THE UNIVERSITY OF CALGARY

Influence of MHC Class II Alleles on Autoimmune Susceptibility and Resistance

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY AND INFECTIOUS DISEASES

CALGARY, ALBERTA

JUNE, 1998

ABSTRACT

This thesis describes the developmental biology of a pancreatic beta cell-reactive, I-A^{g7}-restricted, transgenic T cell receptor (TCR) in nonobese diabetic (NOD) mice and in NOD mice bearing anti-diabetogenic MHC class II genes. These studies show that while this TCR is highly diabetogenic in NOD mice, it undergoes negative selection in diabetes-resistant mice by engaging multiple anti-diabetogenic MHC class II molecules exclusively on bone marrow-derived cells of the thymic medulla. These results provide an explanation as to how protective MHC class II genes on one haplotype can override the genetic susceptibility to autoimmune diseases provided by MHC class II genes on a second haplotype. They also demonstrate that an autoreactive TCR can undergo sequential positive and negative selection on different MHC molecules, and that these two events need not be restricted by the same MHC molecules.

ACKNOWLEDGMENTS

There are a number of important role models, peers, and friends that I would like to thank for their support, understanding, words of advice, criticism, and friendship. First of all, I would like to thank Dr. Pere Santamaria for his time, commitment, guidance, and understanding as my graduate supervisor. I would like to express words of gratitude to the postdocs Drs. Joan Verdaguer and Aziz Amrani for their enormous amount of help as well as their interesting stories of life in their home countries. Words of thanks also go out to Sonny Bou and Nuzhat Averill for their contribution to my work as technicians in our lab. My fellow students and friends Shawn Lewenza and Brad Anderson I am also grateful to for reasons too many to mention. Special thanks to Laurie Robertson for all her help with the flow cytometry in this thesis as well as the many sessions of friendly chat, discussion, and words of advice. I would also like to thank Judi Patterson for making the time to help me with the various paper work that I always seem to need taken care of. To the many friends that I've known and met during my life in Calgary, you know who you are and I don't think I need mention how important you've been (Guri, check the DP with GCs. ITA?). Last of all, I would like to thank my family of aunts, uncles and cousins who've accepted me for who I am even though they may have no idea what it is I do. Oh yeah, I forgot my sister Kyla and my little bro Snowball too.

DEDICATION

I would like to dedicate this thesis to my parents, the ones who've made me who I am today. They have been such an important influence and source of guidance and support that there is no way I can ever thank them. I hope that one day I can be the same parent that they are.

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LIST OF SYMBOLS, ABBREVIATIONS, AND NOMENCLATURE

APC = antigen presenting cell

β2m = beta-2 microglobulin

CD4/CD8 = TCR co-receptors

DNA = deoxyribonucleic acid

D/K/L = murine MHC class I loci

DR/DQ = human MHC molecules

FACs = fluorescence-activated cell sorter

Fc = non-Fab part of Ig

FITC = fluorescein isothiocyanate

H-2 = murine major histocompatibility complex

HLA = human leukocyte antigen (human major histocompatibility complex

I-A/I-E = murine MHC class II loci

Idd = insulin dependent diabetes gene

IDDM = insulin dependent diabetes mellitus

IFN-γ = interferon- γ

Ig = immunoglobulin

IL-2/IL-4 = interleukin-2/interleukin-4

K14 = keratin 14 promoter

LCM = lymphocyte complete media

mAb = monoclonal antibody

MHC = major histocompatibility complex

mRNA = messenger ribonucleic acid

NOD = nonobese diabetic mouse

PBS = phosphate buffer saline

PE = phycoerythrin

PCR = polymerase chain reaction

sAg = superantigen

scid = severe combined immunodeficiency

TH1/2 = T helper cell (subset 1 or 2)

TCR = T cell receptor

vSAg = viral superantigen

EPIGRAPH

Open up your mind

and go your own way

-taken from "Breed Apart" by

Sepultura (Roadblock Music Inc.)

CHAPTER ONE

INTRODUCTION (1.0)

AUTOIMMUNITY (1.1).

The roots and definition of the word "immunity" have been widely used to begin many popular immunology texts (1, 2). Here it will be used once again to reiterate the meaning of the word and to illustrate what relevance this meaning has to the following thesis. As promised, the word immunity has it's roots in the Latin word "immunitas" meaning "freedom from" which in ancient times was a privilege given to Roman senators and was a freedom from legal prosecution or duties of office. In more modern times, this word was used to describe one's protection from disease. This "freedom from" or protection from disease is a simple definition used to explain the complex entity that is our immune system.

One feature of this entity that is often taken for granted is the protection we have from our own immune system. Discriminating and eliminating foreign antigen while not affecting or harming one's own body is an essential but poorly understood feature of the immune system. When this process of self-tolerance, or immune unresponsiveness towards self goes awry, autoimmune disease is often the result (2). Autoimmunity, or immune attack against self, can take many forms involving single or multiple components of the immune system and can affect single organs or can be wide spread (1, 2). The factors that precipitate either susceptibility or resistance to autoimmune disease are also multifaceted and include combinations of genetic, environmental, and dietary factors, as well as viral and/or bacterial infections (1, 2). The mechanisms of how these different factors either predispose or prevent autoimmunity are

generally unknown, however, intense amounts of effort are being taken to understand these processes.

As previously mentioned, autoimmune diseases can take many forms which can target different parts of the body. One particular branch of the "autoimmune tree" are those diseases that are primarily T cell-mediated. In other words, T cells are the primary effectors of autoimmune destruction. Examples of this type of autoimmune disease include rheumatoid arthritis, multiple sclerosis, and insulin-dependent diabetes mellitus among others (1, 2).

INSULIN-DEPENDENT DIABETES MELLITUS (IDDM) (1.2).

Immunopathogenesis (1.2.1).

Insulin-dependent diabetes mellitus (IDDM), or Type-1 diabetes, results from the selective destruction of the insulin-producing pancreatic beta cells by a T lymphocyte-dependent autoimmune process in genetically susceptible individuals (3). It is an autoimmune disease affecting some 20 million people worldwide, many of whom are children or young adults, who cannot survive without daily exogenous insulin replacement (4).

Research into the immunopathogenesis of IDDM has clearly shown that it is a cell-mediated autoimmune process which invariably leads to beta cell destruction (3, 6). T lymphocytes are abundant among those cells infiltrating the islets at diabetes onset with disease recurring in those patients treated with pancreatic isografts or human leukocyte antigen (HLA)-identical allografts (7-10). Most of the cells that infiltrate the pancreatic islets of the non-obese diabetic (NOD) mouse, the predominant animal model of IDDM, are also T lymphocytes (11). In these genetically susceptible animals, IDDM can be prevented with anti-T cell reagents, does not

occur in athymic- or severe combined immunodeficient (*scid)*-NOD mice, and can be adoptively transferred to immunodeficient or young NOD mice by T cells from diabetic animals (12, 13, 14).

Unfortunately, the initial events leading to the autoimmune destruction of pancreatic beta cells are unknown, however epidemiological studies in humans have suggested that environmental, viral, and dietary factors may play an important role (1, 2, 6). What is clear, however, is that the most critical determinant of susceptibility (and resistance) to IDDM is genetic (3, 5, 6).

Genetics of IDDM (1.2.2).

MHC-Linked Susceptibility and Resistance (1.2.2.1). Both genetic susceptibility and resistance to IDDM are associated with genes of the major histocompatibility complex (MHC) on human chromosome 6 or mouse chromosome 17 and, to a lesser extent, with multiple genes on other chromosomes (*idd* genes) (3, 6, 15). Studies of humans and animals have suggested that the MHC-linked susceptibility and resistance to IDDM are inherited as dominant traits with incomplete penetrance and that they are predominantly, but not exclusively, determined by polymorphisms or differences at MHC class II genes (5, 15). In humans, these include alleles carried on the human leukocyte antigen (HLA)-DR3$^+$ and -DR4$^+$ haplotypes, with the most important determinant of either susceptibility or resistance located at the HLA-DQB1 locus (3, 5, 16). Human DQB1 alleles encoding serine, alanine, or valine at amino acid position 57 are associated with susceptibility to IDDM, whereas those encoding aspartic acid at this position are associated with resistance, with varying degrees of dominance (17-20).

The NOD mouse is an inbred strain which spontaneously develops a form of diabetes closely resembling human IDDM (3). This strain is homozygous for a unique MHC haplotype (H-2^{g7}) which encodes a single a MHC class II molecule (I-Aα^{d}/I-Aβ^{g7}), the murine counterpart of human DQβ molecules (19, 20, 21). As in humans, disease susceptibility in the NOD mouse is linked to MHC class II genes. IDDM develops neither in congenic NOD mice expressing anti-diabetogenic MHC haplotypes, nor in NOD mice expressing MHC class II I-E or modified MHC class II transgenes (22-32, 34). These studies have therefore demonstrated that MHC class II molecules encoded on H-2 haplotypes from either NOD or IDDM-resistant mice play a direct role in providing either susceptibility or resistance to IDDM, respectively. Unfortunately, the precise mechanisms by which these molecules afford predisposition or protection are unknown.

Non-MHC-Linked Susceptibility and Resistance (1.2.2.2). As previously stated, IDDM susceptibility and resistance are primarily associated with polymorphisms at MHC class II genes. Genetic risk, however, is also determined by multiple non-MHC-linked genes (*Idd*); 16 of these *Idd* loci have been described so far (5, 15). It appears that development of IDDM requires the presence of certain non-MHC-linked susceptibility alleles (*Idd2-Idd16*) and the appropriate MHC class II genes (*Idd1*). It has also been shown that presence of certain alleles at non-MHC-linked loci can over-ride the genetic susceptibility provided by diabetogenic MHC haplotypes (59). It therefore appears that many of these non-MHC-linked genes are important for susceptibility or resistance to autoimmune diabetes, however, neither the molecular nature nor the mechanism of action of these *Idd* genes are known. For example, in C57BL/6 mice there are at least three anti-diabetogenic *Idd* genes on chromosome 3 (*Idd3*, *Idd10*, and *Idd17*). Within

this chromosome and in the vicinity of these *Idd* genes, are the genes that encode the T cell growth factor IL-2 (*IL2*) and the high affinity Fc receptor for IgG (*Fcgr1*) (60). Studies of subcongenic strains of mice have shown that *Fcgr1* is not the identity of one of these genes and have yet to confirm or rule out whether *IL2* is.

T-LYMPHOCYTE DEVELOPMENT AND THE MAJOR HISTOCOMPATIBILITY COMPLEX (1.3)

The Murine MHC (1.3.1).

There exists in the human and murine genome, a complex of highly polymorphic genes that segregate together upon breeding and which encode protein products that play an important role in the presentation of peptide antigens to cells of the immune system (1, 2). This complex [the major histocompatibility complex (MHC)] is designated HLA (Human Leukocyte Antigen) in humans and H-2 (Histocompatibility-2) in mice (1, 2). The polymorphic nature of the genes within this complex results from the high frequency of variation in the nucleic acid sequences of these genes within populations of individuals. The genes within the MHC complex have various designations with a locus occupying a specific location within the complex, and an allele representing a variant of such locus or gene (2). For example, the MHC complex of the inbred mouse strain C57BL/6 (H-2^b) contains two MHC class II loci (I-Aβ^b and I-Aα^b), encoding the allelic I-A molecule I-A^b (33). It is this inbred nature of most strains of mice that has allowed the study of the identity and function of genes within the MHC complex. As a result, most of the genes encoded within the murine complex have been identified and organized into groups or loci based on structure and function. These include the MHC class I genes (loci K, D, and L),

MHC class II genes (loci I-Aβ and I-Aα, and I-Eβ and I-Eα), complement protein genes, and a variety of others involved in the various aspects of peptide presentation (i.e. processing, loading, etc.) (1, 2).

The combination of alleles at each MHC locus on a single chromosome is defined as a haplotype. For example, the inbred mouse strain C58/J bears two copies of the MHC haplotype given the designation "k" (H-2^k) which carries K^k, D^k, and L^k, and I-Aα^k, I-Aβ^k, I-Eα^k and I-Eβ^k genes (33). This system of classification, or haplotypes, is consistent among the different inbred strains, however, there are exceptions to the rule. Such an exception is the NOD mouse. This inbred strain has a unique MHC haplotype (H-2^{g7}) which only carries two MHC class I genes (K^d and D^b), and two MHC class II genes (I-Aα^d and I-Aβ^{g7}) (5). This unique MHC haplotype is also the primary susceptibility factor for IDDM in the NOD mouse (*Idd1*).

Thymocyte Development (1.3.2).

As stated previously, MHC genes encode proteins, the most prominent of which are expressed as cell-surface receptors (MHC class I and class II) that present short peptide fragments of self and foreign proteins to T lymphocytes and play a pivotal role in the maturation of T cells in the thymus (35, 36). The differences in structure as well as the peptide binding sites on these two classes of MHC molecules determines what peptides are presented and the type of T cells they are presented to (37, 38).

T lymphocytes themselves arise from a common ancestor to all cells in the blood, the bone marrow resident stem cell (39, 40). More specifically, T lymphocytes arise from a common ancestor to B lymphocytes, the lymphoid progenitor. Following departure from the bone

marrow, this progenitor cell enters the thymus to give rise to T cells following maturation within this organ (39, 40, 41).

The thymus, a lymphoid organ present in the upper mediastinum, is compartmentalized into medulla and cortex. These thymic compartments are made up of radiation-sensitive bone marrow-derived dendritic cells and macrophages, and/or radiation-resistant non-lymphoid epithelial cells, respectively (41, 42, 43); the thymic cell types that the lymphoid progenitor will interact with following it's entrance into the thymus. Upon entrance, this pre-T cell will lack expression of a T cell receptor (TCR; the cell surface molecule which allows a T cell to recognize a peptide presented on MHC) and CD4 or CD8 molecules (co-receptors for the TCR that stabilize the interaction of the TCR with MHC/peptide) (39, 40). These TCR^- and $CD4^-$ $CD8^-$, or "double-negative" cells, will first enter into the cortex of the thymus. It is here where TCR expression begins via random rearrangement of TCR genes within the cell. This random rearrangement will in turn produce transcripts that, provided they are functional, will encode the α and β chains of the TCR heterodimer (44). This gene rearrangement leading to protein expression, can produce one of about 10^{18} different possible TCRs that can be expressed on the cell surface, thus giving rise to a heterogeneous population of T cells with different TCR specificities (44).

Along with TCR expression, both CD4 and CD8 co-receptors are expressed on the cell surface of most thymocytes producing "double positive" cells ($CD4^+CD8^+$) (44, 45). These cells then make their way through the inner cortex to the medulla upon engagement of self-peptide/MHC complexes expressed on epithelial cells via their TCRs and associated co-receptors (Fig. 1) (41, 44). It is widely accepted that thymocytes expressing TCRs capable of

engaging self-peptide/MHC complexes on epithelial cells in the cortex with a moderate to intermediate affinity/avidity move on to medulla and are thus "positively-selected" (46-51). Those which subsequently do not engage self-peptide/MHC complexes on bone marrow-derived antigen presenting cells (APCs) in the thymic medulla are thought to leave the thymus and become part of the peripheral T cell repertoire (50, 51, 52). Thymocytes expressing TCRs specific for MHC class II/peptide complexes will lose the CD8 co-receptor while maintaining the CD4 co-receptor ($CD4^+CD8^-$; single positive cell); these cells leave the thymus as mature $CD4^+$ T cells (53). In contrast, those thymocytes bearing TCRs specific MHC class I/peptide complexes will lose the CD4 co-receptor while maintaining the CD8 co-receptor ($CD4^-CD8^+$; single positive cell); these cells leave the thymus as mature $CD8^+$ T cells.

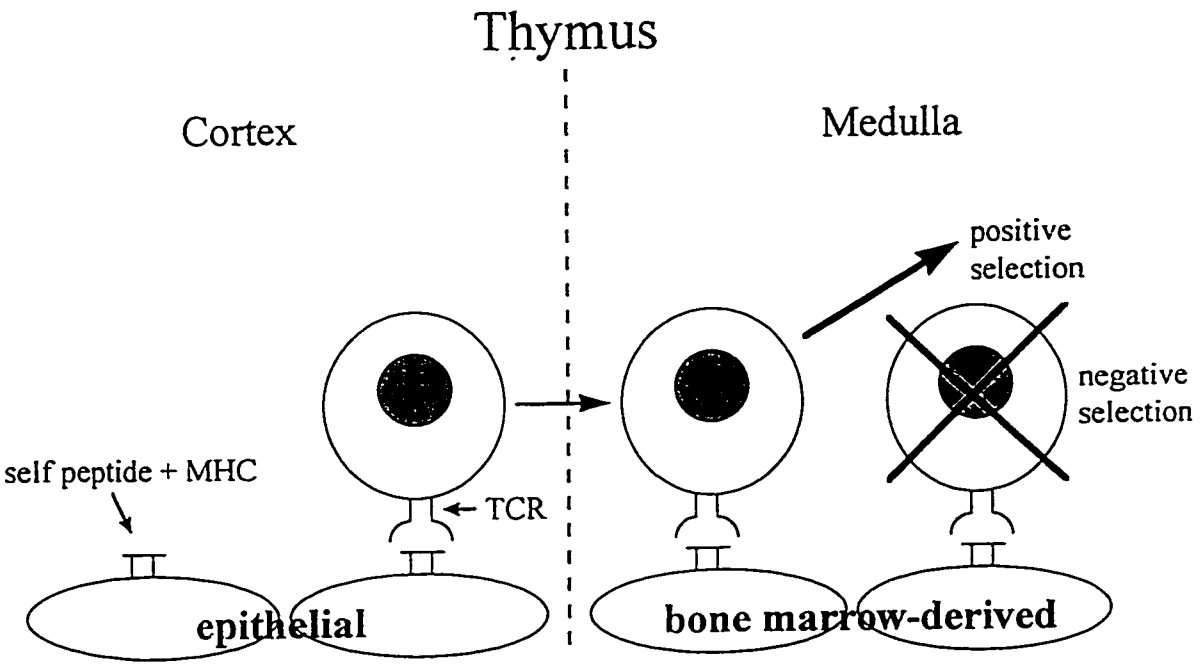

Figure 1. Positive and negative selection of maturing thymocytes. A simple diagram illustrating the selection of T cells within the thymus: those thymocytes expressing T cell receptors (TCRs) that recognize self peptide complexed with self-MHC expressed on epithelial cells within the thymic cortex move on to the thymic medulla, whereas those which do not die of neglect or developmental arrest; those thymocytes which have moved to the thymic medulla and which recognize self peptide/MHC complexes on bone marrow derived cells with a low affinity/avidity leave the thymus and are thus "positively selected", whereas those which recognize self peptide/MHC with a moderate to high affinity/avidity die by apoptosis and are thus "negatively selected". For simplicity, the CD4 or CD8 co-receptors are not shown.

Thymocytes that express TCRs capable of recognizing self-peptide/MHC complexes on medullary APCs with a high affinity/avidity are "negatively selected" and either die via apoptosis, or are rendered anergic (unresponsive to antigenic stimulation) (49-51, 54-57). The result is the removal of those T cells that have the ability to recognize self and thus the potential to cause harm should they be allowed to mature. Of all the lymphoid progenitors with the potential to become T cells, only a fraction (about 1%) actually leave the thymus as mature cells, with most (about 99%) dying (58). The ideal end result is the selection of a mature T cell repertoire that has the ability to recognize foreign antigen (i.e. fight infection) in the context of self-MHC, and to remove those T cells in the thymus with high autoreactive capacity.

CHAPTER TWO

RATIONALE AND OVERALL HYPOTHESIS (2.0)

Both susceptibility and resistance to IDDM are under complex polygenic control, however, the mechanisms through which genes, both MHC and non-MHC-linked, provide either predisposition or protection are unknown. It is well documented that in the NOD mouse (H-2^{g7}) there is a breakdown of T cell tolerance to beta cell antigens (5, 29, 67). It is not known, however, whether genetic resistance to IDDM is the result of T cell tolerance, ignorance of beta cells by autoreactive T cells, or immunoregulation. Studies of experimental animal models expressing transgenic neo-antigens on beta cells and/or transgenic neo-antigen-specific TCRs have produced interesting, but very different results, possibly due to differences in the nature, timing, and/or amount of transgenic neo-antigen expression (63-66). Conclusions based on these results have contributed little to the understanding of the mechanisms underlying the genetic susceptibility and/or resistance to IDDM due to the fact that neither the transgenic antigens, nor the transgenic TCRs employed in these studies are involved in *spontaneous* disease. Thus whether a T cell recognizing a *disease relevant* beta cell autoantigen in NOD mice undergoes tolerance or ignorance in diabetes-resistant mice is unknown.

Studies of unmanipulated animals (mice) do not allow the possibility of addressing this question. The primary reason being that in unmanipulated mice, due to the great heterogeneity of the peripheral T cell repertoire, and to the extremely low frequency of any one antigenic specificity, it is next to impossible to follow the fate of diabetogenic T cell specificities among billions of different TCRs.

To study whether T cells that recognize disease-relevant beta cell autoantigens in NOD mice undergo tolerance in diabetes-resistant backgrounds, Dr. Santamaria's group generated (prior to my arrival) a TCR-transgenic NOD mouse (4.1-NOD) expressing the TCRα and TCRβ chain rearrangements of an I-A^{g7}-restricted, beta cell-reactive, $CD4^+$ T cell clone (NY4.1) that was isolated from the pancreatic islets of an acutely diabetic NOD mouse (73). The uniqueness of this model lies in the fact that the transgenic TCR is specific for a putative non-transgenic beta cell autoantigen presented by the MHC class II molecule that provides IDDM susceptibility in NOD mice (I-A^{g7}).

It has been suggested that the NOD mouse MHC class II molecule I-A^{g7} is inefficient at supporting negative selection of autoreactive TCRs (5, 29, 67). On the basis of this, some authors hypothesized that MHC molecules that provide resistance to IDDM might do so by mediating the negative selection or deletion of autoreactive T cells, or perhaps by rendering them anergic (24, 27, 68). Indeed, diabetes does not develop in congenic and transgenic NOD mice expressing protective I-A- and/or I-E- molecules (27, 29, 34, 69, 70). However, since beta cell-reactive T cells could still be detected in the periphery of these animals, it was proposed that the diabetes resistance provided by protective MHC genes involved the induction of immunoregulatory functions (e.g. generation of suppressor cells) rather than the deletion of autoreactive T cells (26, 31, 34, 62, 69, 70, 71).

This thesis was initiated to test the hypothesis that diabetes-resistant mice bear non-MHC linked genes, other than endogenous superantigens, that are tolerogenic for diabetogenic T cells. Surprisingly, however, we found that diabetogenic T cells undergo dominant negative selection by engaging anti-diabetogenic MHC class II molecules in the thymus of diabetes-resistant H-

$2^{g7/b}$, H-$2^{g7/k}$, H-$2^{g7/q}$, and H-$2^{g7/nbl}$ NOD mice. The studies presented in this thesis focus on this serendipitous discovery. Some of these studied have been published (Schmidt et al., 1997; Verdaguer et al., 1997) or are currently in preparation (Schmidt et al., 1998).

CHAPTER THREE

MATERIALS AND METHODS (3.0)

Generation of TCR Transgenes (3.1).

Sequence analysis of several cDNAs generated by anchor-PCR from the islet-derived $CD4^+$ T cell clone NY4.1 were performed prior to my arrival in the lab. These sequences revealed that the NY4.1 clone transcribed one functional TCRβ chain rearrangement carrying Vβ11 and Jβ2.4 sequences, and one functional TCRα chain rearrangement carrying a novel Vα gene (Vαx4.1) and the Jα33 sequence (this TCR is referred to as the 4.1-TCR). 4.1-TCR V(D)J rearrangements were then subcloned into TCRβ and TCRα shuttle vectors carrying endogenous TCR enhancers and 5'-regulatory sequences. These transgenic constructs (TCRα and/or TCRβ) were then microinjected into fertilized (SJL/J x C57BL/6)F2 eggs and implanted into pseudopregnant female mice and offspring were screened for inheritance of the transgenes by PCR of tail DNA). These transgenes and their production are described in more detail in reference 74.

Mice and Genetic Backgrounds (3.2).

Transgenic founder mice (4.1AN6A3-TCRαβ, 4.1AN6B3-TCRβ and 4.1AN6B7-TCRα) were crossed with the IDDM-susceptible, inbred mouse strain NOD/Lt ($I\text{-}A^{g7}$, $I\text{-}E^-$; The Jackson Laboratory, Bar Harbor, ME) for up to eleven generations to produce TCRαβ-, TCRβ-, and TCRα-transgenic NOD mice, respectively (these mice will be referred to hereafter as 4.1-NOD, 4.1-TCRβ-, and 4.1-TCRα-transgenic NOD mice, respectively; all crosses were performed by Dr. Santamaria). 4.1-TCRα-, 4.1-TCRβ-, or 4.1-NOD mice were crossed with inbred mouse strains SJL/J ($I\text{-}A^s$, $I\text{-}E^-$), C57BL/6 ($I\text{-}A^b$, $I\text{-}E^-$), C58/J ($I\text{-}A^k$, $I\text{-}E^k$), NOD.H-$2^{g7/q}$ ($I\text{-}A^{g7/q}$, $I\text{-}E^-$) or

NOD.H-$2^{g7/nb1}$ (I-$A^{g7/nb1}$, I-E^{nb1}) (The Jackson Laboratory) to generate 4.1-transgenic F1 mice or 4.1-transgenic H-2 heterozygous NOD mice. 4.1-TCRαβ-F1 mice (referred to as 4.1-F1) were backcrossed with NOD, C57BL/6, or C58/J mice to generate H-2^{g7}, H-$2^{g7/b}$, or H-$2^{g7/k}$ animals with 75% NOD genotype, and H-2^{b}, or H-2^{k} animals with 75% C57BL/6 or C58/J genotypes, respectively. 4.1-NOD mice were also crossed with I-Aβ^{b-} C57BL/6 mice (Taconic, Germantown, NY) to generate I-Aβ^{b}-deficient 4.1-F1 mice. Crossing of 4.1-NOD mice with β2 microglobulin mutant (β2m^{-}) NOD/Lt mice (The Jackson Laboratory) produced β2m$^{+/-}$ 4.1-NOD mice which were then crossed with β2m$^{-/-}$ C57BL/6 mice (Taconic) to generate β2m^{-} 4.1-F1 mice. As well, 4.1-NOD mice were crossed with CD8-$\alpha^{-/-}$ C57BL/6 mice (I-A^{b}, I-E^{-}; courtesy of T. Mak, University of Toronto, Toronto, Canada) to generate CD8-$\alpha^{+/-}$ 4.1-F1 mice which were then backcrossed with CD8-$\alpha^{-/-}$ C57BL/6 mice to produce 4.1-H-$2^{g7/b}$ CD8-$\alpha^{-/-}$ mice. I-A^{k}-transgenic 4.1-NOD mice were generated by crossing 4.1-NOD mice with I-Aα^{k}/I-Aβ^{k}-transgenic NOD mice (courtesy of G. Morahan and J.F.A.P. Miller, The Walter and Eliza Hall Institute, Victoria, Australia). NOD/Lt-*scid* mice were purchased from The Jackson Laboratory. 4.1-NOD mice were also bred with I-Aα^{b-}/I-Aβ^{b-}/K14-I-Aβ^{b-} B6 mice (courtesy of L. Glimcher, Harvard School of Public Health, Cambridge, MA) to generate 4.1-I-$A^{g7/o}$, 4.1/K14-I-$A^{g7/o}$, and K14-I-$A^{g7/o}$ mice. Some of these were then intercrossed to generate 4.1-I-$A^{g7/g7}$, 4.1-I-$A^{o/o}$, 4.1/K14-I-$A^{o/o}$, and K14-I-$A^{o/o}$ mice. All mice were screened for inheritance of transgenes, mutations, and MHC haplotypes by PCR of tail DNA (4.1α, 4.1β, β2m, K14-I-Aβ^{b}, I-Aβ^{b-}, I-A^{g7}, I-A^{k}) or by flow cytometry (4.1β, CD8-α, I-Aα^{b}, I-A^{s}, K^{d}, K^{k}) either by myself, or by others in the lab, and were housed in a specific pathogen-free facility in the Animal Resources Center at the University of Calgary.

Lymphocyte Isolation, Flow Cytometry and Antibodies (3.3).

Animals were anesthetized (Metofane, Janssen Pharmaceutica, North York, Ontario) and sacrificed via cervical dislocation prior to removing thymus and spleen from each. Mononuclear cell suspensions of these organs were generated by homogenization using frosted slides. Contaminating erythrocytes were lysed by incubation of cell suspensions in Tris-buffered 0.16M ammonium chloride for 6-8 minutes at room temperature. To isolate cells infiltrating the islets of acutely diabetic animals, pancreata were digested with collagenase (Collagenase Type 4, Worthington Biochemical Corporation, Freehold, NJ) as previously described (75) and isolated islets cultured in lymphocyte complete media [LCM; RPMI 1640 media containing 10% heat-inactivated fetal bovine serum (GIBCO BRL, Gaithersburg, MD), 50 U/ml penicillin, 50 μg/ml streptomycin (Flow Labs, McLean, VA) and 50 μM 2-Mercaptoethanol (Sigma Chemical Co., St. Louis, MO)] containing 0.5 U/mL of human recombinant IL-2 (Takeda, Osaka, Japan) (all collagenase injections performed by other individuals in the lab). $1x10^6$ thymocytes, splenocytes and/or islet-derived T cells were washed once with PBS supplemented with 5% fetal bovine serum and 0.5% sodium azide and incubated at 4°C for 30 min. with one or more of the following mAbs (1μg mAb/10^6 cells): fluorescein isothiocyanate (FITC)-conjugated rat anti-Vβ11 (RR3-15; Pharmingen, Mississauga, Ont.); FITC-conjugated Armenian hamster anti-αβTCR (H57-597; Pharmingen); phycoerythrin (PE)-conjugated rat anti-CD8a (53-6.7; Pharmingen); biotinylated rat anti-CD4 (L3T4; Cedarlane, Hornby, Ont.); biotinylated rat anti-CD5 (53-7.3; Pharmingen); FITC-conjugated rat anti-CD25 (AMT-15; Cedarlane); FITC-conjugated rat anti-CD44 (IM7; Pharmingen); biotinylated rat anti-CD62L (MEL-14; Pharmingen); biotinylated Armenian hamster anti-CD69 (H1.2F3; Pharmingen); FITC-

conjugated mouse anti-H-2K^d (SF1-1.1; Pharmingen); biotinylated mouse anti-I-Aα^b (AF6-120.1; Pharmingen); and purified mouse anti-H-2K^k (11-4.1; Becton-Dickinson, San Jose, CA). Hybridomas secreting the mAbs H57-597 (Armenian hamster anti-TCRβ), 53-6.7 (rat anti-CD8-α), B220 (rat anti-B cells), and M1/70 (rat anti-Mac-1) were all obtained from the American Type Culture Collection (ATCC, Rockville, MD).

Cells were then washed and incubated with streptavidin-PerCP (Becton-Dickinson, San Jose, CA) to reveal biotin-coupled Ab staining, FITC-conjugated goat anti-mouse mAb (Becton-Dickinson) to reveal anti-H-2K^k-coupled Ab staining, or FITC-conjugated goat anti-rat IgG (CALTAG Labs., San Francisco, CA) to reveal rat mAb staining at 4°C for 30 min. Cells were then washed, resuspended in 2% paraformaldehyde in PBS and analyzed on a FACScan flow cytometer (Becton Dickinson) (all cell isolation and flow cytometry was performed by myself).

Proliferation Assays (3.4).

Splenocytes from transgenic and non-transgenic mice were depleted of CD8$^+$ cells via incubation with anti-CD8-α hybridoma (53-6.7) supernatant at 4°C for 30 min. followed by washing with LCM. Cells were then incubated with magnetic beads (Goat anti-rat; Cedarlane, Hornby, Ont.) at 4°C for 30 min. followed by magnetic separation of anti-CD8 labeled cells. Following washing with LCM, efficiency of CD8$^+$ T cell depletion and percentage of CD4$^+$ cells were determined via two-color immunofluorescence staining with PE-conjugated anti-CD8 and FITC-conjugated anti-CD4 mAbs (RM4-5; Pharmingen, Mississauga, Ont.). Single islet cell suspensions were prepared following isolation of islets from non-transgenic NOD mice via collagenase digestion of pancreata and digestion of purified islets with Ca^{2+}- and Mg^{2+}-free PBS containing 0.125 % trypsin- and 3mM EDTA (GIBCO BRL) for 3 min. at 37°C. CD8$^+$-

depleted splenic $CD4^+$ T cells were then plated in triplicate at 2 x 10^4 $CD4^+$ cells/well with γ-irradiated (3,000 rad) NOD islet cells (3-100 x 10^3/well) and unfractionated NOD splenocytes (10^5/well) as sources of antigen and APCs, respectively, in 96-well round bottom tissue culture plates (Falcon, Becton Dickinson) for 3 d at 37°C in 5% CO_2 in LCM. Splenic $CD4^+$ T cells were also purified using the MACs microbead purification system (Miltenyi Biotec, Auburn, CA) and plated as above with γ-irradiated (3,000 rad) NOD, C57BL/6, or I-$A\beta^{b-}$ C57BL/6 islet cells (10^5/well) without exogenous APCs. All cultures were pulsed with 1 μCi of [^{3}H]thymidine during the last 18 h of culture and harvested (PhD Cell Harvester, Cambridge Technology Inc., Cambridge, MA). Incorporated [^{3}H]thymidine was measured by scintillation counting and specific proliferation calculated following subtraction of background proliferation (cpm of cultures containing islet cells with/without APCs and cpm of cultures of T cells alone) from islet cell-induced proliferation (cpm of cultures containing T cells, with/without APCs, and islet cells).

Anti-TCR-induced proliferation was performed by culturing $CD8^+$ T cell-depleted splenocytes in 96-well round-bottom Immulon 1 plates (Dynatech Laboratories Inc., Alexandria, VA) coated with serial dilutions of anti-TCRβ mAb (H57-597). Coating was done by incubating 30μL of a 0.3-10μg/mL solution of the mAb in 50mM Tris-HCl, 150mM NaCl (pH 9.5) coating buffer at 37°C for 90 min. followed by overnight incubation at 4°C. Cells were incubated in the presence or absence of rIL-2 for 3 days at 37°C in 5% CO_2, pulsed with 1μCi/well of [^{3}H]thymidine in the last 18 hours of incubation, harvested, and thymidine incorporation assessed via scintillation counting.

To study the alloreactivity of the 4.1-TCR, 2 x 10^4 $CD8^+$-depleted splenic $CD4^+$ T cells from transgenic and non-transgenic NOD mice were incubated with $1x10^5$ γ-irradiated (3000 rads) splenocytes or lymph node cells from non-transgenic (NOD x C57BL/6)F1 or (NOD x C58/J)F1 mice in 96-well round-bottom plates for 3 and 5 days at 37°C in 5% CO_2. Cultures were pulsed with 1μCi/well of [^{3}H]thymidine in the final 18 hours of incubation, harvested and analyzed for thymidine incorporation as above (all proliferation assay procedures performed by myself).

Histology and Immunopathology (3.5).

Pancreata were fixed in 10% formalin, embedded in paraffin, sectioned at 4.5μm and stained with hematoxylin-eosin. The degree of insulitis was determined by scoring at least 20 islets per mouse using the following criteria: normal or free of infiltrates (0), peri-insulitis (1), mononuclear cell infiltration in <25% of the islets (2), mononuclear infiltration in 25-50% of the islet (3), or >50% of the islet infiltrated (4) (all tissue pathology and H&E staining performed by myself).

For immunohistopathological analysis, pancreata were frozen in liquid nitrogen, embedded in O.C.T. compound (Tissue Tek, Sakura Finetek USA, Inc., Torrance, CA), sectioned at 6-7 μm, incubated with anti-CD4 (ATCC clone GK1.5), anti-CD8 (ATCC clone 53-6.7) or anti-Mac-1 mAbs (ATCC clone M1/70.15.11.5.HL) and FITC-conjugated anti-rat IgG, and analyzed under a fluorescence microscope (91) (frozen tissue sections cut and stained by another individual within the lab).

Adoptive Transfer (3.6).

Cells infiltrating the islets of acutely diabetic transgenic NOD mice were isolated via collagenase digestion as described above. Cells were expanded in culture media containing

0.5U/mL human rIL-2, depleted of contaminating $CD8^+$ T cells, resuspended in 200μL of PBS (pH 7.2) and injected into the tail vein of NOD/Lt-*scid* mice (The Jackson Laboratory). Some experiments employed splenic $CD4^+$ T cells (5-7 x 10^6 cells/mouse) purified (>94% $CD4^+$ purity) by the MACs microbead purification system (Miltenyi Biotec). All transfused mice were followed for development of IDDM by monitoring their urine glucose levels with Diastix (Bayer Corp., Etobicoke, Ontario) twice weekly. Mice with urine glucose levels of 3+ or greater were considered diabetic. Diabetes was confirmed by determining the blood glucose of glycosuric mice with Glucostix and a glucometer (Miles Canada, Etobicoke, Ontario). Diabetic animals were sacrificed by cervical dislocation and used for flow cytometry and pathological studies (all cell isolations performed by myself, while injections were done by another individual).

Bone Marrow Chimeras (3.7).

Bone marrow cell suspensions were prepared using standard protocols (76) from donor mice (4.1-NOD, or 4.1-[NOD x C57BL/6]F1 mice) and 5-10 x 10^6 cells injected into the tail vein of recipient mice (non-transgenic NOD, [NOD x C57BL/6]F1, or [SJL/J x C57BL/6]F1 mice) that had been previously irradiated with two doses of 500 rads from a ^{137}Cs source (Gammacell; Atomic Energy of Canada, Ottawa, Ontario) given 3h apart. Mice were fed autoclaved food and water containing neomycin sulfate (2mg/mL). Chimeras were sacrificed 5-6 wk following bone marrow transplantation for flow cytometry (all bone marrow preparation was performed by myself, while injections were performed by another individual within the lab).

Statistical Analyses (3.8).

All statistical analyses were performed using either Mann-Whitney U or χ^2 tests.

CHAPTER FOUR

RESULTS (4.0)

4.1-NOD Mice and IDDM Susceptibility (4.1).

Prior to my arrival in the lab, the TCR gene rearrangements of the I-A^{g7}-restricted, beta cell-reactive, $CD4^+$ T cell clone NY4.1 were sequenced from several NY4.1-derived TCR cDNAs generated by anchored-PCR. These sequences revealed that this 4.1-TCR transcribed one functional TCRβ rearrangement, carrying Vβ11 and Jβ2.4 elements, and one functional TCRα rearrangement, carrying a novel Vα gene (Vαx4.1) and the Jα33 element. These sequences were then subcloned into TCRβ and TCRα shuttle vectors carrying endogenous TCRβ or TCRα enhancers, respectively, and 5'-regulatory sequences. The genomic constructs were then microinjected into fertilized (SJL/J x C57BL/6)F2 eggs that were subsequently implanted into the uteri of pseudopregnant females. The resulting transgenic founder mice expressing the transgenes (4.1-AN63A-TCRαβ, 4.1-AN6B3-TCRβ, and 4.1-AN6B7-TCRα) were then crossed with NOD mice for several generations to produce 4.1-TCRαβ, 4.1-TCRβ-, and 4.1-TCRα-transgenic NOD mice, respectively.

Expression of the 4.1-TCR gene rearrangements in NOD mice (H-2^{g7}) skewed thymic T cell development towards the $CD4^+CD8^-$ T cell subset (Fig. 2A, right), indicative of positive selection of transgenic thymocytes (see figure legend for specifics). Three-color cytofluorometric analysis showed that >90% of $CD4^+CD8^-$ thymocytes (Fig. 2A, right, bottom histogram), and splenic $CD4^+$ T cells (Fig. 2B, right) from 4.1-NOD mice expressed $V\beta11^+$ TCRs compared to 76% of these same cells from 4.1-TCRβ-transgenic NOD mice (Fig. 2A and B, center) and 6% from non-transgenic littermates (Fig. 2A and B, left), thus indicating TCRβ

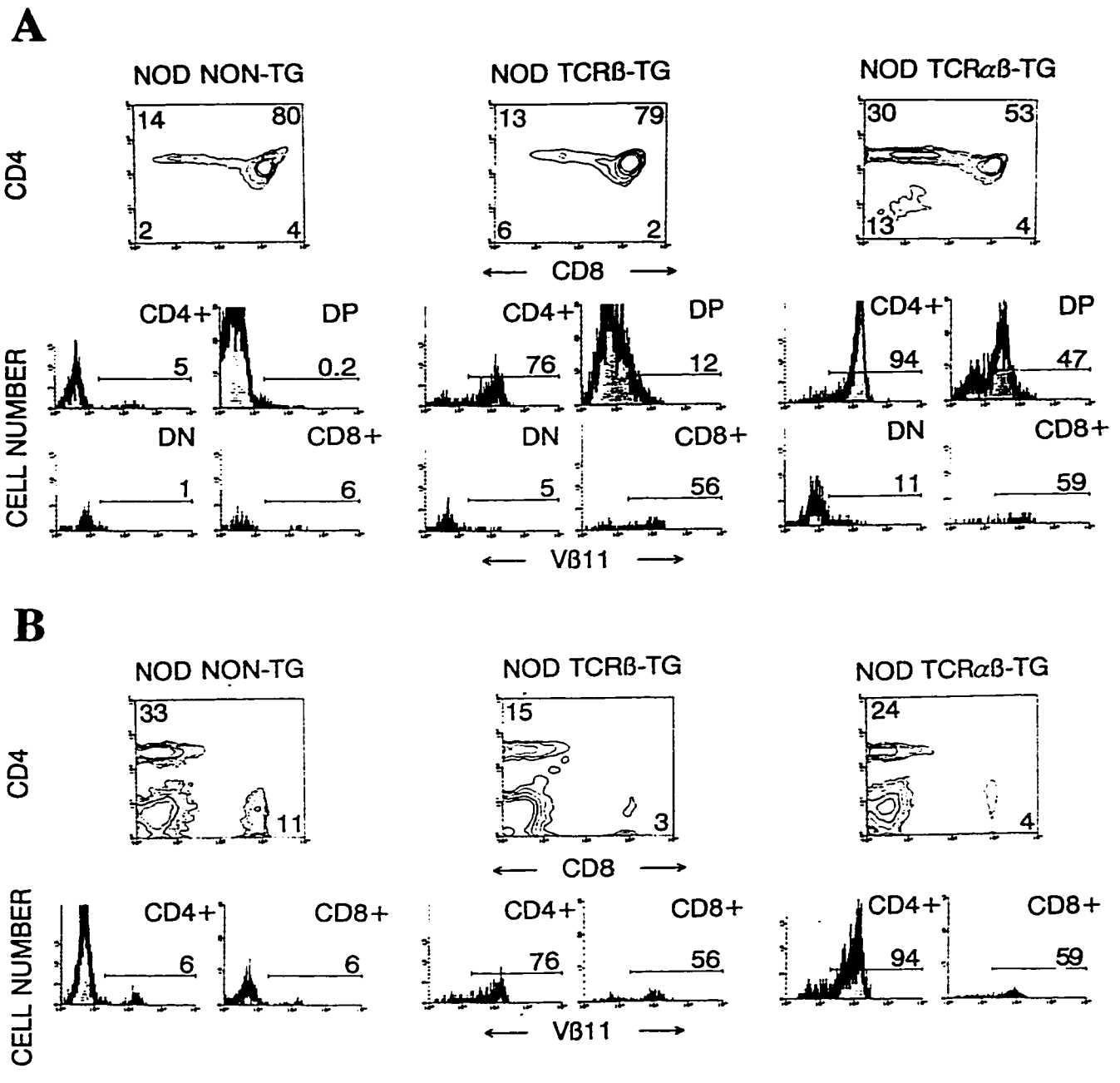

Figure 2. Expression of the 4.1-TCRαβ transgenes in 4.1-NOD mice. (A and B) CD4, CD8 and Vβ11 profiles of thymocytes (A) and splenic cells (B) from transgenic and non-transgenic mice. (Top) CD4 versus CD8 contour plots of cell suspensions stained with anti-CD8-PE, anti-Vβ11-FITC, and anti-CD4-biotin plus Streptavidin-PerCP. (Bottom) Vβ11 fluorescence histograms of each T cell subset after electronic gating. Numbers indicate the average percentage of cells (top) or the average number of $V\beta11^+$ cells (bottom) in each subset. Data correspond to 6-10 3-5 wk-old mice/ group. DP, double positive cells; DN, double negative cells. (C) In vitro proliferation of splenic $CD4^+$ T cells in response to islet cells. Cultures of 2 x 10^4 splenic $CD4^+$ T cell from 4.1- TCRαβ-transgenic, 4.1-TCRβ-transgenic, and nontransgenic NOD mice were incubated with γ-irradiated NOD islet cells and APCs (3-100 x 10^3 well) for 3 d, pulsed with [^{3}H]thymidine, and harvested. Bars show the standard error of the means.

transgene expression. Although there is no transgenic TCRα-specific antibody available and thus a measure of its expression via flow cytometry is not possible, a number of points suggested that the transgenic TCRα chain is expressed appropriately in 4.1-NOD mice. First, thymocyte development in 4.1-TCRαβ-, but not 4.1-TCRβ-NOD mice, was skewed towards the $CD4^+CD8^-$ subset (Fig. 2A, center and right), compatible with the TCRα transgene-dependent positive selection of transgenic $CD4^+CD8^-$ thymocytes. Second, sequence analysis of 37 peripheral TCRα cDNA sequences from splenic $CD4^+$ T cell-derived RNA of 4.1-NOD mice by anchored-PCR, revealed that all were transgene-derived. Third, $CD4^+CD8^-$ thymocytes of 4.1-NOD mice expressed higher levels of total TCRαβ (i.e. total TCRs on the cell surface) than those cells from 4.1-TCRβ-NOD mice (mean fluorescence intensities: 59 ± 12 vs. 31 ± 3, respectively, $p< 0.001$; determined by staining with anti-αβTCR mAb), suggesting early, and appropriate, TCRα chain expression (56). Fourth, skewing of thymocytes to the $CD4^+CD8^-$ subset occurred in transgenic mice expressing the selecting $I\text{-}A^{g7}$ molecule, but not in transgenic mice expressing non-selecting I-A molecules only (i.e. $I\text{-}A^s$, see below). And lastly, splenic $CD4^+$ T cells from 4.1-NOD mice, but not 4.1-TCRβ-transgenic or non-transgenic NOD mice, proliferated in a dose-dependent manner in response to γ-irradiated NOD islet cells and APCs (Fig. 2C). All in all, these results show that in 4.1-NOD mice, the 4.1-TCR is appropriately expressed, and that its expression fosters the positive selection of beta cell-reactive $CD4^+$ T cells.

The pathological significance of positive selection of the beta cell-reactive TCR in 4.1-NOD mice was an acceleration in the age at onset of spontaneous IDDM (Fig. 3A). Both female and male 4.1-NOD mice developed IDDM much earlier in life than non-transgenic littermates

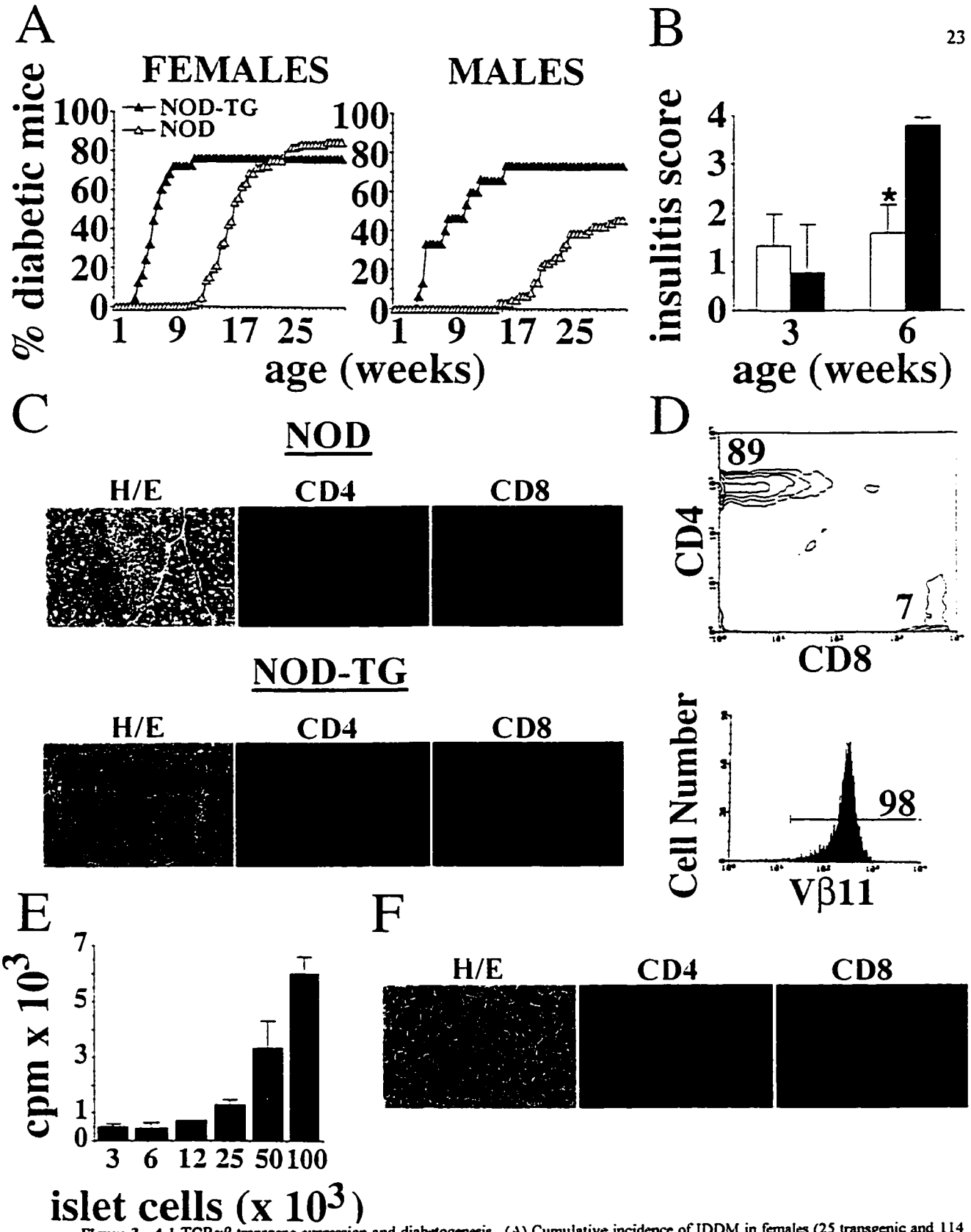

Figure 3. 4.1-TCRαβ transgene expression and diabetogenesis. (A) Cumulative incidence of IDDM in females (25 transgenic and 114 non-transgenic) and male (15 transgenic and 59 non-transgenic) NOD mice. (B) Progression of insulitis in transgenic and non-transgenic NOD mice. Hematoxylin-eosin stained pancreatic sections of 3 and 6 wk-old mice (4-13 mice/age group) were scored for the degree of insulitis as described in Materials and Methods (4 is the maximum score). Bars show the standard deviation of the means. *$p<0.0001$ (χ^2). (c) Phenotype of islet-infiltrating T cells in non-transgenic and transgenic NOD mice. Pancreas sections were stained with anti-CD8 (53.6-7) or anti-CD4 (GK1.5) mAbs and FITC-labeled anti-rat IgG. Original magnification: 200. (D) Flow cytometry profile of islet-derived T cells from diabetic 4.1-NOD mice. (E) Islet cell reactivity of islet-derived $CD4^+$ T cells from 4.1-NOD mice. See legend to Fig. 2C for details. (F) Phenotype of islet-infiltrating T cells in a diabetic *scid*-NOD mice that have been transfused with $CD8^+$ T cell-depleted $CD4^+$ T cells (5×10^6) derived from islets of a diabetic 4.1-NOD mouse.

(IDDM onset: 43.6 ± 13 vs. 119 ± 26 days in females; 56 ± 27 vs. 157 ± 28 days in males; $p<0.0001$). In 4.1-NOD males, transgenic expression also increased the incidence of disease compared to non-transgenic NOD males (73.3% vs. 45.7%, $p<0.05$). Despite this, the kinetics of disease penetrance in both the transgenic and non-transgenic populations were remarkably similar (Fig. 3A). Acceleration of IDDM, however, only occurred in 4.1-NOD mice and required co-expression of the TCRα- and TCRβ-transgenes since the few 4.1-TCRβ-NOD mice that became diabetic (3/7, 43%) did so significantly later than 4.1-NOD mice (103 ± 20 vs 46 ± 19 d, $p<0.01$). These findings contrast with another beta cell-specific TCR-transgenic NOD mouse model (62) which develops IDDM less frequently and much later (10-15% at 6 months) than 4.1-NOD mice when housed under specific pathogen-free conditions (82). Altogether, these results show that the 4.1-TCR is highly diabetogenic in the NOD background and suggest that the events triggering accelerated IDDM in 4.1-NOD mice are similar to those that trigger IDDM in non-transgenic NOD mice.

To investigate the mechanisms underlying disease acceleration in 4.1-NOD mice, we followed the progression of insulitis in prediabetic and diabetic mice. Pathological studies of pancreata from 3 and 6 wk old pre-diabetic 4.1-NOD mice revealed that acceleration of IDDM was not due to an earlier onset of insulitis, but rather to a faster progression of this process (Fig. 3B). The insulitic lesions of diabetic 4.1-NOD mice contained many more $CD4^+$ and fewer $CD8^+$ T cells, but similar numbers of B cells and macrophages (data not shown), than those of diabetic non-transgenic NOD mice (Fig. 3C). Most of the T cells derived from the islets of 4.1-NOD mice were $CD4^+$ and expressed high levels of the transgene-encoded $V\beta11^+$ chain as indicated by flow cytometry (Fig. 3D). These cells proliferated in a dose-dependent manner in

response to γ-irradiated NOD islet cells and APCs *in vitro* (Fig. 3E), and transcribed mRNA for IL-2 and IFN-γ but not IL-4 (data not shown). These results indicated that the majority of insulitic cells in 4.1-NOD mice are transgenic, beta cell-reactive, and are Th1-like, as expected. Furthermore, these cells are highly diabetogenic since purified islet-derived $CD4^+$ T cells from three different diabetic 4.1-NOD mice were able to transfer IDDM into three different *scid*-NOD mice shortly after transfusion (36 ± 12 d) in the absence of $CD8^+$ T cells in the infiltrated islets (Fig. 3F). Based on these and the above findings it can be concluded that expression of the 4.1-TCR in the NOD background promotes the selection of highly diabetogenic $CD4^+$ Th1-like cells and their accelerated recruitment into pancreatic islets, resulting in massive beta cell destruction and IDDM.

4.1-F1 Hybrid and MHC Congenic Mice and IDDM Resistance (4.2).

This unique TCR along with it's exquisite pathogenicity provided a powerful tool with which to investigate our initial hypothesis that diabetes-resistant backgrounds encode non-MHC-linked elements other than endogenous mouse mammary tumor virus superantigens (vSAgs) that are tolerogenic for diabetogenic T cells. These studies were initiated by crossing 4.1-NOD mice (H-2^{g7}, I-E^-) with SJL/J (H-2^s, I-E^-), C57BL/6 (H-2^b, I-E^-), and C58/J (H-2^k, I-E^+) mice to generate 4.1-(NOD x SJL/J)F1 mice (H-$2^{g7/s}$, I-E^-), (NOD x B6)F1 mice (H-$2^{g7/b}$, I-E^-), and (NOD x C58/J)F1 (H-$2^{g7/k}$, I-E^+), respectively; F1 mice resulting from crosses of these strains with non-transgenic NOD mice express the diabetogenic I-A^{g7} molecule but are diabetes-resistant and do not delete $V\beta11^+$ T cells (93). Lack of vSAg-mediated deletion of either $V\beta11^+$ or $V\alpha x4.1^+$ T cells in these backgrounds was confirmed by the fact that the thymocyte profiles of 4.1-TCRβ- or 4.1-TCRα-transgenic F1 mice and those of 4.1-TCRβ- or 4.1-TCRα-transgenic NOD mice,

respectively, were practically identical (Fig. 4); the percentage of thymic and splenic $CD4^+CD8^-$ $V\beta11^+$ thymocytes of both 4.1-TCRβ-transgenic (Fig. 4A) and non-transgenic F1 mice (Fig. 4B) were similar to those of 4.1-TCRβ-transgenic and non-transgenic NOD mice, respectively.

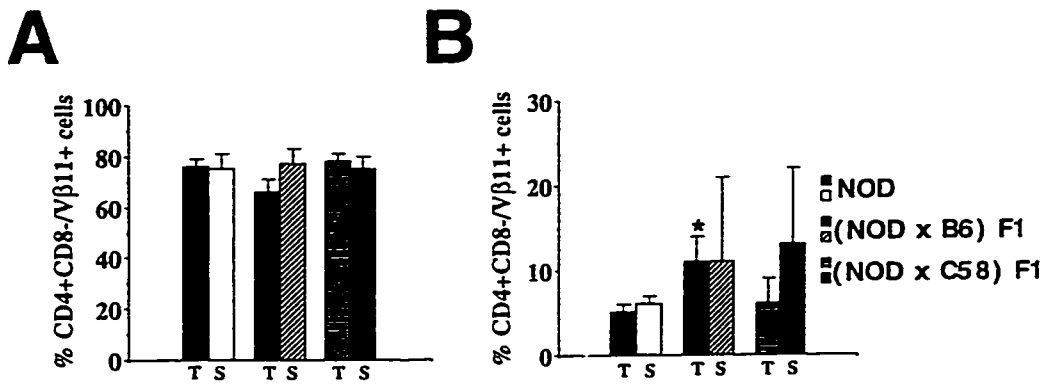

Figure 4. Absence of deletion of $V\beta11^+CD4^+$ T cells in 4.1-TCRβ-transgenic (A) and non-transgenic (B) F1 hybrid mice. Data correspond to average values from 3-6 mice/group. T, thymocytes; S, splenocytes. *$p<0.02$.

Three-color cytofluorometric studies of 4.1-TCR-transgenic offspring from these crosses revealed that thymocytes (Fig. 5A, left) and splenocytes (Fig. 5B) also undergo positive selection in 4.1-(NOD x SJL/J)F1 mice (Fig. 5A and B, right). In contrast, transgenic thymocytes appeared to undergo negative selection in the thymus of all 4.1-(NOD x B6)F1 mice (n=22) and most 4.1-(NOD x C58/J)F1 mice (n=16/19); these mice had fewer total thymocytes than 4.1-NOD mice (and 4.1-[NOD x SJL/J]F1 mice), and displayed a significant reduction in the percentage of $CD4^+CD8^-$ thymocytes, a significant reduction in the percentage of $V\beta11^+CD4^+CD8^-$ T cells, and an increase in the percentage of $CD4^-CD8^-$ thymocytes (Fig. 5A, center and right; statistics in figure legend) when compared to 4.1-NOD mice. In the spleen, there was a reduction in the percentage of $CD4^+$ T cells and a reduction in the percentage of $V\beta11^+CD4^+CD8^-$ T cells (Fig. 5B, center and right) when compared to 4.1-NOD mice. The few $V\beta11^+CD4^+CD8^-$ T cells that matured in these mice expressed half the amount of transgenic

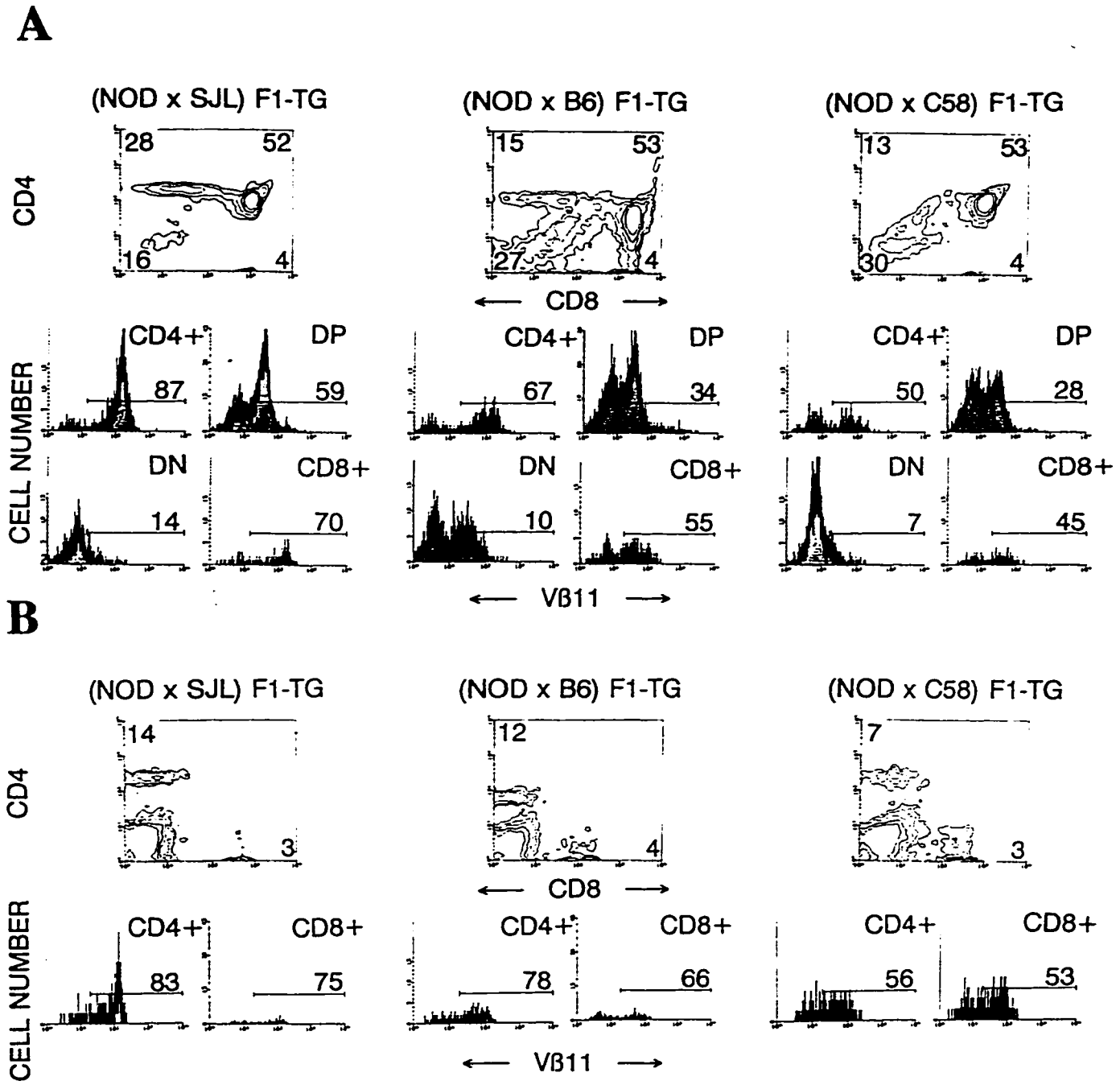

Figure 5. CD4, CD8, and Vβ11 profiles of thymocytes (A) and splenic cells (B) from transgenic F1 hybrid mice. See legend to Fig. 2 for details. Data shown are average values of 7-29 mice/group. In the text, transgenic NOD are referred to as 4.1-NOD; (NOD x SJL)F1-TG as 4.1-(NOD x SJL/J)F1; (NOD x B6)F1-tg as 4.1-(NOD x B6)F1; and (NOD x C58)F1-TG as 4.1-(NOD x C58/J)F1. When compared to 4.1-NOD, 4.1-(NOD x B6)F1 and 4.1-(NOD x C58/J)F1 mice had fewer $CD4^+CD8^-$ thymocytes ($p<0.0002$), fewer $V\beta11^+CD4^+CD8^-$ thymocytes ($p<0.0002$), and more $CD4^-CD8^-$ thymocytes ($p<0.0002$) (A). In the spleen (B), 4.1-NOD mice had more $CD4^+$ T cells ($p<0.0001$) and more $V\beta11^+CD4^+$ T cells ($p<0.002$) than 4.1-(NOD x B6)F1 and 4.1-(NOD x C58/J)F1 mice. All comparisons were done using the Mann-Whitney U test.

TCRβ chains on their cell surface (but comparable numbers of total TCRαβ complexes) than 4.1-NOD mice, both in the thymus and spleen ($p<0.003$; data not shown) suggesting that $CD4^+$ T cells maturing in deleting mice were selected on endogenous TCR chains that had bypassed allelic exclusion, as seen in other models (56, 57, 84). Assays of proliferative activity using splenic $CD4^+$ T cells as responders and γ-irradiated NOD islet cells and splenocytes as antigen and APCs, respectively, revealed the absence of beta cell-reactive $CD4^+$ T cells in the periphery of deleting, but not non-deleting, 4.1-F1 mice (Fig. 6A). The lack of proliferation in these assays was the result of deletion and not due to the induction of anergy, since $CD4^+$ T cells from deleting and non-deleting 4.1-F1 mice proliferated similarly when challenged with plate bound anti-TCRαβ mAb (Fig. 6B).

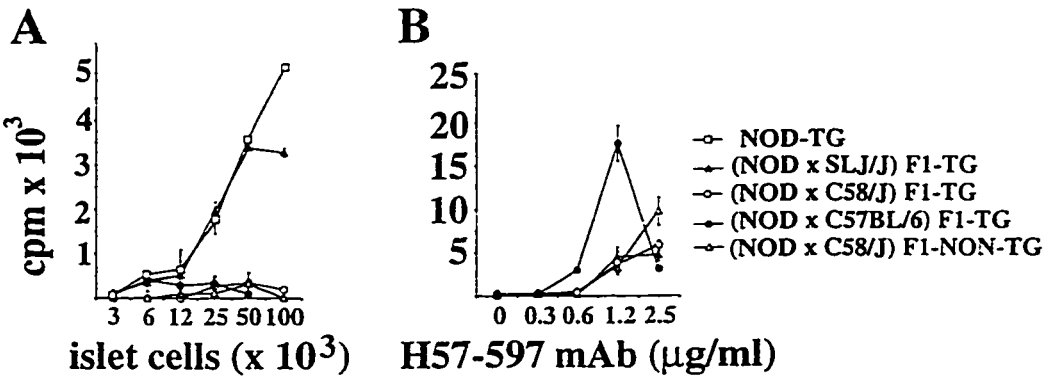

Figure 6. In vitro proliferation of naive splenic $CD4^+$ T cells from F1 hybrid mice to islet cells (A) and immobilized anti-TCRβ mAb (B). Proliferation assays in A were done as in Fig. 2C. For anti-TCRβ-induced proliferation (B), $CD4^+$ T cells (2×10^4) were added in triplicate to wells precoated with serial dilutions of anti-TCRβ mAb (H57-597). Bars show the standard error of the means.

The lack of diabetogenic 4.1-transgenic T cells in the periphery of deleting animals was confirmed by the fact that 4.1-F1 mice, like their non-transgenic littermates, developed neither insulitis, nor diabetes (Table 1). Altogether, these findings indicated that: a) both C57BL/6 and

C58/J mice carry gene(s) encoding elements that provide resistance to insulitis and diabetes via the deletion of the diabetogenic 4.1-TCR in the thymus; b) the genetic element(s) encoding for deletion in these strains has complete or incomplete penetrance, respectively; and c) that these elements are not encoded by vSAgs, and target T cells that co-express both the TCRα and TCRβ chains of the diabetogenic TCR.

Table 1. *Insulitis and Diabetes in NOD versus F1 Hybrid Mice*[*]

	n	IDDM[‡]	Age at Onset[§]	Insulitis Score (*n*)[¶]
			d	$x \pm SD$
NOD-TG	40	30/40[a]	48 ± 20[g]	3.74 ± 0.15[#i](4)
NOD–non-TG	173	123/173[b]	138 ± 27[h]	1.59 ± 0.58[#j](13)
(NOD × B6) F1-TG	10	0/10[c]	–	0.42 ± 0.81[¶k](10)
(NOD × B6) F1–non-TG	12	0/12[d]	–	0[¶l](12)
(NOD × C58) F1-TG	15	0/15[e]	–	0.42 ± 0.70[¶m](15)
(NOD × C58) F1–non-TG	4	0/4[f]	–	0[¶n](4)

[*]Groups include both males and females.
[‡]6-wk-old mice; [§]15-wk-old mice.
Insulitis (15–30 islets/mouse) was scored as described in Materials and Methods.
$x \pm SD$, mean ± standard deviation; TG, transgenic.
[a] versus [c,e], [b] versus [d,f], [g] versus [h], [j] versus [l]: $P < 0.0001$; [i] versus [j] $P < 0.003$; [k] versus [m] $P < 0.0005$; [l] versus [m], [j] versus [n]: $P < 0.0032$.
Data was compared by χ^2 (‡) and Mann-Whitney U test(¶).

Previous studies with NOD mice expressing anti-diabetogenic MHC class II genes did not find evidence of central T cell tolerance and suggested that diabetes-resistance in these mice was the result of immunoregulation rather than deletion or anergy of autoreactive T cells (14, 19, 21, 55, 62-64). Accordingly, we reasoned that the gene(s) responsible for the induction of 4.1-thymocyte tolerance in deleting 4.1-F1 mice would be non-MHC-linked. To test this, deleting

4.1-F1 mice were backcrossed with NOD mice and investigated for thymocyte deletion, IDDM resistance, and H-2 haplotype. Unexpectedly, we found that deletion of 4.1-thymocytes only occurred in H-$2^{g7/b}$ (16/16 mice, 100%) and H-$2^{g7/k}$ (9/15 mice, 60%), but never in H-$2^{g7/g7}$ mice (0/24 mice, 0%; Table 2), signaling that deletion segregated with the diabetes-resistant MHC. And like deleting 4.1-F1 mice, deleting 4.1-F2 mice lacked detectable beta cell-reactive $CD4^+$ T cells in the spleen (data not shown). In addition, deleting H-$2^{g7/b}$ and H-$2^{g7/k}$-4.1-F2 mice developed neither insulitis, nor diabetes, whereas H-$2^{g7/g7}$-4.1-F2 developed severe insulitis (100%) and diabetes (about 50%) within 10 wk (Table 2). These findings indicated that the gene(s) responsible for deletion in C57BL/6 and C58/J mice were linked to or encoded within the H-2^b and H-2^k complexes, respectively, and demonstrated that insulitis- and diabetes-resistance in 4.1-F2 mice carrying these haplotypes is the result of deletion of diabetogenic thymocytes.

The above findings suggested that perhaps the phenomenon of 4.1-thymocyte deletion was mediated by the MHC class I and/or MHC class II molecules encoded by the protective H-2 haplotypes. To investigate the role of MHC class I molecules in the deletion of 4.1-thymocytes, we followed the fate of these cells in CD8α- and β2-microglobulin (β2m)-deficient 4.1-(NOD x B6)F1 mice (H-$2^{g7/b}$) which lack expression of the MHC class I-binding CD8 co-receptor on 4.1-thymocytes, or lack expression of MHC class I molecules encoded by the H-2 complex of B6 mice (H-2D^b, K^b), respectively. The results from these studies, however, showed that these mice deleted transgenic thymocytes as efficiently as wild-type 4.1-F1 mice (data not shown), and therefore indicated that MHC class I molecules do not play a role in transgenic thymocyte deletion.

Table 2. *Cosegregation of Thymocyte Deletion and Resistance to Insulitis and IDDM with H-2^g and H-2^k Haplotypes in 4.1-transgenic Mice**

				Thymocyte profile						
	H-2	*n*	Deleting status	CD4$^+$CD8$^-$ (Vβ11$^+$)	CD4$^+$CD8$^+$	CD4$^-$CD8$^-$	CD4$^-$CD8$^+$	IDDM	Age at onset	Insulitis score ‡‖ (*n*)
				%, $x \pm SD$[‖]				*n*[§]	*d*	$x \pm SD$
(NOD × B6) F1 × NOD	H-2^{g7}	15	−	31 ± 7[a] (92 ± 2)[a]	44 ± 12	22 ± 7[r]	3 ± 1	7/15[k]	53 ± 17	2.4 ± 1.4 (8)[p]
	H-2$^{g7/b}$	16	+	15 ± 3[b] (66 ± 5)[b]	49 ± 12	33 ± 10[s]	3 ± 1	0/16[l]	–	0.4 ± 0.6 (16)[q]
(NOD × C58) F1 × NOD	H-2^{g7}	9	−	35 ± 5[c] (93 ± 1)[c]	43 ± 7	19 ± 4[h]	3 ± 2	5/9[m]	47 ± 16	3.4 ± 0.7 (4)[r]
	H-2$^{g7/k}$	6	−	35 ± 4[d] (89 ± 9)[d]	45 ± 8	16 ± 6[t]	4 ± 2	0/6[n]	–	1.7 ± 1.2(6)[s]
	H-2$^{g7/k}$	9	+	17 ± 3[e] (58 ± 9)[e]	45 ± 6	33 ± 6[j]	4 ± 3	0/9[o]	–	0.2 ± 0.2 (8)[t]

*All mice were killed at IDDM onset or at 10 wk if nondiabetic. Flow cytometry was done as described in the legend to Fig. 1.
‡Nondiabetic mice only.
Groups of mice include both male and female mice (~ 50% each). No differences in the incidence of IDDM nor in the degree of insulitis were noted between female and male mice within groups. Insulitis (15–30 islets/mouse) was scored as described in Materials and Methods.
[a] versus [b],[c] versus [e],[d] versus [e],[f] versus [g],[h] versus [j],[m] versus [j]: $P < 0.0002$; [k] versus [l]: $P < 0.0001$; [m] versus [n], [m] versus [o], [s] versus [t]: $P < 0.002$; [p] versus [q]: $P < 0.0006$; [r] versus [t]: $P < 0.0007$; [e] versus [t]: $P < 0.01$ (compared by χ^2[§] and Mann–Whitney U test [‖]).

Since H-2$^{g7/b}$ mice do not express MHC class II I-E molecules, we reasoned that deletion in 4.1-(NOD x B6)F1 mice might be mediated by the MHC class II molecule I-A^b. Accordingly, the fate of 4.1-thymocytes was followed in I-Aβ^b-deficient 4.1-(NOD x B6)F1 mice which lack I-A^b expression due to a mutation in the I-Aβ^b gene, but are genetically identical to wildtype 4.1-(NOD x B6)F1 mice. Removal of I-Aβ^b expression restored, at least in part, the positive selection of 4.1-thymocytes indicated by: a) skewing of thymocyte development towards the CD4$^+$CD8$^-$ T cell subset that express high levels of transgenic Vβ11 compared to 4.1-(NOD x B6)F1 mice (Table 3), b) significant increases in the ratio of CD4$^+$CD8$^-$ to CD4$^-$CD8$^-$ T cells in

the thymus and in the ratio of $CD4^+$ to $CD8^+$ T cells in the spleen, c) the reemergence of beta cell-reactive $CD4^+$ T cells in the spleen (Fig. 7, left), and d) the reappearance of insulitis (Table 3). Interestingly, the overall positive selection of the 4.1-TCR in I-$A\beta^b$-deficient 4.1-(NOD x B6)F1 mice was less efficient than in 4.1-NOD mice; there was more $CD4^-CD8^-$ thymocytes and half the splenic $CD4^+$ T cells in I-$A\beta^b$-deficient 4.1-(NOD x B6)F1 mice than age-matched (3-5 wk old) 4.1-NOD mice (Table 3). In addition, the peripheral $CD4^+$ T cells of I-$A\beta^b$-deficient 4.1-(NOD x B6)F1 mice proliferated less efficiently against NOD islet cells than those of 4.1-NOD mice (Fig. 7, left). And lastly, the degree of insulitis in I-$A\beta^b$-deficient 4.1-(NOD x B6)F1 mice was less severe than seen in 4.1-NOD mice and did not lead to diabetes in any of the 15 animals. These results suggested the existence of other diabetes-resistance elements other than I-A^b in C57BL/6 mice, however, expression of the I-A^b molecule itself is sufficient to induce deletion of 4.1-thymocytes.

To determine whether the deletion of 4.1-thymocytes in H-$2^{g7/k}$-4.1-F1 and F2 mice was also I-A mediated, 4.1-NOD mice were crossed with I-$A\alpha^k$/I-$A\beta^k$ (I-A^k)-transgenic NOD mice (29) to generate 4.1/I-A^k-trangenic NOD mice. Other than the presence of the I-A^k-transgenes, 4.1/I-A^k-NOD and 4.1-NOD mice are virtually genetically identical. It was found that 4.1/I-A^k-NOD mice had significantly fewer total thymocytes (data not shown), greater ratios of $CD4^-$ $CD8^-$ to $CD4^+CD8^-$ thymocytes, and a greater ratio of $CD8^+$ to $CD4^+$ splenocytes, compared to 4.1-NOD mice (Table 3); these results are compatible with the deletion of 4.1-thymocytes. This negative selection, however, was incomplete and did not occur as efficiently as in deleting H-$2^{g7/k}$ 4.1-F2 mice; the few $CD4^+$ T cells that matured in 4.1/I-A^k-NOD mice expressed high levels of $V\beta11^+$ TCRs, and proliferated in response to beta cells *in vitro* (Tables II and III; and

Table 3. *Influence of I-A^b,I-A^k, I-A^q, and H2^{nb1} MHC Class II Molecules on 4.1 Thymocyte Development*

	n	Organ	Percentage of cells*				Ratios*		IDDM† (*n*)	Insulitis† score (*n*)
			CD4$^+$CD8$^-$(Vβ11$^+$)	CD4$^+$CD8$^+$	CD4$^-$CD8$^-$	CD4$^-$CD8$^+$	CD4$^+$/DN	CD4$^+$/CD8$^+$		
				x ± *SD*						*x* ± *SD*
(NOD × B6) F1	29	T	15 ± 5[a] (67 ± 7)	53 ± 10	27 ± 9	4 ± 3	0.6 ± 0.2[b]	–	0 (10)[ae]	0.42 ± 0.81 (10)[ak]
		S	12 ± 6 (78 ± 12)	–	84 ± 8	4 ± 2	–	2.9 ± 1.5[y]		
(NOD × I-A^{b-}B6) F1	9	T	36 ± 5[h] (93 ± 3)	37 ± 9	23 ± 4[o]	4 ± 1	1.6 ± 0.2[t]	–	0 (15)[af]	1.59 ± 0.45 (10)[al]
		S	12 ± 6[c] (86 ± 11)	–	86 ± 7	2 ± 1	–	4.6 ± 1.4[z]		
NOD	9	T	30 ± 7[d] (94 ± 2)[e]	53 ± 9	13 ± 4[p]	4 ± 2	2.2 ± 0.6[u]	–	30 (40)[ag]	3.74 ± 0.15 (4)[am]
		S	24 ± 4[f] (88 ± 4)[g]	–	72 ± 5	4 ± 1	–	8.7 ± 2.9[aa]		
NOD.H-2^{g7}/I-A^k	7	T	25 ± 3 (93 ± 1)	49 ± 5	21 ± 3[q]	5 ± 1	1.2 ± 0.2[v]	–	0 (9)[ah]	2.2 ± 0.8 (7)[an]
		S	6 ± 1[h] (90 ± 1)	–	92 ± 1	2 ± 1	–	2.9 ± 0.3[ab]		
NOD.H-2$^{g7/q}$	7	T	19 ± 3[i] (88 ± 3)	59 ± 5	19 ± 3	3 ± 1	1.0 ± 0.2[w]	–	0 (7)[ai]	0.7 ± 0.4 (7)[ao]
		S	11 ± 8[j] (87 ± 4)	–	86 ± 10	3 ± 2	–	2.9 ± 1.4[ac]		
NOD.H-2$^{g7/nb1}$	11	T	11 ± 3[k] (37 ± 9)[l]	52 ± 7	34 ± 5[r]	3 ± 1	0.3 ± 0.1[x]	–	0 (11)[aj]	0.1 ± 0.1 (7)[ap]
		S	6 ± 2[m] (51 ± 16)[n]	–	91 ± 4	2 ± 1	–	2.9 ± 0.4[ad]		

Mice were studied at 3–5 (*) or at 10 wk (†) of age. Flow cytometry was done as indicated in the legend to Fig. 1.
DN, double-negative cells (CD4$^-$CD8$^-$); *T*, thymus; *S*, spleen.
Statistics (Mann-Whitney U test): CD4$^+$CD8$^-$: [a] versus [b], $P < 0.0001$; [c] versus [f], $P < 0.003$; [h] versus [f], $P < 0.0009$; [i] versus [d], $P < 0.0001$; [j] versus [f], $P < 0.005$; [k] versus [d], $P < 0.0002$; [m] versus [f], $P < 0.0002$; CD4$^+$CD8$^-$Vβ11$^+$: [l] versus [e], $P < 0.0002$; [n] versus [g], $P < 0.0002$; CD4$^-$CD8$^-$: [o] versus [p], $P < 0.03$; [q] versus [p], $P < 0.015$; [r] versus [p], $P < 0.0002$; CD4$^+$/DN: [s] versus [t], $P < 0.0001$; [u] versus [v], $P < 0.03$; [w] versus [u], $P < 0.002$; [x] versus [u], $P < 0.0002$; CD4$^+$/CD8$^+$: [y] versus [z], $P < 0.015$; [z] versus [aa], $P < 0.002$; [ab] versus [aa], $P < 0.0009$; [ac] versus [aa], $P < 0.0002$; [ad] versus [aa], $P < 0.0002$; IDDM: [ad,ae,ah,ai,aj] versus [ag], $P < 0.0001$; Insulitis Score: [al] versus [ak], $P < 0.0006$; [ak] versus [am], $P < 0.0005$; [al] versus [an], $P < 0.005$; [an] versus [am], $P < 0.03$; [ao] versus [am], $P < 0.008$; [ap] versus [am], $P < 0.006$.

Fig. 7, middle). In addition, unlike deleting H-$2^{g7/k}$ 4.1-F2 mice, 4.1/I-A^k-NOD mice developed a mild to moderate degree of insulitis suggesting that these cells proliferate against beta cell antigen *in vivo* (Table 3). The insulitis that did develop in these animals, however, was less severe than that of 4.1-NOD mice, and did not lead to the development of diabetes in any of the 9 animals studied. Taken together, these results suggested that deletion of thymocytes in H-$2^{g7/k}$-4.1-F1 and F2 mice is somehow triggered, at least in part, by I-A^k molecules, and these molecules may somehow abrogate the diabetogenic potential of the 4.1-T cells that escape deletion.

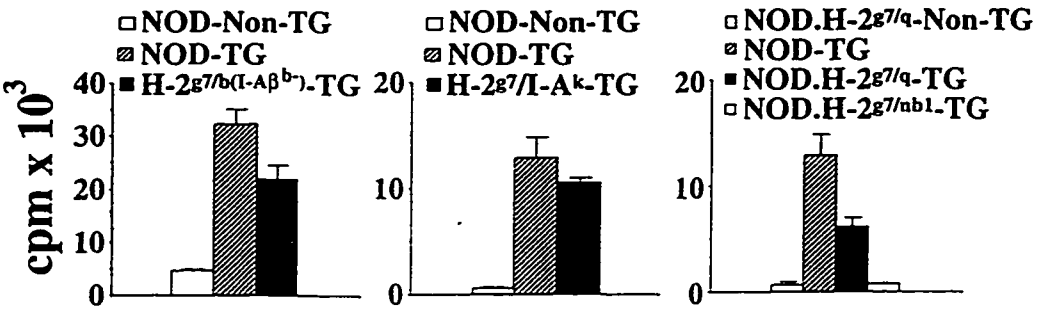

Figure 7. Peripheral beta cell-reactivity in I-$A\beta^{b-}$ 4.1-(NOD x B6)F1, 4.1/I-A^k-NOD, 4.1-NOD.H-$2^{g7/q}$, and 4.1-NOD.H-$2^{g7/nb1}$ mice. Assays were done as in Fig. 2C. Values correspond to cultures of 2 x 10^4 $CD4^+$ T cells with 5 x 10^4 (left) or 10^5 NOD islet cells (middle and right).

Having established that I-A^b and I-A^k molecules could profoundly affect the thymic development of 4.1-thymocytes, the next question was whether the 4.1-TCR could engage additional anti-diabetogenic MHC class II molecules during thymocyte development. Accordingly, we crossed 4.1-NOD mice with NOD mice congenic for H-2^q and H-2^{nb1} haplotypes, both of which have been shown to provide diabetes resistance in non-transgenic animals, (23) and followed the fate of 4.1-thymocytes arising in transgenic offspring. 4.1-NOD.H-$2^{g7/q}$ and 4.1-NOD.H-$2^{g7/nb1}$ mice resulting from these crosses had phenotypes compatible with the partial or complete deletion of the 4.1-TCR, respectively (Table 3, and Fig.

7, right). Like 4.1/I-A^k-transgenic NOD mice, 4.1-NOD.H-$2^{g7/q}$ mice showed thymocyte profiles consistent with the partial deletion of 4.1-thymocytes when compared to 4.1-NOD mice; there was a twofold reduction in the number of total thymocytes (data not shown), an increased percentage of $CD4^-CD8^-$ thymocytes, and reduced percentages of both thymic and splenic $CD4^+CD8^-$ T cells (Table 3). The few $CD4^+$ T cells that escaped deletion in these animals expressed high levels of $V\beta11^+$ TCRs and proliferated, albeit less efficiency than 4.1-NOD, in response to γ-irradiated NOD islet cells and APCs (Fig. 7, right). As well, these mice developed mild perinsulitis, however, none of the animals studied ever became diabetic (Table 3).

In contrast, thymocyte profiles of 4.1-NOD.H-$2^{g7/nb1}$ mice were compatible with complete deletion of 4.1-thymocytes; there was a three- to fourfold reduction in the absolute number of thymocytes (data not shown), a dramatic increase in percentage of $CD4^-CD8^-$ thymocytes, and reduced percentages of both thymic and splenic $V\beta11^+CD4^+CD8^-$ T cells (Table 3). There was a lack of detectable beta cell-reactive $CD4^+$ T cells in these mice indicated by absence of proliferation against NOD islet cells and APCs *in vitro* (Fig. 7, right), and absence of diabetes or detectable insulitis (Table 3). Deletion of 4.1-thymocytes in these mice was not attributable to putative NOD vSAgs (i.e. mouse mammary tumor virus superantigen 17) binding to Vβ11 and H-2^q or H-2^{nb1} MHC class II molecules mice as spleens of non-transgenic NOD.H-$2^{g7/q}$ and NOD.H-$2^{g7/nb1}$ mice had greater percentages of $V\beta11^+CD4^+$ T cells than non-transgenic NOD mice (20 ± 7% and 12 ± 2% vs 6 ± 1%, $p<0.008$ and $p<0.04$, respectively).

4.1-H-2 Homozygous Mice and Bone Marrow Chimeras (4.3)

To determine whether the deletion of 4.1-thymocytes *in vivo* was "restricted" by I-A^{g7} molecules, we followed the fate of transgenic thymocytes in mice homozygous for the diabetes-

resistant MHC haplotypes (i.e. lacked expression of I-A^{g7}). By backcrossing 4.1-F1 animals with the strains that donated the diabetes-resistance genes (i.e. SJL/J, C57BL/6, or C58/J), animals were generated that were 4.1-transgenic and homozygous for either non-selecting/non-deleting (H-2^s) or deleting (H-2^b or H-2^k) MHC haplotypes. As expected, there was neither positive nor negative selection of 4.1-thymocytes in the thymus of H-2^s 4.1 mice; there were significantly fewer $CD4^+CD8^-$ thymocytes, but not more $CD4^+CD8^-$ thymocytes, than H-$2^{g7/s}$ 4.1-F1 mice (Fig. 5, left and Fig. 8, left; $p<0.005$). In contrast, thymocyte profiles of H-2^b and H-2^k 4.1 mice were compatible with deletion; there were considerably fewer total thymocytes and

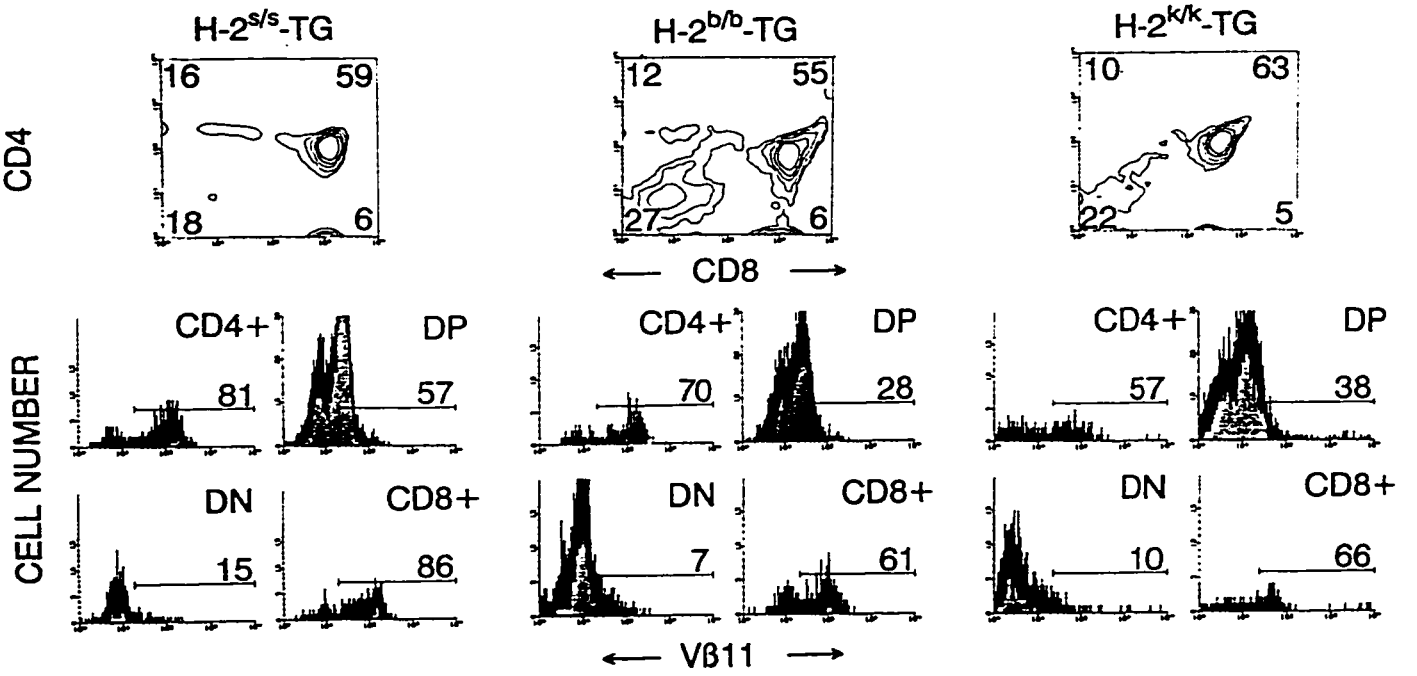

Figure 8. CD4, CD8, and Vβ11 profiles of thymocytes from transgenic mice homozygous for non-selecting or deleting H-2 haplotypes. See legend to Fig. 2 for details. Data are average values corresponding to 7-10 mice/group. H-2^b and H-2^k 4.1 mice had fewer thymocytes ($p<0.006$), and more $CD4^-CD8^-$ thymocytes than H-2^s 4.1 mice ($p<0.02$) (Mann-Whitney U test).

greater percentages of $CD4^-CD8^-$ thymocytes compared to $H\text{-}2^s$ 4.1 mice (Fig. 8, middle, and right). These results showed that unlike the $I\text{-}A^{g7}$-restricted positive selection of 4.1-T cells, the $I\text{-}A^b$- and $I\text{-}A^k$-mediated deletion of 4.1-thymocytes in deleting 4.1-F1 mice was not $I\text{-}A^{g7}$-restricted.

It has previously been shown that factors underlying MHC-linked resistance to IDDM predominantly reside in the bone marrow (32, 43, 70-72, 74-77). Therefore, to determine whether deletion of 4.1-thymocytes in deleting 4.1-F1 mice was mediated by hematopoietic cells or by thymic epithelial cells, bone marrow from either deleting 4.1-(NOD x B6)F1 mice, or positively selecting 4.1-NOD mice were transfused into γ-irradiated non-transgenic NOD or

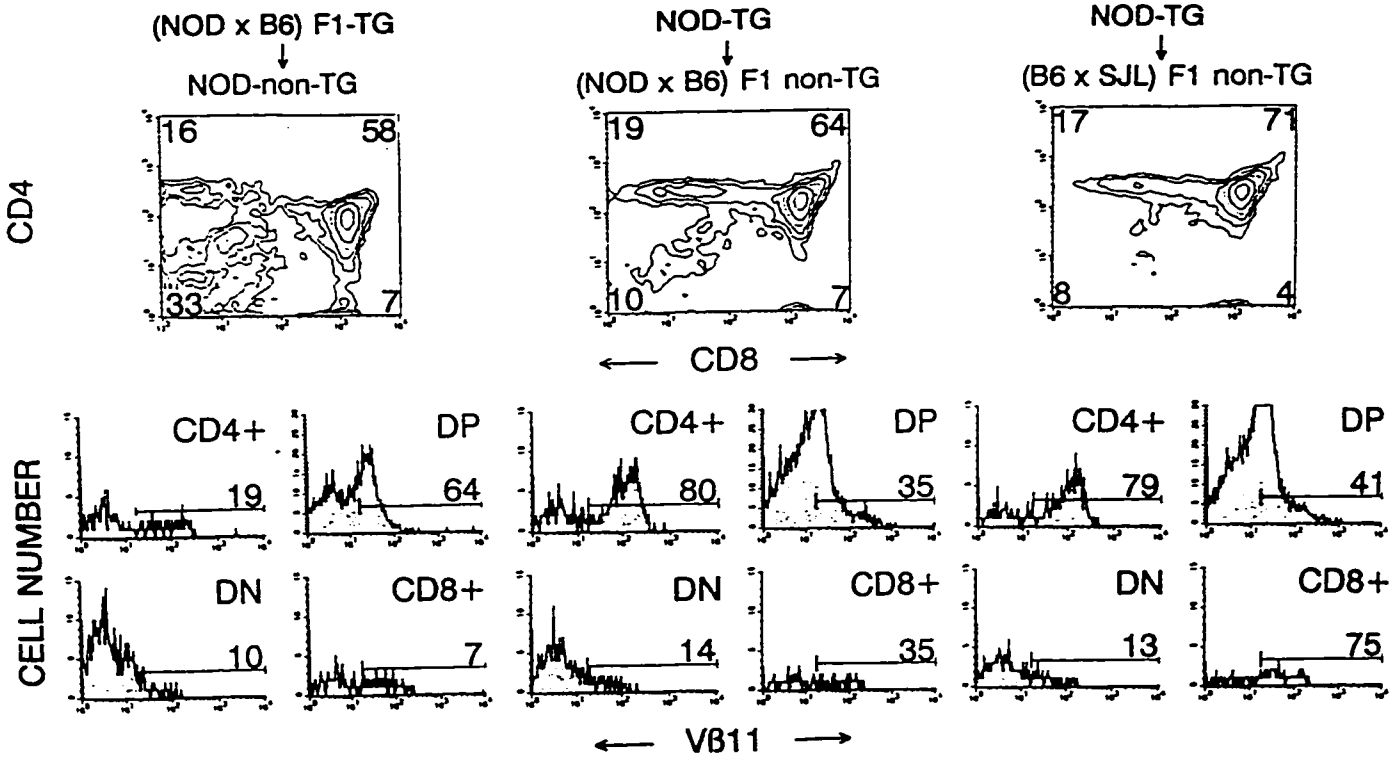

Figure 9. Bone marrow chimeras. Bone marrow cells (5-10 x 10^6) from donor mice (4.1-NOD or 4.1-[NOD x B6]F1 mice) were injected into the tail veins of lethally irradiated recipient mice (non-transgenic NOD, [NOD x B6]F1 or [B6 x SJL/J]F1 mice). Thymi of chimeric mice were analyzed by flow cytometry (as in Fig. 1), 5-6 wk after transplantation. Data represent average values of 2-4 mice/group. The mice that received marrow from $H\text{-}2^{g7/b}$-4.1 mice (left) had lower thymocyte $CD4^+CD8^-/CD4^-CD8^-$ ratios and fewer $V\beta11^+CD4^+CD8^-$ thymocytes than mice which received marrow from $H\text{-}2^{g7}$-4.1 mice (middle) ($p<0.05$).

(NOD x B6)F1 mice, respectively. Mice that received bone marrow from deleting 4.1-(NOD x B6)F1 mice (expressed diabetes-resistant I-A^b molecules only on hematopoietic cells), but not those that received marrow from 4.1-NOD (expressed I-A^b only on thymic epithelial cells), showed thymocyte profiles consistent with the deletion of transgenic thymocytes; there was a low $CD4^+CD8^-/CD4^-CD8^-$ ratio and small percentage of $V\beta11^+CD4^+CD8^-$ thymocytes (Fig. 9, left, and middle) Based on these findings, deletion of 4.1-thymocytes in 4.1-F1 mice is mediated by hematopoietic cells and occurs following their prior positive selection on radioresistant epithelial cells

4.1-Thymocyte Selection on "Deleting" MHC Class II in the Thymic Cortex (4.4).

The promiscuity of the 4.1-TCR for different MHC class II molecules in the thymic medulla suggested that this phenomenon might also occur in the thymic cortex. If that were true, then expression of deleting MHC class II molecules in the thymic cortex should both fail to delete the 4.1-TCR and foster it's positive selection. Indeed, the bone marrow chimeras generated following transfusion of marrow from 4.1-NOD mice into γ-irradiated non-transgenic (B6 x SJL/J)F1 mice suggested that would be the case (Fig. 9, right). To better test this hypothesis, we followed the development of 4.1-thymocytes in I-$A\alpha^{b-}$/I-$A\beta^{b-}$ mice (I-A^o) bearing an I-$A\beta^b$ transgene under control of the K14 (keratin) promoter (referred to as K14; targets transgene expression to stratified squamous epithelium, and occurs in those tissues expressing endogenous I-$A\alpha^b$), both in the presence or absence of one copy of the I-$A\beta^{g7}$ and I-$A\alpha^d$ genes. The K14 promoter was found to restrict transgene expression to the thymic cortical epithelium and thus, mice with the transgene express "deleting" I-A^b molecules (transgenic I-$A\beta^b$ complexed with endogenous I-$A\alpha^b$) exclusively within the thymic cortex (52).

Table 4. *MHC Class II Genotype and Expression in 4.1/K14 mice*

Name	MHC Class II						4.1-Thymocyte	
	Genotype			Phenotype	Site of Thymic Expression		Positive Selection*	Negative Selection
	α Locus	β Locus	Transgene		Cortex	Medulla		
4.1-I-$A^{g7/g7}$	I-Aα^{d}	I-Aβ^{g7}	–	I-A^{g7}	+	+	+	–
4.1-I-$A^{g7/b}$	I-Aα^{d}	I-Aβ^{g7}	–	I-A^{g7}	+	+	+	+
	I-Aα^{b}	I-Aβ^{b}	–	I-A^{b}	+	+		
4.1/K14-I-$A^{g7/o}$	I-Aα^{d}	I-Aβ^{g7}	–	I-A^{g7}	+	+	+	–
	I-Aα^{b}	I-Aβ^{b-}	–	I-A^{o}	–	–		
			K14-I-Aβ^{b}	K14-I-A^{b}	+	–		
4.1-I-$A^{g7/o}$	I-Aα^{d}	I-Aβ^{g7}	–	I-A^{g7}	+	+	+	–
	I-Aα^{b}	I-Aβ^{b-}	–	I-A^{o}	–	–		
4.1/K14-I-$A^{o/o}$	I-Aα^{b}	I-Aβ^{b-}	–	I-A^{o}	–	–	–	–
			K14-I-Aβ^{b}	K14-I-A^{b}	+	–		
4.1-I-$A^{o/o}$	I-Aα^{b}	I-Aβ^{b-}	–	I-A^{o}	–	–	–	–[a]

* 4.1-thymocyte positive selection preceeds negative selection.

[a] Thymocyte cell death in these animals is due to massive developmental arrest of 4.1-thymocytes.

The first set of experiments focused on confirming that the 4.1-TCR undergoes positive selection in I-A^{g7} hemizygous mice, and that I-A^{b} molecules can only negatively select the 4.1-TCR when expressed on bone marrow-derived APCs of the thymic medulla (genotypes and phenotypes of all mice in this portion of the study are summarized in Table 4).

Three color cytofluorometric studies showed that the thymocyte and splenocyte profiles of 4.1-I-A^{g7}-homozygous (referred to as 4.1-I-$A^{g7/g7}$) and 4.1-I-A^{g7}-hemizygous (I-A^{g7}/I-$A\alpha^{b+}$/I-$A\beta^{b-}$; referred to as 4.1-I-$A^{g7/o}$) mice were virtually identical to each other and to those corresponding profiles of 4.1-NOD mice (Fig. 10 and Fig. 2). These results indicated that positive selection of 4.1-$CD4^{+}CD8^{-}$ thymocytes does not require expression of two copies of I-A^{g7}. Subsequent cytofluorometric studies of thymi from 4.1-I-$A^{g7/o}$ mice expressing the K14 transgene (4.1/K14-I-$A^{g7/o}$ mice) revealed that, unlike I-A^{b} molecules expressed on medullary APCs, I-A^{b} molecules expressed on cortical epithelial cells cannot delete the 4.1-TCR (Fig. 11A); the thymi of 4.1/K14-I-$A^{g7/o}$ mice contained many more $V\beta11^{+}CD4^{+}CD8^{-}$ thymocytes and many fewer $CD4^{-}CD8^{-}$ thymocytes than the thymi of deleting 4.1-I-$A^{g7/b}$ mice ($p<0.0009$ and $p<0.0018$, respectively). Likewise, the splenocyte profiles of 4.1/K14-I-$A^{g7/o}$ mice had significantly increased percentages of $V\beta11^{+}CD4^{+}$ T cells when compared to deleting 4.1-I-$A^{g7/b}$ mice ($p<0.003$; Fig. 11B).

To determine whether the $V\beta11^{+}CD4^{+}CD8^{-}$ T cells maturing in 4.1/K14-I-$A^{g7/o}$ mice were responsive to beta cell antigen *in vitro*, we compared the proliferative activity of splenic $CD4^{+}$ T cells from 4.1/K14-I-$A^{g7/o}$, 4.1-I-$A^{g7/g7}$, 4.1-I-$A^{g7/o}$, K14-I-$A^{g7/o}$ and 4.1-I-$A^{g7/b}$ mice in response to stimulation with NOD islet cells and APCs. As expected, $V\beta11^{+}CD4^{+}$ T cells from 4.1/K14-I-$A^{g7/o}$, but not K14-I-$A^{g7/o}$ or 4.1-I-$A^{g7/b}$ mice, proliferated at least as well, if not better than the

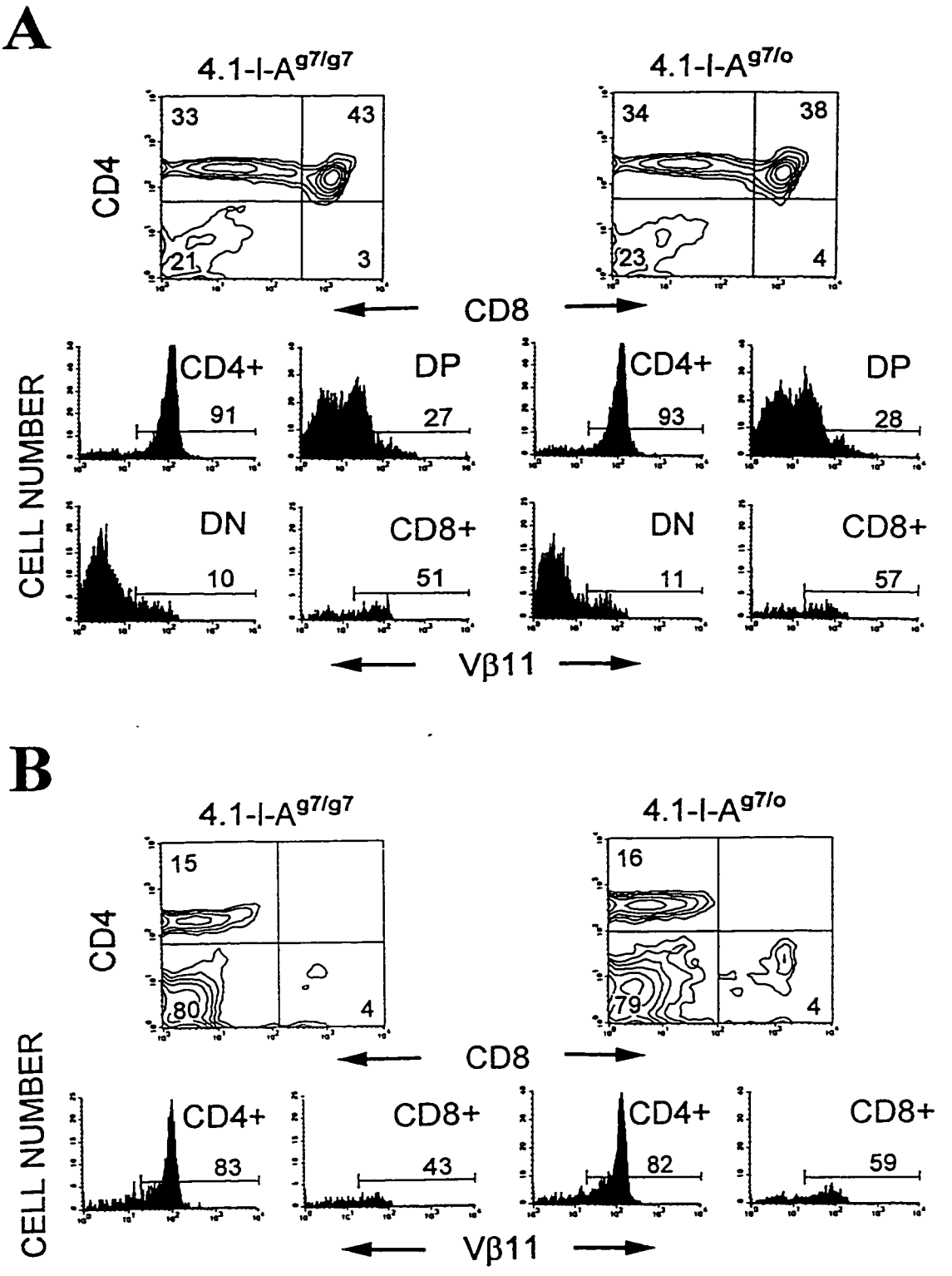

Figure 10. CD4, CD8, and Vβ11 profiles of thymocytes from 4.1-I-A$^{g7/g7}$ and 4.1-I-A$^{g7/o}$ mice. See legend to Fig. 2 for details. Data are average values corresponding to 7-10 mice/group. 4.1-I-A$^{g7/g7}$ and 4.1-I-A$^{g7/o}$ mice had virtually identical thymocyte and splenocyte and were comparable to that of 4.1-NOD mice (Fig. 2).

Vβ11$^+$CD4$^+$ T cells of 4.1-I-A$^{g7/g7}$ and 4.1-I-A$^{g7/o}$ mice (Fig. 12). These results therefore showed that the 4.1-TCR undergoes unopposed positive selection in mice selectively expressing deleting I-A^b molecules on thymic cortical epithelial cells.

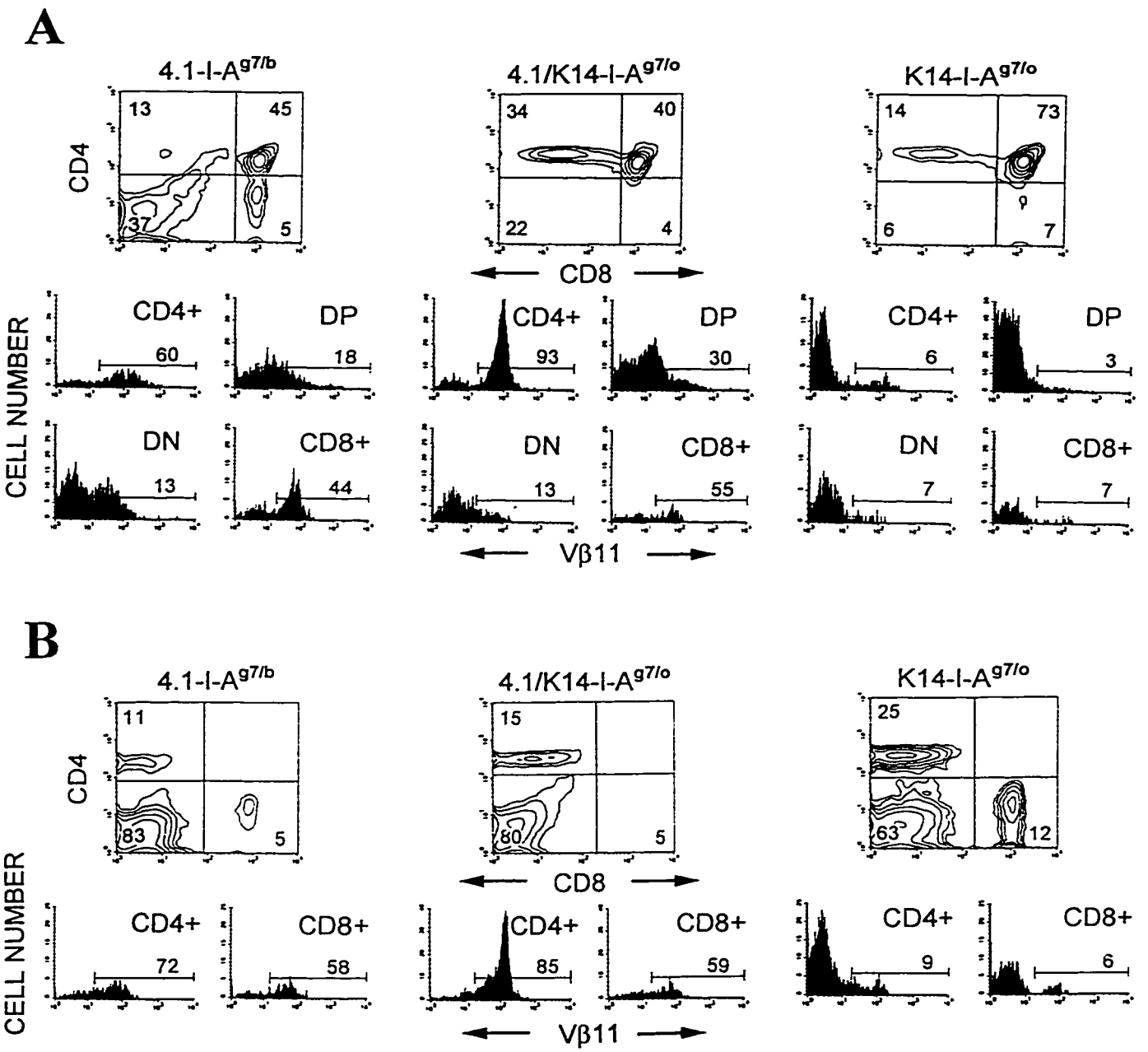

Figure 11. CD4, CD8, and Vβ11 profiles of thymocytes from 4.1-I-A$^{g7/b}$, 4.1/K14-I-A$^{g7/o}$, and K14-I-A$^{g7/o}$ mice. See legend to Fig. 2 for details. Data shown are average values of 6-9 mice/group. When compared with either 4.1-I-A$^{g7/o}$ or 4.1/K14-I-A$^{g7/o}$ mice, 4.1-I-A$^{g7/b}$ mice had fewer CD4$^+$CD8$^-$ thymocytes ($p<0.0011$ and $p<0.0009$, respectively) and many more CD4$^-$CD8$^-$ thymocytes ($p<0.0092$ and $p<0.0018$, respectively) (A). In the spleen (B), 4.1-I-A$^{g7/o}$ and 4.1/K14-I-A$^{g7/o}$ mice, had more Vβ11$^+$CD4$^+$ T cells than 4.1-I-A$^{g7/b}$ mice ($p<0.017$ and $p<0.003$). All comparisons were done using the Mann-Whitney U test.

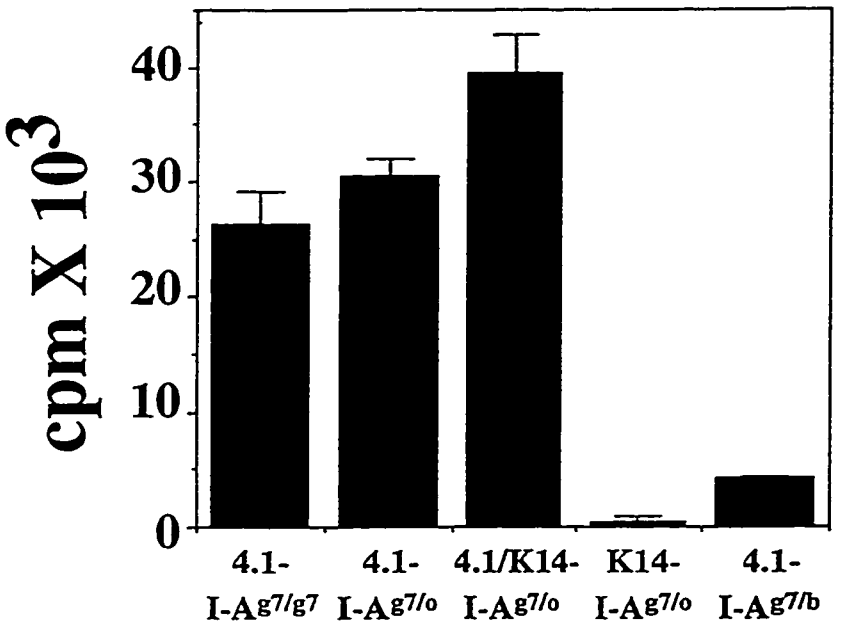

Figure 12. Peripheral beta cell-reactivity in 4.1-I-$A^{g7/g7}$, 4.1-I-$A^{g7/o}$, and 4.1/K14-I-$A^{g7/o}$, but not in K14-I-$A^{g7/o}$ or 4.1-I-$A^{g7/b}$ mice. Assays were done as in Fig. 1C. Values correspond to cultures of 2 x 10^4 $CD4^+$ T cells with 10^5 NOD islet cells and APCs.

To determine whether the 4.1-TCR was able to engage deleting I-A^b molecules in the thymic cortex leading to their positive selection, we followed the fate of 4.1-thymocytes in 4.1-TCR-transgenic, I-Aα^{b-}/I-Aβ^{b-} mice expressing or lacking the K14 transgene (4.1/K14-I-$A^{o/o}$ and 4.1-I-$A^{o/o}$ mice, respectively; Table 4). Studies of 4.1-I-$A^{o/o}$ and 4.1/K14-I-$A^{o/o}$ mice revealed, to our surprise, that cortical I-A^b molecules could not positively select $CD4^+CD8^-$ T cells bearing the highly promiscuous 4.1-TCR; thymi from both types of mice had very few $CD4^+CD8^-$ thymocytes and a significantly increased percentage of $CD4^-CD8^-$ thymocytes (Fig. 13A and B), likely reflecting the massive developmental arrest of 4.1-$CD4^+$ T cells in the absence of selecting I-A^{g7} molecules. Absence of positive selection in these mice was not due to inefficient expression of the K14 transgene, since non-4.1-transgenic K14-I-$A^{o/o}$ mice efficiently selected $CD4^+CD8^-$ T cells; as shown in Fig. 13A and B (right), K14-I-$A^{o/o}$ mice had significantly more

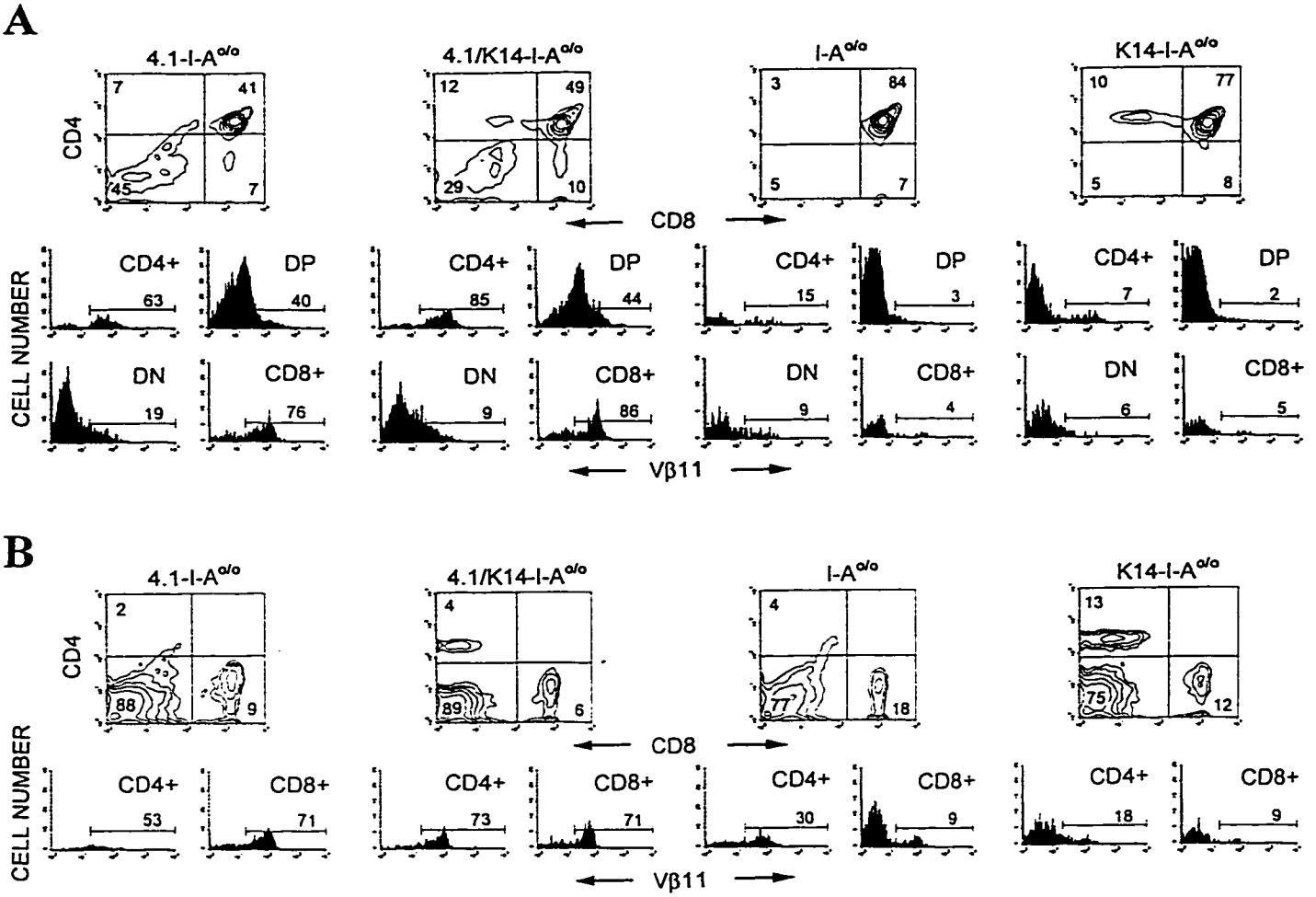

Figure 13. CD4, CD8, and Vβ11 profiles of thymocytes from 4.1-I-A$^{o/o}$, 4.1/K14-I-A$^{o/o}$, I-A$^{o/o}$, and K14-I-A$^{o/o}$ mice. See legend to Fig. 2 for details. Data shown are average values of 7-13 mice/group. When compared to 4.1/K14-I-A$^{g7/o}$ mice, 4.1/K14-I-A$^{o/o}$ had fewer Vβ11$^+$CD4$^+$ T cells ($p<0.0055$) expressing lower levels of Vβ11 on the cell surface (mfi: 78 ± 15 vs 61 ± 13, $p<0.0054$) in the spleen (B).

CD4$^+$CD8$^-$ thymocytes and splenic CD4$^+$CD8$^-$ T cells than I-A$^{o/o}$ mice ($p<0.0012$ and $p<0.0012$, respectively).

A small percentage of Vβ11$^+$CD4$^+$CD8$^-$ T cells did, however, mature in 4.1/K14-I-A$^{o/o}$ mice (Fig. 13B). Proliferation assays employing irradiated NOD islet cells and splenic CD4$^+$ T cells from 4.1/K14-I-A$^{g7/o}$, 4.1/K14-I-A$^{o/o}$ and K14-I-A$^{g7/o}$ mice revealed that, like the CD4$^+$ T cells of 4.1/K14-I-A$^{g7/o}$ mice, the CD4$^+$ T cells of 4.1/K14-I-A$^{o/o}$ mice were beta cell-reactive (Fig. 14).

The splenic CD4$^+$ T cells of 4.1/K14-I-A$^{o/o}$ mice, however, displayed lower islet-reactivity, contained fewer Vβ11$^+$ T cells ($p<0.0055$), and expressed lower levels of the transgenic TCR than those of 4.1/K14-I-A$^{g7/o}$ mice (mean fluorescence intensity: 61 ± 13 vs 78 ± 15, $p<0.0054$) (Fig. 11B, and 13B). These data suggested that the islet-reactive CD4$^+$ T cells of 4.1/K14-I-A$^{o/o}$ mice were selected on endogenous TCRs [i.e. the I-A^b-autoreactive TCRs that mature in K14-I-A$^{o/o}$ mice (Laufer et al., 1996)], rather than on the transgenic 4.1-TCR.

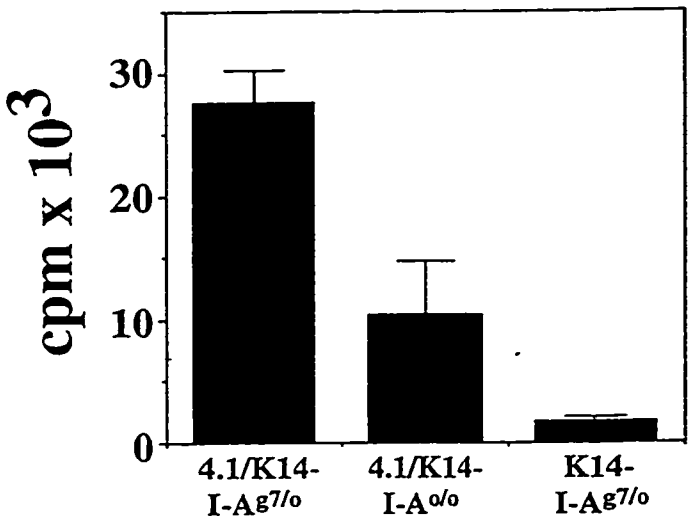

Figure 14. Peripheral beta cell-reactivity in 4.1/K14-I-A$^{g7/o}$, and 4.1/K14-I-A$^{o/o}$, but not in K14-I-A$^{g7/o}$ mice. Assays were done as in Fig. 2C. Values correspond to cultures of 2 x 10^4 CD4$^+$ T cells with 10^5 NOD islet cells and APCs.

Purified splenic CD4$^+$ T cells (APC-deficient) from 4.1-NOD mice engage antigen on intra-islet APCs, but only in the context of I-A^{g7} (unpublished observations). Therefore, to confirm that the CD4$^+$ T cells of 4.1/K14-I-A$^{o/o}$ mice were selected on endogenous, I-A^b-autoreactive TCRs, purified splenic CD4$^+$ T cells from 4.1-I-A$^{g7/o}$, 4.1/K14-I-A$^{o/o}$, and K14-I-A$^{o/o}$ mice were tested for proliferation against irradiated NOD, B6, and I-Aβ^{b-} intra-islet APCs. As shown in Fig. 15A, the CD4$^+$ T cells of all three groups of mice contained cells capable of recognizing islet antigen/I-A^{g7} complexes on intra-islet APCs. In contrast, only the CD4$^+$ T cells from 4.1/K14-I-A$^{o/o}$ and K14-I-A$^{o/o}$ mice proliferated against I-A^{b+} intra-islet APCs in these assays

(Fig. 15B). These responses were I-A^b-restricted since proliferation was not observed in assays employing I-Aβ^{b-} islet cells as stimuli (Fig. 15C).

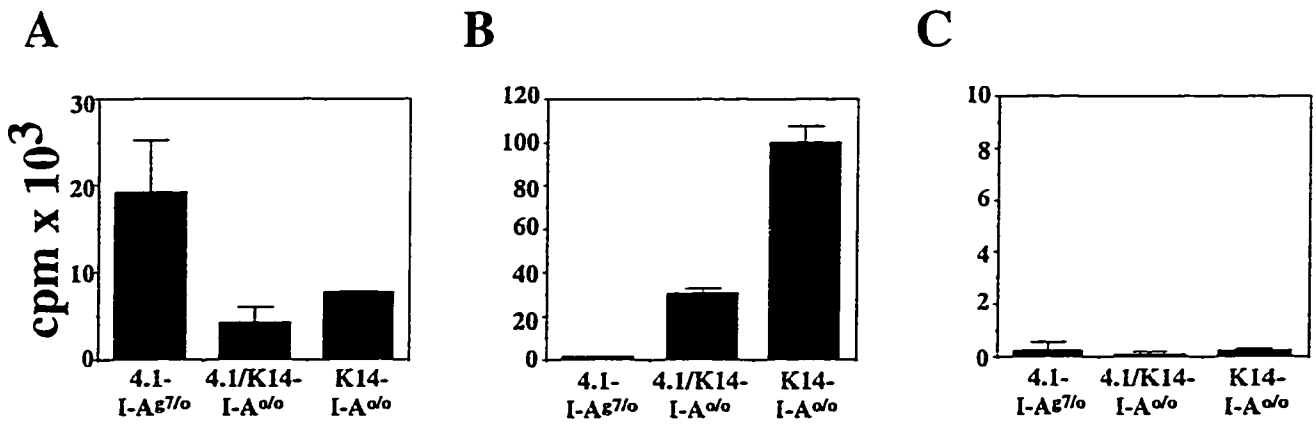

Figure 15. Peripheral beta cell/intra-islet APC-reactivity of purified splenic $CD4^+$ T cells from 4.1-I-$A^{g7/o}$, 4.1/K14-I-$A^{\alpha/o}$, and K14-I-$A^{\alpha/o}$ mice. In vitro proliferation of purified splenic $CD4^+$ T cells (2×10^4) in response to NOD (A), B6 (B), or I-Aβ^{b-} islet cells (C) (10^5/well).

Taken together, these results demonstrated that: a) cortical I-A^b molecules cannot select the 4.1-TCR; b) the few beta cell-reactive Vβ11$^+$$CD4^+$ T cells that mature in 4.1/K14-I-$A^{\alpha/o}$ mice are actually selected on endogenous TCRs; and c) positive selection of 4.1-thymocytes occurs exclusively on I-A^{g7} in the thymi cortex, with negative selection occurring following subsequent engagement of I-A^b in the thymi medulla. These findings therefore suggest that the 4.1-TCR is exclusively MHC monogamous (for the pro-diabetogenic I-A^{g7} molecule) in the thymic cortex and highly promiscuous (for several anti-diabetogenic MHC class II molecules) in the thymic medulla.

CHAPTER FIVE

DISCUSSION (5.0)

The first striking observation of this study was the efficient positive selection of the 4.1-TCR in NOD mice leading to a dramatic acceleration in the onset of IDDM (by about 3 months). This result was surprising in light of previous observations with another beta cell-reactive, I-A^{g7}-restricted transgenic TCR (BDC2.5) (62, 82). These studies found that expression of the BDC2.5-TCR in NOD mice (BDC2.5-NOD mice) did not lead to an accelerated onset or increased incidence of IDDM (IDDM incidence of 15% vs 74% at 6 months of age) (62, 82). Beta cell destruction in 4.1-NOD mice can be confidently attributed to 4.1-$CD4^+$ T cells since islet-derived cells with this phenotype were able to transfer diabetes to *scid*-NOD mice. Furthermore, recombination activating gene (RAG) 2-deficient 4.1-NOD mice, which cannot rearrange endogenous TCR and Ig heavy chain genes, developed IDDM as early and as frequently as $RAG2^+$ 4.1-NOD mice (83).

The most surprising observation of this study, however, was the finding that $CD4^+CD8^-$ thymocytes expressing the 4.1-TCR undergo negative selection in diabetes-resistant H-$2^{g7/b}$-, H-$2^{g7/k}$-, H-$2^{g7/q}$-, and H-$2^{g7/nb1}$-4.1-NOD mice by engaging MHC class II molecules (I-A^b, I-A^k, I-A^q, I-A^{nb1}, and possibly I-E^k, and I-E^{nb1}) on bone marrow-derived APCs of the thymic medulla. These results are particularly surprising, since previous studies could not find evidence for deletion of autoreactive T cells in congenic or transgenic NOD mice expressing these anti-diabetogenic MHC class II molecules (26, 31, 34, 62, 69-71). Deletion of 4.1-thymocytes was not mediated by endogenous superantigens binding to Vβ11, since this phenomenon was clearly absent in 4.1-TCRβ- and non-transgenic F1 mice expressing these deleting MHC haplotypes.

More importantly, however, was the fact that the deleting phenotype segregated in an MHC-linked fashion. Taken together, these results imply that the 4.1-TCR is unique in that it is both highly diabetogenic and highly promiscuous.

Positive selection was clearly restored in I-Aβ^{b-}-4.1(NOD x B6)F1 mice upon selective abrogation of I-A^b, however, negative selection was not completely eliminated when compared with age-matched 4.1-NOD mice. The partial deletion in I-Aβ^{b-}-4.1(NOD x B6)F1 mice may be the result of 4.1-thymocytes engaging heterodimer complexes consisting of I-Aα^b/I-Aβ^{g7} at an affinity/avidity approaching the threshold for deletion, or perhaps to other tolerogenic gene(s)/factor(s) in the C57BL/6 background. Likewise, deletion of 4.1-thymocytes was clearly more efficient in H-$2^{g7/k}$ 4.1-F1 and F2 mice, which both express I-A^k and I-E^k, than in 4.1/I-A^k-transgenic-NOD mice. This finding suggests that thymocyte deletion in the former involves the engagement of I-E^k and/or I-Eα^k/I-Eβ^{g7} molecules by the 4.1-TCR. Alternatively, the timing and levels of expression of I-A^k in 4.1/I-A^k-transgenic-NOD mice might be different than those of endogenous I-A^k molecules in F1 and F2 mice. Indeed, previous experiments with these mice have shown that I-A^k expression on thymic epithelial cells of I-A^k-transgenic-NOD mice is lower than the level of this same molecule in wildtype H-2^k mice. In addition, I-A^k-transgenic-NOD mice do not express I-A^k molecules on bone marrow cells, but do, however, on bone marrow-derived APCs (29). Whatever the explanation for these differences, deletion of 4.1-thymocytes in H-$2^{g7/b}$- and H-$2^{g7/k}$-F1 and F2 mice was complete. Likewise, complete deletion of 4.1-thymocytes was observed in 4.1-NOD.H-$2^{g7/nb1}$ mice, which express both I-A^{nb1} and I-E^{nb1}, and was also present, albeit partially, in 4.1-NOD.H-$2^{g7/q}$ mice, which only express I-A^q. It is therefore apparent that the highly diabetogenic 4.1-TCR can engage several distinct MHC class

II molecules in the thymus leading to totally different consequences, i.e. positive selection or dominant negative selection.

It is the promiscuity exhibited by this receptor that brings to mind a number of intriguing questions. For example: how can an I-A^{g7}-restricted TCR recognize so many different MHC class II molecules? Does this recognition require a common peptide? Or completely different peptides? Does the 4.1-TCR recognize all of these MHC molecules regardless of the molecular nature of bound peptides? Is there anything unique about these MHC class II molecules which may shed some light on why this receptor is so promiscuous? Many of these questions remain to be answered, however, there are some clues which may provide some understanding. For example, we know that the diabetogenic 4.1-TCR does not fit the description of a classical alloreactive TCR; deleting I-A^{b} molecules expressed exclusively on thymic cortical epithelial cells cannot select 4.1-thymocytes, and peripheral APCs expressing the deleting MHC molecules could not stimulate naive or preactivated 4.1-$CD4^{+}$ T cells *in vitro* (data not shown; Alloreactivity Assay described in Materials and Methods). These results do not imply that the putative peptide-MHC class II complexes that mediate thymic deletion of 4.1-thymocytes are expressed solely by specialized thymic APC subpopulations; they may also be present on peripheral APCs but may not be able to trigger peripheral 4.1-$CD4^{+}$ T cells. Indeed, thymic tolerance had been shown to be a more sensitive response than peripheral T cell activation lending credence to the above hypothesis. One thing that does appear certain, however, is that the I-A^{b}-induced deletion of 4.1-thymocytes in the thymic medulla is not mediated by the target antigen in the islets; purified 4.1-$CD4^{+}$ T cells from 4.1-I-$A^{g7/o}$ mice proliferated in response to

irradiated NOD intra-islet APCs but showed no response against B6 intra-islet APCs (Fig. 15A and B).

The 4.1-TCR's promiscuity for multiple MHC class II molecules seems to be relegated strictly to the thymic medulla. While there are examples of TCRs capable of engaging more than one MHC molecule during positive selection (e.g. the MCC_{88-103}/I-E^k-restricted TCR, AND, can undergo positive selection on I-A^b and I-E^k) in the thymus, this was restricted to the thymic cortex (90, 94). Even in the case of alloreactive TCRs [eg. 2C-TCR, which can be selected by one MHC molecule (K^b) and deleted by another (L^d)], the MHC molecules that trigger the deletion of these TCRs can also mediate their prior positive selection (87). Taken together then, the 4.1-TCR is unique in it's ability to engage multiple MHC molecules in the thymic medulla. It is MHC monogamous in the thymic cortex leading to positive selection, is MHC promiscuous in the thymic medulla leading to negative selection, and is MHC monogamous and autoreactive in the periphery leading to autoimmunity (thymic selection of this TCR is summarized in Fig. 16).

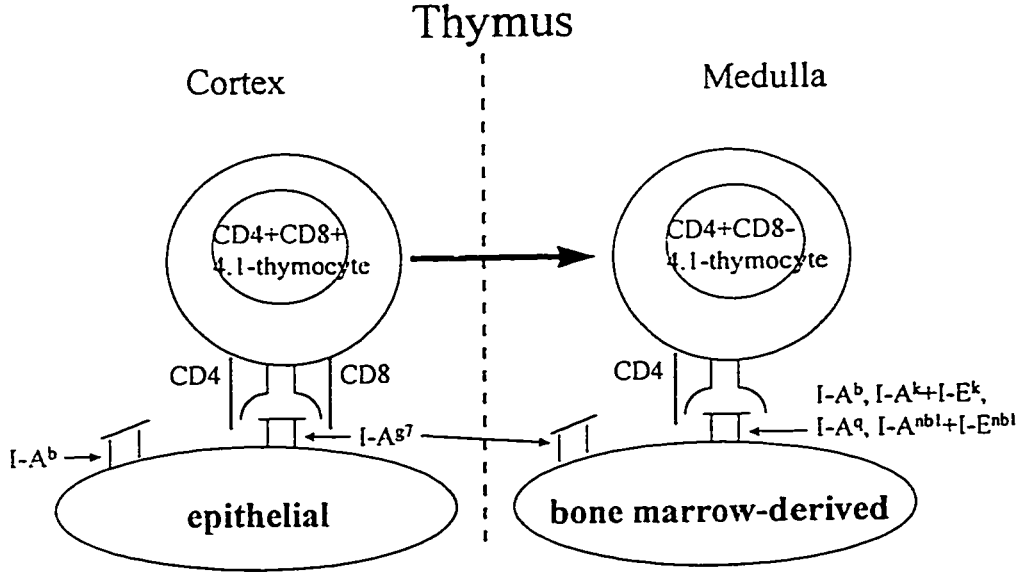

Figure 16. Summary of selection of 4.1-thymocytes in the thymus of an F1 mouse. $CD4^+CD8^+$ 4.1-thymocytes can only recognize self peptide presented on I-A^{g7} allowing their positive selection into the thymic medulla. Here recognition by the 4.1-TCR of self peptide complexed with either I-A^b, I-A^k + I-E^k, I-A^q, or I-A^{nb1} + I-E^{nb1} will deliver the dominant negatively selecting signal leading to the death of the cell and ultimately, resistance to diabetes.

Another clue is the fact that the I-Aβ chains of all the deleting I-A molecules tested thus far ($I\text{-}A^b$, $I\text{-}A^k$, and $I\text{-}A^q$) share amino acid residues at position 57 (aspartic acid; Asp 57) and 61 (tryptophan) (85). Interestingly, the two I-E molecules present in this study, $I\text{-}E^k$ and $I\text{-}E^{nb1}$, also share these characteristics. As well, $I\text{-}A^{nb1}$ also has aspartic acid at position 57, and like the other I-A molecules, has a bulky hydrophobic residue at position 61 (phenylalanine). Presence of Asp 57 alone (which is negatively associated with IDDM susceptibility), however, is not sufficient to endow an MHC molecule with the ability to delete, since $I\text{-}A^s$ molecules also have aspartic acid at position 57 but do not trigger 4.1-thymocyte deletion.

Whatever the specific residues involved, either within the MHC molecules or peptide(s), the unexpected promiscuity of the 4.1-TCR raises an intriguing concept with regards to MHC class II molecules and resistance to autoimmunity. This concept suggests that perhaps the pathogenic potential of a given autoreactive specificity may be related to it's degree of promiscuity for different MHC molecules in the thymic medulla. In other words, the more pathogenic an autoreactive TCR is, the more likely it will react with different MHC haplotypes in the thymic medulla, therefore leading to it's deletion. It is possible that promiscuous autoreactive TCRs, like the 4.1-TCR, are predominantly, but not exclusively, deleted by reaction with MHC residues rather than peptide residues as suggested recently for alloreactive TCRs (94). Like alloreactive receptors, promiscuous autoreactive TCRs may then be able to react with a larger range of peripheral self-peptide-MHC complexes above the level of activation of nonpromiscuous autoreactive TCRs, resulting in a greater opportunity for pathogenicity. This theory would suggest that promiscuity would not be unusual among those autoreactive TCRs with the highest pathogenic potential, like those that trigger IDDM. Concomitantly, MHC molecules providing

dominant resistance to an autoimmune disease would do so by removing only those autoreactive T cells with the most pathogenic potential, rather than removing all autoreactive specificities. This concept would then perhaps explain why beta cell-reactive, I-A^{g7}-restricted receptors like the BDC2.5-TCR are not deleted or tolerized in the presence of anti-diabetogenic MHC haplotypes (62). It would also provide some explanation for the presence of mildly insulitogenic, but not diabetogenic, T cells in certain MHC-congenic NOD mice, and in I-A- and I-E-transgenic NOD mice (23, 28, 29, 31, 34, 71).

Two important considerations are raised upon evaluating the relevance of our findings in 4.1 mice with respect to the MHC-linked susceptibility and resistance to spontaneous IDDM in non-TCR-transgenic NOD mice. First of all, the pathophysiological consequences of 4.1-thymocyte selection in 4.1 mice. There was an absolute correlation between deletion of the 4.1-TCR in H-$2^{g7/b}$ and H-$2^{g7/k}$ 4.1-F2 mice and resistance to development of insulitis and IDDM; none of the deleting offspring of 4.1-F1 mice backcrossed with NOD ever developed insulitis, whereas all non-deleting littermates developed moderate to severe insulitis, with 50% of H-$2^{g7/g7}$ 4.1-F2 mice developing IDDM. The same was also true for 4.1-NOD.H-$2^{g7/q}$ and 4.1-NOD.H-$2^{g7/nb1}$ mice, which developed only mild insulitis or no insulitis at all, respectively, and did not become diabetic. These findings are reminiscent of the insulitis and IDDM resistance of NOD.H-$2^{g7/b}$, NOD.H-$2^{g7/k}$, NOD.H-$2^{g7/q}$, and NOD.H-$2^{g7/nb1}$ congenic mice, and of I-E-, and I-A-transgenic NOD mice (22-34). As well, these finding refute a hypothesis put forth recently that suggested a mechanism of resistance to IDDM provided by protective MHC class II molecules is the positive selection of immunoregulatory cells (88). The fact that 4.1-$CD4^+$ T cells selected in the presence of I-A^b (4.1/K14-I-$A^{g7/o}$ mice) are diabetogenic upon

transfer into *scid*-NOD mice (data not shown) conflicts with this hypothesis and is consistent with the findings of our study.

The second consideration relates to the geography and timing of negative selection of 4.1-thymocytes in deleting 4.1-F1 mice. In concordance with other models of thymocyte selection (52, 86), the negative selection of 4.1-thymocytes was preceded by positive selection and was mediated by hematopoietic cells. These observations are also in accordance with the notion that factors underlying MHC-linked resistance to spontaneous IDDM predominantly reside in the bone marrow (32, 69, 71, 77-80). All in all, these results clearly support the hypothesis that resistance mediated by MHC genes against insulitis and diabetes in non-transgenic mice, and perhaps in humans, is also the result of deletion of highly diabetogenic thymocytes.

An important fact to be taken into consideration here, however, is that negative selection of highly diabetogenic thymocytes does not appear to be the only mechanism of protection afforded by MHC class II molecules against IDDM. As well, positive selection of pathogenic specificities does not necessarily suggest that autoimmunity will result. The basis for these statements lie in the following observations. First, the non-diabetogenic insulitis lesions seen in 4.1/I-A^k-transgenic NOD mice suggests that the cells escaping partial deletion by I-A^k may have differentiated from a TH1-like cell (producing IL-2, IFN-γ, but no IL-4) to a non-diabetogenic TH2-like cell (IL-4, IL-10, no IL-2) as a result of engagement of this MHC class II molecule. Our recent experiments with 4.1-NOD H-$2^{g7/q}$ mice, however, lend some support to this hypothesis; naive splenic $CD4^+$ T cells from 4.1-NOD H-$2^{g7/q}$ mice do not secrete TH2 cytokines in response to NOD islet cells *in vitro*, yet their pancreatic islets contained high levels of IL-4 (unpublished observations). While this observation does not imply that 4.1-

$CD4^+$ T cells undergo immune deviation *in situ*, it indicates that presence of $I\text{-}A^q$ can both delete the 4.1-TCR and promote the recruitment of IL-4-producing cells to the islets. Second, the small group of 4.1-H-$2^{g7/k}$-F2 mice which did not delete 4.1-thymocytes (40%) developed insulitis but remained diabetes-free, the explanation of which may, or may not, be related to the above reasoning with 4.1/$I\text{-}A^k$-transgenic NOD mice. Alternatively, and unlike the positive selection of the 4.1-TCR in thymi of 4.1-$I\text{-}A^{g7/o}$ mice, progression from a moderate to severe form of insulitis, and thus to overt diabetes, may require a double dose of $I\text{-}A^{g7}$ molecules (complexed with target beta cell-antigen) as proposed in non-transgenic models (31). Lastly, $I\text{-}A\beta^b$-4.1(NOD x B6)F1 mice developed insulitis but remained diabetes-free, and 4.1-(NOD x SJL/J)F1 mice, which also positively selected fully functional 4.1-thymocytes, developed neither insulitis nor diabetes. Whether diabetes resistance in these mice is due to hemizygosity for $I\text{-}A^{g7}$, to additional IDDM-resistance factors present in C57BL/6 and SJL/J mice, or to "dilution" of NOD-derived susceptibility factors, however, remains to be determined.

Whatever the nature and mechanism of these additional resistance factors turn out to be, the observations of this study have uncovered what seemed to be an unlikely proposition. Namely, the idea of a relationship between thymocyte selection of highly diabetogenic T cells and MHC-linked susceptibility and resistance to IDDM. The results described here do not suggest that negative selection is the only mechanism of resistance provided by MHC class II molecules, but they may provide an understanding of how individuals with protective MHC haplotypes on one chromosome (e.g. $I\text{-}A^b$ in mice, or DQA1*0102/DQB1*0602 in humans) can provide resistance in the presence of a susceptible MHC haplotype on the other chromosome (e.g. $I\text{-}A^{g7}$ in mice, or DQA1*0301/DQB1*0302 in humans). The processes of

positive and negative selection that the 4.1-TCR undergoes within the thymus also shed some light on our understanding of the rules that govern selection of highly pathogenic autoreactive receptors. The fact that this receptor cannot undergo positive selection on the MHC class II molecules that promote it's deletion in the thymic medulla, put forth the notion that sequential selection events in the thymus, at least for autoreactive TCRs, need not be restricted by the same MHC molecules.

CHAPTER SIX

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IMAGE EVALUATION
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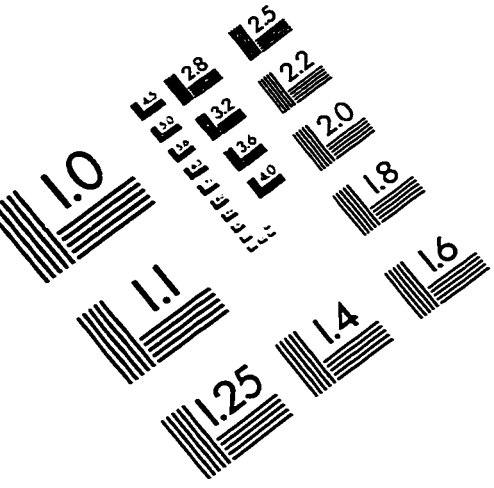

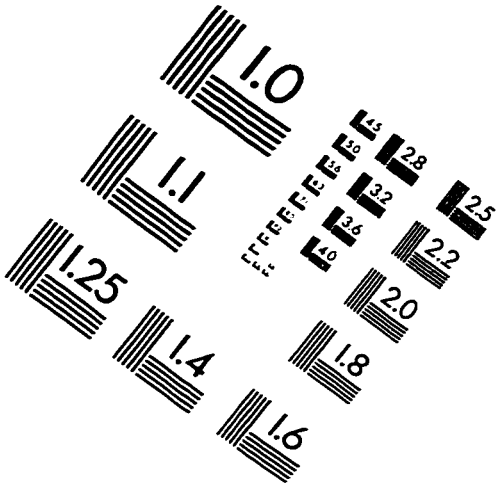

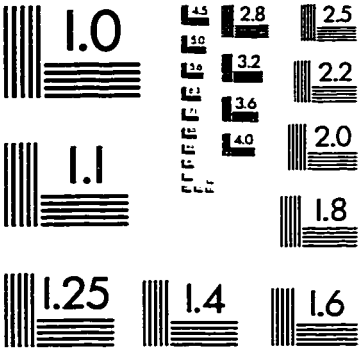

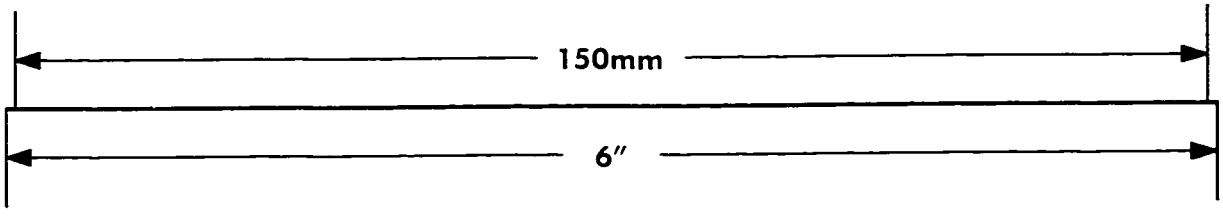

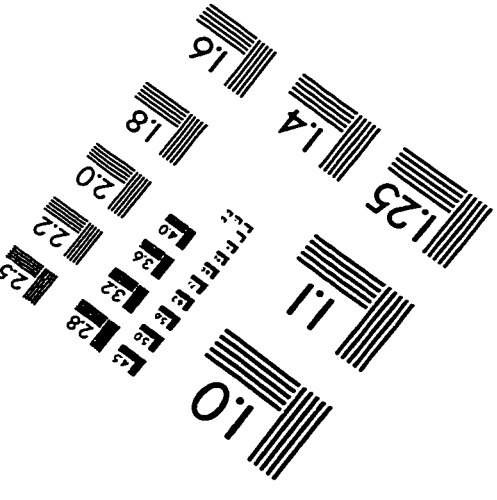

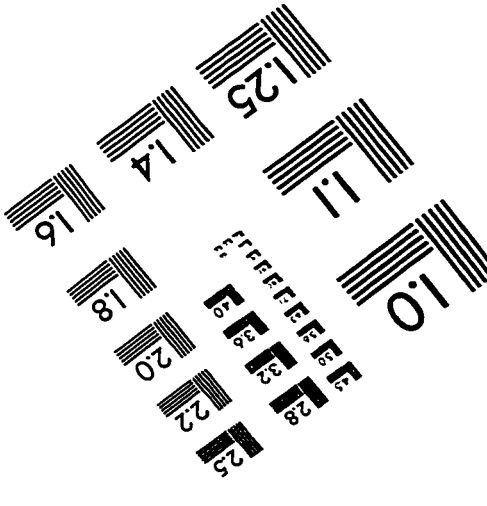

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