2	3	4	2	3	8	5	1.084	ns	1.066	ns	0.516	ns	0.498	ns
9	D9Mit151	.	B	D	B	B	B	D	B	D	D	B	B	D	B	D	2	1	6	2	4	1	5	2	2	4	1	3	8	6	1.012	ns	1.411	ns	0.119	ns	0.518	ns
9	D9Hgu1	.	D	B	B	B	B	D	B	D	D	B	B	D	B	D	2	1	6	2	4	1	5	2	2	4	1	3	8	6	1.012	ns	1.411	ns	0.119	ns	0.518	ns
9	D9Hrb1	D	D	B	B	B	B	D	.	D	D	B	B	D	B	D	2	1	6	2	4	1	5	2	1	5	0	4	7	7	2.167	ns	3.000	0.1	0.500	ns	1.333	ns
9	D9Rp2	D	D	B	B	B	B	D	B	D	D	B	B	D	B	D	2	1	6	2	4	1	5	2	2	5	1	4	8	7	1.290	ns	1.895	ns	0.265	ns	0.869	ns
9	Tel9q	D	D	D	B	B	B	D	.	D	D	B	B	D	B	D	2	1	6	2	4	1	4	3	0	6	0	4	6	8	1.683	ns	3.214	0.1	0.492	ns	2.024	ns
11	Tstap91a	B	D	D	D	B	B	D	B	D	D	B	B	D	B	D	2	1	5	3	3	2	3	4	2	5	2	3	7	8	0.299	ns	0.471	ns	0.401	ns	0.574	ns
11	Krt1	B	D	.	D	B	B	D	B	D	D	B	B	D	B	D	2	1	5	3	3	2	3	3	2	4	2	3	7	7	0.267	ns	0.350	ns	0.167	ns	0.250	ns
11	Gfap	D	D	D	D	B	B	B	D	D	D	B	B	D	B	D	2	1	6	2	4	1	4	3	0	7	0	5	6	9	1.867	ns	3.783	0.1	0.686	ns	2.602	ns
11	Mpmv8	D	D	D	D	?	?	?	D	D	D	B	B	D	B	D	1	1	3	2	2	1	2	3	0	7	0	5	3	9	0.917	ns	2.867	0.1	0.512	ns	2.462	ns
11	Slc4a1	D	D	D	D	B	B	B	D	D	D	B	B	D	B	D	2	1	6	2	4	1	4	3	0	7	0	5	6	9	1.867	ns	3.783	0.1	0.686	ns	2.602	ns
11	D11Nds2	D	D	D	D	B	B	B	D	D	D	B	B	D	B	D	2	1	6	2	4	1	4	3	0	7	0	5	6	9	1.867	ns	3.783	0.1	0.686	ns	2.602	ns
11	Myla	D	D	D	D	B	B	B	D	D	D	B	B	D	B	D	2	1	6	2	4	1	4	3	0	7	0	5	6	9	1.867	ns	3.783	0.1	0.686	ns	2.602	ns
11	Sfrs2	D	B	D	D	B	D	D	B	D	D	D	B	D	B	D	1	2	3	5	2	3	3	4	2	5	1	4	5	10	0.133	ns	0.175	ns	0.095	ns	0.137	ns
11	D11Ncvs61	D	B	D	D	B	D	D	B	D	D	D	B	D	B	D	1	2	3	5	2	3	3	4	2	5	1	4	5	10	0.133	ns	0.175	ns	0.095	ns	0.137	ns
12	Mod1r	B	B	B	D	B	D	B	B	D	D	B	D	B	D	D	0	3	3	5	3	2	5	2	5	2	3	2	8	7	1.648	ns	0.424	ns	2.091	ns	0.867	ns
12	D12Mit1	.	B	B	D	B	D	B	B	D	D	B	D	.	.	.	0	2	3	3	3	1	5	1	3	2	1	2	6	5	1.388	ns	0.320	ns	1.124	ns	0.055	ns
12	Nmyc1	B	B	B	D	D	D	B	B	?	?	B	D	B	D	D	0	3	2	6	2	3	4	3	5	0	3	0	7	6	3.000	0.1	2.621	ns	3.923	0.05	3.544	0.1
12	D12Byu1	B	B	B	D	D	D	B	B	D	D	B	D	B	B	D	0	3	3	5	3	2	5	2	5	2	3	2	8	7	1.648	ns	0.424	ns	2.091	ns	0.867	ns
12	D12Nyu10	B	B	B	D	D	D	B	B	D	D	B	D	B	B	D	0	3	3	5	3	2	5	2	5	2	3	2	8	7	1.648	ns	0.424	ns	2.091	ns	0.867	ns
12	Rnu1a2	D	B	B	D	B	B	B	D	D	D	D	D	B	D	D	1	2	3	5	2	3	4	3	3	4	1	4	6	9	0.367	ns	0.346	ns	0.043	ns	0.022	ns
12	Eif4e	B	B	B	D	B	B	B	D	D	D	D	D	B	D	D	1	2	3	5	2	3	4	3	4	3	2	3	7	8	0.156	ns	0.186	ns	0.258	ns	0.288	ns
12	D12Rik53	B	B	B	D	B	D	B	B	B	D	D	D	B	D	D	0	3	2	6	2	3	4	3	6	1	4	1	8	7	2.362	ns	1.966	ns	3.173	0.1	2.777	0.1

Legend: BXD origin = C57BL/6J x DBA/2J; B = C57BL/6J; D = DBA/2J; . = untyped;
Z = heterozygous; X = new allele; ? = ambiguous typing; * = inconsistent typing

number of lines within group that carry alleles of B or D origin

ns=not significant

		BXD-line															Grouping of BXD lines by symptoms (line nos.)										All lines							
																	I. None (12,27,32)		II. None to Mild (9,11,12,16,25,27,31,32		III. Mild (9,11,16,25,31)		IV. Mild to Moderate (5,6,9,11,16,25,31)		V. Mild to Severe (1,5,6,18,22,24,30)		VI. Moderate to Severe (1,18,22,24,30)				I vs.VI		II vs.VI		I vs.V		II vs.V	
Chr	Marker	1	5	6	9	11	12	16	18	22	24	25	27	30	31	32	B alleles	D alleles	B alleles	D alleles	B alleles	D alleles	B alleles	D alleles	B alleles	D alleles	B alleles	D alleles	B alleles	D alleles	χ2	p-value	χ2	p-value	χ2	p-value	χ2	p-value
12	D12Rik54	B	B	B	D	B	D	B	B	B	D	D	D	B	D	D	0	3	2	6	2	3	4	3	6	1	4	1	8	7	2.362	ns	1.966	ns	3.173	0.1	2.777	0.1
12	Zfp50	B	B	B	D	B	D	B	B	B	D	D	D	B	D	D	0	3	2	6	2	3	4	3	6	1	4	1	8	7	2.362	ns	1.966	ns	3.173	0.1	2.777	0.1
12	D12Mit3	.	B	B	D	B	D	B	B	B	D	D	D	.	.	.	0	2	2	4	2	2	4	2	4	1	2	1	6	5	1.188	ns	0.592	ns	1.804	ns	1.208	ns
12	Hsp70-2	B	B	B	D	B	D	B	B	B	D	D	D	B	D	D	0	3	2	6	2	3	4	3	6	1	4	1	8	7	2.362	ns	1.966	ns	3.173	0.1	2.777	0.1
12	D12Mit5	B	B	B	B	B	D	B	B	B	D	D	D	B	D	D	0	3	3	5	3	2	5	2	6	1	4	1	9	6	2.300	ns	1.175	ns	2.957	0.1	1.832	ns
12	Mtv9	B	B	B	B	B	D	B	D	B	B	D	D	B	D	D	0	3	3	5	3	2	5	2	6	1	4	1	9	6	2.300	ns	1.175	ns	2.957	0.1	1.832	ns
12	Chga	B	B	.	B	B	B	D	B	B	D	D	D	D	B	D	1	2	4	4	3	2	4	2	4	2	3	2	8	6	0.307	ns	0.081	ns	0.425	ns	0.198	ns
12	D12Mit7	B	B	D	D	D	B	D	B	B	D	D	D	D	B	D	1	2	2	6	1	4	2	5	4	3	3	2	6	9	0.367	ns	0.783	ns	1.186	ns	1.602	ns
12	Spi2	B	B	D	D	D	B	D	B	B	D	D	D	D	B	D	1	2	2	6	1	4	2	5	4	3	3	2	6	9	0.367	ns	0.783	ns	1.186	ns	1.602	ns
12	Bcga	D	B	D	D	D	D	D	D	B	D	.	D	D	D	D	0	3	0	7	0	4	1	5	2	5	1	4	2	12	0.448	ns	1.019	ns	1.095	ns	1.667	ns
12	Mpmv24	B	B	D	D	D	D	D	B	B	D	D	D	D	D	D	0	3	0	8	0	5	1	6	4	3	3	2	4	11	1.558	ns	2.891	0.1	2.713	0.1	4.046	0.05
12	Ckb	B	B	D	D	D	D	D	B	B	D	D	D	D	D	D	0	3	0	8	0	5	1	6	4	3	3	2	4	11	1.558	ns	2.891	0.1	2.713	0.1	4.046	0.05
12	D12H14S17	B	B	D	D	D	D	D	B	B	D	D	D	D	D	D	0	3	0	8	0	5	1	6	4	3	3	2	4	11	1.558	ns	2.891	0.1	2.713	0.1	4.046	0.05
12	D12Nyu14	B	B	D	D	D	D	D	B	B	D	D	D	D	D	D	0	3	0	8	0	5	1	6	4	3	3	2	4	11	1.558	ns	2.891	0.1	2.713	0.1	4.046	0.05
12	Xmmv50	D	D	B	D	B	D	B	D	B	B	D	D	B	D	D	0	3	2	6	2	3	3	4	4	3	3	2	6	9	1.533	ns	0.783	ns	2.352	ns	1.602	ns
12	Crip	B	B	D	D	D	D	D	D	B	D	B	D	D	D	D	0	3	1	7	1	4	2	5	3	4	2	3	4	11	0.921	ns	0.723	ns	1.687	ns	1.489	ns
12	Igh-Nbp	B	B	D	D	D	D	D	D	B	D	B	B	D	D	D	1	2	2	6	1	4	2	5	3	4	2	3	5	10	0.033	ns	0.200	ns	0.595	ns	0.762	ns
12	Igh-Np	B	B	D	D	D	D	D	D	B	D	B	B	D	D	D	1	2	2	6	1	4	2	5	3	4	2	3	5	10	0.033	ns	0.200	ns	0.595	ns	0.762	ns
12	Igh-Sa2	B	B	D	D	D	D	D	D	B	D	D	D	D	D	D	0	3	0	8	0	5	1	6	3	4	2	3	3	12	0.850	ns	1.850	ns	1.807	ns	2.807	0.1
12	Igh-Sa4	B	B	D	D	D	D	D	D	B	D	D	B	D	D	D	1	2	1	7	0	5	1	6	3	4	2	3	4	11	0.171	ns	0.723	ns	0.937	ns	1.489	ns
12	Igh-Src	B	B	D	B	D	D	D	D	B	D	B	B	B	D	D	1	2	3	5	2	3	3	4	4	3	3	2	7	8	0.281	ns	0.311	ns	0.919	ns	0.949	ns
13	Hist1	D	B	B	B	B	D	B	D	B	B	D	D	B	D	D	0	3	3	5	3	2	5	2	5	2	3	2	8	7	1.648	ns	0.424	ns	2.091	ns	0.867	ns
13	D13Byu1	B	B	D	D	B	D	D	D	B	D	D	B	D	D	B	2	1	3	5	1	4	2	5	3	4	2	3	6	9	0.533	ns	0.013	ns	0.876	ns	0.355	ns
13	Tpmt	B	D	B	D	D	D	D	B	B	B	D	D	D	D	D	0	3	0	8	0	5	1	6	5	2	4	1	5	10	2.633	ns	4.300	0.05	3.881	0.05	5.548	0.025
13	Msx2	B	B	D	D	?	D	D	B	B	D	D	D	D	D	D	0	3	0	7	0	4	1	5	4	3	3	2	4	10	1.549	ns	2.691	ns	2.657	ns	3.800	0.1
13	D13Rik49	B	B	D	D	B	D	D	B	B	D	D	D	D	D	D	0	3	1	7	1	4	2	5	4	3	3	2	5	10	1.533	ns	1.575	ns	2.524	ns	2.565	ns
13	Iapls1-13	B	B	D	D	B	D	D	B	B	D	D	D	D	D	D	0	3	1	7	1	4	2	5	4	3	3	2	5	10	1.533	ns	1.575	ns	2.524	ns	2.565	ns
13	Il9	B	B	D	D	B	D	D	B	B	D	D	D	D	D	D	0	3	1	7	1	4	2	5	4	3	3	2	5	10	1.533	ns	1.575	ns	2.524	ns	2.565	ns
13	D13Rik54	B	B	D	.	B	D	D	B	B	D	D	.	D	D	D	0	2	1	5	1	3	2	4	4	3	3	2	5	8	1.146	ns	1.118	ns	2.005	ns	1.977	ns
13	Hmg1-rs3	B	B	D	D	D	D	D	B	B	D	D	D	D	D	.	0	2	0	7	0	5	1	6	4	3	3	2	4	10	1.263	ns	2.691	ns	2.371	ns	3.800	0.1
13	Hmg1-rs5	B	B	D	D	D	D	D	B	B	.	D	D	.	.	.	0	2	0	6	0	4	1	5	4	1	3	0	4	7	2.636	ns	4.091	0.05	3.909	0.05	5.364	0.025
13	Pmv41	B	B	D	D	D	D	D	B	B	D	D	D	D	D	D	0	3	0	8	0	5	1	6	4	3	3	2	4	11	1.558	ns	2.891	0.1	2.713	0.1	4.046	0.05
13	Sui1-rs2	B	B	D	D	D	D	D	B	B	D	D	D	D	D	D	0	3	0	8	0	5	1	6	4	3	3	2	4	11	1.558	ns	2.891	0.1	2.713	0.1	4.046	0.05
13	D13Byu2	B	B	D	D	D	D	D	B	B	.	D	D	D	B	D	0	3	1	7	1	4	2	5	4	2	3	1	5	9	2.032	ns	2.167	ns	3.188	0.1	3.324	0.1
13	D13Byu3	B	B	D	D	D	D	D	B	B	D	D	D	D	B	D	0	3	1	7	1	4	2	5	4	3	3	2	5	10	1.533	ns	1.575	ns	2.524	ns	2.565	ns
13	D13Rik51	B	B	D	D	D	D	D	B	B	D	D	D	D	B	.	0	2	1	6	1	4	2	5	4	3	3	2	5	9	1.173	ns	1.359	ns	2.103	ns	2.289	ns
13	D13Mit148	D	B	D	D	D	D	D	B	D	D	B	D	B	D	D	0	3	1	7	1	4	2	5	3	4	2	3	4	11	0.921	ns	0.723	ns	1.687	ns	1.489	ns
13	Iapls3-14	D	B	D	D	D	D	D	B	D	D	B	D	B	D	D	0	3	1	7	1	4	2	5	3	4	2	3	4	11	0.921	ns	0.723	ns	1.687	ns	1.489	ns
13	D13Rp1	.	D	D	D	D	D	D	B	D	.	B	D	B	D	D	0	3	1	7	1	4	1	6	2	3	2	1	3	10	1.433	ns	1.129	ns	2.798	0.1	2.494	ns
13	D13Rp2	D	D	D	D	D	D	D	B	D	D	B	D	B	D	D	0	3	1	7	1	4	1	6	2	5	2	3	3	12	0.850	ns	0.475	ns	1.807	ns	1.432	ns
13	D13Rp4	D	D	D	D	D	D	D	B	D	D	B	D	B	D	D	0	3	1	7	1	4	1	6	2	5	2	3	3	12	0.850	ns	0.475	ns	1.807	ns	1.432	ns
14	D14Nds1	.	B	D	D	D	B	B	D	D	B	B	B	.	.	.	2	0	4	2	2	2	3	3	2	3	1	2	6	5	1.055	ns	0.459	ns	0.790	ns	0.194	ns
14	Plau	D	B	D	D	D	B	B	D	D	B	B	B	B	D	D	2	1	4	4	2	3	3	4	3	4	2	3	7	8	0.299	ns	0.061	ns	0.401	ns	0.163	ns
14	D14Mit1	.	B	D	D	D	B	D	D	D	D	B	B	.	.	.	2	0	3	3	1	3	2	4	1	4	0	3	4	7	2.851	0.1	0.930	ns	2.238	ns	0.317	ns
14	D14Mit2	.	B	D	D	D	B	B	D	D	B	B	B	.	.	.	2	0	4	2	2	2	3	3	2	3	1	2	6	5	1.055	ns	0.459	ns	0.790	ns	0.194	ns
14	D14Pas1	B	B	D	D	D	B	D	B	D	B	B	B	B	D	D	2	1	3	5	1	4	2	5	5	2	4	1	8	7	0.862	ns	1.138	ns	1.673	ns	1.949	ns
14	Glud	B	B	B	D	D	B	B	B	.	B	B	B	.	B	D	D	1	1	3	4	2	3	4	3	6	0	4	0	9	4	1.338	ns	1.934	ns	1.953	ns	2.549	ns
14	Mtv11	B	B	D	D	D	B	D	B	D	B	D	B	B	D	D	2	1	2	6	0	5	1	6	5	2	4	1	7	8	1.299	ns	1.846	ns	2.258	ns	2.806	0.1
14	Ms15-7	B	B	D	D	D	B	D	D	D	B	B	B	B	D	D	2	1	3	5	1	4	2	5	4	3	3	2	7	8	0.424	ns	0.311	ns	1.062	ns	0.949	ns
14	Ms6-5	B	.	D	D	D	D	D	D	B	B	B	B	B	D	D	1	2	2	6	1	4	1	5	4	2	4	1	6	8	1.271	ns	1.802	ns	1.784	ns	2.315	ns
14	Np	B	B	D	D	D	D	D	D	B	B	B	B	B	D	D	1	2	2	6	1	4	2	5	5	2	4	1	7	8	1.156	ns	1.846	ns	2.115	ns	2.806	0.1
14	Nrl	B	B	D	D	D	D	D	D	B	B	B	B	B	D	D	1	2	2	6	1	4	2	5	5	2	4	1	7	8	1.156	ns	1.846	ns	2.115	ns	2.806	0.1
14	Tcra	B	B	D	D	D	D	D	D	B	B	B	B	B	D	D	1	2	2	6	1	4	2	5	5	2	4	1	7	8	1.156	ns	1.846	ns	2.115	ns	2.806	0.1
14	Xmv19	B	B	D	D	D	D	D	D	B	B	B	B	B	D	D	1	2	2	6	1	4	2	5	5	2	4	1	7	8	1.156	ns	1.846	ns	2.115	ns	2.806	0.1
14	Ang	B	B	D	D	D	D	D	D	B	B	B	B	B	D	D	1	2	2	6	1	4	2	5	5	2	4	1	7	8	1.156	ns	1.846	ns	2.115	ns	2.806	0.1
14	D14Byu2	B	B	D	D	D	D	D	D	B	.	B	B	.	D	D	1	2	2	6	1	4	2	5	3	2	2	1	5	8	0.408	ns	0.765	ns	1.422	ns	1.779	ns

Legend: BXD origin = C57BL/6J x DBA/2J; B = C57BL/6J; D = DBA/2J; . = untyped;
Z = heterozygous; X = new allele; ? = ambiguous typing; * = inconsistent typing

number of lines within group that carry alleles of B or D origin

ns=not significant

																Grouping of BXD lines by symptoms (line nos.)																			
																I. None (12,27,32)		II. None to Mild (9,11,12,16,25,27,31,32		III. Mild (9,11,16,25,31)		IV. Mild to Moderate (5,6,9,11,16,25,31)		V. Mild to Severe (1,5,6,18,22,24,30)		VI. Moderate to Severe (1,18,22,24,30)		All lines		I vs.VI		II vs.VI		I vs.V		II vs V	
		BXD-line														B	D	B	D	B	D	B	D	B	D	B	D	B	D							
Chr	Marker	1	5	6	9	11	12	16	18	22	24	25	27	30	31	32		alleles	alleles	alleles	alleles	alleles	alleles	alleles	alleles	alleles	alleles	alleles	alleles	alleles	alleles	χ^2	p-value	χ^2	p-value	χ^2	p-value	χ^2	p-value
14	D14Byu3	B	B	D	D	D	D	D	D	B	B	B	B	B	D	D		1	2	2	6	1	4	2	5	5	2	4	1	7	8	1.156	ns	1.846	ns	2.115	ns	2.806	0.1
14	D14Byu4	B	B	D	D	D	D	D	D	B	B	B	B	B	D	D		1	2	2	6	1	4	2	5	5	2	4	1	7	8	1.156	ns	1.846	ns	2.115	ns	2.806	0.1
14	Mcpt1	B	B	D	D	D	D	D	D	D	B	B	B	B	D	D		1	2	2	6	1	4	2	5	4	3	3	2	6	9	0.367	ns	0.783	ns	1.186	ns	1.602	ns
14	Mcpt4	B	B	D	D	D	D	D	D	D	B	B	B	B	D	D		1	2	2	6	1	4	2	5	4	3	3	2	6	9	0.367	ns	0.783	ns	1.186	ns	1.602	ns
14	Mcpt5	B	B	D	D	D	D	D	D	D	B	B	B	B	D	D		1	2	2	6	1	4	2	5	4	3	3	2	6	9	0.367	ns	0.783	ns	1.186	ns	1.602	ns
14	Myhca	B	B	D	D	D	D	D	D	B	B	B	B	B	D	D		1	2	2	6	1	4	2	5	5	2	4	1	7	8	1.156	ns	1.846	ns	2.115	ns	2.806	0.1
14	Rib1	B	B	D	D	D	D	D	D	B	B	B	B	B	D	D		1	2	2	6	1	4	2	5	5	2	4	1	7	8	1.156	ns	1.846	ns	2.115	ns	2.806	0.1
14	Gzme	B	B	D	D	D	D	D	D	D	B	B	B	B	D	D		1	2	2	6	1	4	2	5	4	3	3	2	6	9	0.367	ns	0.783	ns	1.186	ns	1.602	ns
14	D14Mit5	.	B	D	D	D	B	D	D	D	B	B	B	.	.	.		2	0	3	3	1	3	2	4	2	3	1	2	5	6	1.390	ns	0.108	ns	1.503	ns	0.221	ns
16	D16Rik29	.	B	.	.	D	B	.	B	B	D	D	D	D	B	D		1	2	2	4	1	2	2	2	3	2	2	2	5	6	0.112	ns	0.209	ns	0.291	ns	0.388	ns
16	D16Rik30	B	D	B	B	D	D	B	B	B	D	D	D	B	B	D		0	3	3	5	3	2	4	3	5	2	4	1	8	7	2.362	ns	1.138	ns	3.173	0.1	1.949	ns
16	D16Rik31	B	D	B	B	D	D	B	B	B	D	D	D	B	B	D		0	3	3	5	3	2	4	3	5	2	4	1	8	7	2.362	ns	1.138	ns	3.173	0.1	1.949	ns
16	D16Rik32	B	B	B	B	D	B	B	B	B	D	D	D	B	B	B		2	1	5	3	3	2	5	2	6	1	4	1	11	4	0.102	ns	0.211	ns	0.421	ns	0.530	ns
16	Prm1	B	B	D	B	D	B	B	D	B	D	D	B	D	B	D		2	1	5	3	3	2	4	3	3	4	2	3	8	7	0.290	ns	0.317	ns	0.122	ns	0.148	ns
16	D16Ros2	B	B	B	B	D	B	D	D	B	D	D	B	B	B	D		2	1	4	4	2	3	4	3	5	2	3	2	9	6	0.022	ns	0.133	ns	0.251	ns	0.362	ns
16	D16Mit47	B	B	B	B	D	B	D	B	B	D	D	B	B	B	D		2	1	4	4	2	3	4	3	6	1	4	1	10	5	0.267	ns	0.600	ns	0.762	ns	1.095	ns
16	Pmv14	B	B	B	B	D	D	D	B	B	D	D	D	B	B	D		0	3	2	6	2	3	4	3	6	1	4	1	8	7	2.362	ns	1.966	ns	3.173	0.1	2.777	0.1
16	D16H21S16	B	B	B	B	D	D	D	B	B	D	D	D	B	B	D		0	3	2	6	2	3	4	3	6	1	4	1	8	7	2.362	ns	1.966	ns	3.173	0.1	2.777	0.1
16	D16Mit50	B	D	B	B	D	D	D	B	B	D	D	D	B	B	D		0	3	2	6	2	3	3	4	5	2	4	1	7	8	2.442	ns	1.846	ns	3.401	0.1	2.806	0.1
16	Gabpa	B	D	B	B	D	B	D	B	B	D	D	D	D	B	D		1	2	3	5	2	3	3	4	5	2	4	1	8	7	0.987	ns	1.138	ns	1.798	ns	1.949	ns
16	App	B	D	B	B	D	B	D	B	B	D	D	D	B	B	D		1	2	3	5	2	3	3	4	5	2	4	1	8	7	0.987	ns	1.138	ns	1.798	ns	1.949	ns
17	D17Byu1	D	D	D	B	B	D	B	B	D	D	B	B	D	D	D		1	2	5	3	4	1	4	3	1	6	1	4	6	9	0.367	ns	1.346	ns	0.043	ns	1.022	ns
17	D17Leh507	D	D	D	B	B	D	B	D	B	B	D	B	D	D	D		1	2	4	4	3	2	3	4	2	5	2	3	6	9	0.033	ns	0.200	ns	0.376	ns	0.543	ns
17	D17Rp17e	D	D	D	B	B	D	B	D	D	D	B	B	D	D	D		1	2	5	3	4	1	4	3	0	7	0	5	5	10	0.833	ns	2.875	0.1	0.024	ns	2.065	ns
17	Plg	D	D	D	B	B	D	B	D	D	D	B	B	D	D	D		1	2	5	3	4	1	4	3	0	7	0	5	5	10	0.833	ns	2.875	0.1	0.024	ns	2.065	ns
17	Tcp1	D	D	D	B	B	D	B	D	D	D	B	B	D	D	D		1	2	5	3	4	1	4	3	0	7	0	5	5	10	0.833	ns	2.875	0.1	0.024	ns	2.065	ns
17	D17Leh66D	D	D	D	B	B	D	B	D	D	D	B	B	D	D	D		1	2	5	3	4	1	4	3	0	7	0	5	5	10	0.833	ns	2.875	0.1	0.024	ns	2.065	ns
17	D17Tu30	D	D	D	B	.	D	B	D	D	D	D	B	D	.	.		1	1	3	2	2	1	2	3	0	7	0	5	3	9	0.917	ns	2.867	0.1	0.512	ns	2.462	ns
17	D17Pas1	D	D	D	B	B	D	B	D	D	D	D	B	D	D	D		1	2	4	4	3	2	3	4	0	7	0	5	4	11	0.535	ns	2.118	ns	0.053	ns	1.637	ns
17	D17Leh111	D	D	D	B	B	D	B	D	D	D	D	B	D	D	D		1	2	4	4	3	2	3	4	0	7	0	5	4	11	0.535	ns	2.118	ns	0.053	ns	1.637	ns
17	D17Pri1	D	D	D	B	B	D	B	D	D	D	D	B	D	.	D		1	2	4	3	3	1	3	3	0	7	0	5	4	10	0.595	ns	2.571	ns	0.024	ns	2.000	ns
17	D17Leh94	D	D	D	B	B	D	B	D	D	D	D	B	D	D	D		1	2	4	4	3	2	3	4	0	7	0	5	4	11	0.535	ns	2.118	ns	0.053	ns	1.637	ns
17	Zfp51	D	D	D	B	B	D	B	D	D	D	D	B	D	.	.		1	1	4	2	3	1	3	3	0	7	0	5	4	9	0.924	ns	3.197	0.1	0.245	ns	2.518	ns
17	Zfp52	D	D	D	B	B	D	B	D	D	D	D	B	D	.	.		1	1	4	2	3	1	3	3	0	7	0	5	4	9	0.924	ns	3.197	0.1	0.245	ns	2.518	ns
17	D17Tu50	D	D	D	B	.	D	B	D	D	D	B	B	D	.	.		1	1	4	1	3	0	3	2	0	7	0	5	4	8	1.000	ns	4.100	0.05	0.190	ns	3.290	0.1
17	D17Tu36	D	.	D	B	B	D	.	D	D	D	.	B	.	.	.		1	1	3	1	2	0	2	1	0	5	0	4	3	6	0.833	ns	2.750	0.1	0.300	ns	2.217	ns
17	Zfp40	D	D	D	B	B	D	B	D	D	D	D	B	D	.	.		1	1	4	2	3	1	3	3	0	7	0	5	4	9	0.924	ns	3.197	0.1	0.245	ns	2.518	ns
17	D17Leh54M	D	D	D	B	B	D	B	D	D	D	D	B	D	D	D		1	2	4	4	3	2	3	4	0	7	0	5	4	11	0.535	ns	2.118	ns	0.053	ns	1.637	ns
17	D17Leh180	D	D	D	B	B	D	B	D	D	D	D	B	D	D	D		1	2	4	4	3	2	3	4	0	7	0	5	4	11	0.535	ns	2.118	ns	0.053	ns	1.637	ns
17	Hba-ps4	D	D	D	B	B	D	B	D	D	D	D	B	D	D	D		1	2	4	4	3	2	3	4	0	7	0	5	4	11	0.535	ns	2.118	ns	0.053	ns	1.637	ns
17	Ly39	D	D	D	B	.	D	D	D	B	B	D	B	D	D	D		1	2	2	5	1	3	1	5	2	5	2	3	4	10	0.115	ns	0.091	ns	0.824	ns	0.800	ns
17	Glo1	D	D	D	D	D	B	D	D	D	D	D	B	D	D	D		2	1	2	6	0	5	0	7	0	7	0	5	2	13	6.503	0.025	0.919	ns	6.588	0.025	1.004	ns
17	Glo1-r	D	D	D	D	D	B	D	D	D	D	D	B	D	.	.		2	0	2	4	0	4	0	6	0	7	0	5	2	11	9.448	0.005	1.396	ns	9.452	0.005	1.400	ns
17	D17Leh26	D	D	D	D	D	B	D	D	D	D	D	B	D	D	D		2	1	2	6	0	5	0	7	0	7	0	5	2	13	6.503	0.025	0.919	ns	6.588	0.025	1.004	ns
17	Pim1	D	D	D	D	D	B	D	D	D	D	D	B	D	D	D		2	1	2	6	0	5	0	7	0	7	0	5	2	13	6.503	0.025	0.919	ns	6.588	0.025	1.004	ns
17	D17H21S56	D	D	D	D	D	B	D	D	D	D	D	B	D	D	D		2	1	2	6	0	5	0	7	0	7	0	5	2	13	6.503	0.025	0.919	ns	6.588	0.025	1.004	ns
17	Cryal	D	D	D	D	D	D	D	D	D	D	D	B	D	D	D		1	2	1	7	0	5	0	7	0	7	0	5	1	14	3.224	0.1	0.432	ns	3.560	0.1	0.768	ns
17	H-2	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D																							
17	Gln3-4	D	D	D	D	D	B	D	D	D	D	D	D	D	D	D		1	2	1	7	0	5	0	7	0	7	0	5	1	14	3.224	0.1	0.432	ns	3.560	0.1	0.768	ns
17	Gln3-7	D	D	D	D	D	B	D	D	D	D	D	D	D	D	D		1	2	1	7	0	5	0	7	0	7	0	5	1	14	3.224	0.1	0.432	ns	3.560	0.1	0.768	ns
17	D17Leh173	D	D	D	D	D	B	D	D	D	D	D	D	D	D	D		1	2	1	7	0	5	0	7	0	7	0	5	1	14	3.224	0.1	0.432	ns	3.560	0.1	0.768	ns
17	Ms15-3	D	D	D	D	D	B	D	D	D	D	D	D	D	D	D		1	2	1	7	0	5	0	7	0	7	0	5	1	14	3.224	0.1	0.432	ns	3.560	0.1	0.768	ns
17	D17Mit11	.	D	D	.	D	B	D	D	D	D	D	D	.	.	.		1	1	1	4	0	3	0	5	0	5	0	3	1	9	3.233	0.1	0.533	ns	3.700	0.1	1.000	ns
17	D17Tu16	D	D	D	?	D	B	D	?	D	D	D	D	D	D	.		1	1	1	5	0	4	0	6	0	6	0	4	1	11	4.197	0.05	0.530	ns	4.576	0.05	0.909	ns
17	Tpx1	D	D	D	D	.	B	D	B	D	D	D	D	D	.	.		1	1	1	4	0	3	0	5	1	6	1	4	2	10	1.340	ns	0.040	ns	1.910	ns	0.610	ns
17	Tcte1	D	D	D	B	D	B	D	D	D	D	D	D	D	D	D		1	2	2	6	1	4	1	6	0	7	0	5	2	13	1.003	ns	0.919	ns	1.088	ns	1.004	ns

Legend: BXD origin = C57BL/6J x DBA/2J; B = C57BL/6J; D = DBA/2J; . = untyped;
Z = heterozygous; X = new allele; ? = ambiguous typing; * = inconsistent typing

number of lines within group that carry alleles of B or D origin

ns=not significant

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Publications

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