Failure to Censor Forbidden Clones of CD4 T Cells in Autoimmune Diabetes

Sylvie Lesage, Suzanne B. Hartley, Srinivas Akkaraju, Judith Wilson, Michelle Townsend, and Christopher C. Goodnow

Australian Cancer Research Foundation Genetics Lab, Medical Genome Centre, John Curtin School of Medical Research, Australian National University, Canberra ACT 2601, Australia

Abstract

Type 1 diabetes and other organ-specific autoimmune diseases often cluster together in human families and in congenic strains of NOD (nonobese diabetic) mice, but the inherited immunoregulatory defects responsible for these diseases are unknown. Here we track the fate of high avidity CD4 T cells recognizing a self-antigen expressed in pancreatic islet β cells using a transgenic mouse model. T cells of identical specificity, recognizing a dominant peptide from the same islet antigen and major histocompatibility complex (MHC)-presenting molecule, were followed on autoimmune susceptible and resistant genetic backgrounds. We show that non-MHC genes from the NOD strain cause a failure to delete these high avidity autoreactive T cells during their development in the thymus, with subsequent spontaneous breakdown of CD4 cell tolerance to the islet antigen, formation of intra-islet germinal centers, and high titre immunoglobulin G1 autoantibody production. In mixed bone marrow chimeric animals, defective thymic deletion was intrinsic to T cells carrying diabetes susceptibility genes. These results demonstrate a primary failure to censor forbidden clones of self-reactive T cells in inherited susceptibility to organ-specific autoimmune disease, and highlight the importance of thymic mechanisms of tolerance in organ-specific tolerance.

Key words: autoimmune disease • diabetes mellitus type I • clonal deletion • T lymphocytes • genetic predisposition to disease

Introduction

Autoimmune diseases that target specific organs such as the pancreatic islets, thyroid, adrenal glands, stomach, or nervous system, often cluster together in either different family relatives or occur simultaneously in one individual. High concordance among identical twins indicates a large inherited component in susceptibility to these diseases, and genetic studies reveal a complex pattern of interactions between MHC and non-MHC chromosomal regions (1). The nature of the inherited immunological defect(s) that leads to these diseases nevertheless remains obscure. On the one hand, a variety of lines of evidence suggest that these diseases may reflect excessive stimulation of autoreactive T cells by viruses, costimulatory molecules, or antigen-presenting cells that promote Th1 T cells (2–4). Alternatively these diseases may be related to observed heritable decreases in Th2 cells (5), regulatory T cells (3, 6), NKT cells

(7, 8), CD4+CD25+ regulatory T cells (9), or diminished thymocyte signaling (10) or thymocyte apoptotic responses measured in vitro (11). Defects in antigen presentation and peripheral censoring of autoreactive T cells by processes such as clonal anergy or activation-induced death have also been proposed (12–16). One of the principal barriers to identifying the primary inherited defects, however, has been the inability to track the fate of organ-reactive T cells directly in vivo and identify primary inherited aberrations in this fate in autoimmune susceptible individuals.

Inherited susceptibility to autoimmune disease in the NOD (nonobese diabetic)* mouse strain represents a key model to begin identifying the primary immunological lesions underlying the clustering of different organ-specific autoimmune diseases (17). NOD mice spontaneously develop type 1 diabetes due to autoreactive T cell destruction of pancreatic islet β cells. A particular allelic product of the NOD strain MHC, I-A^{g7}, is a key diabetes susceptibility

Address correspondence to Christopher C. Goodnow, Australian Cancer Research Foundation Genetics Laboratory, Medical Genome Centre, John Curtin School of Medical Research, Mills Rd., PO Box 334, The Australian National University Canberra, ACT 2601 Australia. Phone: 61-26125-3621; Fax: 61-2-6125-8512; E-mail: Chris.Goodnow@anu.edu.au

Abbreviations used in this paper: HEL, hen egg lysozyme; insHEL, transgenic mice expressing HEL under the rat insulin promoter; NOD, nonobese diabetic.

gene and shares unique sequence and structural motifs with diabetes-susceptible MHC gene products in humans (18, 19). Like humans, diabetes in NOD mice nevertheless requires many other susceptibility genes other than the MHC, and elegant mapping and congenic breeding strategies have delineated the complex nature of inherited susceptibility (1, 20, 21).

C57BL/6 or C57BL/10 strain mice, which are not especially prone to organ-specific autoimmunity, do not develop insulitis or diabetes when the NOD MHC haplotype is introduced by congenic breeding, underscoring the importance of other non-MHC genes. In fact, substituting a number of different non-MHC chromosomal regions from the C57BL/10 strain into the NOD strain by congenic breeding, either individually or in combination, is sufficient to partially or completely prevent development of insulitis or diabetes despite the continued presence of the NOD MHC alleles (1, 21, 22). Conversely, when a different MHC type is introduced into the NOD strain, as in the NOD.H-2^k congenic derivative, insulitis and diabetes are prevented but autoimmune thyroiditis is exaggerated (21, 23, 24). Introducing a defective B7.2 (CD86) costimulatory gene into NOD suppresses insulitis and diabetes but leads instead to spontaneous autoimmune damage of the peripheral nerves (25). These results point to a general susceptibility to organ-specific autoimmunity in NOD caused by a complex set of non-MHC genes, with alleles of MHC or costimulatory molecules influencing the specific organ targets of this general immunological defect (1, 21).

In addition to the complicated inheritance of autoimmune susceptibility, the complexity and heterogeneity of the normal T cell repertoire also represents a barrier to visualizing the primary immunological defects underlying autoimmune susceptibility. Self-reactive T cells can be readily demonstrated in the circulation and peripheral tissues of animals and individuals with no apparent predisposition to autoimmune disease, but the heterogeneity in TCR specificity and affinity and the need to use functional response assays to measure these cells makes it difficult to interpret the presence of these cells. Censoring of self-reactive T cells through the induction of clonal deletion or clonal anergy in the thymus and in peripheral lymphoid tissues requires a critical threshold of antigen/MHC and TCR affinity to be triggered. For organ-specific antigens that are present in only trace amounts in the thymus or circulation, many T cells fall below this threshold and escape thymic deletion (26–32) because either the T cells have lower affinity or TCR density, the peptide/MHC combinations they recognize are less efficiently presented, or they recognize unique epitopes that are only generated in the peripheral organ.

Three different TCR transgenic models that employ TCRs from islet reactive clones that could be grown in vitro from autoimmune mice represent examples of clones that fall below this thymic selection threshold, although the islet antigens recognized by these TCRs are not yet known (33, 34). Peripheral deletion or other regulatory mechanisms may normally prevent T cells of this type from be-

coming activated (15, 35). Nevertheless, many organ-specific antigens do reach the circulation and thymus in low amounts (eg. insulin, thyroglobulin), and a great number appear to be made there by rare thymic medullary epithelial cells (36, 37). Efficiently presented dominant peptides from these self-antigens can be shown to censor reactive T cells by thymic deletion and by causing selective export of anergic CD4$^+$CD25$^+$ cells that may be responsible for regulating the clones that fall below these thymic selection screens (32, 38–46). The relative roles of thymic and peripheral mechanisms for achieving organ-specific tolerance and the pathogenesis of autoimmune disease thus remains a key unresolved issue.

To study how the (non-MHC) general autoimmune susceptibility genes in NOD mice affect the acquisition of tolerance by organ-specific CD4 T cells, we sought to compare the fate of islet-reactive CD4 T cells bearing a well-defined high avidity TCR in a TCR transgenic mouse model, when bred to H-2 matched strains with an underlying autoimmune susceptible or resistant background. By holding the TCR, the islet antigen and the H-2–presenting molecules constant, and varying the non-MHC background genes, we show here that non-MHC genes from the NOD strain act within high avidity autoreactive CD4 T cells to cause them to escape clonal deletion in the thymus and reach the peripheral lymphoid organs and pancreatic islets in greatly elevated numbers.

Materials and Methods

Mice, Antibodies, Histology. 3A9 TCR transgenic (47) and ILK-3 Ins-HEL transgenic mice (32) produced in C57BL/6J were backcrossed to B10.Br/SgSnJ (JAX) or NOD.H2^k (48) (gift from L. Wicker, Cambridge University, Cambridge, UK). Data presented are from N5-N10 mice. TCR, transgenic mice expressing HEL under the rat insulin promoter (insHEL), and H-2 genotype of each mouse was tested twice by PCR (32). Urine glucose was tested using Testape at biweekly intervals or when cage was wet. Mice with two successive positive Testape were called diabetic, euthanized, and diabetes confirmed by blood glucose measurement. Nondiabetic mice were culled at 6 mo. From each mouse, one-half pancreas was fixed in 10% formalin, paraffin embedded, and stained with H&E, while the other half was snapped frozen in OTC (Tissue Tek) in liquid nitrogen. Frozen sections were stained with peanut agglutinin biotin/streptavidin-alkaline phosphatase/Fast Blue (all from Vector Labs) and with sheep anti–mouse IgD/anti–sheep HRP (both from The Binding Site)/DAB. HEL-binding IgG was measured in serum by ELISA on Nunc maxisorp plates coated with 100 μg/ml HEL, developed with sheep anti–mouse IgG-alkaline phosphatase (Southern Biotech Associates, Inc.) and Sigma Substrate 104. A reference pool of immune sera from HEL primed and boosted B6 mice was set to contain 100 arbitrary units. Radiation chimeras were constructed as described previously (32), using a total of 2 × 10^6 bone marrow cells to reconstitute recipients irradiated with 5.5Gy twice separated by 3 h. Recipients were analyzed 6–10 wk after reconstitution, therefore diabetes was not monitored in these mice.

Flow Cytometry. 6–12-wk old nondiabetic mice (Testape negative) were analyzed. Thymus and spleen were passed

through a sterile cell strainer. Lymphocytes from pancreatic lymph nodes were obtained by pressing between two glass slides. Liberase R1 (Boehringer) was injected into the common bile duct of double transgenic mice to inflate the pancreas. The pancreas was then incubated 20 min at 37°C, and washed with a solution containing FCS. Islets were handpicked and dissociated using a protein gel-loading pipette tip. The following antibody stains were used: mouse IgG1 anti-clonotypic 1G12 antibody (49) (gift of E. Unanue and D. Peterson, Washington University, St. Louis, MO) as culture supernatant followed by rat monoclonal anti–mouse IgG1 conjugated to FITC or allo-phycocyanin (BD PharMingen); anti–CD8-PE (Caltag); anti-CD4 allo-phycocyanin, anti-CD3, anti-CD69, anti-Va2, anti-CD45.1-biotin, and anti-CD45.2-biotin (all from BD PharMingen), anti-CD24 biotin (Caltag), anti-Vb8 biotin (clone F23.1), and streptavidin-PerCP (BD). Data was collected on a Becton Dickinson LSR, and analyzed with WinList™, CELLQuest™ PRO, and FlowJo™.

Results

To track how the acquisition of CD4 T cell tolerance to organ-specific self-antigens is altered by the general autoimmune susceptibility genes from NOD, we used an experimental model involving a cross between two transgenic mouse strains. In the 3A9 TCR transgenic strain (47) many CD4 T cells carry the same TCR recognizing a normally foreign antigen, hen egg lysozyme (HEL) peptide 46–61 bound to I-A^k (50). HEL 46–61 is the dominant peptide epitope presented by I-A^k, and its high avidity binding to I-A^k and recognition by the 3A9 TCR (Vb8.2/Va3) are very well characterized (50). T cells bearing the 3A9 TCR can be readily detected in vivo with a clonotypic anti-TCR mAb developed by Peterson and Unanue (reference 49, Fig. 2). The insHEL transgenic strain synthesizes high concentrations of membrane-bound HEL as a self-antigen in pancreatic islet β cells under control of the rat insulin promoter (32). Trace quantities of cleaved HEL are present in the circulation, mirroring the distribution of insulin itself (32). When the two transgenic strains are intercrossed to make TCR/insHEL double-transgenic progeny, the large population of HEL-reactive T cells now reacts with the islet-specific self-antigen, and the fate of these T cells can be readily studied.

Previously we showed that TCR/insHEL double transgenic mice on the (B6×B10.BR)F1 background remain free of diabetes, but develop early and extensive insulitis that remains subclinical or "benign" (32). The insulitis was explained by thymic export of large numbers of CD4 T cells that had reduced but not lost their responsiveness to HEL so that high concentrations of HEL such as exist in the islet could elicit a weak response by most of the cells (32). The weakened responsiveness of the T cells reflected two changes: (i) selective downregulation of the transgene-encode TCR Va3/Vb8.2 pairs, while still retaining a sufficient density of TCRs to allow survival by coexpressing a second TCR-α chain; and (ii) an independent diminished responsiveness of the T cells to TCR stimulation revealed by stimulating all the surface TCRs with anti-Vb8.2 antibody (32).

Spontaneous Progression of Insulitis to Form Germinal Centers and Autoantibody. We backcrossed the TCR and insHEL transgenic strains to two H-2^k matched congenic partner strains: NOD.H-2^k (reference 48; referred to below as NOD^k) which carries all the autoimmune susceptibility alleles of NOD except the H-2, and hence normally lacks insulitis or diabetes but is susceptible to autoimmune thyroiditis and sialitis; and B10.BR (referred to below as B10^k), which carries diabetes resistance alleles at most non-MHC diabetes loci (51). Then, we intercrossed mice carrying TCR and insHEL transgenes to produce TCR/insHEL double transgenic mice of three genetic makeups: NOD^k, B10^k, and (NOD^k × B10^k)F1.

The effects of the different strain backgrounds were first determined by studying large cohorts of female TCR/insHEL animals and littermate controls until 24 wk old. No autoimmune diabetes or insulitis developed in NOD^k, B10^k, or F1 hybrid progeny carrying either the TCR or insHEL transgenes alone, or neither transgene. The lack of insulitis or diabetes in the NOD^k animals reflects the inhibitory effects of this H-2 haplotype on islet-specific autoimmunity in NOD mice (but not on thyroid or salivary autoimmunity; references 21 and 51). By contrast all TCR/insHEL double transgenic mice had extensive lymphocytic infiltrates in pancreatic islets by 6 wk old regardless of the strain background, and this inflammation persisted to the 24-wk time point (Fig. 1 A). As discussed above, the spontaneous insulitis in double-transgenic mice on the autoimmune resistant strain backgrounds (B10^k and F1) can be explained by the presence of HEL-reactive TCRs on most of the peripheral CD4s, albeit expressed at reduced cell surface density and weak responsiveness to antigen. It is important to note, however, that the populations of T cells and IgD$^+$ resting B lymphocytes in these chronically inflamed islets reflects the lymphocyte populations in the circulation (Fig. 1, and data not shown), and the majority probably accumulate in these sites secondary to chronic inflammation and independently from activation or any specificity for the inciting HEL antigen. The presence of infiltrating cells therefore give little information about the frequency or responsiveness of the minority of HEL-specific Th cells among them.

In contrast to the absence of autoimmune diabetes in insHEL or TCR animals on the NOD^k background, 90% of TCR/insHEL double transgenic mice developed diabetes on this background. Diabetes onset occurred over a broad age range from 8–24 wk old (Fig. 1 B) and was accompanied by formation of large germinal centers in the pancreatic islets (Fig. 1 A) and spontaneous secretion of high titre anti-HEL IgG1 autoantibodies in the serum (Fig. 1 C). Only 20% of B10^k TCR/insHEL animals developed diabetes, and this appeared to reflect a distinct disorder because it developed only during a narrow age window in mice <12 wk old, and none of these animals produced measurable anti-HEL IgG autoantibody in their serum (Fig. 1 C) or had discernible germinal centers in the islets. Consistent with a different genetic basis, the B10^k form of low-incidence/antibody-free diabetes was completely suppressed by

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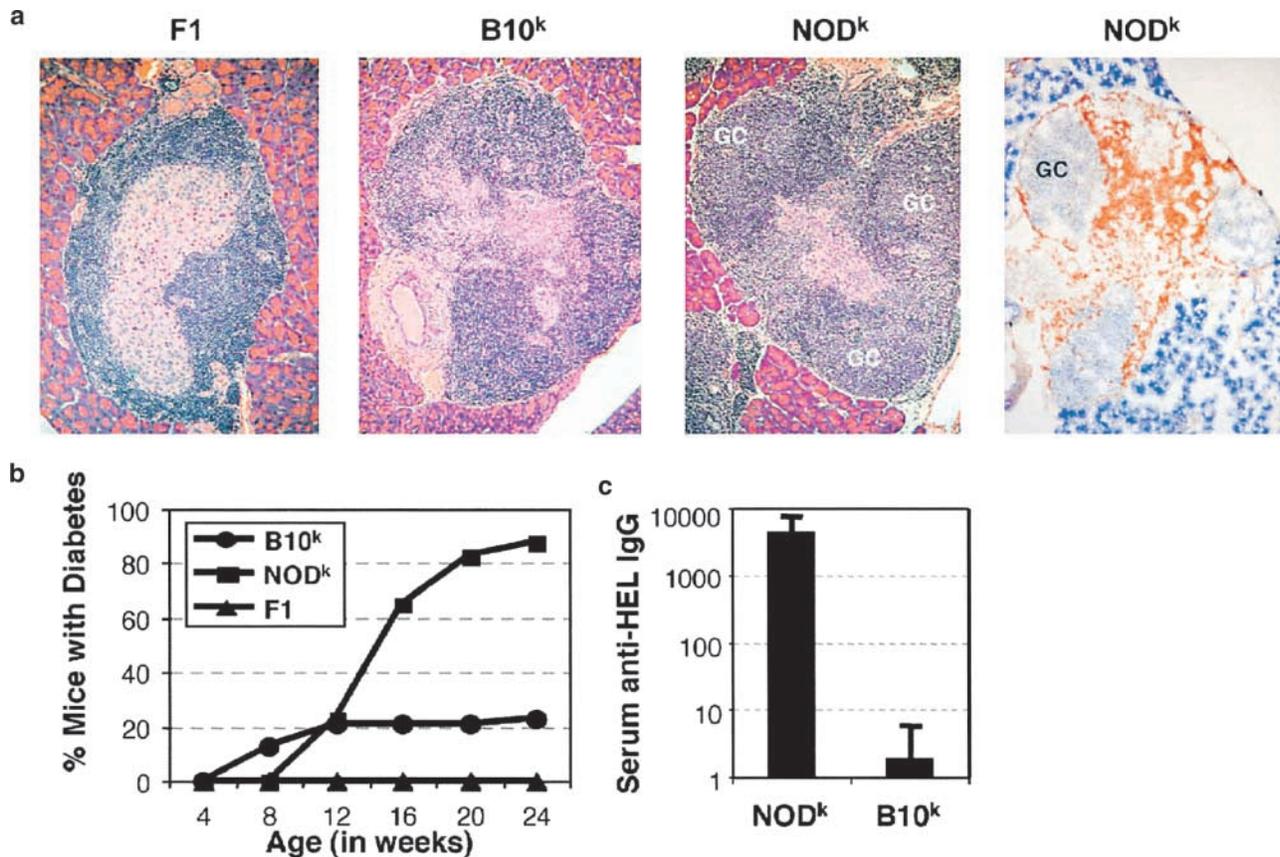
Figure 1. Islet-reactive T cells cause germinal center formation, autoantibodies, and diabetes in NOD[k] mice. (a) Pancreatic islets stained with H&E from TCR/InsHEL mice of the indicated genotypes. Right panel is a frozen section stained with peanut agglutinin (blue) and anti-IgD (brown). GC, germinal center. (b) Onset of diabetes in TCR/insHEL female mice of the indicated genotypes. Data represent >20 mice per group. (c) HEL-binding IgG titre in serum from diabetic double transgenic mice, collected within 2 wk of diabetes onset. Titres are expressed in arbitrary units relative to a reference immune sera from HEL-immunized nontransgenic mice set at 100 units.

one full set of non-MHC genes from NOD in F1-hybrids, and likewise the NOD[k] form of high incidence/high autoantibody disease was fully suppressed by non-MHC B10 genes (Fig. 1 B).

Defective Censoring of Islet Reactive CD4 Cells in NOD[k]. The progression to diabetes, formation of germinal centers, and secretion of anti-HEL IgG in NOD[k] double transgenic mice indicated that the 3A9 HEL-reactive CD4 T cells either failed to be tolerized or broke out of tolerance as a result of non-MHC NOD genes. To explore this issue, CD4 T cells bearing the 3A9 anti-HEL TCR were measured in different tissues of young mice before the onset of diabetes by flow cytometric staining with the 1G12 anti-clonotypic antibody (Fig. 2). In TCR-transgenic mice lacking insHEL antigen, a high frequency of CD4$^+$ TCR clonotype$^+$ cells was present in the spleen and lymph nodes regardless of the strain background. By contrast, while the frequency of peripheral CD4$^+$ TCR clonotype$^+$ cells was reduced in all TCR/insHEL double-transgenic mice, much higher numbers of islet reactive T cells were present in the spleen, pancreatic lymph nodes, and pancreatic islets in the NOD[k] animals. The failure to eliminate islet reactive T cells in NOD

appears to be a recessive defect as it was fully corrected in (NOD[k] × B10[k])F1 hybrid animals.

Receptor downregulation is an important means of diminishing antigen reactivity, and almost all CD4$^+$ T cells in the periphery of TCR/insHEL B10[k] mice display half the surface density of CD3 and Vβ8 that are found on TCR transgenic mice without the negative-selecting antigen (Fig. 3 A and Fig. 4). Most of these T cells carry the transgenic Vα3 chain on their surface, but at 10-fold reduced levels compared with TCR cells (Fig. 3 B) so that they are difficult to detect by staining with the clonotypic 1G12 antibody (Fig. 3 A). Since transgenic Vβ8 chains on these cells are only reduced by 50%, the remaining receptors must contain a second TCR-α chain. This conclusion is supported by the fact that Vα2 chains are expressed by a fraction of these cells, and by the finding that the level of Vα2 displayed on their surface is about half that found on nontransgenic mice (Fig. 3 C). By contrast, very little decrease in CD3 or Vβ8 occurred on peripheral CD4 T cells from NOD[k] TCR/insHEL mice, and a large fraction still express high surface densities of clonotypic receptor 1G12 (Fig. 3 A). Thus, insHEL caused a dramatic reduction in
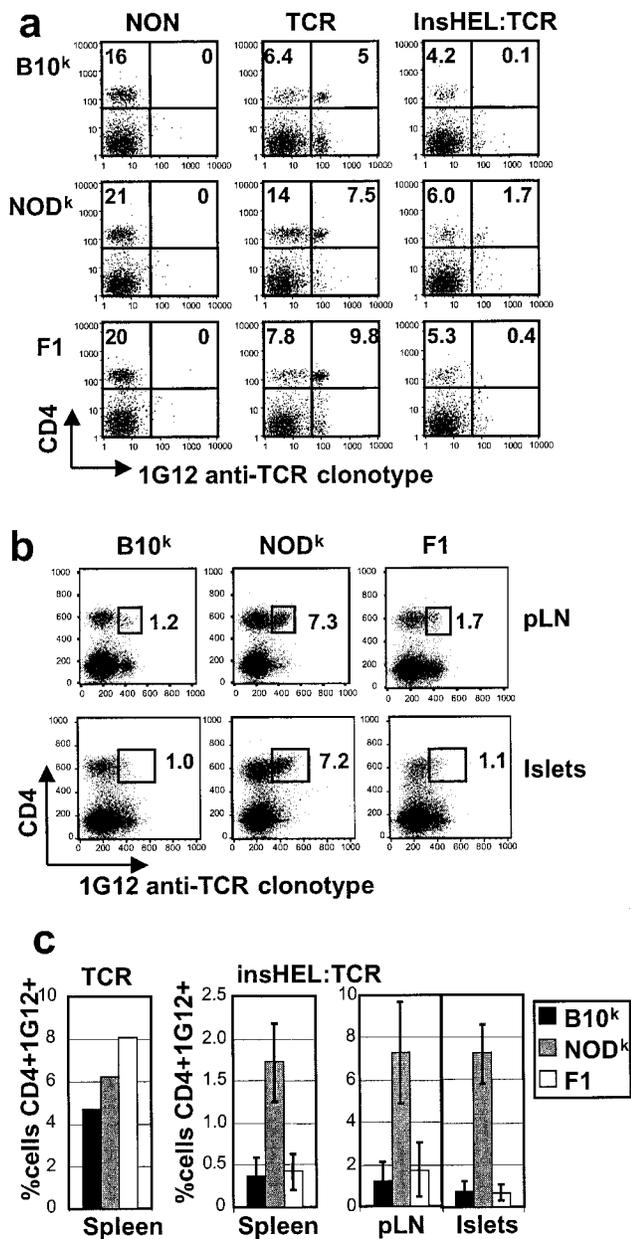
Figure 2. Failure to eliminate islet-reactive CD4 T cells in NOD[k] mice. (a) Spleen cells from 6–12-wk old nondiabetic female mice of the indicated genotypes were stained with 1G12 anti-TCR clonotype and antibody to CD4. The percentage of lymphocytes falling within the quadrants is shown. (b) The same analysis performed on cells from pancreatic lymph nodes (pLN) and pancreatic islets of 6–12-wk old nondiabetic TCR/insHEL mice. (c) Data collected as above compiled from multiple animals of the indicated genotypes.

the avidity of peripheral T cells reacting with HEL on the B10[k] background, but induced little change compared with TCR-transgenic animals without HEL on the NOD[k] background.

A similar difference in HEL–reactive TCR expression was exhibited by the lymphocytes that infiltrated pancreatic islets of NOD[k] and B10[k] mice. Like the circulating T cells, infiltrating CD4^{+} T cells show less reduction in CD3 or Vb3 in

NOD[k] TCR/insHEL animals compared with the B10[k] counterparts (Fig. 4). Consistent with a difference in responsiveness to HEL made in the pancreatic islets, clonotype-positive CD4 cells specifically increased expression of CD69 in the islets relative to their levels in lymph nodes or spleen (Fig. 5). Clonotype-negative cells in the same islet infiltrates showed no induction of CD69 (Fig. 5). Likewise, no specific increase in CD69 occurred on clonotype-positive CD4 cells in the islets of B10k double transgenic mice (Fig. 5). While these data indicate a difference in the number of high avidity autoreactive T cells homing to the islet, the majority of islet infiltrating cells are nevertheless likely to have been attracted independently of their antigen specificity, since the infiltrate is broadly composed of B cells, clonotype-positive and -negative CD4 and CD8 cells (Fig. 1, and data not shown).

Defective Thymic Deletion of Islet Autoreactive T Cells. The presence of large numbers of high avidity T cells in NOD[k] insHEL/TCR animals could reflect expansion of these cells as a result of dysregulated peripheral tolerance mechanisms, or failure to delete these cells in the thymus. Analysis of thymocytes from the double-transgenic mice showed a failure to eliminate high avidity islet reactive T cells during their development in the thymus (Fig. 6).

In TCR-transgenic mice lacking insHEL, strong positive selection is evidenced by the skewing to form a large population of CD4^{+}8^{−} single-positive thymocytes (Fig. 6 A). In both NOD[k] or B10[k] backgrounds most of these single-positive thymocytes bear high surface densities of the 3A9 TCR based on 1G12 staining (Fig. 6 B). In double-transgenic B10[k] mice expressing insHEL, high avidity HEL-reactive T cells are negatively selected during the CD4^{+}8^{+} double-positive and early single-positive stages. Thus, the total number of double-positive cells is reduced ~50% and the number of CD4 single-positive cells is dramatically decreased (Fig. 6, A and C). The CD4 single positives that mature in these thymi carry greatly reduced clonotypic TCR (Fig. 6 B and Fig. 7) and a smaller reduction in surface CD3 and Vβ8 levels (Fig. 7), comparable to the CD4 cells found in the periphery of these mice (Fig. 3).

In double transgenic mice on the NOD[k] background, however, there is no reduction in the numbers of double-positive cells and CD4 single-positive cells continue to be formed in approximately the same frequency as in TCR littermates (Fig. 6 A). These autoreactive single positive thymocytes show no evidence of selection for low avidity cells, as they continue to express high densities of clonotypic TCR (Fig. 6 B) and high surface levels of CD3 and Vβ8 (Fig. 7). As a result, whereas almost no high avidity CD4 T cells recognizing islet HEL are formed in the thymus of B10[k] double transgenic mice, these cells continue to be formed in NOD[k] double transgenic thymi in numbers that are only slightly reduced compared with animals lacking the autoantigen (Fig. 6 D).

In theory, the presence of CD4^{+}1G12^{+} cells in the thymus of TCR/insHEL NOD[k] mice could have resulted from a peripheral tolerance defect, allowing recirculation of mature CD4^{+} cells back to the thymus after activation and expansion in the periphery. Three lines of evidence exclude
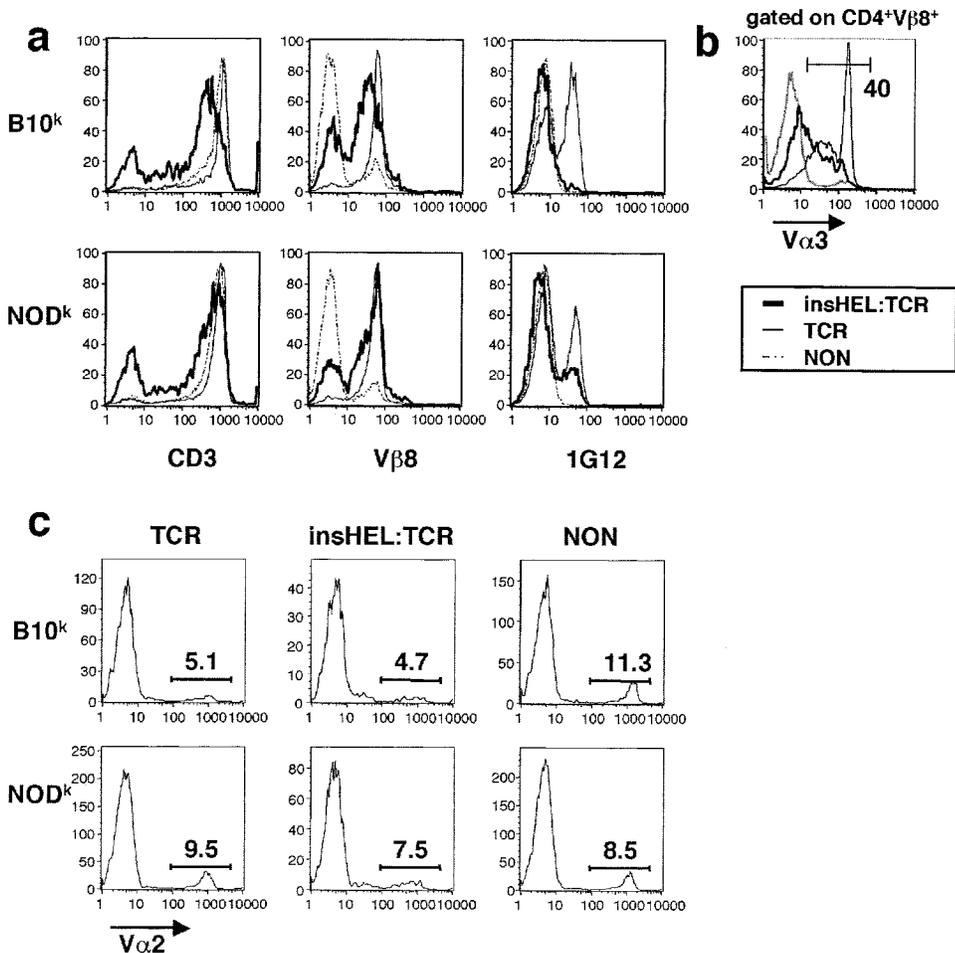
Figure 3. Failure to downregulate TCR expression in splenocytes of NOD[k] animals. (a) Spleen cells from nondiabetic female mice of various genotypes were stained with antibodies to CD4, CD3, Vβ8, and 1G12 clonotype. Histograms show profiles of CD4-positive cells from mice of the indicated genotypes. (b) Vα3 expression on CD4^{+}Vβ8^{+} spleen cells from B10[k] mice of the indicated genotypes. (c) Staining for an endogenous TCR-α chain, Vα2, on CD4^{+} spleen cells. The percentage of CD4^{+} cells that are Vα2^{+} is indicated.

this possibility. First, negative selection is evident within the double-positive population in B10[k] but not in NOD[k] thymi. Second, high avidity autoreactive cells are present in much higher numbers in NOD[k] thymi of mice of various ages, and they do not accumulate with age as would be expected for peripheral expansion (Fig. 8 A). Third, all of the clonotype-high single positive thymocytes are CD24 (heat stable antigen)[hi], indicating that they are immature recently formed T cells that have failed to be deleted, and not mature cells that have homed back (Fig. 8 B).
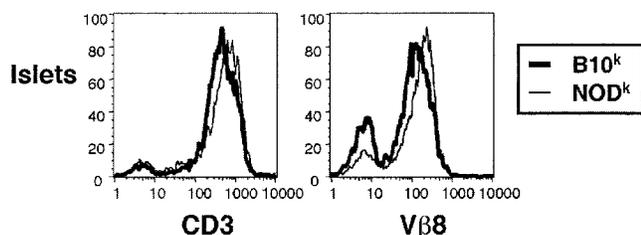
Figure 4. High TCR levels on lymphocytes in the islets of NOD[k] mice. Islets from nondiabetic female mice were purified and analyzed as in Fig. 3 a.

T Cell Intrinsic Action of NOD Genes on Thymic Deletion to Islet Antigen. The failure to delete islet-HEL specific T cells in the NOD[k] double-transgenic mice could reflect differences in the expression or presentation of insHEL to T cells in the thymus, or differences in the way the immature T cells respond to self-antigen. To distinguish between these possibilities, we constructed radiation chimeras using mixtures of bone marrow from TCR-transgenic mice of B10[k] and NOD[k] backgrounds to reconstitute lethally irradiated insHEL or nontransgenic recipients (Fig. 9 A). Leukocytes from the two donor strains could be distinguished by expression of different allelic forms of the Ly5 (CD45) surface protein. F1 hybrid recipients were used in most experiments, thus allowing any radioresistant host T cells to be distinguished as Ly5[ab] double-positive, compared with the donor-derived T cell populations which carry only one or other marker. In preliminary studies using nontransgenic donor bone marrow, we found that NOD[k]-derived T cells preferentially expand within the CD4^{+}8^{+} double-positive population of mixed chimeras (Fig. 9 B). The preferential expansion of NOD[k]-derived cells could be offset by reconstituting with bone marrow mixtures containing a 4:1 ratio of
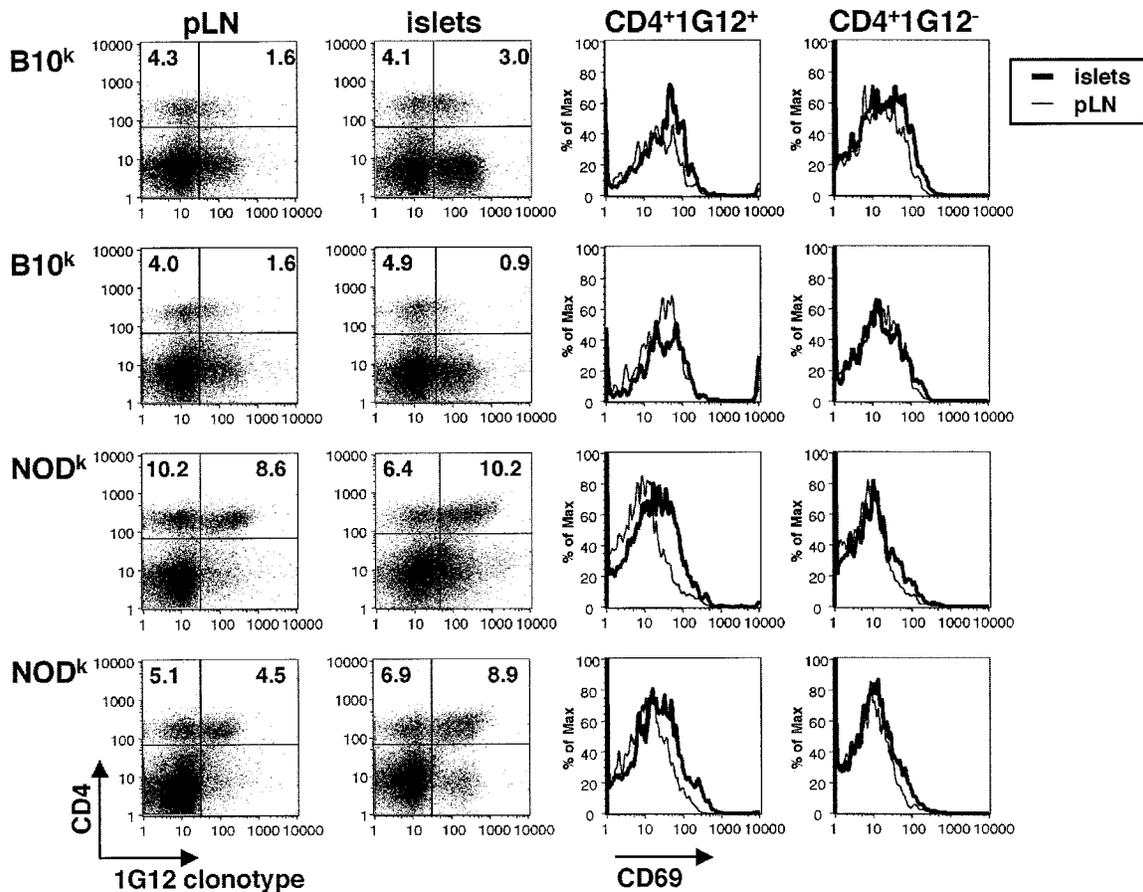
Figure 5. T cell activation in the islets is specific to CD4$^+$clonotype$^+$ NOD^k T cells. Lymphocytes from pancreatic lymph nodes and islets of two non-diabetic TCR/insHEL female mice of B10^k or NOD^k background were stained with CD4, 1G12 clonotype, and the activation marker CD69. The percentage of lymphocytes falling within the quadrants is indicated. Histograms show profiles of CD69 expression on clonotype-positive or clonotype-negative CD4 cells, overlaying cells from the islet and pancreatic lymph node of the same animal.

B10^k to NOD^k marrow (Fig. 9 C). Using 4:1 mixtures of TCR transgenic marrow, chimeras were obtained that formed comparably sized populations of single-positive T cells and peripheral CD4$^+$ 1G12$^+$ T cells in the absence of insHEL (InsHEL$^-$; Fig. 9, D and E).

When the same mixtures reconstituted negatively selecting thymuses of insHEL-transgenic recipients, by contrast, B10^k-derived T cells were selectively reduced in frequency in the double-positive compartment and very few B10^k single-positive T cells or CD4$^+$clonotype$^+$ T cells remained. NOD^k-derived DP and single-positive T cells in the same thymuses were not markedly deleted despite bearing the same TCR and recognizing the same antigen/MHC combination (Fig. 9 D). As a result, despite the chimeric mice being reconstituted with a fourfold excess of B10^k bone marrow, islet-reactive CD4 T cells in spleen and pancreatic lymph node of the chimeric mice were almost entirely of NOD^k origin (Fig. 9 E). Thus, non-MHC genes from NOD act cell autonomously within high avidity autoreactive T cells to make them insensitive to thymic negative selection.

Discussion

This study shows that self-reactive CD4 T cells bearing a high affinity TCR for a dominant epitope of HEL fail to be effectively censored during their development in the thymus of NOD^k mice that are making HEL in pancreatic islet β cells. The primary effect of the non-MHC genes from NOD is within the high affinity autoreactive T cells, causing them to be insensitive to clonal deletion stimuli in the thymus at the double-positive and CD4$^+$CD24^{high} single-positive stages. In conjunction with this NOD defect in thymic deletion, HEL-specific T cells specifically respond to antigen in the pancreatic islets where T cell help becomes available, after a variable time-lag of weeks or months, as evidenced by spontaneous development of germinal centers and large amounts of HEL-binding IgG1 autoantibody, and gradual progression to diabetes. These results define an important primary defect in the thymic selection and acquisition of T cell self-tolerance that is likely to play an important part in the general autoimmune susceptibility of NOD mice. The findings focus attention on thymic selection mechanisms for organ specific toler-
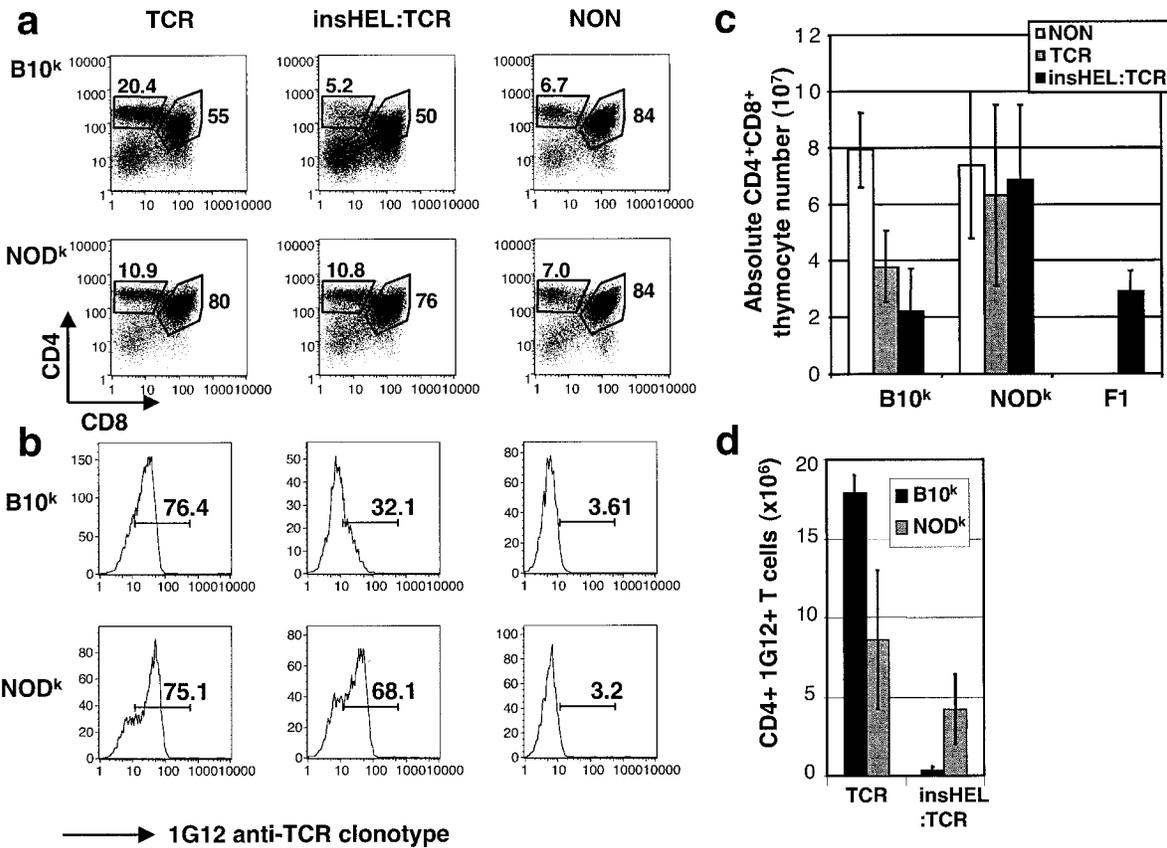
Figure 6. Failure to delete islet-reactive T cells in the thymus of NOD^k animals. (a) Thymocytes from 6–12-wk old nondiabetic female mice of the indicated genotypes were stained with 1G12 anti-TCR clonotype, and antibodies to CD4 and CD8. The percentage of CD4$^+$CD8$^-$ and CD4$^+$CD8$^+$ thymocytes are shown. (b) The 1G12 profiles are shown for CD4$^+$CD8$^-$ mature thymocytes. 1G12 staining cannot be detected on CD4$^+$CD8$^+$ thymocytes. (c) Absolute number of CD4$^+$CD8$^+$ thymocytes was calculated for each group. From left to right, there were exactly 4, 6, 16, 3, 5, 13, and 10 mice, respectively. (d) Compilation of the number of CD4$^+$1G12$^+$ thymocytes in each group.

ance, and raise subsequent questions about how this defect might arise and how it is connected to other genetic and immunological defects described in mice and humans with organ-specific autoimmune disease.

Negative selection of self-reactive thymocytes is strikingly altered by non-MHC NOD genes acting within the T cells. In the presence of insHEL self-antigen, HEL-specific T cells bearing B10 genes showed a marked deletion
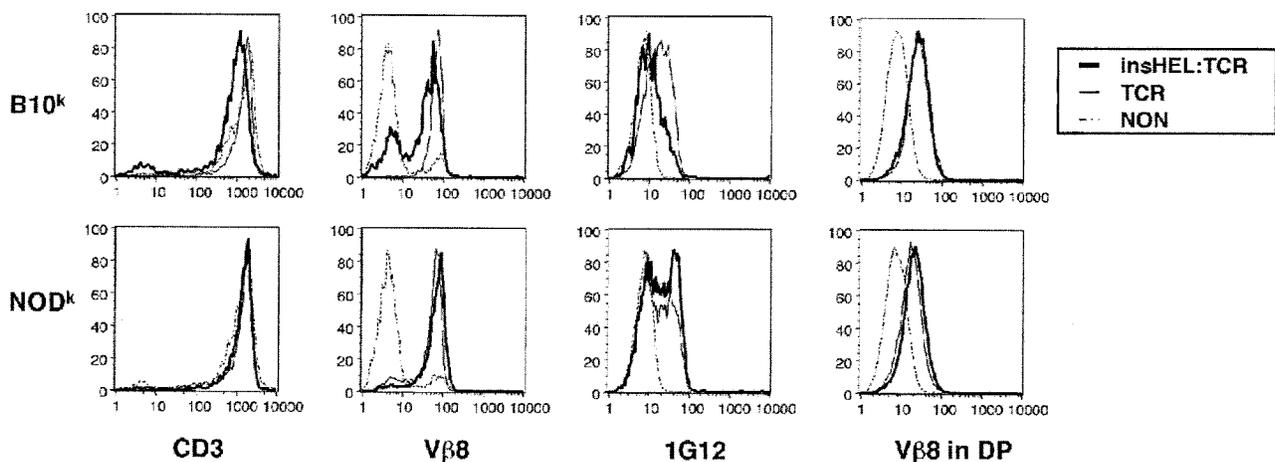
Figure 7. Failure to downregulate TCR expression in CD4$^+$ thymocytes of NOD^k animals. (a) Thymocytes from nondiabetic female mice of various genotypes were stained with antibodies to CD4, CD8, and 1G12 clonotype, together with either CD3 or Vβ8. All histograms show profiles of CD4$^+$CD8$^-$ cells, except for the graphs on the right which are gated on CD4$^+$CD8$^+$ (DP) thymocytes.
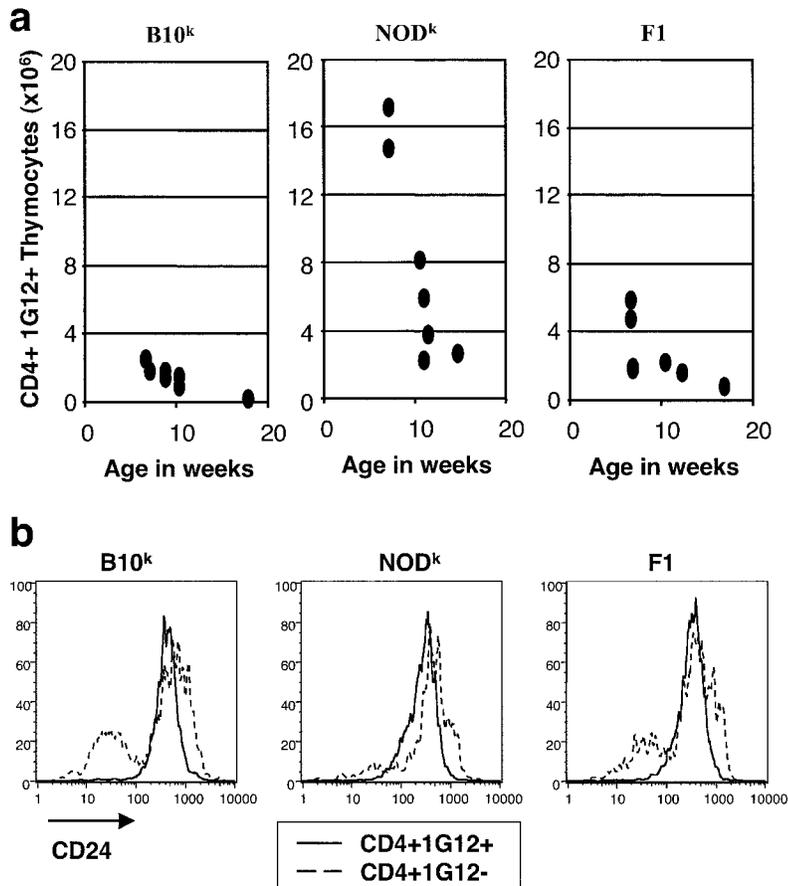
Figure 8. Increased CD4^{+}1G12^{+} thymocytes in NOD[k] insHEL/TCR mice is not due to mature T cell recirculation to the thymus. (a) Absolute number of CD4^{+}1G12^{+} mature thymocytes in insHEL/TCR double transgenic on B10[k], NOD[k], and F1 background plotted versus age. (b) Thymocytes from insHEL/TCR double transgenic mice were stained with 1G12, CD4, CD8, and CD24. CD24 profiles are shown for 1G12-positive or –negative CD4^{+}CD8^{−} mature thymocytes.

in their numbers at the double-positive stage and relatively few reached the single-positive stage (Figs. 6 and 7). By contrast, T cells bearing the NOD genes showed little reduction in double-positive thymocytes and only a modest decrease in single-positive thymocytes bearing the HEL-specific TCR. Three lines of evidence establish that thymic negative selection is the primary defect, and exclude the possibility that the elevated numbers of CD4 single-positive T cells in the NOD[k] thymus represent mature T cells that have expanded in the periphery and then homed back to the thymus. Foremost, the CD4^{+}1G12^{+} cells of NOD[k] InsHEL/TCR thymus are entirely immature CD24^{hi} cells, indicating they are recently differentiated rather than mature cells that have returned (Fig. 8). Second, deletion is evident within the less mature CD4^{+}8^{+} cells in the B10[k] animals but not in NOD[k] (Fig. 6). Third, the numbers of escaped CD4^{+}1G12^{+} thymocytes is highest in very young mice an diminishes with age (Fig. 8), whereas the opposite might be expected if the cells must first be expanded in the periphery. The mixed bone marrow chimera experiments exclude the possibility that differences in the types of antigen-presenting cells or efficiency or types of antigens presented to the T cells account for the failure to censor the thymic and peripheral autoreactive T cell populations, and demonstrate that NOD[k] genes act within individual immature T cells to dramatically change the way

they respond to TCR engagement by negative selecting autoantigen.

The thymic selection defects defined here are paralleled by results from Kishimoto and Sprent (11) who have shown that thymocytes from NOD mice are selectively resistant to in vitro or in vivo negative selection induced by anti-TCR and anti-CD28 antibodies or by bacterial superantigens, when compared with B6-H-2[nod] congenic controls. Since it is unclear how well anti-TCR or superantigen responses mirror the acquisition of tolerance to self-antigens, our findings clearly establish that NOD genes dramatically interfere with tolerance by negative selection in vivo, as well as showing that these inherited defects act cell autonomously within individual autoreactive CD4 cells.

The chief difference between the two analyses is the finding by Kishimoto and Sprent that NOD thymocyte apoptosis defects are only detectable in the DP-SP transitional cell population in vitro, with normal apoptosis of double-positive thymocytes. By contrast, NOD genes prevented islet antigen induced deletion in DP and SP populations in vivo. This may reflect differences in the strength and quality of signals provided by plate-bound antibodies compared with antigen-presenting cells bearing trace amounts of self-antigen. Of potentially greater significance, however, is the finding here that peripheral autoreactive T cells with the NOD genes fail to exhibit the modulation of
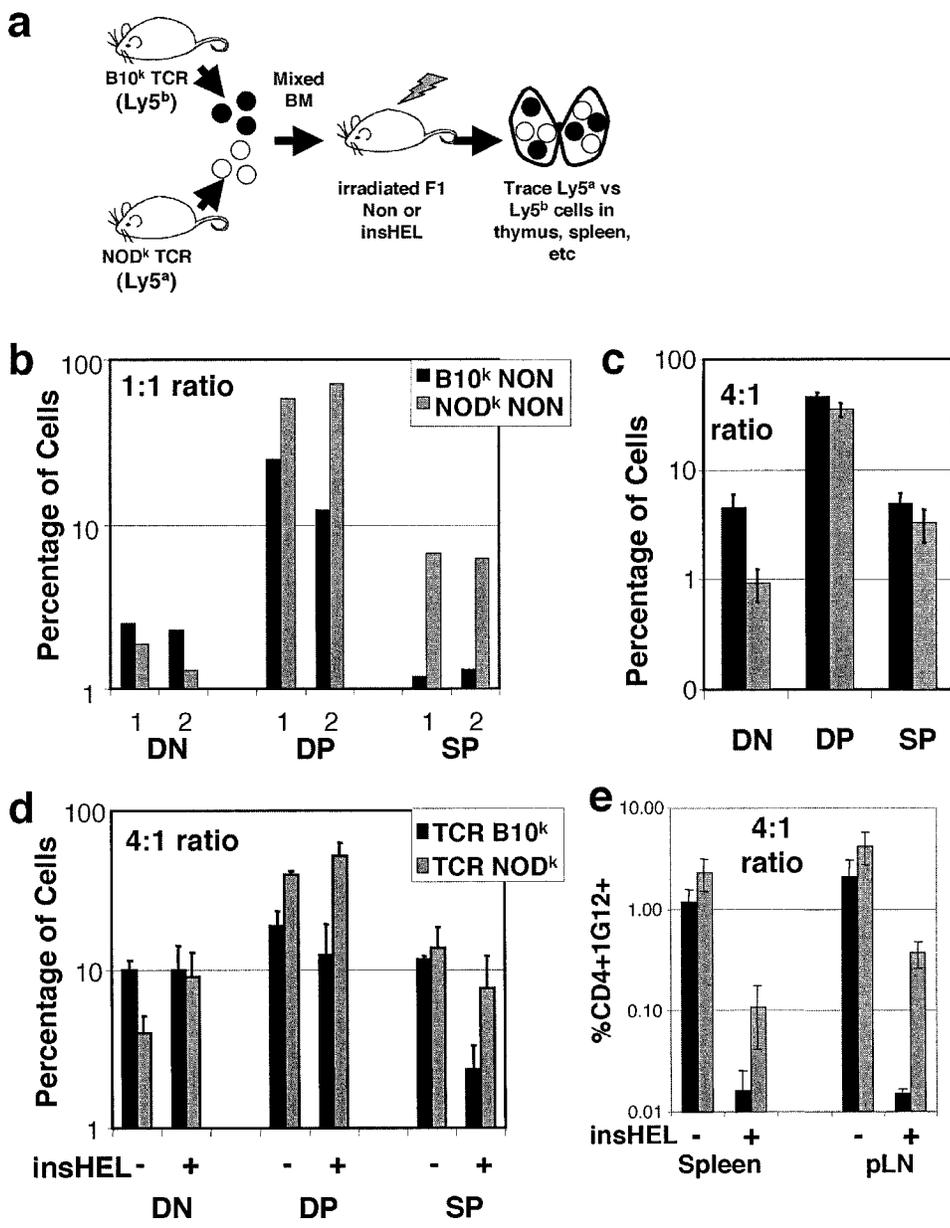
Figure 9. Failure to delete islet-reactive cells in NOD^k is T cell intrinsic. (a) Design of bone marrow chimera experiment. (b) Nontransgenic Ly5^{ab} recipients were reconstituted with a 1:1 mixture of bone marrow from B10^k and NOD^k nontransgenic mice. The thymocytes were stained with CD4, CD8, Ly5^a and Ly5^b. Percentage of each thymocyte subsets is shown for two mice. (c) Percentage is as in b, except that the ratio of B10^k/NOD^k bone marrow cells is 4:1. (d) InsHEL-positive or –negative recipients were reconstituted with a 4:1 ratio of B10^k/NOD^k marrow. Percentage of thymocyte subsets is shown from analysis of four nontransgenic and eight insHEL-transgenic recipients. (e) Percentage of peripheral lymphocytes bearing CD4 and the HEL-reactive TCR from each donor genotype from the mice in days.

surface TCR density that is maintained by B10 T cells (Fig. 3). This result is at odds with the conclusion that NOD genes selectively altered in vitro responses of thymocytes but not of mature peripheral T cells (11) and indicates that the inherited NOD T cell defect in sensitivity to autoantigen may carry through to alter peripheral T cell regulation as well. In this regard, non-MHC NOD genes have been shown to render thymocytes, peripheral T cells and B cells resistant to dexamethasone or cyclophosphamide-induced apoptosis (12, 52, 53).

Biochemical differences in TCR signaling in thymocytes from NOD mice have been previously shown by Delovitch and colleagues (10). In particular they showed that, compared with BALB/c thymocytes, the TCR on NOD thymocytes is less efficient at activating the ZAP70/ras/ERK pathway, which is known to be important for in-

ducing T cell–positive and –negative selection. It will be important in future work to investigate whether inefficient activation of this pathway explains the selection defects identified here, and whether these signaling differences reflect inherited differences in components of the TCR pathway itself or in modulating factors such as costimulatory or inhibitory receptors. The latter are interesting candidates since the genes for CD28 and CTLA-4 lie in chromosomal regions of inherited diabetes susceptibility in NOD (12, 54).

CD28 signaling is a potent enhancer of negative selection in double-positive thymocytes, and complete deficiency of CD28 or its B7.1 ligand greatly accelerates the onset of diabetes in NOD mice while deficiency of the B7.2 ligand triggers autoimmune neuritis (9, 25). The CTLA-4 receptor for B7 ligands also modulates TCR signaling ap-

parently acting at the level of TCR phosphorylation upstream of ZAP70 (55). In future experiments it will be important to map the inheritance of the thymic deletion defect by intercrosses and use of congenic strains carrying individual resistance loci.

It is interesting to consider that the intrinsic differences in NOD thymocyte response to TCR signals may also account for reduced activity of regulatory cells (3, 6), NKT cells (7, 8), and CD4$^+$CD25$^+$ regulatory T cells (9) in this strain. NKT cells are selected from double-positive thymocytes to carry a particular TCR in response to an unknown self-antigen presented by CD1 (56). Similarly, anergic CD4$^+$CD25$^+$ regulatory T cells are selected by self-antigen during the double-positive to single-positive transition (43), at the same stage that negative selection occurs. The intrinsic defects in thymocyte response to self-antigen observed here might therefore also diminish the selection of NKT and CD4$^+$CD25$^+$ cells. Thus, a single primary defect in thymocyte selection could conceivably create two separate secondary abnormalities contributing to autoimmune susceptibility: increased numbers of high avidity T cells recognizing organ-specific self-antigens and decreased numbers of NKT cells or anergic CD4$^+$25$^+$ cells capable of regulating the autoreactive population.

The thymic selection defects described here could account for the observed increase in T cells capable of proliferating to self antigens in NOD mice (13, 14) by allowing more autoreactive T cells to leave the thymus. Because the actual frequency of high avidity autoreactive T cells is not measured by proliferation assays, it is nevertheless also possible that enhanced proliferation in these assays reflects the deficiencies of NKT cells or CD4$^+$25$^+$ regulatory cells discussed above. In the Ridway and Kanagawa studies, inefficient negative selection of thymic or peripheral T cells was proposed but via a different mechanism, namely reduced presentation of self-antigens by the NOD I-A^{g7} molecule. The defect in negative selection defined here is clearly established to reside in the responding T cells and not in the antigen-presenting cells, but it is interesting to consider that the two heritable deficits may be additive or multiplicative: an MHC-linked deficit in presentation of islet autoantigens by I-A^{g7} may compound the non-MHC linked deficit in TCR-induced deletion in response to those antigens.

The insensitivity of NOD T cells to autoantigen may apply both during thymic development and after emigration to the periphery (see above). If this can be confirmed, the intrinsic T cell insensitivity caused by non-MHC NOD genes may also diminish peripheral deletion of CD8 T cells that recognize organ specific autoantigens (16, 57). Interestingly, Verdegauer found that diabetes resistance genes from NOR caused fewer CD8 T cells with an islet-reactive TCR to accumulate in the periphery (15). It is unclear if the two results are related, however, since Verdaguer et al. found no evidence for thymic deletion in the resistant background, homozygosity for resistance genes was required, and the reduced number of islet reactive cells may have been secondary to absence of helper cells

promoting expansion or to diminished presentation of the islet autoantigen.

Is the failure to delete HEL-reactive T cells in the thymus the sole cause of progression to diabetes in the NOD^k double-transgenic mice? It is a straightforward extrapolation to connect the increased number of high avidity HEL-reactive T cells in the peripheral lymphoid tissues and pancreas to the formation of high titred IgG1 against HEL and pancreatic germinal centers in these mice, since both of these processes depend on cognate T cell help and no autoantibody is made in insHEL animals without the HEL-specific TCR transgene. It is also a reasonable extrapolation to assume that β cell destruction also results from the increased numbers of HEL-reactive CD4 cells, since diabetes in the NOD^k female mice requires both the TCR and insHEL genes. Nontolerant T cells from 3A9 TCR transgenic are capable of rapid β cell destruction, because they induce diabetes within 14 d after adoptive transfer into unirradiated B10^k insHEL recipients provided the recipients are immunized with HEL in adjuvant (unpublished data). Nevertheless, it is striking that diabetes and anti-HEL IgG antibody production in NOD^k double transgenic mice occurs stochastically between 3 and 6 mo of age, despite all of the mice having increased numbers of HEL-reactive T cells and extensive insulitis at 6–8 wk old. Breakage of tolerance to HEL may take a variable period of time simply because the high avidity HEL-reactive T cells must first organize the insulitis into supportive structures like germinal centers, or it may require a separate deficit such as the regulatory abnormalities discussed above or an unknown exogenous insult.

It is interesting that progression to diabetes occurs by an apparently distinct genetic and cellular mechanism in a subset of B10^k double transgenic mice (Fig. 1). In these animals, diabetes develops only at an 8–12 wk age window and is not accompanied by any IgG1 secretion to insHEL, and this form of diabetes is fully suppressed by a single set of NOD^k chromosomes in F1 hybrids. The early onset disease linked to B10 genes has parallels to the early onset diabetes that occurs in BDC2.5 TCR transgenic mice on the C57BL/6.H-2^{NOD} strain background (35). A region on chromosomes 7 from the related B6 or B10 strains has been shown to contain a diabetes susceptibility genes, but it is not yet known what gene or defect may be involved (35, 58, 59).

Despite the conceptually attractive idea that peripherally induced mechanisms are critical for acquiring self-tolerance to organ-specific antigens, the primary defect in thymic negative selection found here indicates that thymic selection may play a more critical role than has been thought. Indeed a growing body of evidence implicates thymic selection as a critical step for organ-specific tolerance. In quail/chicken chimeras, tolerance to a transplanted quail wing bud is only acquired if some of the T cells develop in a quail thymus (60, 61). Neonatal thymectomy in rodents has long been known to lead to organ-specific autoimmune diseases, notably autoimmune gastritis and oophoritis. Thymic-derived T cells, notably a CD4$^+$25$^+$ subset, have been

shown to be capable of suppressing autoimmune and immunopathologic reactions in thymectomized or T cell lymphopenic recipients (43–46). Deficiency of CD4$^+$25$^+$ cells and autoimmune disease in mice without IL-2 or IL-2R may reflect a primarily thymic function of IL-2 in tolerance, because the IL-2Rb deficiency disorder is corrected when expression of this receptor is limited to developing thymocytes (62). A growing list of organ-specific genes has been shown to be expressed in the thymus, especially in thymic medullary epithelial cells (36, 37), and inheritance of an insulin gene allele that causes lower thymic expression is associated with diabetes susceptibility in humans (63, 64). Finally, multiple organ-specific autoimmune diseases develop in people with the inherited Mendelian disorder, Autoimmune Polyendocrinopathy, resulting from loss of a putative transcription factor primarily expressed in subsets of thymic epithelial or dendritic cells (65–67).

Our results have implications for diabetes prevention and therapy efforts. If similar defects in thymocyte or peripheral T cell responses to self-antigens contribute to human type 1 diabetes, then agents that further lessen TCR signaling such as cyclosporin or CD28/B7 blockade may be contraindicated. These maneuvers may exaggerate the failure to censor self-reactive T cells and lead to worsening of disease or development of other autoimmune diseases, as occurs when NOD mice are made deficient for B7 or CD28 molecules (9, 25). Agents that nonspecifically augment costimulation, stimulate negative selection selectively, or that stimulate formation of regulatory T cells may be better at correcting the primary defect in individuals with organ-specific autoimmunity.

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