THE UNIVERSITY OF CALGARY

MHC Class II Polymorphism and Central Tolerance in Autoimmune Diabetes

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY AND INFECTIOUS DISEASES

CALGARY, ALBERTA

MAY, 2001

ABSTRACT

Insulin-dependent diabetes mellitus is a complex disease characterized by the destruction of the pancreatic beta cells which produce insulin. A strong genetic link exist between autoimmunity and MHC class II polymorphism where different alleles infer either susceptibility or resistance from disease. The NOD mouse spontaneously develops IDDM and therefore provides an appropriate genetic background with which to study this disease. Previously it has been shown that T cells bearing an I-A^{g7}-restricted, beta cell reactive, highly diabetogenic T cell receptor (4.1-TCR) underwent negative selection when non-NOD MHC class II molecules were expressed in the 4.1-NOD background. Here it is shown that when 4.1-thymocytes interact with I-A^{d}, I-A^{g7PD}, or I-$E\alpha^{k}$ MHC class II molecules (expressed as transgenes) they undergo different levels of tolerance. Therefore, anti-diabetogenic MHC class II molecules may provide protection from disease by having a tolerogenic affect on highly pathogenic, 4.1-like $CD4^{+}$ T cells.

ACKNOWLEDGEMENTS

I would like to thank Dr. Pere Santamaria for teaching and supporting me throughout my time of study. Dr. Aziz Amrani deserves recognition and many thanks for sharing his experimental knowledge as well as his aid during long days in the laboratory. My gratitude to my supervisory committee, Dr. Julie Deans and Dr. Chris Mody, for their helpful suggestions, guidance, and encouragement during my program. A big thank you to my fellow graduate student, Pau Serra, for his help with certain experiments. My gratitude to Marcela Deuma, Angie Gignac, Shelley Stewart, and Sonny Bou for their technical services and animal care. For FACS services, a special thank you to Laurie Robertson who was always a joy to see and very accommodating with my many appointments. I would also like to acknowledge Daniela Minardi for keeping the paperwork under control, making countless overheads, and for always having something interesting to share. For those who helped me proof-read my thesis and who encouraged me during the writing period, I am very grateful. Finally, I would like to thank Shelley Stewart, Marcela Deuma, and Angie Gignac for keeping a smile on my face and for contributing to some of the most enjoyable times that I have had thus far. To the many others who have shared their knowledge with me throughout the years, I appreciate what you have taught me.

DEDICATION

I would like to dedicate my thesis to my parents. It is because of their encouragement and love that I found a field which I enjoy. I thank you, mom and dad, for teaching me the importance of hardwork, determination, and finding something which you can do everday and enjoy.

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LIST OF ABBREVIATIONS

APC	Antigen Presenting Cell
BrdU	5-bromo-2'-deoxyuridine
CD4/CD8	T cell co-receptors
CDR	complementarity determining region
CLIP	class II-associated invariant-chain peptide
DN	double negative thymocytes
DP	double positive thymocytes
ELISA	enzyme-linked immunoabsorbant assay
FACS	fluorescence activated cell sorting
FITC	fluorescein isothiocyanate
HBSS	Hank's balanced salt solution
H/E	hemotoxylin/eosin staining
HLA	human leukocyte antigen
IDDM	insulin dependent diabetes mellitus
IFN	interferon
IL	interleukin
mAbs	monoclonal antibodies
MHC	major histocompatibility complex
NK	natural killer cell
NOD	non-obese diabetic
OCT	optimal cutting temperature compound
PBS	phosphate-buffered saline
PE	phycoerythrin
PMA	phorbol-myristate acetate
rIL-2	recombinant IL-2
RAG	recombination-activating gene
RBC	red blood cell

MHC Class II Polymorphism and Central Tolerance in Autoimmune Diabetes

I Introduction

A Insulin-dependent Diabetes Mellitus

i Pathogenesis

Insulin dependent diabetes mellitus (IDDM) is an autoimmune disease that involves the destruction of the insulin-producing pancreatic beta-cells (1,2). The beta cells are destroyed following infiltraton of the pancreatic islets by mononuclear cells. The primary cells involved in the pathogenesis of IDDM include $CD4^+$ and $CD8^+$ T cells, as well as professional antigen presenting cells (APCs), such as macrophages and dendritic cells (1). Other cells involved are cells which secrete certain cytokines (such as interleukin-1, interferon-γ, tumor necrosis factor α, and IL-12), and B cells which secrete autoantibodies (1,3). Although both humoral and cell-mediated immunity have distinct roles in diabetes, the latter is more prominent in the disease (1). Autoantibodies seem to be secondary to cell-mediated immunity in that autoantibodies are not involved in pathogenesis and appear after tissue damage has already been effected by T cells.

Insulitis, the infiltration of the pancreatic islets by mononuclear cells, is essential for diabetes development (4). Insulitis eventually results in mononuclear cells destroying the islet beta cells, thereby abolishing insulin production (5). Macrophages and dendritic cells infiltrate islets early and act as professional APCs, which present autoantigens to $CD4^+$ T cells as well as $CD8^+$ T cells (1, 6). If greater than 90% of the islet beta cells are damaged then insulin production ceases, resulting in IDDM.

There are important roles for both $CD4^+$ and $CD8^+$ T cells in the pathogenesis of

IDDM. Effective transfer of diabetes requires both $CD4^+$ and $CD8^+$ T cells (7-10) and both are involved in beta cell destruction (2, 11). Diabetes requires both T cell subsets for disease to occur (12); however, it appears that $CD8^+$ T cells are more important during the initiation of disease (13-15) while $CD4^+$ T cells are involved as disease progresses (16). This does not exclude $CD8^+$ T cells from acting as effectors of beta cell damage since IDDM does not occur in the absence of $CD8^+$ T cells as shown by studies of β2-microglobulin-deficient NOD mice (17-19) and anti-CD8 mAb-treated NOD mice (20). $CD8^+$ T cells are able to mature normally and differentiate into cytotoxic T cells (21) in the absence of $CD4^+$ T cells but are not efficient mediators of beta cell destruction when $CD4^+$ T cells are absent. This was shown using a highly pathogenic TCR (8.3-TCR) that is K^d-restricted and beta cell reactive which was expressed in RAG 2-deficient NOD mice which do not have any $CD4^+$ T cells or B cells (22). In the presence of only $CD8^+$ T cells, the incidence of diabetes decreased as compared to 8.3-NOD mice which have RAG-2 activity and therefore have $CD4^+$ T cells and B cells (22). For a certain highly pathogenic $CD8^+$ T cell population, it was shown that the T cells underwent avidity maturation where $CD8^+$ T cells matured over time and those with a greater avidity for the peptide were selected and were more pathogenic (23). Therefore, $CD8^+$ T cells have a distinct role in diabetes pathogenesis but do not fulfill their full potential in the absence of $CD4^+$ T cells.

On the other hand, $CD4^+$ T cells have many roles in disease pathogenesis in that they can act as helper T cells, secrete cytokines, and have a CTL effect on beta cells. To exert their effect, $CD4^+$ T cells do not necessarily require $CD8^+$ T cells (7, 15, 24-29). This was demonstrated by using a highly pathogenic, I-A^{g7}-restricted, beta cell reactive TCR (4.1-TCR) in the NOD background either with or without a RAG-2-deficiency (22). RAG-2-deficient-4.1-NOD mice developed diabetes in the absence of $CD8^+$ T cells and B cells and did so at a more efficient rate than RAG-2^+-4.1-NOD mice (22). These results demonstrated that $CD4^+$ T cells are capable of causing disease in the absence of $CD8^+$

CTLs and that $CD4^+$ T cells can kill beta cells *in vivo*. $CD4^+$ T cells are able to differentiate into CTLs and effect beta cell damage through the Fas-FasL interaction, provided that the beta cells have upregulated Fas in response to certain pro-inflammatory cytokines such as IFNγ (30). Activated $CD4^+$ T cells of the Th1 phenotype secrete pro-inflammatory cytokines such as IFNγ, TNFα, and IL-2 which then activate other effector cells. These effector cells include macrophages, $CD8^+$ CTLs, and B cells which in turn augment the immune response generated by activating more effector cells (31). As well as producing cytokines which activate effector cells, $CD4^+$ T cells act as helper T cells to stimulate $CD8^+$ T cells to differentiate into CTLs. Therefore, even though $CD4^+$ and $CD8^+$ T cells have different roles in IDDM pathogenesis, efficient beta cell destruction requires both T cell subsets (2, 11, 14).

Bone marrow-derived macrophages and dendritic cells play an important role in mediating disease pathogenesis. Bone marrow from mouse strains expressing anti-diabetogenic H-2 haplotypes protect lethally irradiated non-obese diabetic (NOD) mice from developing diabetes when the marrow is transplanted (32-35). Bone marrow from NOD mice injected into irradiated (NODxB10)F1 mice, which are normally resistant to disease, developed insulitis and became diabetic (34) and the same results were obtained when the irradiated hosts were C57BL/6 or B10.BR/cd where the chimeras developed insulitis and a small percentage became diabetic (33). These results show that bone marrow derived cells are involved in mediating MHC-induced disease susceptibility and resistance. The fact that diabetes protection occurs when anti-diabetogenic MHC molecules are expressed on bone marrow-derived cells and not on thymic epithelial cells shows that the mediating factors of disease reside in the marrow (32, 36). These observations suggest that disease resistance as well as susceptibility involve bone marrow-derived hematopoietic cells.

ii Genetics

Genetic factors play a key role in the development of diabetes. Diabetes is considered a polygenic disease, involving both genes linked to the MHC and non-MHC genes. In humans, HLA-DQ and HLA-DR HLA class II alleles have been linked to IDDM susceptibility or resistance. Alleles that provide protection tend to code for aspartic acid at position 57 of the beta chain, whereas susceptibility HLA class II molecules tend to have neutral residues, such as serine, valine, or alanine at this position. Specifically, HLA-DQA1*0301/DQB1*0302 demonstrates susceptibility in humans, while HLA-DQA1*0102/DQB1*0602 is protective (37). The importance of the aspartic acid residue at position 57 stems from its ability to modify the conformation of the MHC class II beta chain. Aspartic acid at position 57 forms a salt bridge with the arginine at position 76 of the alpha chain which makes the MHC class II molecule more stable and able to bind peptides more efficiently. Susceptibility alleles do not have aspartic acid and therefore do not have the salt bridge (38). It has been postulated that this conformational difference caused by the absence of aspartic acid affects the stability of the MHC class II molecule thereby affecting the interaction with peptides (37, 38). The less stable I-A^{g7} MHC class II molecule binds peptides poorly and causes inefficient negative selection in the thymus during T cell development (39, 40).

The association between HLA-DQ and IDDM is stronger than of that between HLA-DR and IDDM. It is often difficult to distinguish between HLA-DQ and HLA-DR involvement due to linkage disequilibrium; the close proximity of HLA-DR to HLA-DQ prevents recombination, making it difficult to discern which gene is involved (1, 2, 41). In humans, when serologic typing was used to determine susceptibility and resistance, DR3 and DR4 were linked to susceptibility and DR2 was associated with resistance (41). In mice, the susceptible allele is I-$A\beta^{g7}$, whereas other alleles such as I-$A\beta^{b}$ and I-$A\beta^{d}$ are protective.

The NOD mouse spontaneously develops a form of IDDM which greatly resembles human IDDM, making it an excellent animal model with which to study the disease. As in humans, the murine MHC plays a key role in susceptibility and resistance to disease. The NOD mouse has a unique MHC class II haplotype due to the unusual beta chain (11, 42-44). This haplotype consists of an I-Aα^d/I-Aβ^{g7} heterodimer, as well as a dysfunctional I-E molecule - this is caused by an unproductive I-Eα gene (11, 42, 45). The Eα gene is unproductive because there is a deletion in the promoter region precluding transcription (42). The I-E molecule is a heterodimer expressed on the cell surface, consisting of an alpha and beta chain. The NOD mouse is capable of producing I-Eβ; however, the absence of I-Eα prevents heterodimer formation. Therefore, the I-E molecule is not displayed on the cell surface. The use of the NOD mouse in genetic studies of IDDM is ideal because the environment can be controlled so that the results observed are due solely to the animal's genetics (46).

Diabetes development in the NOD mouse reflects that observed in humans. The two major similarities are the polygenic nature of the disease, to which the MHC genes are central, and the involvement of autoreactive T cells (47, 48). In both human and NOD IDDM, the MHC plays a key role in disease susceptibility and resistance. The protective affect of aspartic acid at position 57 of the beta chain of the MHC class II molecule is the same in humans and mice since susceptibility alleles do not have aspartic acid (49). The primary effector cells in NOD mice reflects those involved in human diabetes pathogenesis which cause insulitis to occur (47). The presence of insulitis which can progress to diabetes is also common between the NOD mouse and human disease (50). Another similarity is the appearance of autoantibodies even though they have no pathogenic affect (47).

B The Major Histocompatibility Complex

The major histocompatibility complex (MHC) is highly polymorphic and plays an important role in determining the immune response mounted against different antigens. The ability of the immune system to distinguish self from nonself is afforded by the MHC (51). Recognition of an antigen by T cells is done in the context of self-MHC and is known as MHC restriction. Further restraint stems from the fact that $CD4^+$ T cells recognize antigen presented by MHC class II molecules and $CD8^+$ T cells recognize antigen in the context of MHC class I. Class I MHC is expressed on all nucleated cells, whereas MHC class II is expressed only on professional APCs, such as B cells, dendritic cells, and macrophages (31).

The MHC loci are the HLA (human leukocyte antigen) genes in humans and the H-2 genes in mice. The HLA and H-2 genes code for MHC classes I and II. In humans, the MHC class II loci is made up of DP, DQ, and DR genes, while the MHC class I consists of A, B, and C genes. In mice, the MHC class II genetic region is made up of I-Aα and I-Aβ genes, which have homology to the human DQ genes, and I-Eα and I-Eβ loci, which are similar to human DR genes. The MHC class I genes in mice are K, L and D (Figure 1) (31).

The three main functions of the MHC are as follows: to present antigen to T cells, to help shape the repertoire of T cells during development, and to dictate which type of T cell is stimulated to mount the immune response to a particular antigen. Since MHC class II is recognized by $CD4^+$ T cells and MHC class I by $CD8^+$ T cells, the MHC determines which T cell subset responds based on which class of MHC presents the antigen (31).

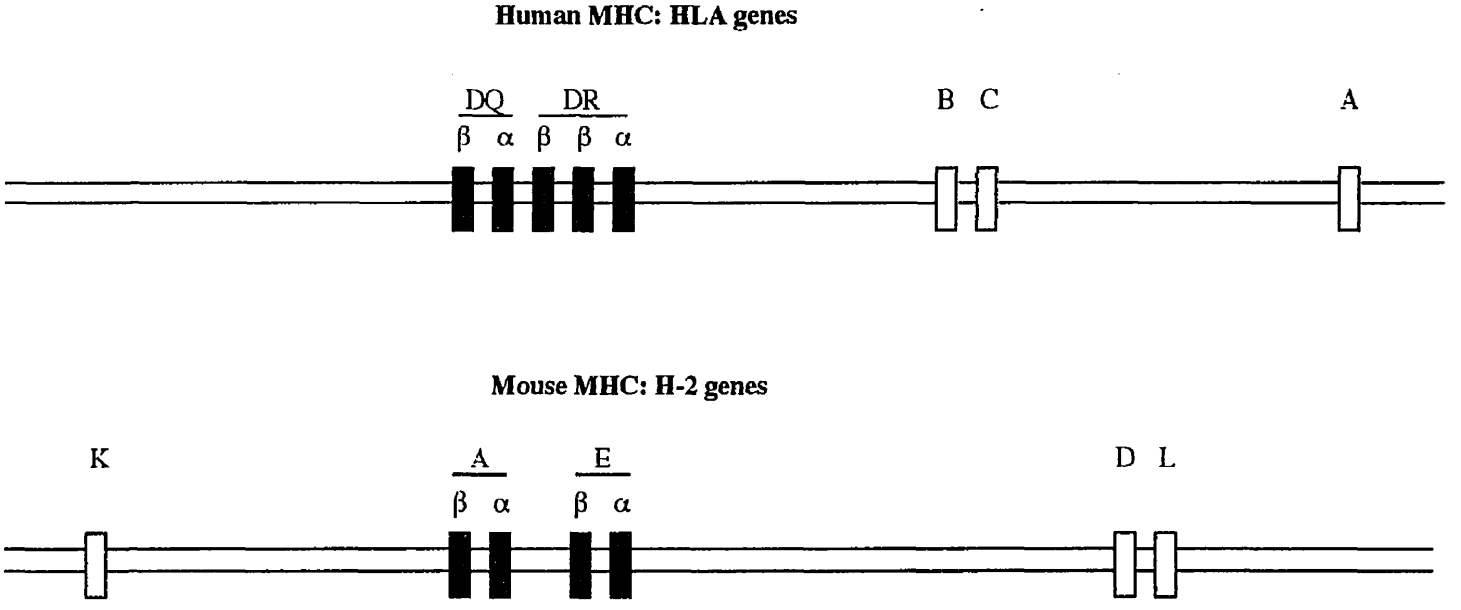


Figure 1: An overview of the major histocompatbility complex (MHC) in humans and mice. The primary MHC genes are shown for both human (where the MHC is referred to as HLA) and mouse (where the MHC is called H-2). The black boxes represent the MHC class II genes, whereas the white boxes demonstrate MHC class I genes.

C MHC Molecules and T Cell Development

i Positive and Negative Selection

T (thymus-dependent) cell development involves bone marrow-derived precursors homing to the thymus, where maturation then occurs via positive and negative selection. Immature T cells do not have either CD4 or CD8 coreceptors and are referred to as double negative (DN) thymocytes. During the course of maturation, DN thymocytes acquire both CD4 and CD8, becoming double positive (DP) thymocytes. T cells also begin to rearrange the T cell receptor (TCR) genes in order to generate a functional receptor; a functional TCR is required for positive and negative selection to proceed. These DP thymocytes then undergo positive and negative selection in the thymus. Positive selection ensures that the T cells leaving the thymus are capable of recognizing self-MHC, while negative selection eliminates autoreactive T cells by clonal deletion (52). Positive selection occurs in the cortex of the thymus when the functional TCR engages self-MHC (expressed on cortical epithelial cells) with intermediate affinity/avidity (52). The affinity with which the TCR interacts with the MHC/peptide complex is important and can affect thymocyte selection (53). If the affinity is too low, thymocytes do not receive a survival signal to continue maturing while an affinity which is too high will trigger the negative selection of thymocytes (31). This process selects a T cell repertoire capable of recognizing antigens in the context of self-MHC (54). Positive selection is often less stringent than negative selection because many different peptides can be presented by self-MHC which includes self peptides (55-66). The same MHC molecule is usually involved in restricting positive and negative selection since self-MHC is recognized in both processes (56, 60, 61, 66). However, there are exceptions to this as seen with an I-A^{g7}-restriced, beta cell reactive transgenic TCR (4.1-TCR) where the MHC class II molecule that triggered negative selection (I-A^{b}) was not able to positively select thymocytes (67). After positive selection,

the thymocytes leave the cortex of the thymus for the medulla (Figure 2). Negative selection occurs here, where the TCR interacts with peptide/MHC complexes expressed on bone marrow-derived macrophages and dendritic cells (52). T cells which are potentially autoreactive are deleted during negative selection, since they are capable of recognizing self peptide presented by self-MHC (52, 54, 68-70). Recognition of self peptides by T cells in the context of self-MHC leads to the destruction of self tissue, resulting in autoimmunity. Negative selection prevents the maturation of DP thymocytes into single positive cells and these cells are eliminated (54, 68-70). Cells which are eliminated during negative selection undergo apoptosis and lose both CD4 and CD8 co-receptors thus becoming double negative thymocytes. T cells engaging antigen/MHC complexes on APCs with high affinity/avidity also undergo negative selection (45, 52, 54, 69-72). This process ensures that the T cells exiting the thymus are not autoreactive; this makes negative selection an important mechanism for self tolerance (45, 54).

Determination of whether the T cell matures into a $CD4^+CD8^-$ or a $CD4^-CD8^+$ T cell depends on which co-receptor is lost when the T cell engages peptide/MHC complexes. If the T cell engages MHC class II and retains the CD4 co-receptor while losing CD8, then it matures into a $CD4^+CD8^-$ T cell. The same is true for MHC class I and CD8. Either CD4 or CD8 is downregulated during the TCR engagement of the MHC. Only cells that downregulate the proper co-receptor corresponding to the engaged MHC will receive the required survival signal for further maturation. Roughly two percent of all thymocytes mature into single positive cells that actually exit the thymus into the periphery; the rest of the thymocytes are lost during maturation (31).

ii TCR and TCR-transgenic mice

The TCR consists of an alpha and beta chain which form a heterodimer on the cell surface. The beta chain is composed of variable (V), diversity (D), and joining (J) gene

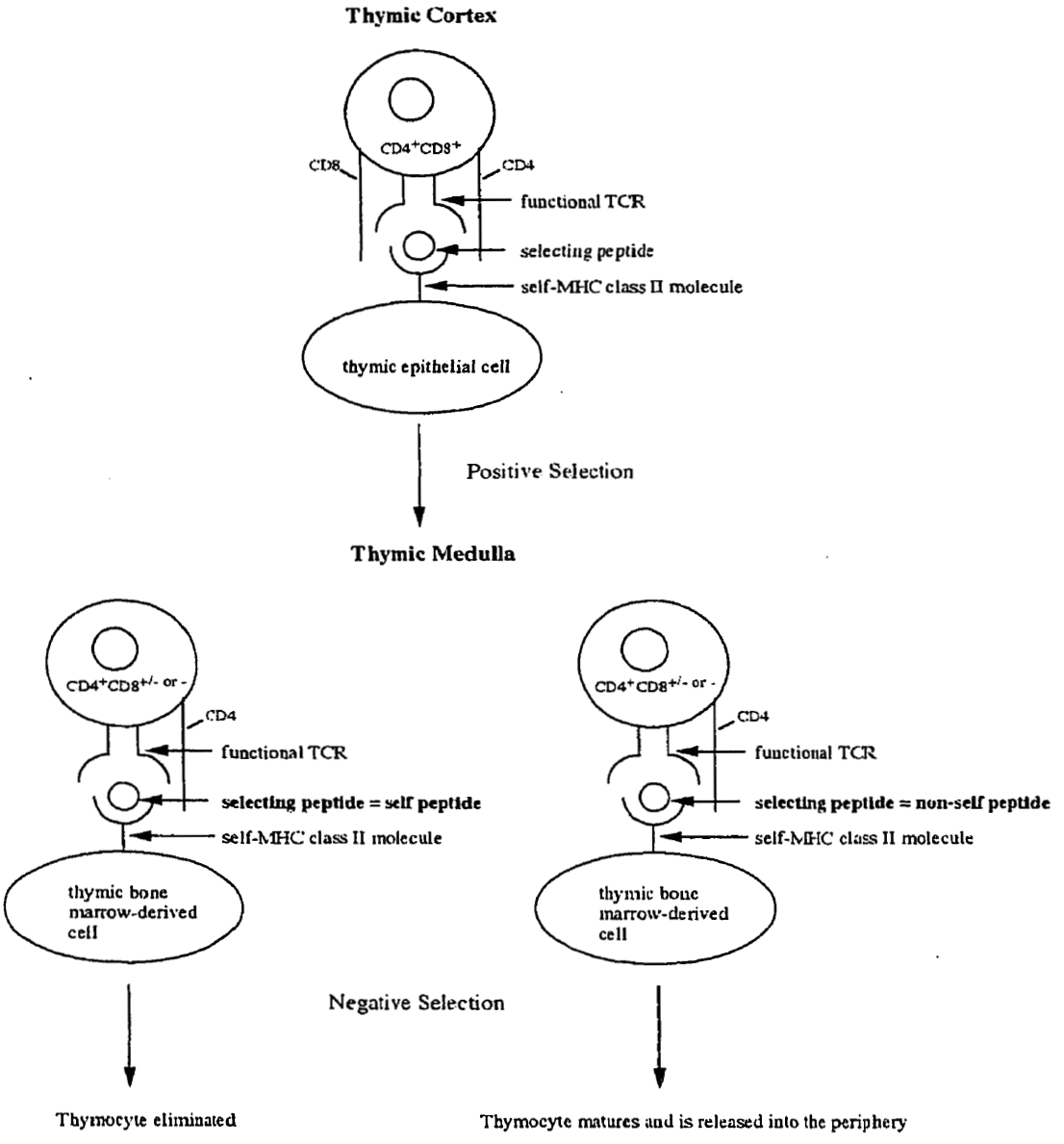


Figure 2: An overview of positive and negative selection during T cell development. Positive selection (which occurs in the thymic cortex) occurs when the TCR recognizes a selecting peptide in the context of self-MHC. Negative selection (which occurs in the thymic medulla) eliminates maturing thymocytes with a TCR capable of recognizing self-peptide in the context of self-MHC.

segments which rearrange to form a functional chain. Once a functional rearrangement has occurred, the T cell is signaled to stop any further rearrangments, a process referred to as allelic exclusion. The alpha chain consists of V and J segments and is not allelically excluded during rearrangement. Random addition of nucleotides between the different gene segments during rearrangment increases the diversity of the TCR. This diversity is most prominent in the CDR3 region since it is found in the peptide binding groove, the portion of the TCR which interacts with antigen. Once a functional TCR is rearranged it is expressed on the cell surface (31).

The thymocytes of T cell receptor-transgenic mice express the same TCR alpha and beta chains; therefore, the T cells have the same specificity as each other, resulting in a limited repertoire (73). This is because the T cell receptor transgenes for the alpha and beta chains do not undergo gene rearrangement because they are inserted in the desired rearrangement. The alpha and beta transgenes code for the transgenic TCR expressed. The presence of a functional TCRβ transgene leads to the suppression of endogenous TCRβ rearrangements due to allelic exclusion. Even though allelic exclusion does not occur with the alpha chain of the TCR, the transgenic alpha chain segregates with the transgenic beta chain so that the majority of the T cells express the alpha and beta chain of the transgenic TCR (43). The TCR specificity of transgenic mice will determine whether CD4 or CD8 will be lost during T cell maturation. The expression of a transgenic, MHC class II-restricted TCR will skew positive selection of DP thymocytes towards $CD4^+CD8^-$ thymocytes with greater efficiency than in non-transgenic mice (73).

TCR-transgenic mice are used to study the mechanisms involved in disease pathogenesis. Use of a transgenic TCR allows examination of a system in a reduced, disease-relevant manner. Employment of a transgenic T cell receptor limits the specificity by decreasing the variability of the T cell repertoire, as most of the T cells exiting the thymus now have a TCR with the same specificity.

The 4.1-transgenic TCR is an I-A^{g7}-restricted, beta cell reactive TCR which is highly pathogenic when introduced into the NOD background. This transgenic TCR consists of both the alpha and beta chain transgenes and is non-pathogenic when only the beta transgene is expressed (43). This transgenic TCR triggers the positive selection of T cells in the NOD background since it is I-A^{g7}-restricted and accelerates the onset of diabetes making it highly pathogenic. T cells expressing this same transgenic TCR also undergoes negative selection in F1 hybrid strains where non-NOD MHC class II molecules were expressed in the 4.1-NOD background resulting in the absence of diabetes. This deletion process involved the expression of MHC class II molecules on bone marrow-derived cells (43). These results demonstrate the promiscuity of the 4.1-TCR in that it recognizes different MHC class II molecules and either undergoes positive or negative selection depending on the MHC molecule present.

E Autoimmunity, MHC Class II and IDDM

Autoimmunity is defined by immune responses directed against self antigens and is often due to a breakdown in self tolerance. Self tolerance ensures that the immune system is unresponsive to self antigens and results from a combination of central and peripheral tolerance. Central tolerance involves positive selection followed by negative selection in the thymus, which prevents the maturation and release of autoreactive T cells into the periphery. A breakdown in thymic selection can give rise to autoimmunity, as autoreactive cells are not eliminated and thus are able to exit the thymus (74). Peripheral tolerance involves inducing unresponsiveness in mature cells at peripheral sites. This often results from antigen presentation in the absence of co-stimulation which leads to anergy; that is, the cells are rendered unresponsive (70).

For many years, a strong association between the major histocompatibility complex loci and autoimmune disease has been shown. Almost every autoimmune disease (such as

multiple sclerosis and systemic lupus erythematosus) seems to involve MHC genes, and diabetes is no exception (13). In particular, the MHC class II region in both humans and mice has been implicated in these diseases (75, 76). In many cases, the polymorphic nature of the MHC affects the immune response where different alleles of the MHC genes have been found to infer either susceptibility or resistance to disease. Polymorphisms within the MHC can affect what peptides are presented and the selection of the T cell repertoire which in turn influences whether there is susceptibility or resistance to disease (41).

This MHC class II link to autoimmunity is observed in IDDM. The HLA-DQβ MHC class II molcule in humans and the I-Aβ^{g7} MHC class II molecule in mice play an important role in IDDM susceptibility (77). In the NOD mouse, the presence of this unique I-Aβ^{g7} MHC class II molecule (78) and the absence of an I-E molecule (78, 79) predisposes the animal for diabetes development. In diabetes, certain human and mice MHC class II molecules without aspartic acid at position 57 of the beta chain have been linked to susceptibility to IDDM, while those having aspartic acid at position 57 have been shown to have a protective effect (2).

II Rationale and Hypothesis

The fact that certain MHC class II molecules can provide resistance to diabetes is confirmed by studying congenic NOD mice having non-NOD MHC haplotypes (80) and by the introduction of non-NOD MHC class II transgenes into the NOD background (76) which results in protection from diabetes development (81). Such transgenes include I-Aβ^{d} (82), I-Eα (24, 77, 79, 83, 84), I-A^{k} (85), and I-A^{g7PD} (86, 87). The I-Aβ^{d} transgene decreased the incidence of diabetes in the NOD background and those mice which developed IDDM displayed a delayed onset. The protective effect was not complete in that

there was no reduction in the degree of insulitis as compared to non-transgenic littermates and some mice did develop disease (82). The I-A^k MHC class II molecule protected NOD mice from diabetes development when introduced as a transgene and even though insulitis did occur, it was decreased in comparison to non-transgenic littermates (78, 85, 88). The introduction of the I-Eα transgene resulted in the absence of both insulitis and diabetes occurence showing a high degree of protection against disease (79, 83, 84). The I-A^{g7PD} molecule is similar to I-A^{g7} except that it is mutated at positions 56 and 57 of the beta chain, replacing the histidine and serine for proline and aspartic acid (11). This change to proline and aspartic acid resulted in protection from diabetes but also appears to be incomplete because there is insulitis and diabetes occurring with varying levels of protection (83, 87). In each case, when these different MHC class II molecule transgenes were introduced into the NOD background they had varying protective effects; however, the mechanism(s) involved in this protection remain(s) unclear due to the complexity of this disease. The NOD background has a massively variable T cell repertoire due to many different endogenous TCR specificities with varying alpha and beta chains. This makes it very difficult to state for certain what the mechanism(s) is(are) behind the observed protection because of the many different T cell specificities. It is therefore important to study the effect of these different transgenes in mice that express a relevant transgenic TCR, such as the 4.1-TCR. The 4.1-TCR is a disease relevant TCR which is representative of a group of highly pathogenic $CD4^+$ T cells which are involved in diabetogenesis. The presence of a disease-relevant transgenic TCR narrows the focus of the system by ensuring that T cells express a TCR of one specificity and so enables conclusions to be drawn regarding the involved mechanisms.

Many different mechanisms have been proposed for explaining how certain MHC class II molecules are capable of protecting NOD mice from diabetes. These include the induction of a protective Th2 response (2, 36, 82, 85, 87, 89) and epitope stealing (36,

85), neither of which have been definitively shown to provide resistance. Induction of $CD4^+$ T cells towards a Th2 phenotype inhibits cell mediated immunity by suppressing the production of pro-inflammatory cytokines which activate effector cells, such as T cells and macrophages. Epitope stealing involves the protective MHC class II molecule drawing peptide away from the I-A^{g7} molecule so that I-A^{g7} cannot present autoantigens to autoreactive T cells. Other suggested mechanisms are the triggering of negative selection or the induction of anergy, leading to the absence of autoreactive T cells (24, 36, 83, 85, 70).

It was previously observed in our lab that the introduction of non-NOD MHC class II molecules or haplotypes into the 4.1-NOD background resulted in protection from IDDM. These non-NOD MHC class II backgrounds included C57BL/6 (which expresses I-A^b), and SWR (which expresses I-A^q) (43). Other haplotypes, such as H-2^{nb1} and H-2^k, also provided protection from diabetes when introduced into NOD mice expressing the 4.1-TCR. The diabetes resistance afforded by I-A^b, I-A^q, H-2^{nb1}, and H-2^k occurred via the deletion of the highly pathogenic $CD4^+$ thymocytes which bear the 4.1-TCR. Other MHC class II molecules, such as I-A^s, were also studied and it was found that they did not delete T cells bearing the 4.1-TCR. Therefore, the 4.1-TCR is either positively or negatively selected by structurally diverse MHC class II molecules. This led to a question of whether those protective MHC class II molecules triggering negative selection in the thymic medulla could mediate positive selection in the cortex of the thymus (67). This was tested by using a K14 promoter which only allowed the expression of I-A^b in the cortex of the thymus which resulted in the absence of positive selection (67). This suggested that the promiscuity of the 4.1-TCR for different MHC class II molecules may only occur in the thymic medulla.

As previously mentioned, the I-E MHC class II molecule, as well as the I-$A\beta^d$ and I-$A\beta^{g7PD}$ molecules, has been shown to be protective against the development of diabetes

in NOD mice; however, the mechanism of this protection remains unknown. Based on the observations made in our lab, MHC class II molecules of diabetes resistant mouse strains may provide protection by triggering the deletion of certain highly diabetogenic thymocytes which are 4.1-like. Therefore, it is the hypothesis of this thesis that if the I-A^b molecule protects from diabetes by triggering central tolerance of a certain group of autoreactive $CD4^+$ T cells (that is, 4.1-like), then other protective MHC class II molecules such as I-E, I-A^d, and I-A^{g7PD} should protect via a similar mechanism. Provided that this hypothesis is true, then a common mechanism exists between protective MHC class II molecules involving the tolerization of certain highly pathogenic $CD4^+$ T cells, even though these MHC class II molecules are structually diverse.

III Materials and Methods

A Creation of Transgenic Mice

This project employed single transgenic (TCR only) and double transgenic (TCR and MHC class II) NOD mice. By definition, transgenic mice have an extra copy of a gene or a copy of the gene of interest is introduced into a system where it is normally absent in order to see its effect. Transgenic mice are generated by microinjecting the cloned gene into fertilized oocytes. Fertilized eggs are obtained by removing the oocytes from a female which has been induced to superovulate and then mated. These microinjected oocytes are then implanted into a pseudopregnant female. Since the gene of interest integrates randomly into the genome of the offspring, only some of the offspring have the transgene. The presence of the introduced transgene allows one to study the effect of this gene on the system of interest.

B Mice

The 4.1-TCR is a beta cell specific, I-A^{g7} restricted T cell receptor that was isolated from the NY4.1 clone obtained from the pancreatic islets of a diabetic NOD mouse. The 4.1-TCR is positively selected in the cortex by the I-A^{g7} MHC class II molecule expressed by epithelial cells. The 4.1-TCR is highly diabetogenic and greatly accelerates the onset of diabetes when expressed in the NOD background (43). 4.1-NOD mice were created by expressing the transgenic 4.1-TCR in the NOD background. NOD.I-Eα^k mice were generated by backcrossing I-Eα^k-transgenic C57BL/6/129 mice (Jackson Laboratory) with NOD mice for 10 generations (N10). To ensure that C57BL/6 *Idd* loci were not transfered along with the I-Eα^k-transgene, N8 mice were typed for microsatellites linked to known *Idd* loci. Mice homozygous for NOD *Idd* loci were then used for further breeding. NOD.I-A$\alpha\beta^k$ mice came from G. Morahan (Walter and Eliza Hall Institute, Melbourne, Australia). I-Aβ^d-transgenic NOD mice (NOD.I-A^d) were obtained from H. McDevitt (Stanford University, Palo Alto, CA). NOD.I-Aβ^{g7PD} mice were created by backcrossing BALB/c.I-A^{g7PD} mice (from O. Kanagawa, Washington University, St. Louis, MO) with NOD for seven generations and typed for NOD *Idd* regions. 4.1-NOD.I-Eα^k, 4.1-NOD.I-A^d, 4.1-NOD.I-A^{g7PD}, 4.1-NOD.I-A^k, and F1[4.1-(NODxC58/j)] (4.1-H-$2^{g7/k}$) mice were produced by crossing 4.1-NOD (I-Aα^d/I-Aβ^{g7}) with either NOD.I-Eα^k, NOD.I-Aβ^d, NOD.I-Aβ^{g7PD}, NOD.I-A$\alpha\beta^k$, or C58/j (H-2^k) mice. H-$2Ma^{-/-}$B6/129 mice were from L. van Kaer (Vanderbilt University, Nashville, TN). I-Aβ^b-deficient C57BL/6 mice came from Taconic Farms (Germantown, New York). B10.H-2^{g7} (H-2^{g7}-congenic C57BL/10) mice were obtained from L. Wicker (Merck Research Labs, Rahway, NJ). Transgenic mice were screened for the inheritance of the transgenes via polymerase chain reaction (PCR) of tail DNA. Mice hemizygous for MHC and TCR transgenes were studied. All mice were housed in a specific pathogen-free facility.

C Antibodies and Flow Cytometry

i Antibodies

Anti-Lyt-2 (CD8-α)-PE (53-6.7), anti-L3T4 (CD4)-FITC (RM4-5), anti-Vβ11-FITC (RR3-15), anti-I-Aβ^b-biotin (AF6-120.1), anti-I-E^k-biotin (17-3-3), and anti-I-Aβ^{g7}-biotin (10-3.6) were purchased from PharMingen (San Diego, CA). Anti-L3T4 (CD4)-biotin (YTS191.1) was purchased from Cedarlane. Streptavidin-PerCP was obtained from Becton Dickinson.

ii Flow cytometry

Thymi and spleens were analyzed by three-color flow cytometry using a FACScan®. The thymus and the spleen of studied mice were removed and disrupted into single cell suspensions. Single cells obtained from the thymus were washed with Hank's balanced salt solution (HBSS), then resuspended in HBSS and counted. $5x10^5$ thymocytes were washed twice using fluorescence activated cell sorting (FACS) buffer and then triple stained with anti-Lyt-2(CD8-α)-PE, anti-L3T4(CD4)-biotin, and anti-Vβ11-FITC. The cells were then incubated for 30 minutes at 4°C. Cells were then washed again with FACS buffer and incubated with Streptavidin-PerCP (which binds to biotin stained cells and fluoresces) for 30 minutes at 4°C. After this second incubation, cells were again washed and resuspended in FACS buffer for analysis using a FACScan® (Becton-Dickinson). Cell percentages in the different subsets were determined by gating on the cells. The gates were set using 4.1-NOD mice and were kept consistent when analyzing MHC class II-transgenic-4.1-NOD mice.

Single cell suspensions obtained from the spleen were treated similarly, with the added step of lysing the red blood cells (RBCs). RBCs were lysed for seven minutes using an ammonium chloride solution warmed to 37°C. The remaining splenocytes were

then washed and resuspended in HBSS for counting. $5x10^5$ splenocytes were used for staining and were treated as above for triple staining. Single staining was also done on the splenocytes to ensure the presence of the I-E^k molecule. Splenocytes used in the single stain were incubated with I-$A\beta^b$-biotin (negative control), I-$A\beta^{g7}$-biotin (positive control), and I-E^k-biotin for 30 minutes at 4°C, then washed before staining with streptavidin-PerCP. These cells were then washed again and resuspended in FACS buffer for flow cytometric analysis as above.

D Preparation of CD8+ T Cell-depleted Splenic T Cells

Spleens were disrupted into single cell suspensions and red blood cells lysed using ammonium chloride. After hemolysis, cells were washed with complete medium (RPMI 1640 media containing 10% heat-inactivated fetal bovine serum, 50 U/mL penicillin, 50μg/mL streptomycin, 50 μM 2-ME, and sodium pyruvate). After washing, splenocytes were incubated with Lyt-2 at 4°C for 30 minutes. The cells were washed after incubation and then combined with washed goat-anti-rat IgG magnetic beads and further incubated for 30 minutes at 4°C, with occasional inverting to mix. A magnetic particle concentrator was then used for five minutes to remove the $CD8^+$ T cells bound to the magnetic beads. The CD8-depleted cells were then washed, resuspended in complete medium and counted.

Successful depletion was determined by two-color staining of $5x10^5$ splenic T cells with anti-L3T4(CD4)-FITC and anti-Lyt2(CD8α)-PE for 30 minutes at 4°C. Stained cells were then resuspended in FACS buffer after washing and analyzed by FACScan®.

E Islet Isolation

Collagenase (3mL, 470 U/mL; Worthington Biochemical Corp cat #4189) was injected into the bile duct of mice, from there it entered and digested the pancreas. The injected pancreas was then removed and incubated for 21 minutes in a 37°C waterbath.

The digestion process was stopped with the addition of cold HBSS and then the pancreas was disrupted by pipetting up and down. The pancreatic digests were then passed through a 600μm mesh to remove the undigested material. The remaining digested tissue was washed and then the islets were separated from the endocrine tissue by using a ficoll density gradient. Ficoll (Sigma cat#F4375) was used at 25%, 23%, 21.5%, 20.5%, and 11%, then the ficoll (containing pancreatic tissue) was centrifuged for 10 minutes. The islets stayed in the first two layers; these were removed and placed into petri dishes. The islets were then handpicked using a microscope and a pipet, and then washed. Single cells were then made from the islets by disrupting them with cell dissociation buffer (Gibco cat# 13150-016) for three minutes at 37°C. The single cells were then washed and counted. On average, this process yielded 150 islets and each islet had roughly 1000 beta cells; however, this process is highly variable and resulting numbers were not always consistent.

F Proliferation Assays

Beta cells, the antigen source used in the proliferation assay, were obtained via islet isolation. The beta cells were then γ-irradiated (3,000 rads) using a Cs-source. $2x10^4$ splenic T cells depleted of $CD8^+$ T cells (see above) from responder mice were incubated, in duplicate, with islet cells ($1x10^5$/well) as a source of antigen and APCs, respectively. These cells were cultured in a 96-well round-bottomed tissue culture plate for 3 days at 37°C in 5% CO_2, either in the presence or absence of IL-2. Cultures were pulsed with 1μCi of [^{3}H]thymidine during the last 18 hrs of culture and then harvested. The incorporation of thymidine was measured by scintillation counting. Specific proliferation was calculated by subtracting the background proliferation (cpm of cultures containing islet cells alone and of cultures of T cells alone) from islet-induced proliferation (cpm of cultures containing islets and T cells). The background, or spontaneous, proliferation was similar between NOD, 4.1-NOD, and MHC class II transgenic-4.1-NOD mice.

G Cytokine Assay

Single cell suspensions were generated from islets isolated from 4.1-NOD.I-E^k, 4.1-NOD.I-A^d, 4.1-NOD.I-A^{g7PD}, and 4.1-NOD mice as above. $2x10^4$ islet-T cells were plated in 96-well round-bottom tissue culture plates and activated with phorbol-myristate acetate (PMA, 10ng/mL) and calcium ionophore (250 ng/mL) for 12 hrs at 37°C, 5% CO_2. After activation, the supernatants were harvested for use in cytokine ELISAs. The supernatants (100μL/well) were assayed for IL-2, IL-4, and IFNγ, using commercially available cytokine ELISA kits (IL-4 from Genzyme Diagnostics and IL-2, IFNγ from R&D Systems).

H Bone Marrow Chimeras

Bone marrow chimeras were generated as following: the bones from the hind legs were removed from the donor mice and the bone cavities flushed; the collected cells were then washed, counted and resuspended in PBS. Bone marrow suspensions ($5-10x10^6$ cells) from donor mice (transgenic NOD mice) were injected into the tail vein of lethally-irradiated recipient NOD or Balb/c.I-A^{g7PD} mice (two doses of 500 rads, 3 hrs apart from a ^{137}Cs source). The bone marrow chimeras were followed for 7 weeks in order to allow reconstitution of the irradiated recipient with the donor marrow; they were then sacrificed.

I Histology and Immunopathology

i Histology

Each pancreas was divided into two pieces. One piece was fixed in 10% formalin, embedded in paraffin, sectioned at 4.5μm, and stained with hematoxylin and eosin. The degree of insulitis was determined by scoring 12-30 islets per mouse using the following criteria: 0=normal islet; 1=peri-insulitis; 2=mononuclear cell infiltration in <25% of the

islet; 3=mononuclear cell infiltration in 25-50% of the islet; 4=mononuclear cell infiltration in >50% of the islet. The second piece of pancreas was immersed in OCT and rapidly frozen using dry ice and acetone. These frozen blocks were then sectioned at 6-7 μm, and stored at -80°C for immunopathology.

ii Bromodeoxyuridine Immunopathology

4.1-NOD, 4.1-NOD.I-E^k, and 4.1-NOD.I-A^d were injected with 200μL of a 4mg/mL solution of 5-bromo -2'-deoxyuridine (BrdU; Calbiochem, La Jolla, CA). These mice were injected twice via the tail vein, four hours apart. The mice were then sacrificed twelve hours after the last injection and the pancreas and spleen were taken for immunopathology. The pancreas was divided into two pieces as above, one for hematoxylin/eosin staining and the other quickly frozen. The frozen pancreas was then sectioned at 6-7μm as above and the sections were fixed in cold acetone for ten minutes. These sections were stained for the presence of BrdU using a commercially available kit (Calbiochem). A blocking reagent was used to inhibit non-specific binding before staining with the biotinylated anti-BrdU monoclonal antibody. They were then stained with streptavidin-peroxidase, which binds to biotin and allows visualization of $BrdU^+$ cells. The DAB mixture, provided by the kit, was used to develop the color; then the sections were counterstained using hematoxylin. The percentage of $BrdU^+$ cells, islet-infiltrating cells which incorporated BrdU, was then determined.

J Diabetes Incidence

Diabetes was monitored by measuring urine glucose levels with Diastix (Bayer). Animals were considered diabetic after two consecutive readings of +3 on a 0-3 scale.

K Purification of spenic B cells and dendritic cells, thymic dendritic cells, and peritoneal macrophages

B cells were purified via positive selection using B220-coated microbeads in the MiniMACS system (Miltenyi Biotec, Auburn, CA), with the resulting purity of >95% $CD19^+$ or $B220^+$ cells. Dendritic cells were also purified using microbeads. Thymi or spleens (n=10) were dissected in 10mL of 100U/mL of collagenase type IV on ice in HBSS. Another incubation with 400U/mL of collagenase was done for 30 minutes at 37°C. Cells were then purified using anti-CD11c-coated microbeads (Miltenyi-Biotech) resulting in >95% $CD11^+$ cells. Peritoneal macrophages were collected via peritoneal lavage from mice that were injected intraperitoneally three days prior with 1mL of 3% thyoglicollate in PBS. Staining with anti-F4/80 and anti-CD11b monoclonal antibodies revealed a purity of >80%.

L Dulling Assay

Dulling assays were done by culturing $1x10^5$ APCs with $1x10^5$ thymocytes from RAG-2-deficient 4.1-NOD mice in triplicate for 20 hours in 96-well U-bottomed plates at 37°C and 5%CO_2. Cells were then collected at the end of the incubation period, washed, and stained with anti-CD4-PE, anti-CD8-FITC, and anti-CD11c-biotin, anti-CD11b-biotin, or anti-B220-biotin. Streptavidin-PerCP was used to visualize biotin-stained cells. Samples were then analyzed by flow cytometry as described above.

M Statistical analyses

Statistical analyses were peformed using the Mann-Whitney U test and the $\chi 2$ test using Statview.

IV Results

A I-E Molecules Trigger Tolerance in the 4.1-NOD Background

Previously in our lab, it was demonstrated that I-A^b MHC class II molecules were capable of triggering the deletion of autoreactive $CD4^+$ T cells (43). To determine whether or not I-E molecules were similar to I-A^b in their ability to remove T cells bearing the 4.1-TCR, the fate of the 4.1-TCR in 4.1-NOD.I-Eα^k mice was followed. Three-color staining was used for FACS analysis of thymocytes and splenocytes to determine the T cell profile in these animals. Results from staining thymocytes reflect T cell development, whereas splenocytes are representative of peripheral T cells. We observed that the I-E^k molecule was capable of triggering the deletion of diabetogenic thymocytes bearing the 4.1-TCR in 33% of the mice (7 of 21). The following criteria was used in defining deletion: a decrease in the percentage of $CD4^+$ T cells, a decrease in the percentage of Vβ11$^+$$CD4^+$ T cells, and an increase in the percentage of double negative T cells (DN; $CD4^-CD8^-$). At least two of the three criteria had to be met in order to classify a FACS profile as a deleting profile. When compared to the percentages from 4.1-NOD FACS profiles, those classified as deleting differed by at least two standard deviations in order for the differences to be considered significant. The remaining 67% (14 of 21 mice) showed a profile similar to that of the 4.1-NOD mouse and therefore showed positive selection of $CD4^+$T cells instead of deletion. (Figure 3a and 3b; Table I) Mice displaying a deletion profile will be referred to as deleters, whereas those which do not are nondeleters.

The deletion observed in this population of mice was due to a specific interaction and not resulting from superantigens nonspecifically binding to the Vβ-element of the TCR. This was determined by looking at the percentage of Vβ11$^+$$CD4^+$ cells in NOD, NOD.I-Eα^k, and NOD.H-$2g^{7/k}$ mice. Superantigen binding to the Vβ11 element would result in a decreased precentage of Vβ11$^+$$CD4^+$ cells in TCR-nontransgenic mice due to

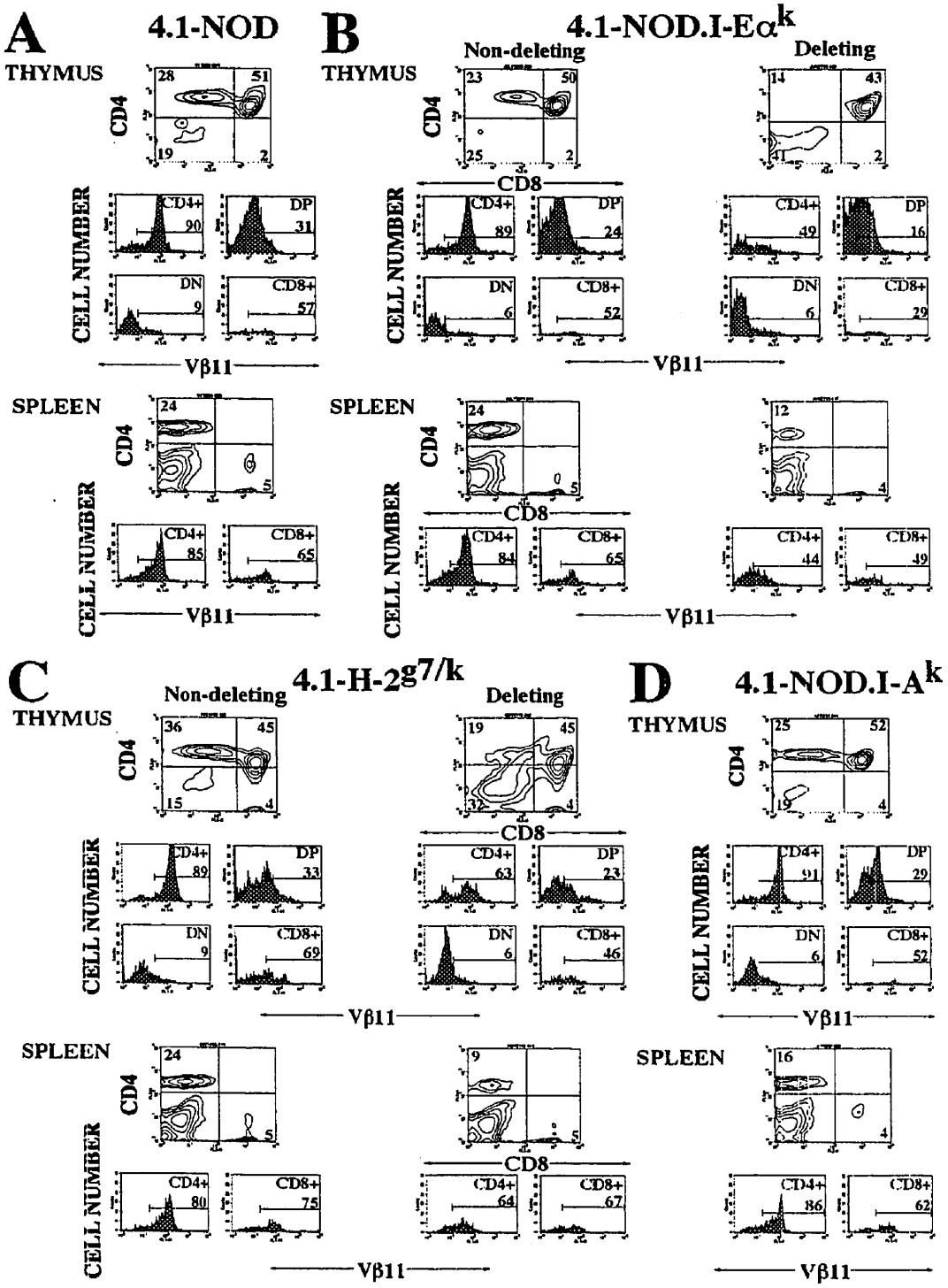


Figure 3: CD4, CD8, Vβ11 profiles of thymocytes and splenocytes from 4.1-NOD (A), 4.1-NOD.I-Eα^k (B), 4.1.H-2g$^{7/k}$ (C), and 4.1-NOD.I-A^k (D) mice. Data shown are average values of 6-14 mice/group. CD4 versus CD8 dot plots of cell suspensions stained with anti-CD8-PE, anti-Vβ11-FITC, and anti-CD4-biotin plus Streptavidin-PerCP. Vβ11 fluorescence histograms of each T cell subset after electronic gating. Numbers indicate the average percentage of T cells or Vβ11$^+$ cells in each subset. DP-double positive, DN-double negative cells.

Table I: The Influence of I-A^k, I-$E\alpha^k$, I-A^d, and I-Ag^{7PD} MHC class II molecules on 4.1-Thymocyte Development

	Deletion	n	organ	cell # (± SD)	$CD4^+CD8^-$ (± SD)	$V\beta11^+CD4^+$ (± SD)	DP (± SD)	DN (± SD).	$CD8^+CD4^-$ (± SD)	CD4/DN (± SD)	CD4/CD8 (± SD)
4.1-NOD	-	12	T	19 ± 8.1	28 ± 8.1[a]	90 ± 7.5[i]	51 ± 10.6	19 ± 4.4[r]	2 ± 0.6	1.45 ± 0.4[am]	
			S	47 ± 28	24 ± 5.2[e]	85 ± 6.8[m]			5 ± 1.8		9.15 ± 12.8[ac]
4.1-H-$2g^{7/k}$	+	8	T	10 ± 4.6[w]	19 ± 6.6[b]	63 ± 12.9[j]	45 ± 6.6	32 ± 6.3[s]	4 ± 2.5	0.65 ± 0.3[an]	
			S	117 ± 48	9 ± 1.6[f]	64 ± 13.5[n]			5 ± 0.5		1.73 ± 0.3[ad]
4.1-H-$2g^{7/k}$	-	6	T	24 ± 14[x]	36 ± 3.5	89 ± 7.9	45 ± 8.2	15 ± 5.2	4 ± 1.7	2.57 ± 0.7[ap]	
			S	95 ± 22	24 ± 14.3	80 ± 21.5			5 ± 1.7		10.1 ± 15.8[ae]
4.1-NOD.I-A^k	-	15	T	16 ± 3.9	25 ± 2.9	91 ± 2.7	52 ± 5.2	19 ± 2.9	4 ± 1.5	1.30 ± 0.2	
			S	67 ± 20	16 ±10.2	86 ± 3.6			4 ± 1.9		4.16 ± 1.4
4.1-NOD.I-$E\alpha^k$	+	7	T	16 ± 8.4	14 ± 14.4[c]	49 ± 18.5[k]	43 ±13.2	41 ± 13.3[t]	2 ± 0.7	0.38 ± 0.21[aq]	
			S	43 ± 20[y]	12 ± 2.6[h]	44 ± 11.2[p]			4 ± 1.3		3.30 ± 1.0[ag]
4.1-NOD.I-$E\alpha^k$	-	14	T	28 ± 13	23 ±3.0	89 ± 1.4	50 ± 7.0	25 ± 8.7[u]	2 ± 0.5	1.07 ± 0.5[ar]	
			S	71 ± 26.3[z]	24 ± 3.0	84 ± 4.9			5 ± 0.7		5.21 ± 0.74
4.1-NOD.I-A^d	+	8	T	13 ± 6.2	15 ± 6.6[d]	70 ± 8.6[l]	41 ± 18.7	41 ± 14.7	3 ± 2.2	0.40 ± 0.2[as]	
			S	54 ± 13.6	21 ± 3.1	77 ± 6.4[q]			6 ± 0.6		3.76 ± 0.5[ai]
4.1-NOD.I-Ag^{7PD}	-	6	T	21 ±7.9	21 ± 1.6	88 ± 1.5	56 ± 4.1	22 ± 5.7	2 ± 0.3	1.02 ± 0.3	
			S	62 ± 21.7	28 ± 5.2	84 ± 4.7			5 ± 1.3		5.08 ± 0.5

Mice were studied at 8-16 wk of age. DN: double negative; DP: double positive; T: thymus; S: spleen
Statistics (Mann-Whitney U test): a vs b p=0.0055; a vs c p=0.0007; a vs d p=0.0026; e vs f p=0.0002; e vs h p=0.0004; i vs j p=0.0009; i vs k p=0.0007; i vs l p=0.0009; m vs n p=0.0043; m vs p p=0.0004; m vs q p=0.0136; r vs s p=0.0012; r vs t p=0.0041; r vs u p=0.0020; am vs an p=0.0012; am vs ap p=0.0087; am vs aq p=0.0004; am vs ar p=0.0372; am vs as p=0.0002; ac vs ad p=0.0142; ac vs ae p=0.0192; ac vs ag p= 0.0142; ac vs ai p=0.0043; w vs x p=0.0201; y vs z p=0.0277

nonspecific binding to Vβ11 which triggers deletion. Decreased levels of $V\beta11^{+}CD4^{+}$ cells were not seen in NOD, NOD.I-$E\alpha^{k}$, or NOD.H-$2g^{7/k}$ mice (Figure 4), which demonstrates that superantigens were not responsible for the observed deletion.

To address the question of whether or not T cells from 4.1-NOD.I-$E\alpha^{k}$ mice are able to respond to antigenic stimulation, proliferation assays were performed. If these T cells are capable of recognizing β cell autoantigens in the context of I-A^{g7}, then proliferation will occur. T cells from 4.1-NOD mice proliferate in response to NOD islet stimulation because the 4.1-TCR is I-A^{g7}-restricted; 4.1-thymocytes are positively selected by I-A^{g7} and recognize antigen in the context of this MHC class II molecule. As expected, $CD4^{+}$ T cells from deleter 4.1-NOD.I-$E\alpha^{k}$ mice cannot proliferate in response to islet stimulation (Figure 5a). The absence of proliferation was expected with deleter 4.1-NOD.I-$E\alpha^{k}$ mice, because deletion occurs in the thymus; therefore, T cells capable of recognizing islet antigen are not exported into the periphery. T cells from nondeleter 4.1-NOD.I-$E\alpha^{k}$ mice were unable to proliferate when challenged with NOD islet cells (Figure 5b, left panel). This was surprising because the T cells bearing the 4.1-TCR were not deleted in the thymus. Since deletion does not occur, T cells are released into the periphery and should be able to react to antigen, and yet T cells from nondeleter 4.1-NOD.I-$E\alpha^{k}$ mice are unresponsive. However; when T cells from nondeleter 4.1-NOD.I-$E\alpha^{k}$ mice were cultured as above in the presence of rIL-2, the proliferative ability was restored (Figure 5b, right panel). Together, these results suggest that the nondeleter 4.1-NOD.I-$E\alpha^{k}$ T cells were anergic to antigenic stimulation *in vitro*.

The presence of both deleters and nondeleters within the 4.1-NOD.I-$E\alpha^{k}$ mouse population was not due to a difference in the level of expression of the I-E molecule since the mean fluorescence intensities were almost identical at 260.99 for deleters and 260.06 for nondeleters (Figure 6). There was, however, a statistically significant difference between the percentage of cells expressing the I-E molecule. A greater percentage of cells

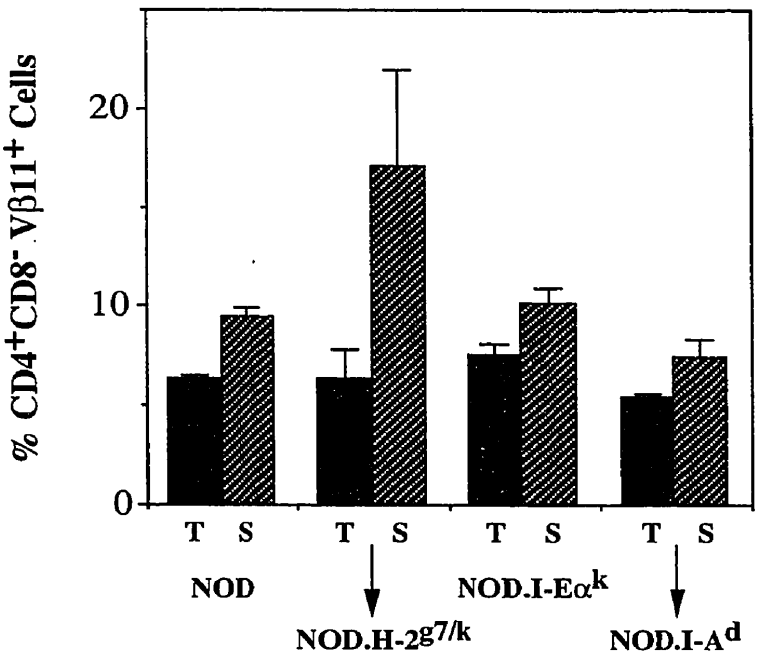


Figure 4: Absence of deletion of $CD4^+V\beta11^+$ T cells in T-cell receptor non-transgenic, MHC class II transgenic mice. Data correspond to average values of 2-8 mice/group. Numbers are average values obtained from 3 color flow cytometric analysis as described in the legend for figure 3. Bars represent the standard error of the means. T-thymocytes; S-splenocytes.

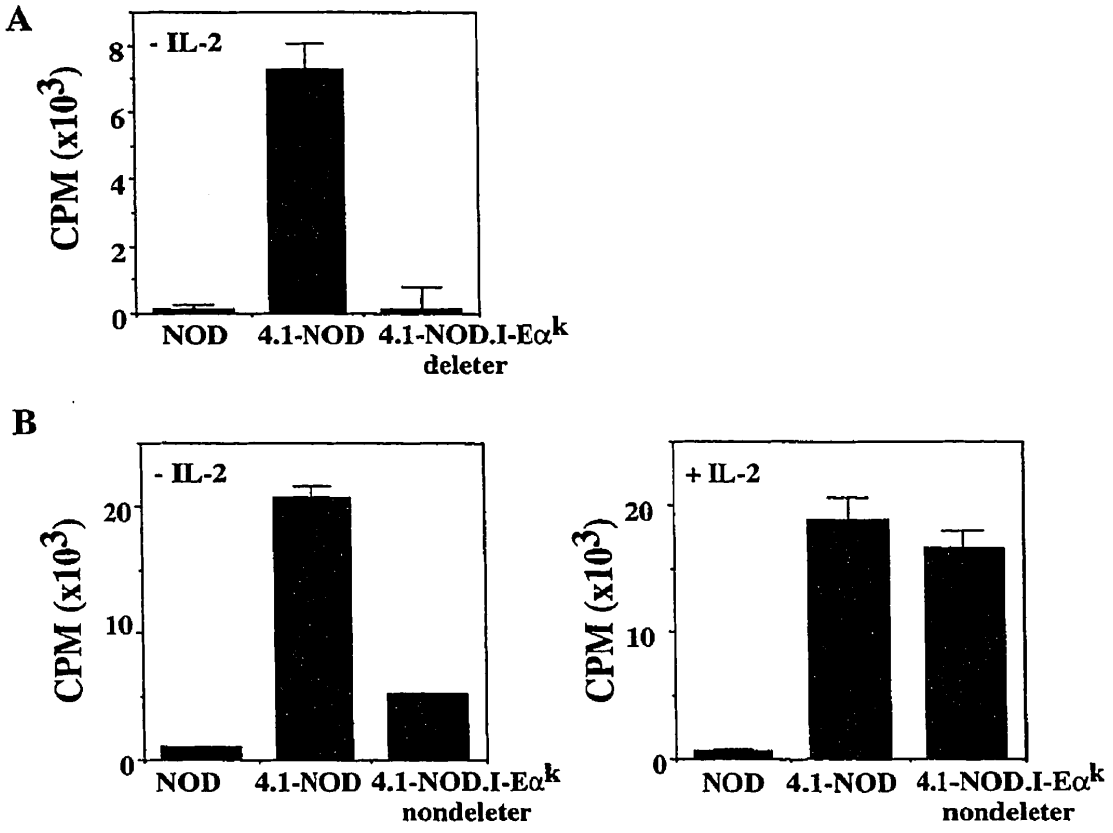
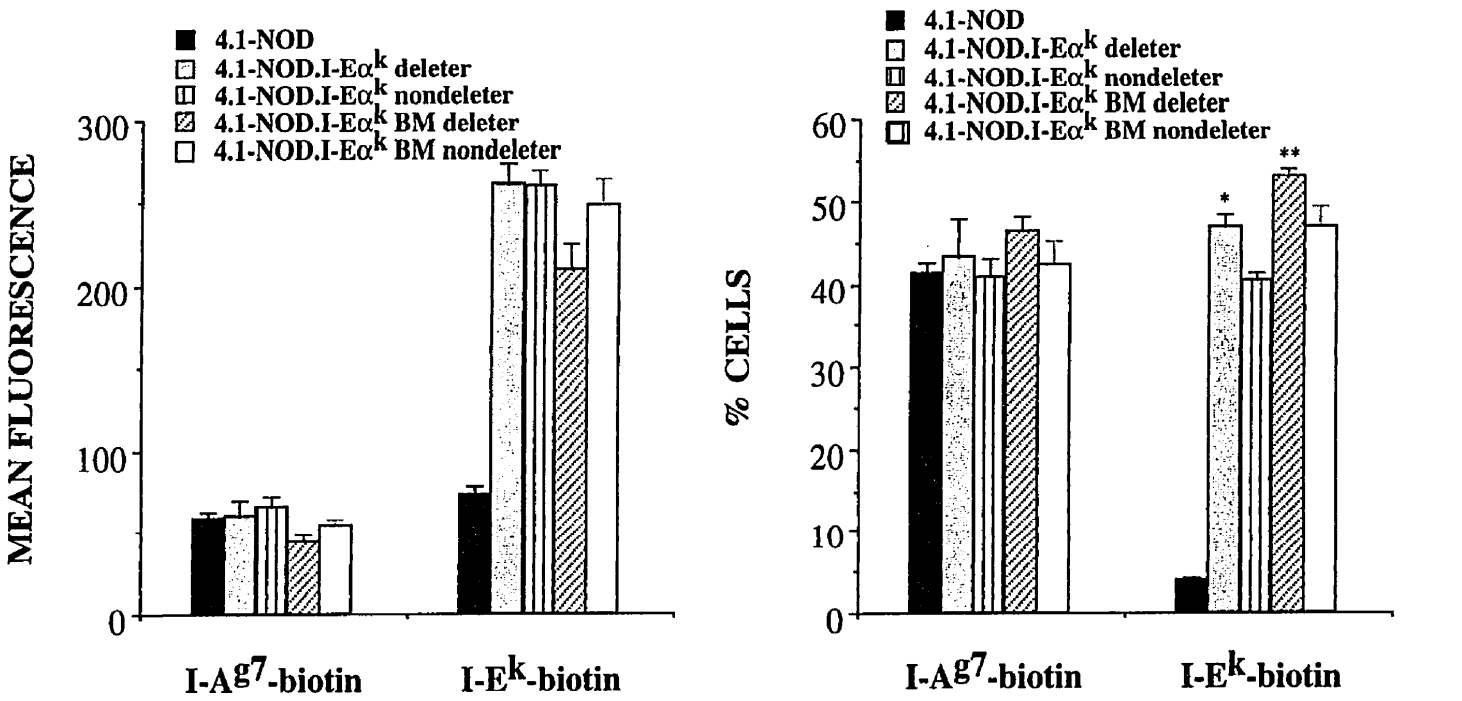


Figure 5: *In vitro* proliferation of naive splenic CD4$^+$ T cells from 4.1-NOD.I-Eα^k mice. CD4$^+$ T cells from deleting (A) and nondeleting (B, left panel) 4.1-NOD.I-Eα^k mice in response to NOD islet cells in normal culture media. Recovery of proliferation when splenocytes from nondeleter 4.1-NOD.I-Eα^k mice were cultured with IL-2 (B, right panel). Cultures of $2x10^4$ splenic CD4$^+$ T cells from 4.1-NOD.I-Eα^k mice were incubated with γ-irradiated NOD islet cells (10^5/well) for 3 d, pulsed with [^{3}H]thymidine, and harvested. Bars show the standard error of the means. The background proliferation (islets alone + T cells alone) was 1000cpm or lower without IL-2 and 3500cpm or lower when cultured with IL-2. Data shown is representative of three separate experiments.



* (del vs nondel) p=0.0041; ** (BMdel vs BMnondel) p=0.0457

Figure 6: Mean Fluorescence Intensity and Percentage of cells expressing I-A^{g7} and I-E^{k} MHC class II molecules. Cell suspensions were stained with I-A^{g7}-biotin or I-E^{k}-biotin plus Streptavidin-PerCP. Numbers indicate the average fluorescence intensity or the average percentage of cells expressing either I-A^{g7} or I-E^{k}. Data shown are average values of 6-17 mice/group. BM: bone marrow chimeras

in deleter 4.1-NOD.I-Eα^k mice display the I-E molecule on the cell surface than in nondeleter animals (Figure 6).

Transgenes integrate randomly when introduced into the genome. How and where the transgene locates within the genome can affect its expression and influence on the system. It is possible that the transgene's effect does not reflect what is observed with the endogenous gene. To address this issue, we looked at a background which normally expresses the I-Eα^k molecule, that is has an endogenous Eα gene. The C58/j mouse expresses the H-2^k haplotype, which consists of I-A^k and I-E^k molecules. F1 generations were generated by crossing 4.1-NOD with C58/j mice in order to create 4.1-H-$2g^{7/k}$ mice, and three-color flow cytometric analysis was done to study the 4.1-TCR during T cell development. A similar pattern to 4.1-NOD.I-Eα^k mice was observed in these mice; two phenotypes were present which were classified as deleters and nondeleters. The same results were obtained whether or not the gene was transgene encoded, suggesting that the results were not an artifact of transgenesis. (Figure 3b and 3c; Table I) Since 4.1-NOD.H-$2g^{7/k}$ mice have both I-A^k and I-E^k, we also looked at 4.1-NOD.I-A^k transgenic mice to determine if I-A^k was involved with deletion. 4.1-NOD.I-A^k mice showed flow cytometric profiles similar to 4.1-NOD mice (Figure 3a and 3d; Table I) and so the deletion observed in 4.1-NOD.H-$2g^{7/k}$ mice was due to the I-E^k molecule and not I-A^k.

B I-E Triggered Deletion is induced by Hematopoietic cells and is a Stochastic Process

Cells derived from the bone marrow have been shown to be important mediators in diabetes susceptibility and resistance. Bone marrow chimeras were created to determine if the deletion observed in 4.1-NOD.I-Eα^k mice arose from the bone marrow-derived hematopoietic cells. Bone marrow from 4.1-NOD.I-Eα^k transgenic mice was injected into the tail vein of lethally irradiated NOD mice. These chimeras were then sacrificed and

FACS analysis was performed on the thymi and spleens. Flow cytometric analysis of the chimeras showed that deletion is mediated by the bone marrow-derived hematopoietic cells of the thymic medulla (Figure 7). As in the donor mice, the two phenotypes of deletion and nondeletion were observed in the bone marrow chimeras. In general, the phenotype of the donor marrow was a good predictor of the chimera phenotype. However, exceptions did exist, with nondeleter marrow giving rise to a deleter phenotype and vice versa (Figure 7; Table II). This suggested that the deletion process involves some stochastic element, as the donor marrow does not always dictate the phenotype of the chimera. Since the donor marrow does not always determine the chimera phenotype, there is a random element involved to some degree. However, this process is not completely stochastic in that the chimera phenotype was usually similar to that of the donor marrow (Table II).

C I-E protects from diabetes development

4.1-NOD.I-Eα^k mice were followed for diabetes development in order to determine the effect of the I-E-triggered tolerance on CD4$^+$ T cell-induced disease. Mice were followed for 25 weeks for diabetes development, and as shown in Table III, the I-E molecule protects 4.1-NOD mice from developing diabetes. Compared to 4.1-NOD mice, the presence of the I-Eα^k transgene greatly decreased the incidence of diabetes (Table III).

D Insulitis in 4.1-NOD.I-Eα^k mice

Insulitis is a measure of the mononuclear cell infiltration into the pancreatic islets and often correlates with diabetes development. Usually a high insulitis score reflects a high incidence of diabetes, whereas a low score suggests a low incidence of disease development. As expected, the insulitis scores from deleter 4.1-NOD.I-Eα^k mice were very low, as T cells are deleted in the thymus and not exported into the periphery (Table III). However, the insulitis scores from the nondeleter 4.1-NOD.I-Eα^k mice were

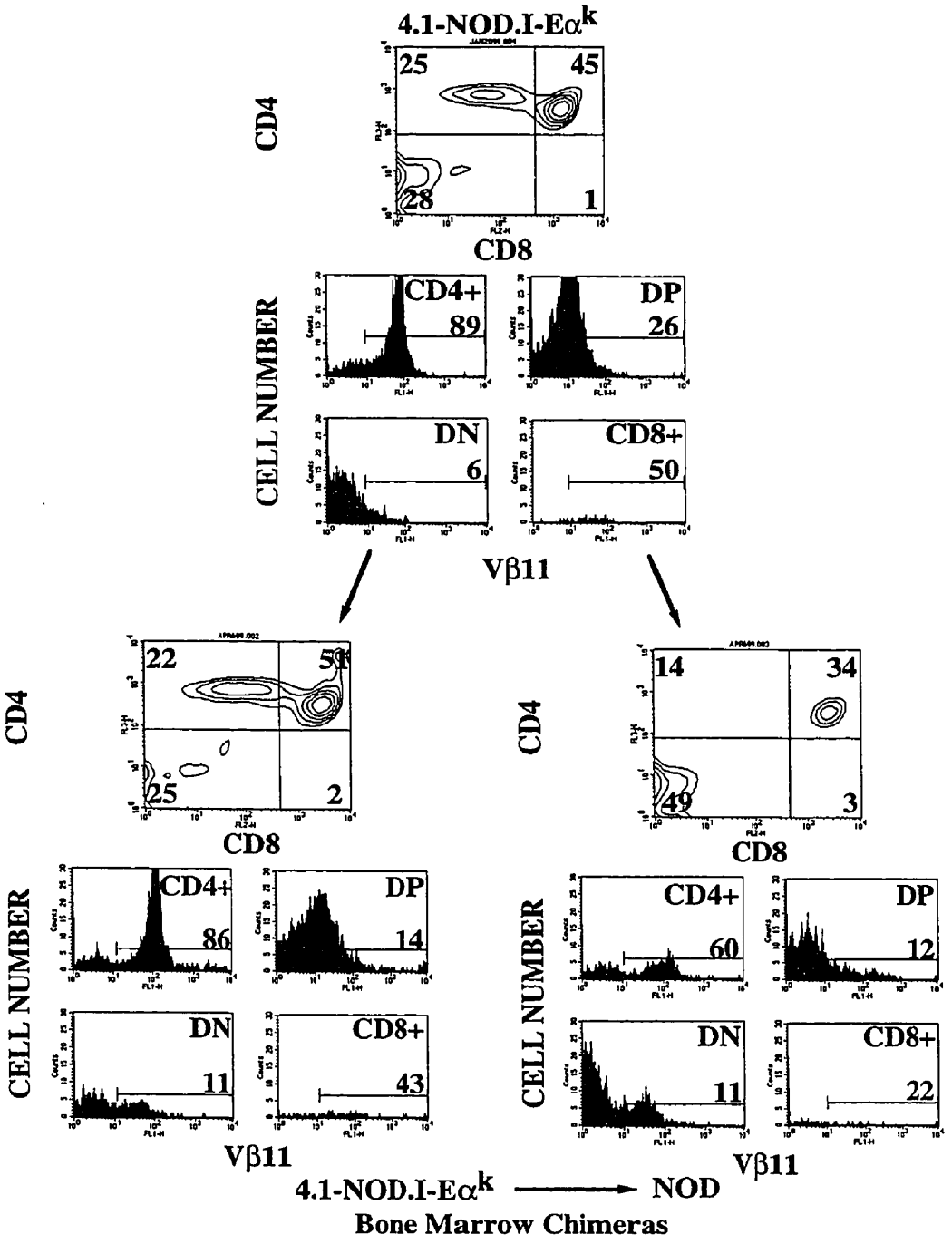


Figure 7: 4.1-NOD.I-Eα^k bone marrow chimeras. Bone marrow cells (6-10x10^6) from 4.1-NOD.I-Eα^k donor mice were injected into the tail veins of lethally irradiated recipient mice (nontransgenic NOD). Thymi of chimeric mice were analyzed by flow cytometry 7-8 weeks after transplantation. The top panels show the donor profile and the lower panels represent the chimera profiles. See legend of figure 3 for details.

Table II: Bone Marrow Chimeras

Donor	Deletion	Host	n	Deleting Status
4.1-NOD	-	NOD	7	0/7[a]
4.1-NOD.I-Eα^k	+	NOD	8	7/8[b]
4.1-NOD.I-Eα^k	-	NOD	20	4/20[c]
4.1-NOD.I-A^d	+	NOD	8	8/8[d]

a vs b p=0.0076; a vs c p=0.0001; a vs d p=0.0082; b vs c p=0.0003

Table III: Insulitis and Diabetes Incidence in 4.1-NOD, 4.1-NOD.I-A^k, 4.1-NOD.I-$E\alpha^k$, 4.1-NOD.I-A^d, and 4.1-NOD.I-A^{g7PD} Mice

	Deletion	n	IDDM	Age of Onset (wks) (± SD)	Insulitis (± SD)
4.1-NOD	-	32	21[d]	7 ± 2.5	2.95 ± 0.78 (n=10)[a]
4.1-NOD.I-A^k	-	9	0[e]	-	2.20 ± 0.8 (n=7)
4.1-NOD.I-$E\alpha^k$	+	5	0[f]	-	0.80 ± 0.54 (n=4)[b]
4.1-NOD.I-$E\alpha^k$	-	9	1[g]	7	2.58 ± 1.06 (n=8)
NOD.I-$E\alpha^k$	-	19	0	-	0.06 ± 0.11 (n=9)
4.1-NOD.I-A^d	+	19	5[h]	19 ± 8.2	2.30 ± 0.56 (n=15)[c]
NOD.I-A^d	-	9	0	-	1.02 ± 0.63 (n=5)
4.1-NOD.I-A^{g7PD}	-	17	7[i]	11 ± 2.6	2.17 ± 0.85 (n=6)
NOD.I-A^{g7PD}	-	18	0	-	0.85 ± 0.22 (n=2)

insulitis: 15-30 islets scored
a vs b p=0.0089; a vs c p=0.0305
d vs e p=0.0004; d vs f p=0.0030; d vs g p=0.0010; d vs h p=0.0002; d vs i p=0.0019
IDDM was followed in female mice only

somewhat surprising; the scores were comparable to those of 4.1-NOD mice even though there was still protection from diabetes development (Table III). This shows that the T cells exported from the thymus into the periphery can home to the pancreas but are not capable of causing significant beta cell damage.

A high degree of insulitis in nondeleter 4.1-NOD.I-Eα^k mice, along with the absence of diabetes development and the presence of anergy *in vitro,* raised the question of whether or not anergy was occurring *in vivo*. Cells proliferating *in vivo* were detected using BrdU, which incorporates itself into actively proliferating cells. Surprisingly, nondeleter 4.1-NOD.I-Eα^k mice had as many BrdU$^+$ cells as 4.1-NOD mice (Figure 8). This suggests that even though there is insulitis with actively proliferating cells, they are incapable of effecting beta cell damage. The cells that invade the islets seem to be nonpathogenic, or perhaps there is a required threshold for disease occurrence which had not been reached.

To determine whether or not the recruitment of immune-deviated T cells was responsible for the inability of the islet-infiltrating T cells to cause beta cell damage, the ability of these T cells to secrete IL-2, IFNγ, and IL-4 when stimulated with PMA and ionomycin was compared. The islet-associated T cells from 4.1-NOD and nondeleting 4.1-NOD.I-Eα^k mice produced comparable levels of IL-2 and IFNγ while there were minimal levels of IL-4 (Figure 9). These results suggested that even though the islet-infiltrating T cells were unable to effect beta cell damage, it was not due to regulatory cells, because the islet-associated T cells produced pro-inflammatory cytokines. Thus, these islet-infiltrating T cells proliferate *in vivo* but cannot cause significant beta cell damage; therefore, diabetes does not occur.

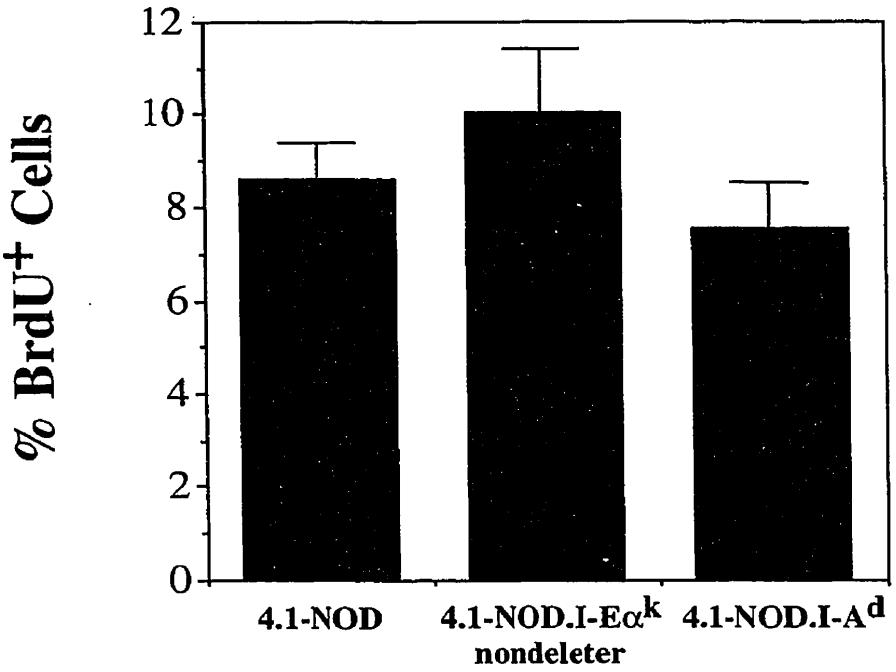


Figure 8: Percentage of BrdU$^+$ cells in the pancreatic islets of 4.1-NOD, nondeleting 4.1-NOD.I-Eα^k, and 4.1-NOD.I-A^d mice. Sections were made from frozen blocks containing pancreata from 4.1-NOD, 4.1-NOD.I-Eα^k, and 4.1-NOD.I-A^d mice. These sections were then stained for the presence of BrdU. The percentage of BrdU$^+$ cells was determined by dividing the number of BrdU$^+$ cells by the total number of infiltrating cells within an islet. Error bars show the standard error of the means. Numbers are representative of 3-5 mice per group.

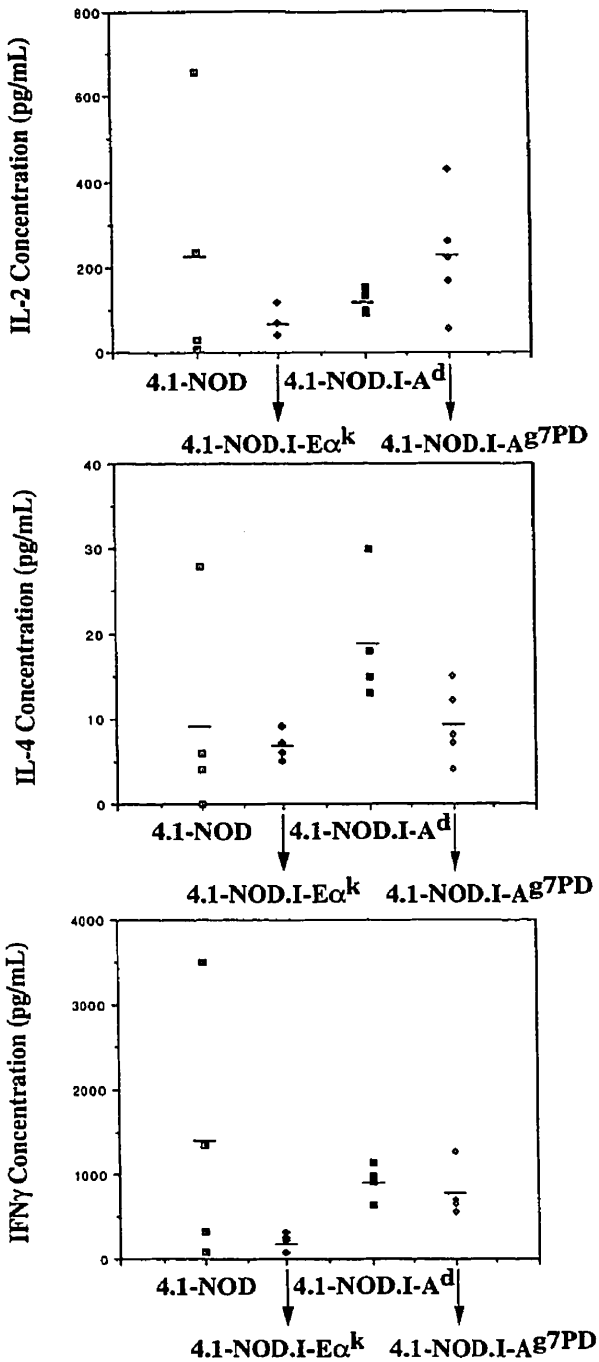


Figure 9: Cytokine profiles of islet-associated T cells from 4.1-NOD, nondeleting 4.1-NOD.I-Eα^{k}, 4.1-NOD.I-A^{d}, and 4.1-NOD.I-A^{g7PD} mice. Single cells were made from islets from either 4.1-NOD, 4.1-NOD.I-Eα^{k} nondeleter, 4.1-NOD.I-Aβ^{d}, or 4.1-NOD.I-Aβ^{g7PD} mice. Cultures of $2x10^{4}$ single cells were activated with 10ng/mL PMA and 250ng/mL Calcium ionophore overnight. The supernatants were collected and the levels of produced cytokines were detected using cytokine ELISAs. Lines show the mean of the values represented by the individual points. Data shown came from 4-5 mice per group.

E A structurally different MHC class II molecule is capable of inducing tolerance in 4.1-NOD mice

Since tolerance is triggered in the 4.1-NOD mouse by I-A^b and I-E, then it is possible that the same results occur in a structurally different MHC class II molecule that has been shown to be protective in the NOD background, such as I-A^d. Flow cytometric analysis revealed partial deletion of thymocytes bearing the 4.1-TCR in 4.1-NOD.I-A^d mice (Figure 10 left panels, Table I). When compared to deleting 4.1-NOD.I-Eα^k mice, 4.1-NOD.I-A^d mice had a slightly higher percentage of Vβ11$^+$CD4$^+$ cells; this suggested that the deletion was partial. However, when compared to 4.1-NOD mice, the FACS profiles of 4.1-NOD.I-A^d mice show the deletion profile previously described. As with 4.1-NOD.I-Eα^k mice, the deletion observed was not due to the binding of endogenous superantigens, because NOD.I-A^d mice did not exhibit decreased numbers of Vβ11$^+$CD4$^+$ cells (Figure 4).

In the periphery, T cells from 4.1-NOD.I-A^d are not capable of recognizing the beta cell autoantigen as shown by proliferation assays cultured either in the presence or absence of rIL-2 (Figure 11a and 11b). As expected, there was no proliferative response observed because deletion was observed in the thymus.

F The role of I-A^d in insulitis and diabetes development

I-A^d-transgenic-4.1-NOD mice were followed for diabetes development and scored for insulitis. 4.1-NOD.I-A^d mice were moderately protected from diabetes, showing a low incidence of disease with a delayed onset (Table III) Despite this moderate protection, these mice still showed a fair degree of insulitis. This result was unexpected because deletion occurs in the thymus, and peripheral T cells do not respond to antigenic stimulation (Table III). This suggests that a small percentage of cells may be exported into the periphery and infiltrate the islets, but are not significant enough in number to cause disease.

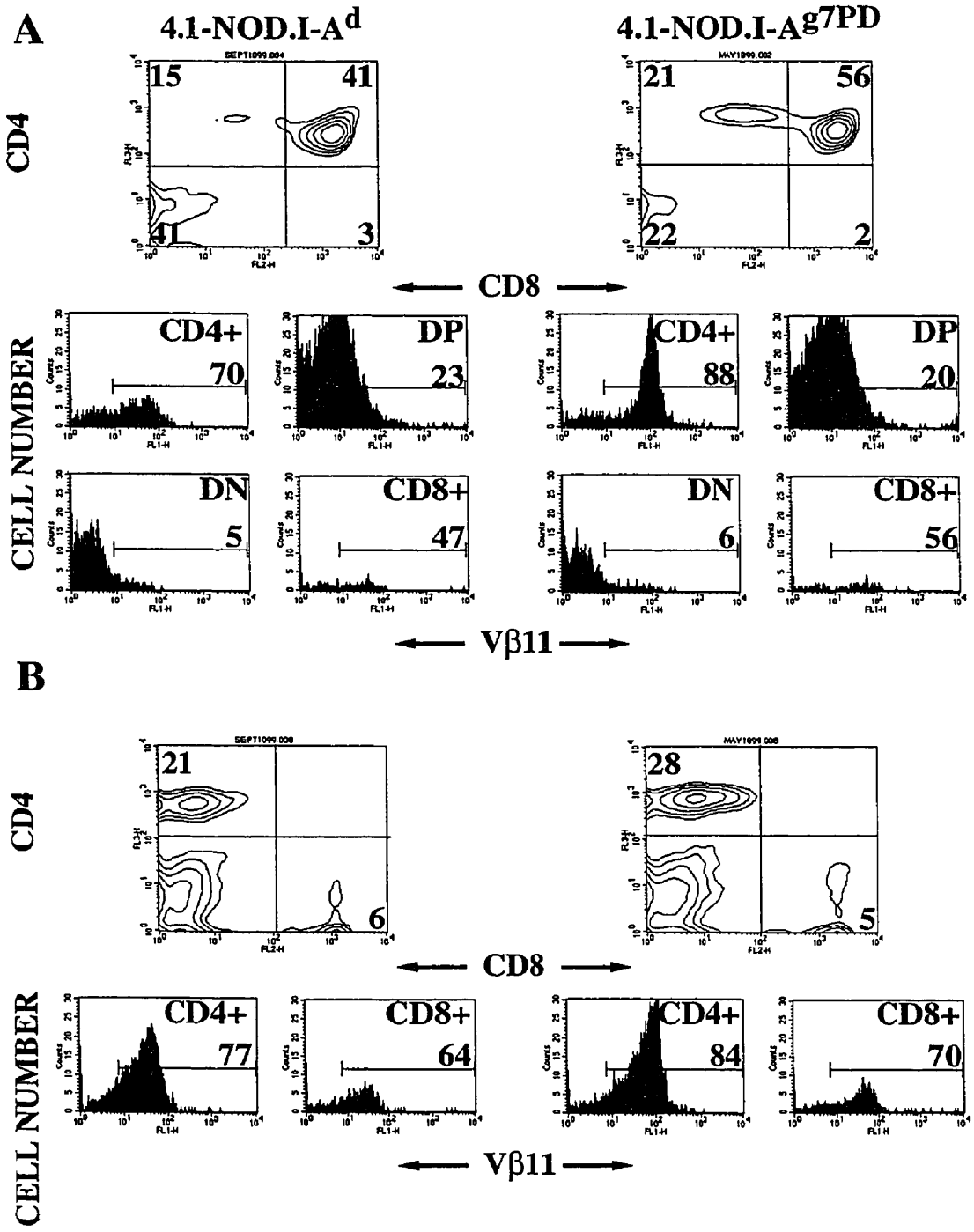


Figure 10: CD4, CD8, Vb11 profiles showing 4.1-CD4$^+$ T cell development in 4.1-NOD.I-A^d and 4.1-NOD.I-A^{g7PD} mice. Figure shows thymocytes (A) and splenocytes (B) from 4.1-NOD.I-A^d and 4.1-NOD.I-A^{g7PD} mice. Data shown are average values of 6-8 mice/group. See the legend from figure 3 for details. DP-double positive cells, DN-double negative cells.

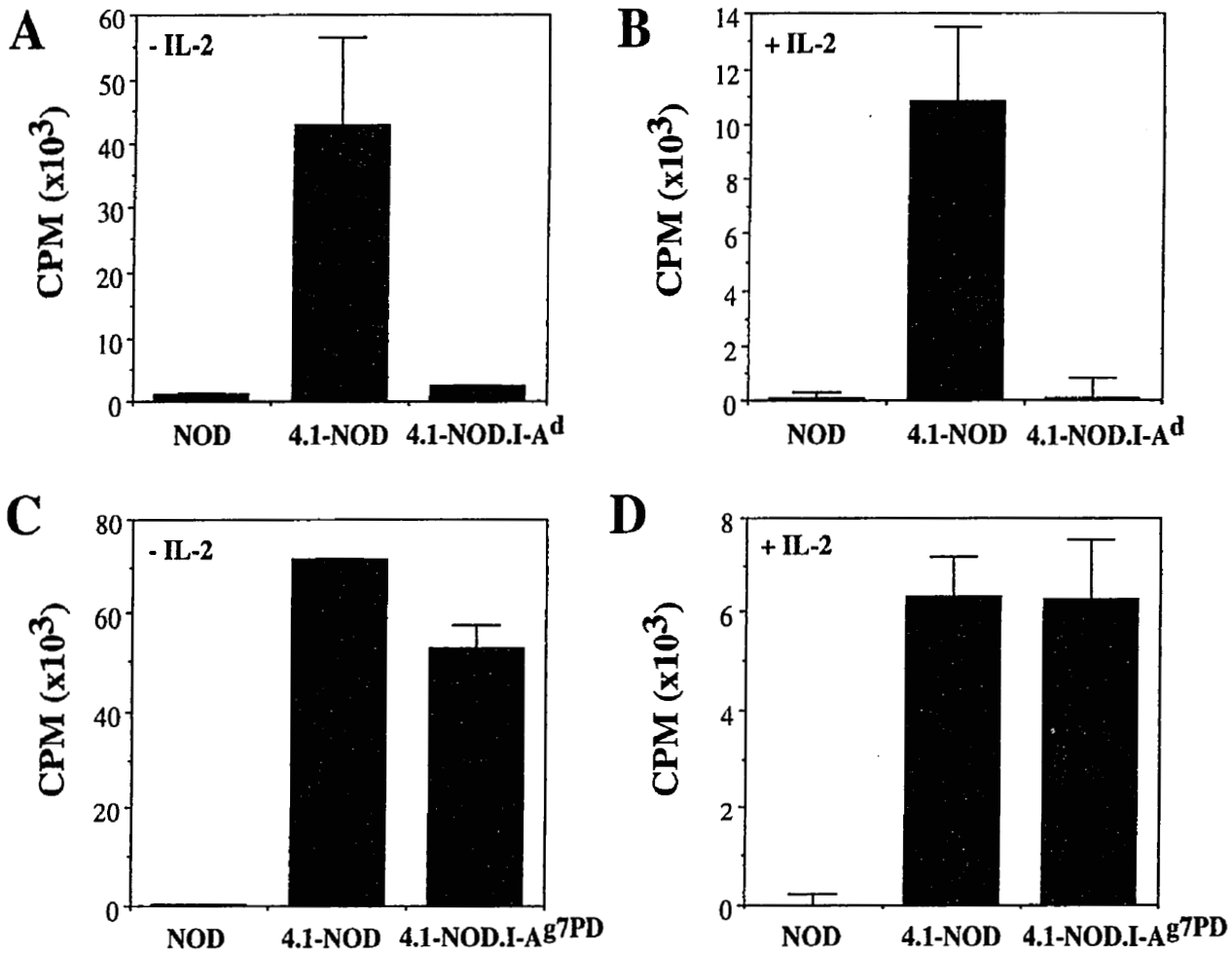


Figure 11: ***In vitro*** **proliferation of splenic CD4$^+$ T cells from 4.1-NOD.I-A^d and 4.1-NOD.I-A^{g7PD}.** T cells from 4.1-NOD.I-Aβ^d cultured without (A) and with (B) rIL-2 and from 4.1-NOD.I-A^{g7PD} without (C) and with (D) rIL-2 in response to NOD islet cells. See the legend of figure 5 for details. Bars show the standard error of the means.

BrdU staining of pancreatic tissue from 4.1-NOD.I-A^d mice revealed that there were similar numbers of positively proliferating cells as in 4.1-NOD mice (Figure 8). Similar to cells from 4.1-NOD.I-Eα^k mice, these islet-infiltrating cells were not immune deviated; the islet-associated T cells secreted levels of IL-2, IFNγ, and IL-4 that were similar to 4.1-NOD mice (Figure 9). Therefore, these results suggest that 4.1-NOD.I-A^d mice export a small number of autoreactive T cells into the periphery which can home to the pancreas and infiltrate the islets but cannot efficiently cause disease.

G The importance of proline and aspartic acid at positions 56 and 57 of the MHC class II β chain for diabetes resistance

Through studying the sequences of the β-chain of the MHC class II molecule it was clear that there was a recurring motif in the protective molecules. This motif includes proline at position 56 and aspartic acid at position 57 of the β-chain (Table IV). When the I-A^{g7} molecule was mutated at positions 56 and 57 and then introduced into the NOD background, the I-A^{g7PD} molecule proved to be protective against diabetes development; however, the mechanism of this protection is unknown (86, 87). The importance of proline and aspartic acid at positions 56 and 57 to the protective nature of certain MHC class II molecules (such as I-A^b, I-A^d, and I-E) was investigated by transgenically introducing the I-A^{g7PD} molecule into the 4.1-NOD background. Flow cytometric analysis of the thymus and spleen of 4.1-NOD.I-A^{g7PD} mice showed that deletion was not triggered in the thymus of these mice; rather, positive selection occurs resembling the profile of 4.1-NOD mice (Figure 10 right panels, Table I). However, 4.1-NOD.I-A^{g7PD} mice showed a reduced $CD4^+$:DN thymocyte ratio compared to 4.1-NOD mice (Table I). Peripheral T cells from 4.1-NOD.I-A^{g7PD} mice respond to antigenic stimulation by NOD islet cells but did not exhibit as strong a response as 4.1-NOD mice (Figure 11c). The addition of rIL-2 to the culture conditions resulted in a stronger proliferative response, similar to that seen in

Table IV: Amino Acid Sequences, from positions 51-73 of the Beta Chain, of MHC class II Molecules

				MHC class II transgenic/congenic 4.1-TCR-transgenic Mice			
I-A/I-E Beta Chain Sequence		Tg	H-2	Deletion	Anergy	Insulitis	IDDM
	51----------73						
$I\text{-}A^{g7}$	TELGRHSAEYYNKQ*Y*LERTRA	-	$I\text{-}A^{g7/g7}$	-	-	+++	+++
$I\text{-}A^{s}$	-----**PD**----------**Q**---	-	$I\text{-}A^{s/g7}$	-	-	?	?
$I\text{-}A^{g7PD}$	-----**PD**----------	$I\text{-}A\beta^{g7PD}$	$I\text{-}A^{g7/g7}$	+/-	?	+	+
$I\text{-}A^{k}$	-----**PD**---**W**----------	$I\text{-}A\beta^{k}$	$I\text{-}A^{g7/g7}$	+/-	?	+	-
$I\text{-}A^{q}$	-----**PD**---**W**-**S**-**PEI**------	-	$I\text{-}A^{q/g7}$	++	?	-	-
$I\text{-}A^{d}$	-----**PD**---**W**-**S**-**PEI**------	$I\text{-}A\beta^{d}$	$I\text{-}A^{g7/g7}$	+++	?	+	+
$I\text{-}A^{b}$	-----**PD**---**W**-**S**-**PEI**------	-	$I\text{-}A^{b/g7}$	++++	-	-	-
$I\text{-}E^{g7}$	-----**PD**--**NW**-**S**-**PEI**--**QK**--	$I\text{-}E\alpha^{k}$	$I\text{-}A^{g7/g7}$	++++	-	-	-
				-	+++	+	-
$I\text{-}E^{k}$	-----**PD**--**NW**-**S**-**PEF**--**QK**--	-	$I\text{-}A^{g7/k}/I\text{-}E^{k}$	++++	-	-	-
				-	+++	+	-

Tg: transgene, H-2: murine MHC

4.1-NOD mice (Figure 11d). This suggests that T cells from I-A^{g7PD}-transgenic 4.1-NOD mice respond to islet stimulation but at a reduced level; this may reflect a minor state of anergy.

4.1-NOD.I-A^{g7PD} mice showed moderate protection from insulitis and diabetes development. The incidence of diabetes was reduced, and the time of onset was delayed, compared to 4.1-NOD (Table III). When insulitis was scored, moderate infiltration was observed (Table III). As with the 4.1-NOD.I-A^{d} and nondeleting 4.1-NOD.I-Eα^{k} mice, the islet-associated T cells from 4.1-NOD.I-A^{g7PD} mice showed similar cytokine profiles to 4.1-NOD mice (Figure 9). Therefore, having proline and aspartic acid at positions 56 and 57 was protective against autoreactive CD4^{+} T cell-induced diabetes, even though there was no massive deletion or immune deviation of 4.1-CD4^{+} T cells.

H I-Aβ^{g7PD}, but not I-A^{d} nor I-E^{d}, can positively select 4.1-NOD thymocytes on thymic epithelial cells

It was shown previously that even though I-A^{b} was capable of triggering the deletion of 4.1-thymocytes in the thymic medulla, it was not able to mediate positive selection in the thymic cortex (Figure 12) (67). Since it has been shown that different MHC class II molecules function in a similar matter in inducing tolerance, it is possible that other protective molecules should also be incapable of positively selecting 4.1-thymocytes. To determine if the same would hold true for other MHC class II molecules (such as I-A^{d}, I-E^{d}, and I-A^{g7PD}), bone marrow chimeras were generated by injecting 4.1-NOD marrow into lethally irradiated NOD (I-A^{g7}), Balb/c (I-A^{d}, I-E^{d}), or Balb/c.I-A^{g7PD} (I-A^{d}, I-E^{d}, I-A^{g7PD}) hosts. These chimeras expressed either I-A^{g7}, I-A^{d} and I-E^{d}, or I-A^{d}, I-E^{d}, and I-A^{g7PD} on the radioresistant thymic epithelial cells; the radiosensitive bone marrow-derived APCs in the medulla only expressed I-A^{g7}. After reconstitution, the chimeras were sacrificed and flow cytometric analysis was performed on their thymi. Thymi of 4.1-

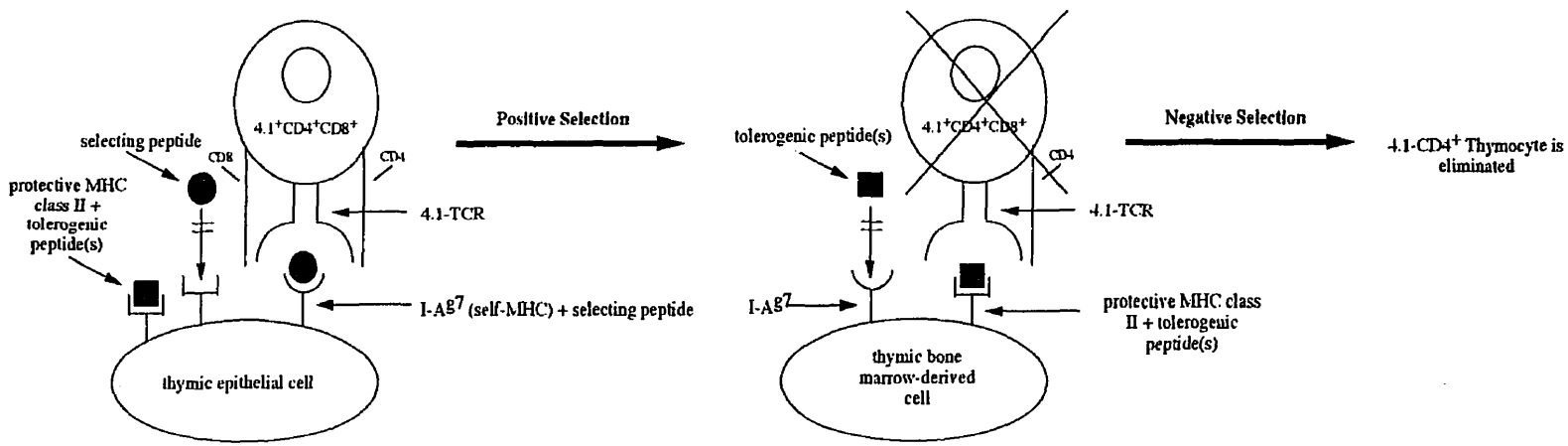


Figure 12: Protective MHC class II molecules cannot mediate positive selection in the thymic cortex; rather they are only recognized by the 4.1-TCR *in the thymic medulla*. 4.1-DP thymocytes undergo positive selection (in the thymic cortex) when engaging I-A^{g7}, but not anti-diabetogenic (such as I-A^{b}, and possibly I-E^{d} and I-A^{d}) MHC class II molecules on thymic epithelial cells. Negative selection, which occurs in the thymic medulla, is mediated by the 4.1-TCR recognizing many different peptide/protective MHC class II complexes. displayed on bone marrow-derived APCs.

NOD→Balb/c chimeras appeared to have a similar profile to that of MHC class II-deficient 4.1-NOD mice (4.1-I-$A^{o/o}$; Figure 13, upper and lower right panels), suggesting developmental arrest of 4.1-thymocytes instead of positive selection. On the other hand, thymi from 4.1-NOD→Balb/c.I-A^{g7PD} mice, which are similar to Balb/c except for the presence of I-A^{g7PD}, had a profile comparable to that of 4.1-NOD→NOD chimeras where positive selection of 4.1-thymocytes occurs. In contrasting 4.1-NOD→Balb/c and 4.1-NOD→Balb/c.I-A^{g7PD} chimeras, the latter had a greater number of thymocytes ($22 \pm 4 \times 10^6$ vs $5 \pm 0.7 \times 10^6$; $p<0.003$) as well as a larger $CD4^+CD8^+$:$CD4^-CD8^-$ thymocyte ratio (3.2 ± 0.5 vs 1 ± 0.3; $p<0.007$) (Figure 13). Since these chimeras are able to positively select 4.1-thymocytes, it was not surprising that their splenic $CD4^+$ T cells proliferated in response to islet antigen almost as well as splenic $CD4^+$ T cells from 4.1-NOD mice, regardless of whether rIL-2 was present or absent (Figure 14). Taken together, this data suggests that 4.1-thymocytes can recognize I-A^{g7PD} expressed on thymic epithelial cells, but neither I-A^d nor I-E^d is seen.

I Only I-Aβ^{g7} is able to present the target autoantigen to T cells

As shown above, moderate protection from diabetes is observed in I-A^{g7PD}-transgenic-4.1-NOD mice despite the absence of 4.1-thymocyte deletion. The only difference between the I-A^{g7} and I-A^{g7PD} molecules is the mutated amino acids at positions 56 and 57 of the β chain. Since I-A^{g7PD} has anti-diabetogenic properties, it was of interest to determine whether this change affected the ability of this MHC class II molecule to present autoantigen to T cells bearing the 4.1-TCR, thus affecting proliferation. To ascertain whether or not this molecule was capable of presenting the target autoantigen, splenic $CD4^+$ T cells from 4.1-NOD mice were cultured with islet cells from either NOD, Balb/c, or Balb/c.I-A^{g7PD} mice. Islet cells from these mice included the beta cell autoantigen and professional APCs which express I-A^{g7} (NOD), I-A^d and I-E^d (Balb/c), I-

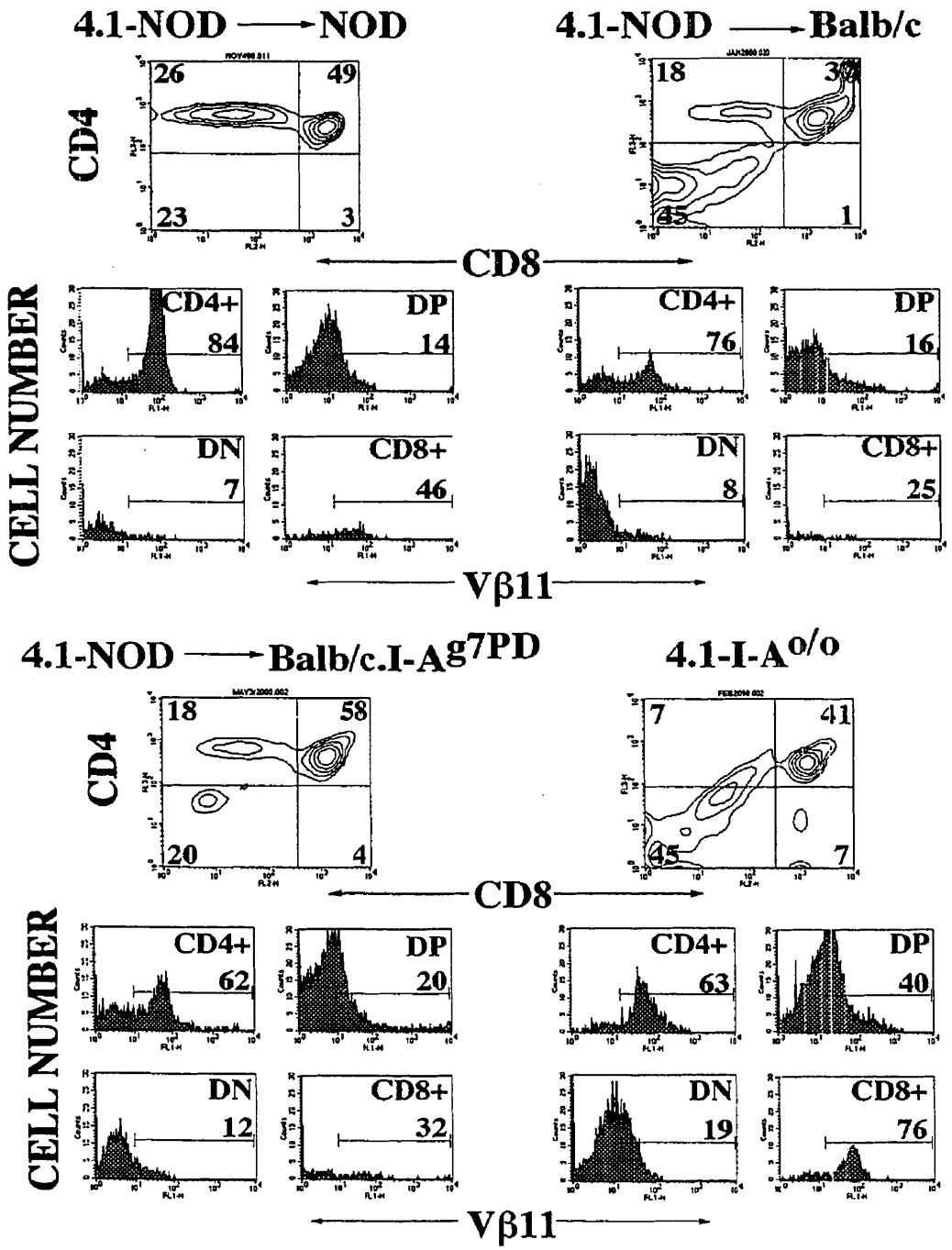


Figure 13: Bone marrow chimeras demonstrating that I-A^{g7PD} is capable of positively selecting 4.1-thymocytes in the thymic cortex. Bone marrow cells (6-10x10^6) from 4.1-NOD mice were injected into the tail veins of lethally irradiated recipient mice: NOD, Balb/c, or Balb/c.I-A^{g7PD}. Thymi of chimeric mice were analyzed by flow cytometry 7-8 weeks after transplantation. See the legend of figure 3 for details. The profile of MHC class II deficient mice (4.1.I-A$^{o/o}$) was compared to the thymocyte profiles of the chimeras. Data shown are average values of 6-7 chimeras/group.

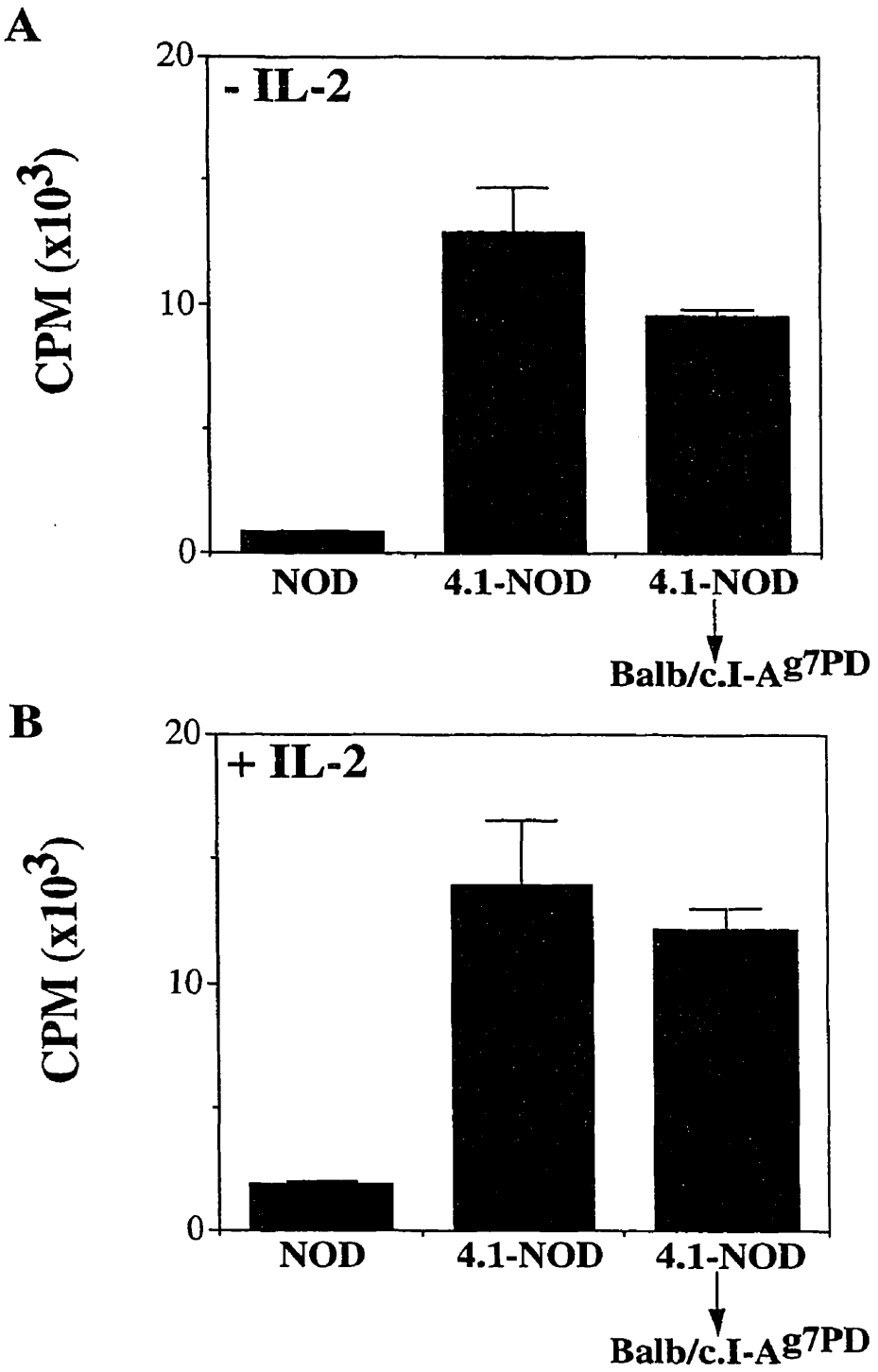


Figure 14: Proliferation of splenic CD4$^+$ T cells from 4.1-NOD->Balb/c.I-A^{g7PD} bone marrow chimeras *in vitro*. Proliferation of 4.1-NOD->Balb/c.I-A^{g7PD} bone marrow chimeras in response to NOD islet cells in the absence (A) or presence (B) of rIL-2. See the legend for figure 5 for details. Bars show the standard error of the means.

A^d, I-E^d, and I-A^{g7PD} (Balb/c.I-A^{g7PD}) on the cell surface. Proliferation occurred in response to NOD islets but not in response to islets from either Balb/c or Balb/c.I-A^{g7PD} mice (Figure 15). These results show that I-A^d, I-E^d, and I-A^{g7PD} were incapable of presenting the target autoantigen to the 4.1-TCR, since there was no proliferation. In contrast, the 4.1-TCR was capable of recognizing antigen in the context of I-A^{g7} because NOD islets were able to generate a proliferative response.

J Macrophages and Dendritic Cells, but not B cells, are able to present the target autoantigen

As shown above, protective MHC class II molecules induce tolerance of 4.1-thymocytes via bone-marrow derived APCs located in the thymic medulla. In order to determine the nature of the tolerogenic APC population, the ability of thymic and splenic dendritic cells, splenic B cells, and peritoneal exudate macrophages to downregulate the CD4 and CD8 co-receptors was compared. This process is termed "dulling" (90) when the interaction between 4.1-$CD4^{hi}CD8^{hi}$ thymocytes and the different APCs may or may not result in the occurrence of co-receptor downregulation. H-2^{b+} mouse strains were used because I-A^b triggers the strongest tolerogenic response of the 4.1-TCR: complete deletion occurred in 100% of the mice. Only dendritic cells from C57BL/6 mice (I-A^b) triggered dulling of $CD4^+CD8^+$ thymocytes from RAG-2-deficient 4.1-NOD mice, which express only the 4.1-TCR. Dendritic cells from NOD (I-A^{g7}), I-$A\beta^b$-deficient C57BL/6 (I-$A^{-/-}$), and C57BL/10.H-2^{g7} (I-A^{g7}) were incapable of dulling the double positive thymocytes from 4.1-$RAG^{-/-}$NOD mice (Figure 16a-b). To determine whether or not this dulling process was peptide-specific, dendritic cells from H-2Ma-deficient C57BL/6 were also tested. The peptide repertoire presented by the professional APCs of H-2Ma-deficient C57BL/6 mice is greatly restricted since most of the I-A^b molecules present CLIP (91); also, these mice express normal levels of I-A^b on the cell surface (Figure 16d). Dendritic

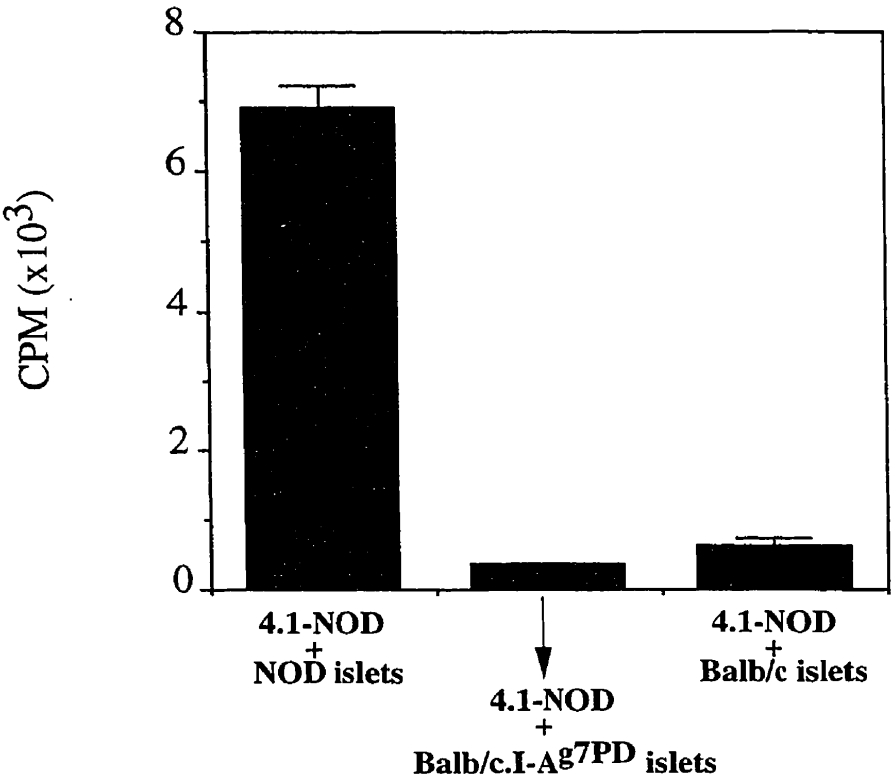


Figure 15: Proliferation, *in vitro*, of splenic CD4$^+$ T cells from 4.1-NOD in response to NOD, Balb/c.I-A^{g7PD}, or Balb/c islet cells. See the legend for figure 5 for details. Bars show the standard error of the means.

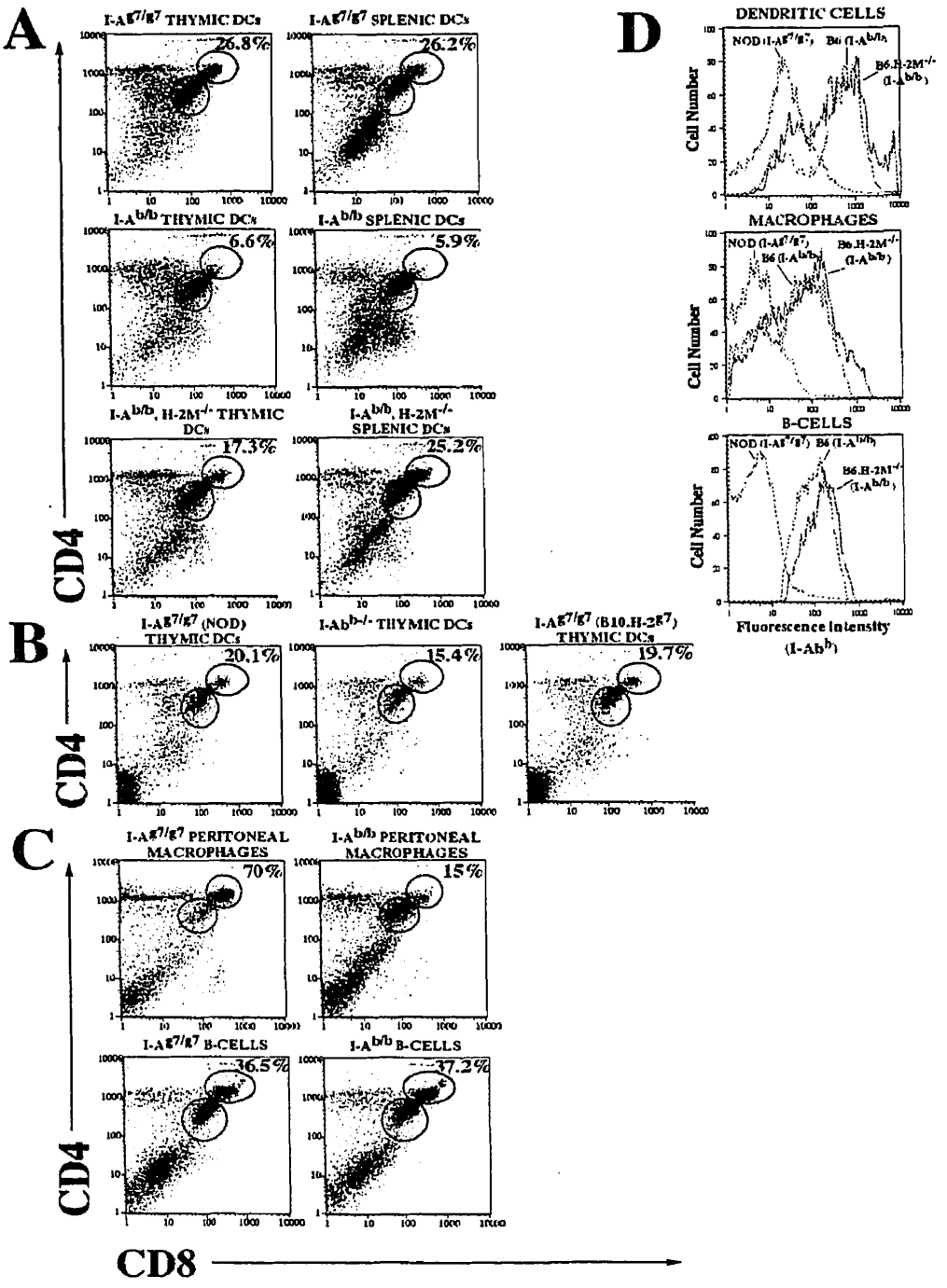


Figure 16: Dulling affect of different APCs on 4.1-CD4$^+$CD8$^+$ thymocytes from RAG-2-deficient 4.1-NOD mice. A-C Dulling Assays. These assays involved co-culturing 10^5 APCs with 10^5 thymocytes from RAG-2-deficient 4.1-NOD mice for 20 hours. These cells were stained with anti-CD4-PE, anti-CD8-FITC, and anti-CD11c-, anti-CD11b-, or anti-B220-biotin/streptavidin-PerCP mAbs. A,B, and C correspond to different experiments. Figure shows ungated results, however, similar results were obtained when CD11c$^+$, CD11b$^+$, and B220$^+$ cells were electronically gated. Values shown represent the percentage of CD4^{hi}CD8^{hi} thymocytes within the DP population. Figure represents 1 of 3 experiments. Panel D I-A^b levels on thymic dendritic cells from NOD, C57BL/6, and H-2M$^{-/-}$C57BL/6 mice. Cells were stained with anti-I-Aβ^b-biotin (AF6-120.1) plus streptavidin-PerCP.

cells from H-2Ma-deficient mice perform similarly to those from NOD, I-Aβ^b-deficient C57BL/6, and C57BL/10.H-2^{g7} mice; they could not downregulate CD4 and CD8 from double positive 4.1-thymocytes (Figure 16a). Therefore, 4.1-thymocytes recognize I-A^b molecules expressed on dendritic cells in a peptide-specific manner.

Further experiments performed using peritoneal macrophages and splenic B cells suggested that not all professional APCs from C57BL/6 mice were capable of downregulating CD4 and CD8 from 4.1-CD4$^+$CD8$^+$ thymocytes. Macrophages from the peritoneum, but not the splenic B cells, had the ability to dull double positive thymocytes (Figure 16c) even though I-A^b was expressed at similar levels on the cell surface of both APCs (Figure 16c). These results imply that the deletion of 4.1-thymocytes is triggered by the recognition of a tolerogenic I-A^b/peptide complex on dendritic cells and macrophages, but not on B cells.

V Discussion

A Summary

The focus of this thesis was to answer the question of whether or not diabetes protection afforded by structurally different MHC class II molecules occurs via a common mechanism. The idea of a general mechanism was tested by studying the ability of three different protective MHC class II molecules (I-Eα^k, I-A^d, and I-A^{g7PD}) to tolerize 4.1-CD4$^+$ T cells. The results *in vitro* and *in vivo* support the hypothesis that there is one mechanism of resistance which involves central tolerance triggered by bone marrow derived APCs, either by deletion of autoreactive T cells or by rendering them anergic.

The first protective MHC class II molecule studied was I-E. The presence of the I-Eα^k transgene restored the expression of the I-E molecule in the NOD mouse which is normally incapable of I-E expression. I-E expression in 4.1-NOD.I-Eα^k mice resulted in

two different phenotypes characterized by deletion in one-third of the population and anergy in the other two-thirds. Since deletion occurred in only 33% of the population, this suggested that deletion happens with incomplete penetrance; however, when deletion did occur it was complete. Complete deletion means that the majority of the autoreactive $CD4^+$ T cells were deleted in the thymus during negative selection. Why deletion occurs with incomplete penetrance has yet to be determined. This phenomenon may be due to the number of cells which express I-E; deleter 4.1-NOD.I-Eα^k mice tended to have a greater percentage of I-E^+ cells. The presence of a greater number of cells expressing I-E may trigger negative selection more efficiently. If there is a certain threshold needed for deletion to occur then a larger number of cells expressing I-E may surpass the required threshold. It is less likely that this difference is due to the number of I-E molecules expressed per cell, as there was not a significant difference in the mean fluorescence intensities between nondeleter and deleter mice. The presence of two different phenotypes mirrored what was observed in the 4.1-H-$2g^{7/k}$ mice, showing that the protection afforded by the I-Eα^k transgene is not an artifact of transgenesis. Studying the FACS profiles of the 4.1-H-$2g^{7/k}$ mice also showed that deletion triggered in these mice is due to the I-E^k molecule and not I-A^k as there was no deletion observed in 4.1-NOD.I-A^k mice. As expected, thymocyte deletion resulted in a failure to respond to antigenic stimulation. Unexpectedly, the absence of deletion in 4.1-NOD.I-Eα^k mice also resulted in unresponsiveness when splenocytes from nondeleters were challenged with NOD islets. Surprisingly, this absence of deletion resulted in a number of autoreactive $CD4^+$ T cells in the periphery, which are incapable of causing beta cell destruction. However, when the same cells were cultured in the presence of IL-2, the proliferative ability was restored. This suggested that *in vitro* the splenocytes were not deleted but rendered anergic; this is another mechanism of tolerance.

The I-E molecule proved to be protective against diabetes in both deleter and nondeleter 4.1-NOD.I-Eα^k mice. These results corresponded to what has been previously

recorded in the literature about the protective nature of the I-E molecule in the NOD background (79, 83, 84). Diabetes resistance was expected for deleter 4.1-NOD.I-Eα^k mice, as was the protection from insulitis, since the autoreactive T cells are eliminated in these mice during T cell development. Surprisingly, even though I-E protected nondeleter 4.1-NOD.I-Eα^k mice from diabetes development, moderate insulitis still occurred. Even though insulitis occurs in the nondeleting population, these results do not conflict with previous literature in that there have been reports of the presence (92) and absence (79, 83, 84) of insulitis in NOD mice with an Eα transgene. Therefore, the anergy observed *in vitro* does not occur *in vivo*, as there is islet infiltration by lymphocytes which are actively proliferating (as shown by BrdU staining). Compared to 4.1-NOD mice (where there is diabetes susceptibility), islet-associated T cells from nondeleting 4.1-NOD.I-Eα^k mice produced similar levels of the pro-inflammatory cytokines IL-2 and IFNγ without an increase in IL-4; therefore, protection from diabetes was not caused by the induction of Th2 regulatory cells. IL-4 often reflects the presence of a Th2-type response where the immune response is directed away from cell-mediated immunity. An increase in the amount of IL-4 produced by the islet-associated T cells would reflect a protective Th2 response. Since this increase in IL-4 is not observed in nondeleting 4.1-NOD.I-Eα^k mice and the amounts of pro-inflammatory cytokines are similar to those seen in 4.1-NOD mice, then the protection from diabetes observed in these animals is unlikely due to a protective Th2 response. Therefore, the stimulus received by the T cells in nondeleting 4.1-NOD.I-Eα^k mice is not strong enough to prevent the proliferation of lymphocytes *in vivo*. This is seen in the presence of moderate insulitis and the detection of proliferating cells with anti-BrdU staining. The stimulus received *in vivo* may be stronger than that received *in vitro,* which would explain why there is anergy only *in vitro*. Another possibility is that some autoreactive T cells with a lower avidity/affinity for MHC/peptide exit the thymus; these T cells may cause insulitis but not diabetes because there would not be enough highly

diabetogenic T cells in the periphery to trigger the disease. It was shown with a certain, highly pathogenic population of $CD8^+$ T cells that avidity maturation occured where there was selective expansion of high avidity T cells as the disease progressed (23). When NOD mice were injected with an antigenic peptide, deletion of the most pathogenic $CD8^+$ T cells occurred, sparing lower avidity (less pathogenic) cells which were unable to cause diabetes (23). It is possible that this also occurs in $CD4^+$ T cells where the most pathogenic cells are deleted in the thymus and the cells which do escape into the periphery are less pathogenic. These less pathogenic T cells can still infiltrate the islets and proliferate but may not be capable of destroying the beta cells because the TCR has a lower avidity/affinity for MHC/peptide complexes.

Similar tolerogenic effects were observed between $I\text{-}A^b$ and I-E, which have similar structures but different amino acid sequences. Since these two different molecules are both protective, then other MHC class II molecules such as $I\text{-}A^d$ may be capable of having a tolerogenic affect. It was found that $I\text{-}A^d$ demonstrated a phenotype consistent with deletion, as was seen with I-E. There were slight differences observed between 4.1-NOD.I-A^d mice and deleting 4.1-NOD.I-$E\alpha^k$ mice; this entailed a small increase in the percentage of $V\beta11^+CD4^+$ T cells, an increase in insulitis, and an increase in the incidence of diabetes in 4.1-NOD.I-A^d mice when compared to deleting 4.1-NOD.I-$E\alpha^k$ mice. These differences between the two transgenic populations may be due to differences in expression, either in the levels or timing, as they are different MHC class II molecules. The expression patterns may be different between I-A and I-E class II molecules. Depending on when these MHC class II molecules are expressed during T cell development or the level at which they are expressed can affect T cell development. If the MHC class II molecule is expressed late in T cell development, negative selection may not occur as efficiently because the molecule is not expressed in time to trigger deletion. Consistent with the deletion phenotype of 4.1-NOD.I-$E\alpha^k$, $CD4^+$ T cells from 4.1-NOD.I-A^d mice

did not respond to antigenic stimulation, even in the presence of rIL-2; this shows that the majority of the autoreactive T cells were eliminated. However, a few animals developed the disease which suggests that some T cells escaped into the periphery and were capable of infiltrating the islets, even though 4.1-NOD.I-A^d mice were mostly protected from disease. Occasionally these infiltrating cells were capable of causing the progression from insulitis to diabetes. Since only a few mice developed diabetes, there may be a threshold which needs to be reached for disease to occur. 4.1-NOD.I-A^d mice did show a moderate degree of insulitis, which only occasionally progressed to disease. These results reflected the degree of protection against diabetes observed in I-A^d-transgenic NOD mice where insulitis still occurs but the diabetes incidence is reduced with a delayed onset (82). This protection is similar to that seen in 4.1-NOD.I-Eα^k mice in that regulatory Th2 cells do not appear to play a role. Islet-associated T cells from 4.1-NOD.I-A^d mice produced similar levels of IL-2, IFNγ, and IL-4 compared to those from 4.1-NOD mice. If regulatory cells were involved as a major mechanism for diabetes resistance, one would expect an increase in the level of IL-4, as it is a Th2 cytokine.

To understand the importance of proline and aspartic acid at positions 56 and 57 of the β-chain to the diabetes protection of certain MHC class II molecules, 4.1-NOD.I-A^{g7PD} mice were studied. 4.1-NOD.I-A^{g7PD} mice showed a decreased incidence of diabetes and insulitis compared to 4.1-NOD mice; however, deletion was not observed in these mice, and $CD4^+$ T cells from these animals were able to respond to islet stimulation. Even though the incidence of diabetes and insulitis were decreased in these mice, there was a moderate degree of insulitis and a small percentage of mice became diabetic. Once again these results follow those previously reported in that NOD mice expressing an I-A^{g7PD} transgene exhibited insulitis and diabetes development occurred in a small number of the animals (86, 87). These results suggested that the presence of proline and aspartic acid at positions 56 and 57 of the β-chain is important for diabetes resistance but is not sufficient

for complete tolerance to occur.

We determined that in specific circumstances, the I-A^{g7PD} molecule had a similar function to that of I-A^{g7}. It is capable of positively selecting 4.1-NOD thymocytes even though 4.1-NOD peripheral T cells are incapable of responding to antigen in the context of I-A^{g7PD}. This property was previously shown with another MHC class II-restricted, beta cell reactive transgenic TCR (BDC2.5) where I-A^{g7PD} could positively select thymocytes but could not present antigen to T cells in the periphery (86). Protection is afforded by the presence of proline and aspartic acid instead of histidine and serine at positions 56 and 57 of the beta chain. This change in the sequence causes the molecule to act inefficiently. Therefore, the protection against diabetes is not due to I-A^{g7PD} interfering with the positive selection of, or by triggering the deletion of autoreactive T cells. Rather, the I-A^{g7PD} molecule is altered enough that it is weakly tolerogenic and is incapable of acting as I-A^{g7} does in immune responses.

The 4.1-TCR is capable of interacting with many structurally different MHC class II molecules. This promiscuity results from the ability of the 4.1-TCR to see tolerogenic MHC/peptide complexes which are presented by dendritic cells and macrophages but not B cells or thymic epithelial cells. When deleting molecules are expressed on thymic epithelial cells there is neither positive nor negative selection of 4.1-thymocytes. The exception to this is I-A^{g7PD}, which triggers weak positive selection of 4.1-thymocytes when it is expressed on thymic epithelial cells. The 4.1-TCR promiscuity is peptide dependent, since H2Ma-deficient thymic and splenic dendritic cells cannot trigger the dulling of double positive thymocytes. H2Ma-deficient mice express, almost exclusively, I-A^{b} molecules bound to the class II-associated invariant chain peptide (CLIP), thus preventing other peptides from binding to I-A^{b} molecules. Whether the 4.1-TCR recognizes one tolerogenic peptide in the context of MHC class II molecules or many is not yet known.

There was no sequence homology in the alpha chains of the different MHC class II

molecules, but homology was observed between positions 55-67 of the beta chain. Interestingly, a commonality was found in the sequences of those MHC class II molecules shown to exhibit a protective effect against diabetes. A common motif was observed in the beta chain of protective MHC class II molecules which was not present in weakly tolerogenic or non-tolerogenic MHC class II molecules (Table IV). This motif consists of proline and aspartic acid at positions 56 and 57, a bulky hydrophobic residue at position 61, and insertions at positions 65 and 67. Proline and aspartic acid alone are not sufficient for deletion to occur, even though the presence of these two residues has an anti-diabetogenic affect. These two residues are capable of moderately protecting 4.1-NOD mice from disease but are incapable of providing complete resistance. All of the components of the motif seen in protective molecules must combine and work together to have a tolerogenic effect. Other factors may also affect the tolerogenic ability to varying degrees; such as, interactions with other beta chain residues, interactions with the corresponding alpha chains, or the timing and levels of MHC expression. Aspartic acid at position 57 of the beta chain has been linked to diabetes resistance in both humans and mice. However, there is no substantial evidence that aspartic acid alone is responsible for resistance, and as shown here aspartic acid and proline together are insufficient for complete protection. It does appear that aspartic acid and proline are not sufficient for complete resistance, since I-A^k and I-A^{g7PD} both contain these residues at positions 56 and 57 of the beta chain, but both molecules are only weakly tolerogenic and no massive deletion of 4.1-$CD4^+$ T cells was observed. Differences in the affinity/avidity with which peptide/I-A^{g7PD} complexes interact with the 4.1-TCR may approach, but not reach, the threshold required for deletion to occur. Other residues seen in highly tolerogenic molecules (such as the highly hydrophobic residue at position 61 and the insertions at positions 65 and 67) may need to be present in order to change the affinity/avidity of the interaction, and thus trigger deletion.

It seems plausible that this motif, which is only present in protective MHC class II molecules, affects the conformation of the peptide binding cleft of the MHC class II molecule, thus affecting the repertoire of T cells selected in the thymus during T cell development. Along with positions 56 and 57, the other residues within this region (such as 61) interact to change the shape of the P9 pocket structure (38, 93-96). The amino acids present in protective MHC class II molecules affect the conformation of the P9 pocket, such that it is deeper and less accessible to certain peptides. On the other hand, molecules such as the I-A^{g7} molecule have a shallow and easily accessible P9 pocket. Therefore, the differences between the conformations of the P9 pocket may lead to differences in the peptide repertoire selected, which in turn affects the specificity of the T cells exiting the thymus. The I-A^{g7} molecule is capable of binding a greater number of peptides than other protective MHC class II molecules. The I-A^{g7} MHC class II molecule has a more open peptide binding groove due to its unique interactions, making it more promiscuous during negative selection; this leads to poor central tolerance. This allows autoreactive T cells to undergo positive selection and then exit the thymus, which increases their number in the periphery. The shape change that occurs in the peptide binding groove with the protective motif may aid in making the negative selection of autoreactive T cells more stringent, and so the T cells are deleted in the thymus or rendered anergic. The ability of protective MHC class II molecules, but not I-A^{g7}, to present tolerogenic peptides may result from these structural differences. Molecules such as I-A^{k} and I-A^{g7PD} may be weakly tolerogenic because they share some protective amino acid residues but since they do not have the full motif tolerogenic peptides may be presented inefficiently. This may explain why the presence of proline and aspartic acid alone are not sufficient for deletion to occur.

These results taken together point towards a common mechanism for the MHC-linked protection from diabetes involving central tolerance. This mechanism would involve MHC-associated resistance of non-TCR-transgenic mice to diabetes resulting from the

induction of tolerance of certain highly diabetogenic thymocytes. This ability to trigger central tolerance is rooted in the molecular structure of the β-chain of the MHC class II molecule. Therefore, multiple, structurally diverse MHC class II molecules would provide resistance to diabetes via a single mechanism as opposed to multiple MHC class II molecules operating via multiple mechanisms. A better understanding of the mechanism behind diabetes protection will enable a better understanding of the disease itself and may provide new avenues for therapeutic intervention.

B Importance of this Study

The main importance of this study is that it provides a better understanding of the mechanisms involved in the diabetes disease process. In order to properly study the mechanisms, a disease-relevant system, such as the one used in this study, must be used to obtain knowledge about how the disease develops. The use of transgenic TCR and MHC class II molecules provides a relevant and focused study of how the MHC interacts with the TCR in order to provide resistance or susceptibility to disease. Once the mechanisms of the disease are known then methods can be developed that will block disease occurrence. A better understanding about how the disease develops and progresses, and the mechanisms involved, will provide a better chance for developing more effective treatment, or perhaps even a cure.

C Future Considerations

The next step to be taken regarding the results presented here is to investigate which residues of the beta chain are required for the complete tolerance of these autoreactive $CD4^+$ T cells that are 4.1-like. To do this, two of the MHC class II molecules will be mutated from their original residues to be more like the other. The I-A^b molecule is the most tolerogenic and the I-A^{g7} molecule is the most diabetogenic. Mutating the I-A^{g7}

molecule towards the sequence of I-A^b and vice versa will show which residues are necessary for protection since it appears that proline and aspartic acid at positions 56 and 57 are not sufficient. These mutated MHC class II molecules would then be introduced into the 4.1-NOD background as transgenes to see their effect on T cell development and diabetes development. This would contribute to further understanding the molecular basis behind the mechanism for diabetes resistance.

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APPENDIX A: MEDIA and BUFFERS

Hanks Balanced Salt Solution (HBSS)
10L ddH_2O
1 package HBSS powder (Gibco catalogue #61200-093)
3.5g $NaHCO_3$
23.83g Hepes
pH 7.0 (pH rises 0.1-0.3 when filter sterilized)

RPMI 1640
10L ddH_2O
1 package RPMI 1640 powder (Gibco catalogue #31800-089)
20g $NaHCO_3$
23.83g Hepes
pH 7.0 (pH rises 0.1-0.3 when filter sterilized)

Complete Media
450mL RPMI 1640
5mL sodium pyruvate
5mL L-glutamine
5mL penicillin/streptomycin
50mL heat-inactivated fetal calf serum
500μL β-2-mecaptoethanol

Phosphate Buffered Saline (PBS)
in 3.2L ddH_2O add: 32g NaCl
0.8g KCl
5.76g Na_2HPO_4
0.96g KH_2PO_4
bring volume up to 4L with ddH_2O
pH to 7.4

Fluorescence Activated Cell Sorting (FACS) Buffer
500mL PBS
1% heat-inactivated fetal calf serum (5mL)
0.1% NaN_3 (0.5g)

Hemolysis Buffer (ammonium chloride solution)

Combine	4.12g Tris
	200mL ddH_2O
Combine	14.94g NH_4Cl
	1800mL ddH_2O

add the two solutions together
pH buffer to 7.2