The Journal of Immunology

Two Mechanisms for the Non-MHC-Linked Resistance to Spontaneous Autoimmunity

Joan Verdaguer2, Abdelaziz Amrani2, Brad Anderson, Dennis Schmidt and Pere Santamaria

J Immunol 1999; 162:4614-4626; ;
http://www.jimmunol.org/content/162/8/4614

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Two Mechanisms for the Non-MHC-Linked Resistance to Spontaneous Autoimmunity[1]

Joan Verdaguer,[2] Abdelaziz Amrani,[2] Brad Anderson, Dennis Schmidt, and Pere Santamaria[3]

Genetic susceptibility and resistance to most autoimmune disorders are associated with highly polymorphic genes of the MHC and with non-MHC-linked polygenic modifiers. It is known that non-MHC-linked polymorphisms can override or enhance the susceptibility to an autoimmune disease provided by pathogenic MHC genes, but the mechanisms remain elusive. In this study, we have followed the fate of two highly diabetogenic β cell-specific T cell receptors (K^d and I-A^{g7} restricted, respectively) in NOR/Lt mice, which are resistant to autoimmune diabetes despite expressing two copies of the diabetogenic MHC haplotype *H-2^{g7}*. We show that at least two mechanisms of non-MHC-linked control of pathogenic T cells operate in these mice. One segregates as a recessive trait and is associated with a reduction in the peripheral frequency of diabetogenic $CD8^+$ (but not $CD4^+$) T cells. The other segregates as a dominant trait and is mediated by IL-4- and TGF-β1-independent immune suppressive functions provided by lymphocytes that target diabetogenic $CD4^+$ and $CD8^+$ T cells, without causing their deletion, anergy, immune deviation, or ignorance. These results provide explanations as to how non-MHC-linked polymorphisms can override the susceptibility to an autoimmune disease provided by pathogenic MHC haplotypes, and demonstrate that protective non-MHC-linked genes may selectively target specific lymphoid cell types in cellularly complex autoimmune responses. *The Journal of Immunology*, **1999, 162: 4614–4626.**

Insulin-dependent diabetes mellitus (IDDM)[4] is a prototypic organ-specific spontaneous autoimmune disease that results from selective destruction of the β cells of the pancreas by a $CD4^+$ and $CD8^+$ T cell-dependent autoimmune process (1). Susceptibility and resistance to most spontaneous autoimmune diseases, including IDDM, are associated with highly polymorphic genes of the MHC and with multiple non-MHC-linked polygenic modifiers (2–4).

The diabetes-prone nonobese diabetic (NOD) mouse, which spontaneously develops a form of diabetes closely resembling human IDDM, is homozygous for a unique MHC haplotye (*H-2^{g7}*) that ecodes a single MHC class II molecule (I-A^{g7}) with unique structure and biochemical behavior (5, 6). Genetic studies have shown that, while necessary, *I-A^{g7}* homozygosity is insufficient for development of diabetes, and that diabetogenesis requires interactions between pathogenic MHC molecules and putative products encoded on as many as 17 different non-MHC-linked loci (2–4). The mechanisms through which protective non-MHC-linked loci afford diabetes resistance, however, remain poorly understood.

It has been shown that in T cell-dependent autoimmune disorders, such as IDDM, there is a breakdown of T cell tolerance to self autoantigens (7–10). Studies of T cell tolerance in nonautoimmune disease-prone mice expressing transgenic neo-Ags and neo-Ag-specific TCR transgenes have revealed that autoreactive T cells may undergo intrathymic or peripheral clonal deletion, functional inactivation (anergy), receptor desensitization, down-regulation of TCRs or associated coreceptors, immune deviation, or ignorance (11). A logical hypothesis arising from these studies is that antidiabetogenic genes encode elements that promote the induction of tolerance or ignorance of diabetogenic T cells. Unfortunately, however, the relevance of these protective mechanisms to spontaneous autoimmunity is difficult to test with currently available animal models; most of these models employ TCRs and/or antigenic systems that are either not involved in spontaneous autoimmune diseases (12–19) or are not highly pathogenic in genetically susceptible backgrounds (20–22). To overcome some of these limitations, we have generated transgenic mice expressing the MHC class I (K^d)- or MHC class II-restricted (I-A^{g7}) β cell-reactive TCR genes of diabetogenic $CD4^+$ and $CD8^+$ T cell clones isolated from pancreatic islets of diabetic NOD mice. These TCRs are involved in spontaneous diabetes, are restricted by diabetogenic MHC molecules, target native, nontransgenic β cell autoantigens, are highly pathogenic when expressed in the diabetes-prone NOD mouse, and fail to trigger disease in diabetes-resistant mice (23, 24). These are, therefore, useful models with which to probe the mechanisms of action of protective genetic elements.

Our previous studies with I-A^{g7}-restricted β cell-reactive TCR-transgenic mice expressing antidiabetogenic MHC class II haplotypes have shown that protective MHC class II molecules may function by inducing the deletion of certain highly pathogenic TCRs (23). These studies, however, also revealed that positive selection of pathogenic TCRs in diabetes-resistant backgrounds

Department of Microbiology and Infectious Diseases, and Julia McFarlane Diabetes Research Centre, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada

Received for publication September 18, 1998. Accepted for publication January 27, 1999.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This research was supported by grants from the Medical Research Council of Canada, the Natural Sciences and Engineering Research Council of Canada, and the Juvenile Diabetes Foundation International. J.V. is supported by a Canadian Diabetes Association postdoctoral fellowship. P.S. is a Senior Scholar of the Alberta Heritage Foundation for Medical Research.

[2] J.V. and A.A. made equal contributions to this paper.

[3] Address correspondence and reprint requests to Dr. Pere Santamaria, Department of Microbiology and Infectious Diseases, Faculty of Medicine, University of Calgary, 3330 Hospital Drive N.W., Calgary, Alberta, Canada T2N 4N1. E-mail address: psantama@acs.ucalgary.ca

[4] Abbreviations used in this paper: IDDM, insulin-dependent diabetes mellitus; β_2m, β_2-microglobulin; BrdU, 5-bromo-2′-deoxyuridine; CDR, complementarity-determining region; CM, complete medium; DAB, diaminobenzidine; HRP, horseradish peroxidase; NOD, nonobese diabetic; NOR, nonobese resistant; RAG-2, recombination-activating gene-2; PerCP, peridinin chlorophyll protein.

0022-1767/99/$02.00

expressing diabetogenic MHC haplotypes does not imply autoreactivity, and suggested that the diabetes resistance of these mice is controlled by non-MHC-linked genetic elements. The studies presented in this work were initiated to elucidate the mechanisms of action of non-MHC-linked antidiabetogenic genes. This was done by following the fate of our two β cell-specific TCRs (4.1, I-A^{g7} restricted; and 8.3, K^d restricted) in NOR and (NOD $\times$ NOR)F_1 mice, both of which are resistant to islet inflammation (insulitis) and diabetes, despite deriving $\approx$88% of their genome from the NOD mouse, including two copies of the H-2^{g7} haplotype (25, 26). These studies have resulted in the discovery of two mechanisms of non-MHC-linked control of diabetogenic T cells. One of these mechanisms is recessive and induces a reduction in the peripheral frequency of diabetogenic $CD8^+$, but not $CD4^+$, T cells. The other is dominant and is mediated by immune suppressive functions provided by endogenous lymphocytes that do not involve the deletion, anergy, or immune deviation of diabetogenic T cells, nor their ignorance of β cells, and that target pathogenic TCRs regardless of their MHC restriction or fine antigenic specificity. These results provide an explanation as to how non-MHC-linked gene polymorphisms can override the susceptibility to an autoimmune disease provided by pathogenic MHC haplotypes and demonstrate that protective non-MHC-linked genes may selectively target specific cellular elements in cellularly complex pathogenic autoimmune responses.

Materials and Methods

Mice

8.3-NOD and 4.1-NOD mice, expressing K^d- or I-A^{g7}-restricted β cell-reactive TCR transgenes, derived from the $CD8^+$ and $CD4^+$ T cell clones NY8.3 and NY4.1, respectively, have been described (23, 24). NOR/Lt mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Diabetic 8.3-NOD or 4.1-NOD mice were outcrossed with NOR/Lt mice (*H-2^{g7}* and ~88% genetically identical to NOD mice, including the diabetes susceptibility loci *Idd2*, *idd3*, *Idd6*, *Idd7*, *Idd8*, and *Idd10*, but homozygous for diabetes-resistance alleles at *Idd5*, *Idd9/11* and *Idd13*) (26) to generate 8.3-F_1 and 4.1-F_1 mice, respectively. NOR/Lt mice are also homozygous for *Idd4^r*, but this locus does not afford diabetes resistance in NOR mice (26). Diabetic 8.3-F_1 and 4.1-F_1 mice were then backcrossed with NOR/Lt mice for up to seven generations, to generate 8.3-NOR and 4.1-NOR mice. Mice of the N5 backcross were homozygous for NOR alleles at *Idd5*, *Idd9/11*, and *Idd13* loci. *RAG-2$^-$* NOR mice were generated by backcrossing the *RAG-2* mutation of *RAG-2$^-$* C57BL/6/129 mice (a gift from F. Alt, Boston Children's Hospital, Boston, MA) onto the NOR background for 10 generations, followed by intercrossing N10 heterozygotes (typed as homozygous for NOR alleles at *Idd5*, *Idd9/11*, and *Idd13*) (the *RAG-2* locus lies 13 cM away from the *Idd13*-containing region on chromosome 2). *RAG-2$^-$* 8.3-F_1 and *RAG-2$^-$* 4.1-F_1 mice were generated by intercrossing *RAG-2$^-$* 8.3-NOD or *RAG-2$^-$* 4.1-NOD mice (24) with *RAG-2$^-$* NOR or *RAG-2$^{+/-}$* NOR mice. 8.3-TCRβ-transgenic NOR mice were produced by backcrossing 8.3-TCRβ-transgenic (NOD $\times$ NOR)F_1 mice (27) with NOR mice for up to nine generations. $Fas^{+/-}$ NOD.*lpr* mice were generated by backcrossing the *Fas^{lpr}* gene of B6.MRL-*Fas^{lpr}* mice (from The Jackson Laboratory) onto the NOD background for up to seven generations. *Fas$^-$* NOD.*lpr* mice were generated by intercrossing heterozygous mice of the N3 to N7 generations. Mice were screened for inheritance of the transgenes and mutated and wild-type *RAG-2* or *Fas* alleles by PCR of tail DNA. NOR/NOD polymorphisms at *Idd5*, *Idd9/11*, and *Idd13* loci were determined by PCR using primers for *D1Mit46* (*Idd5*), *D4 Mit11* (*Idd9/11*), *D2Mit144*, and *D2Mit490* (*Idd13*) (26), in the absence (for *D2Mit490*) or presence of [^{32}P]dCTP (for all other markers). *D2 Mit490* lies between the *Idd13* (3cM) and *RAG-2* loci (10 cM). PCR products were resolved in 4% NuSieve agarose gels (*D2Mit490*) or in 7 M urea/6% acrylamide gels (other markers). All mice were housed in a specific pathogen-free facility.

Diabetes

Diabetes was assessed by measuring urine glucose levels with Diastix strips (Miles, Ontario, Canada) twice weekly. Animals were considered diabetic after two consecutive readings $\geq 3^+$.

Cell lines, Abs, and flow cytometry

L1210-Fas^+ and L1210-Fas^- cells were provided by Dr. P. Goldstein (Centre National de la Recherche Scientifique, Marseille, France). NIT-1 NOD insulinoma cells were a gift from Dr. E. Leiter (The Jackson Laboratory). L929-K^d transfectants were provided by Dr. J. Yewdell (National Institutes of Health, Bethesda, MD). Hybridomas secreting mAbs GK1.5 (anti-CD4) and 53-6.7 (anti-CD8) were obtained from the American Tissue Culture Collection (Manassas, VA). A hybridoma secreting the Vβ8.1/8.2-specific mAb KJ16 was a gift from P. Marrack (National Jewish Center, Denver, CO). Anti-Lyt-2 (CD8α) phycoerythrin (53-6.7), anti-L3T4 FITC (IM7), anti-L3T4 biotin (CD4) (H129.19), anti-CD2 biotin (RM2-5), anti-CD5 (53-7.3) biotin, anti-CD11a biotin (M17/4), anti-CD24 biotin (M1/69), anti-CD28 biotin (37.51), anti-CD44 FITC (IM7), anti-CD45RB FITC (23G2), anti-L-selectin biotin (CD62L) (Mel-14), anti-CD69 biotin (H1.2F3), anti-Vβ8.1/8.2 FITC (MR5-2), and anti-H-$2K^d$ FITC (SF1-1.1) were purchased from PharMingen (San Diego, CA). Anti-IL-2R FITC (CD25) (AMT13) was purchased from Cedarlane Laboratories (Hornby, Ontario, Canada). Mouse IgG-absorbed FITC- or biotin-conjugated goat anti-rat IgG, and FITC-conjugated goat anti-mouse IgG were obtained from Caltag (San Francisco, CA) and Becton Dickinson (San Jose, CA), respectively. Streptavidin-PerCP (peridinin chlorophyll protein) was obtained from Becton Dickinson. Goat polyclonal anti-granzyme B IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Guinea pig polyclonal anti-swine insulin antisera and biotinylated anti-guinea pig IgG were obtained from Dako (Carpinteria, CA). Biotinylated swine anti-goat IgG was obtained from Cedarlane Laboratories. Thymi, spleens, and islet-derived T cell lines were analyzed by three-color flow cytometry using a FACScan (Becton Dickinson), as described by Verdaguer et al. (27).

Proliferation and limiting dilution assays

Splenocytes from 8.3-NOD mice or 4.1-NOD were depleted of $CD4^+$ or $CD8^+$ T cells, respectively, using anti-CD4 mAb (GK-1.5)- or anti-CD8 mAb (53-6.7)-coated magnetic beads (27), adjusted to 10^4 $CD8^+$ or $CD4^+$ T cells/100 μl of complete medium (CM: RPMI 1640 media containing 10% heat-inactivated FBS (Life Technologies, Grand Island, NY), 50 U/ml penicillin, 50 μg/ml streptomycin (Flow Laboratories, McLean, VA), and 50 μM 2-ME (Sigma, St. Louis, MO)) and incubated, in triplicate, with γ-irradiated (3000 rad) islet cells (3–100 $\times$ 10^3/well) in 96-well tissue culture plates for 3 days at 37°C in 5% CO_2. Cultures of 4.1-$CD4^+$ T cells received 10^5 irradiated splenocytes from nontransgenic NOD mice, as feeders. Cultures were pulsed with 1 μCi of [^{3}H]thymidine during the last 18 h of culture and harvested. Thymidine incorporation was measured by scintillation counting, and specific proliferation was calculated as described previously (27). Proliferation assays using plate-bound anti-Vβ8.1/8.2 mAb (KJ16) were done as described earlier (24), except that IL-2 was omitted. To determine the frequency of β cell-reactive $CD8^+$ T cells, 12 replicate cultures of four 10-fold serial dilutions of splenocytes (10^1–10^5 cells/well) were stimulated with irradiated NOD islets (8/well) for 4 days, expanded in rIL-2 for 10 days, and restimulated with islets and rIL-2. The resulting cultures were split and challenged with NIT-1 or L929-K^d cells for 24 h, and the supernatants were collected to measure the contents of TNF-α. Cultures that secreted TNF-α in response to NIT-1, but not L929-K^d, cells were considered to contain β cell-reactive $CD8^+$ T cells (24). To determine the frequency of β cell-reactive $CD4^+$ T cells, four 12 replicate cultures of 10-fold serial dilutions of $CD8^+$ T cell-depleted splenocytes (10^1–10^5 cells/well) were stimulated with 2.5 $\times$ 10^3 irradiated islet cells and 2 $\times$ 10^5 irradiated NOD splenocytes for 4 days, followed by rIL-2 for 10 days and a second restimulation with islet cells, splenocytes, and rIL-2 (27). Control plates received rIL-2 and splenocytes, but not islet cells. Growth was scored microscopically. Frequencies were calculated with Poisson statistics.

Generation of spleen- and islet-derived $CD8^+$ T cell lines and clones

$CD4^+$ T cell-depleted spleen cells were stimulated with irradiated NOD islets for 3 to 4 days, and the activated cells expanded with 0.5 U/ml rIL-2 (Takeda, Osaka, Japan) for 10 to 14 days. Growing cultures were assayed for serine esterase content (27) and used as effectors in cytotoxicity assays, or restimulated twice with irradiated NOD islets and rIL-2, to generate β cell-specific $CD8^+$ T cell lines. Islet-derived $CD8^+$ T cell lines and clones were generated as described by Verdaguer et al. (24). Growing clones were assayed within 15 days of cloning for serine esterase content, and serine $esterase^+$ clones were expanded by stimulation with irradiated NOD islets and rIL-2. Some experiments employed islet cell suspensions (containing endocrine cells and infiltrating T cells) as effectors in cytotoxicity assays.

^{51}Cr release assays

Target cells (L1210-Fas$^+$, L1210-Fas$^-$, and single NOD or NOR islet cells) (5×10^5) were labeled with ^{51}Cr sodium chromate (DuPont/NEN, Boston, MA) and seeded at 10^4 cells per 100 μl/well. Some experiments (those in Fig. 5*F*) employed islet cells derived from islets that had been preincubated overnight with supernatants from 3-day-old islet-derived CD8$^+$ T cell lines, or with rIL-1α (10^3 U/L), to increase their susceptibility to Fas-mediated cytotoxicity (28). Effector cells (islet-infiltrating T cells, islet-derived serine esterase$^-$ CD8$^+$ T cell lines, or islet-derived serine esterase$^+$ CD8$^+$ T cell clones; 100 μl) were added to each well, in duplicate, at several E:T ratios. Plain medium or 1% Triton X-100 was added to sets of target cells for examination of spontaneous and total cell lysis, respectively. The plates were incubated at 37°C for 8 h, and the supernatants were collected at this point for determination of specific ^{51}Cr release (24).

Cloning and sequencing of TCRα mRNAs

The TCRα-chain cDNA molecules of islet-stimulated splenic CD8$^+$ T cells from 8.3-TCRβ-transgenic NOD and 8.3-TCRβ-transgenic NOR mice were amplified by anchor PCR, cloned, and sequenced, as previously described (27).

Cytokine RT-PCR

Total cellular RNA from islet-derived, CD4$^+$ T cell-depleted T cell lines was reverse transcribed using oligo(dT)$_{12-18}$ (Life Technologies) as a primer, and the resulting cDNAs were amplified by PCR (27).

In vitro and in vivo cytokine secretion

Splenic CD4$^+$ T cells (2×10^4/well) were incubated with γ-irradiated NOD islet cells (10^5/well) and splenocytes (10^5/well) in 96-well plates for 48 h at 37°C in 5% CO_2. The supernatants (100 μl/well) were assayed for IL-2, IL-4, IFN-γ, and/or TGF-β1 content by ELISA using commercially available kits (Genzyme Diagnostics, Cambridge, MA). Determination of intraislet cytokine content was done as described in Cameron et al. (29), with modifications. Briefly, purified islets from nondiabetic mice were homogenized in PBS containing protease inhibitors (PMSF (1 mM), leupeptin (20 μM), pepstatin A (10 μM), sodium azide (0.2%), and EDTA (100 μM)), and centrifuged at 10,000 rpm to remove debris. The supernatants were adjusted at 200 μg of total protein/ml, and 100 μl of each sample was used in duplicate to measure the cytokine content by ELISA.

Histopathology and immunohistochemistry

Pancreata (one-half) were fixed in formalin, embedded in paraffin, sectioned at 4.5 μm, stained with hematoxylin and eosin, and examined for inflammation. The degree of insulitis was evaluated by scoring 15–30 islets/mouse using previously described criteria (24). The second half of each pancreas was snap frozen in liquid nitrogen, immersed in OCT, sectioned at 6–7 μm, fixed in cold acetone for 10 min, incubated with hydrogen peroxide to block endogenous peroxidase activity, and stained with anti-CD4 (GK1.5) and anti-CD8 (53-6.7) mAbs, followed by biotinylated anti-rat Ig Abs and horseradish peroxidase (HRP)-streptavidin conjugate (Dimension Laboratories, Mississauga, ON, Canada). The slides were developed with diaminobenzidine (DAB; Sigma).

To determine the percentages of granzyme B$^+$ cells in islets, mice were euthanized and perfused with PBS and 4% paraformaldehyde in PBS. The pancreas of each mouse was then fixed overnight in 4% paraformaldehyde at 4°C, immersed in 20% sucrose overnight at 4°C, snap frozen in liquid nitrogen, and processed for immunopathology, as described above. Tissue sections were incubated with goat polyclonal anti-granzyme B IgG, biotinylated swine anti-goat IgG, and HRP-streptavidin; developed with DAB; and counterstained with hematoxylin.

In vivo bromodeoxyuridine (BrdU) labeling

Mice were given two i.v. injections of 200 μl of a 4 mg/ml solution of 5-bromo-2′-deoxyuridine (BrdU; Calbiochem, La Jolla, CA) 4 h apart. The pancreas and spleen of each mouse were collected 12 h later. Quantitation of the percentage of islet-infiltrating cells and splenocytes incorporating BrdU was done on frozen tissue using a BrdU staining kit (Calbiochem).

Statistical analyses

Statistical analyses were performed using Mann-Whitney *U* and χ^2 tests and by simple regression.

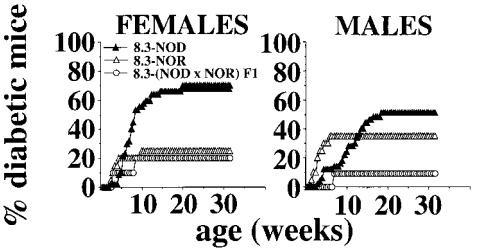


FIGURE 1. Diabetogenesis in 8.3-NOR and 8.3-F$_1$ mice. Cumulative incidence of IDDM in female (8.3-NOD (n = 83), 8.3-F$_1$ (n = 19), and 8.3-NOR (n = 20) (N5-N7 generations)) and male (8.3-NOD (n = 79), 8.3-F$_1$ (n = 24), and 8.3-NOR (n = 20) (N5-N7 generations)) mice. The average age at onset of diabetes for 8.3-NOR, 8.3-F$_1$, and 8.3-NOD mice was: 37 ± 21 vs 68 ± 31 and 61 ± 24 days, for females; and 30 ± 10 vs 41 ± 15 vs 83 ± 35 days, for males. See text for statistical comparisons.

Results

IDDM resistance in 8.3-(NOD × NOR)F$_1$ and 8.3-NOR mice

We have shown that 8.3-CD8$^+$ T cells are highly diabetogenic in 8.3-NOD mice (24). Since these cells require the assistance of endogenous CD4$^+$ T cells to accelerate diabetes (24), we reasoned that studies with the 8.3-TCR would be the most informative. To test the hypothesis that non-MHC-linked genes control the diabetogenic activity of 8.3-CD8$^+$ T cells, we first crossed 8.3-NOD mice with NOR/Lt mice to generate 8.3-(NOD × NOR)F$_1$ (8.3-F$_1$) mice. NOR/Lt mice are homozygous for the prodiabetogenic *H-2^{g7}* haplotype and are ~88% genetically identical to NOD/Lt mice, but unlike NOD/Lt mice and the 8.3-NOD mice used for these studies (homozygous for NOD alleles at the three *Idd* regions that provide diabetes resistance in NOR mice), are homozygous for antidiabetogenic alleles at *Idd5*, *Idd9/11*, and *Idd13* loci. The incidence of diabetes in 8.3-F$_1$ mice was significantly lower than in 8.3-NOD mice, both in females and in males ($p < 0.0002$) (Fig. 1). These differences in diabetes incidence between 8.3-NOD and 8.3-F$_1$ mice were not due to genetic heterogeneity in the 8.3-NOD mouse population used as donor of the transgenes for this study, since the incidence of diabetes in the 8.3-TCR-transgenic littermates of the transgene donors was similar to the current incidence of diabetes in our 8.3-NOD colony (78% vs 71% in females, and 60% vs 52% in males, respectively). As expected, none of the 20 female nontransgenic F$_1$ littermates that were followed developed diabetes (data not shown). We then backcrossed the 8.3-TCR of a diabetic 8.3-F$_1$ male mouse onto the NOR/Lt background for up to seven generations, to generate 8.3-NOR mice. Cohorts of mice from each backcross were followed for diabetes development. As expected, female 8.3-NOR mice of the N5-N7 backcrosses (homozygous for NOR alleles at *Idd5*, *Idd9/11*, and *Idd13*) also developed diabetes less frequently than female 8.3-NOD mice ($p < 0.0002$) (Fig. 1). Surprisingly, however, 8.3-NOR males of the N5-N7 backcrosses displayed an increased incidence of diabetes when compared with 8.3-F$_1$ (but not 8.3-NOD) mice ($p < 0.02$) (Fig. 1). In both female and male 8.3-NOR mice (N5-N7 generations), there was a decrease in the average age at onset of the disease when compared with 8.3-NOD and/or 8.3-F$_1$ mice (37 ± 22 days vs 43 ± 26 days and 68 ± 31 days for females, $p < 0.04$; 30 ± 11 days vs 83 ± 35 and 41 ± 15 days for males, $p < 0.0001$). No obvious differences in disease incidence or age at diabetes onset were noted between N2, N3, or N4 mice and N5-N7 mice. In males, for example, the incidence and age at onset of diabetes for each generation were: N2, 2/4 mice at 39 ± 6 days; N3, 3/11 mice at 34 ± 6 days; N4, 2/5 mice at 35 ± 4 days. It therefore appears that NOR/Lt mice bear one or more chromosomal regions that, either

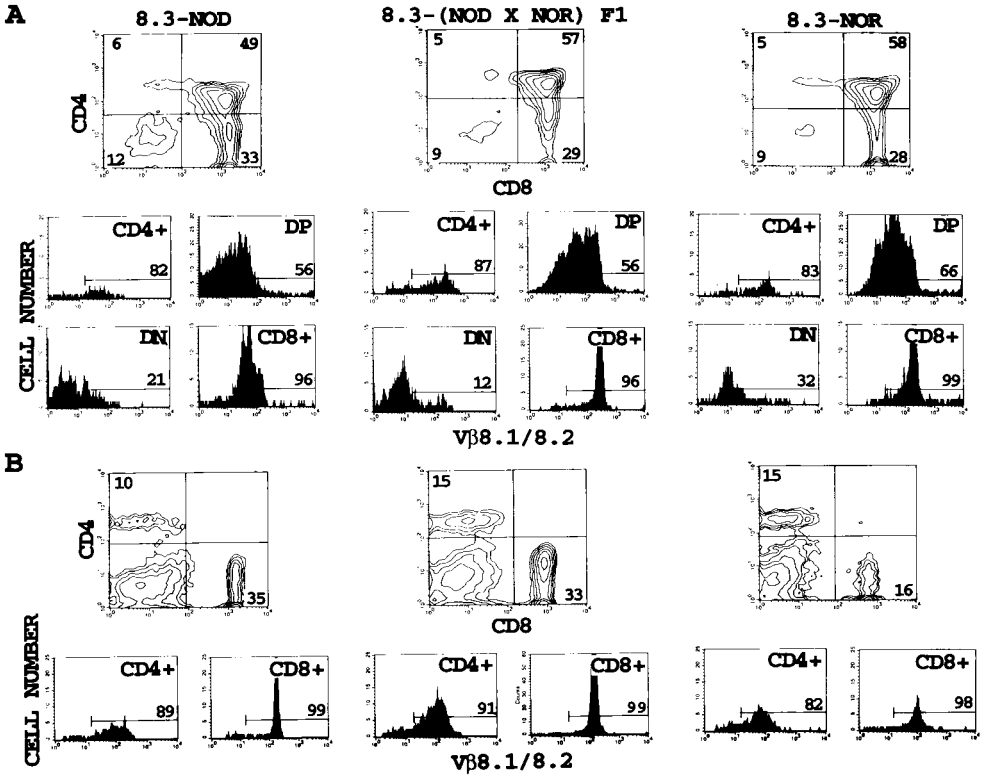


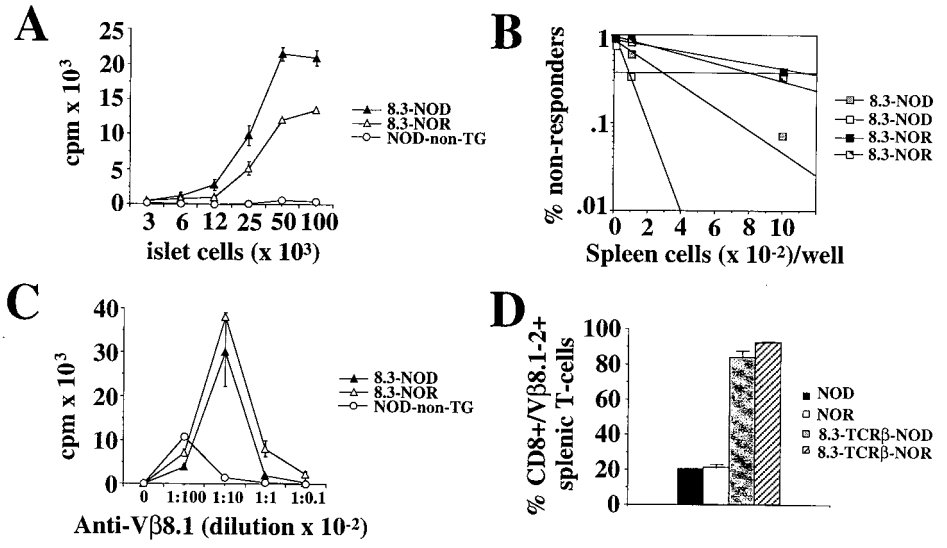
FIGURE 2. T cell development in 8.3-NOR and 8.3-F_1 mice. CD4, CD8, and Vβ8.1/8.2 profiles of thymocytes (*A*) and splenic cells (*B*) from 8.3-NOD ($n = 25$; age = 90 ± 57 days), 8.3-F_1 ($n = 6$; age = 107 ± 38 days), and 8.3-NOR mice ($n = 13$ N4-N5 mice; age = 89 ± 40 days). *Upper panels* show CD4 vs CD8 dot plots of cell suspensions stained with anti-CD8-phycoerythrin, anti-Vβ8.1/8.2 FITC, and anti-CD4-biotin plus streptavidin-PerCP. The *lower panels* show the Vβ8.1/8.2 fluorescence histograms of each T cell subset after electronic gating. Numbers indicate the average percentage of cells (*upper panels*) or number of Vβ8.1/8.2$^+$ cells (*lower panels*) in each subset. DP, double-positive cells; DN, double-negative cells. The percentage of splenic CD8$^+$ T cells in 8.3-NOR mice (16 ± 6%) was significantly lower than in 8.3-NOD mice (35 ± 6%) or 8.3-F_1 mice (33 ± 9%) ($p < 0.0005$ and $p < 0.002$, respectively).

alone or in combination, afford dominant protection from 8.3-CD8$^+$ T cell-induced diabetes in 8.3-F_1 mice. The fact that this dominant protective effect is lost in male 8.3-NOR mice suggests that NOR/Lt mice also bear recessive prodiabetogenic genes that in males, but not females, can override the antidiabetogenic function of these dominant protective elements.

Reduction in the peripheral frequency of 8.3-CD8$^+$ T cells in 8.3-NOR mice, but not in 8.3-F_1 mice

Three-color cytofluorometric studies of thymocytes revealed that the resistance of 8.3-F_1 and 8.3-NOR mice to 8.3-CD8$^+$ T cell-induced diabetes was not a result of deletion or abnormal maturation of 8.3-CD8$^+$ thymocytes. The thymi of these mice contained similar numbers of thymocytes and similar percentages of the four major thymocyte subsets as the thymi of 8.3-NOD mice (Fig. 2*A*). Likewise, no differences were found between the individual thymocyte subsets of all of these mice with respect to cell surface levels of the transgenic TCR (Fig. 2*A*) and several differentiation markers, including CD5, MHC class I (K^d), CD24, CD44, and CD69 (data not shown).

The cytofluorometric profiles of the spleens of 8.3-F_1 mice were also remarkably similar to those of 8.3-NOD mice (Fig. 2*B*); however, the spleens (and, to a lesser extent, the lymph nodes) of 8.3-NOR mice contained significantly fewer CD8$^+$ T cells than the spleens (or lymph nodes) of 8.3-NOD mice (Fig. 2*B*, and data not shown) ($p < 0.0005$). This reduction in the percentage of splenic CD8$^+$ T cells in 8.3-NOR mice (defined as a splenic CD4$^+$:CD8$^+$ T cell ratio greater than the average plus 2 SDs of the 8.3-NOD values) segregated as a recessive trait: it occurred in none of 6 F_1 mice, in 4 of 10 N2 mice, and in 11 of 13 N4-N5 mice (8.3-F_1 vs 8.3-N2, $p < 0.009$; 8.3-N2 vs 8.3-N4/N5, $p < 0.0001$). This phenotype was not due to CD8 coreceptor down-regulation by 8.3-CD8$^+$ T cells, since virtually all of the CD4$^-$CD8$^-$ splenic cells of these mice were Vβ8.1$^-$ (data not shown), and could not be accounted for by disproportionate expansion of 8.3-CD8$^+$ T cells in the spleens of 8.3-NOD vs 8.3-NOR mice, since the splenic CD8$^+$ T cells of 8.3-NOD (and 8.3-NOR) mice did not express activation markers ((24) and data not shown). Furthermore, the splenic periarteriolar lymphoid sheaths of 8.3-NOD mice contained very few actively proliferating cells, as determined by immunopathologic analyses of BrdU-injected mice, and the number of BrdU-incorporating cells in the spleens of 8.3-NOD and 8.3-NOR mice was similar (data not shown). Interestingly, this reduction in the percentage of splenic CD8$^+$ T cells was accompanied by a reduction in the overall ability of the splenic CD8$^+$ T cells of 8.3-NOR vs 8.3-NOD mice to proliferate in response to islet stimulation (Fig. 3*A*). Subsequent limiting dilution (Fig. 3*B*) and proliferation assays (Fig. 3*C*) using islet Ag or a plate-bound anti-Vβ8.1 mAb as stimuli, respectively, demonstrated that the lower proliferative responsiveness of 8.3-NOR-derived CD8$^+$ T cells to islet stimulation was not due to anergy, but rather to a reduction in the peripheral frequency of β cell-reactive CD8$^+$ T cells (~1/70–90 vs 1/17–30 CD8$^+$ T cells in 8.3-NOR and 8.3-NOD mice, respectively). Paradoxically, however, 8.3-NOR mice developed an accelerated onset (but not an increased incidence) of diabetes. This suggested that this reduction in the peripheral frequency of β



E

DONOR CTL	Vα-Jα	N*	Vα	Nα CDR3		Jα
NY8.3	Vαn1.1-Jα34	-	CA	MRD	S	GGSNAKLTFGKGTKLSVKS
CTL LINES (female NOD spleen-derived)						
P4	Vαn1.1-Jα34	17/28	CA	MRD	S	GGSNAKLTFGKGTKLSVKS
	Vαn1.1-Jα33	1/28	CA	MRED		VNTGNYKYVFGAGTRLKVIA
	Vα1-Jα11	7/28	CA	AS S	NS	GTYQRFGTGTKLQVVP
	VαnP4-Jα45	2/28	CA	A	S	GGSNYKLTFGKGTLLTVTP
	Vα10-Jα40	1/28	CA	ASTTMP		TFGAGTKLTVKP
P7	Vαn1.1-Jα34	10/13	CA	MRD	S	GGSNAKLTFGKGTKLSVKS
	Vαn3-Jα15	2/13	CA	VRG		GSALGRLHFGAGTQLIVIP
	Vα8-Jα17	1/13	CA	LSER	S	GSWQLIFGSGTQLTVMP
L1	Vαn1.1-Jα34	19/19	CA	MRD	S	GGSNAKLTFGKGTKLSVKS
		46/60				
CTL LINES (female NOR spleen-derived)						
N1	Vα1-Jα11	16/16	CA	ASES	NS	GTYQRFGTGTKLQVVP
N3	Vα3-Jα11	3/12	CA	LRE	NS	GTYQRFGTGTKLQVVP
	Vα5-Jα33	3/12	CA	VSD	T	GNYKYVFGAGTRLKVIA
	Vαn1.1-Jα15	1/12	CA	MREGGR	S	ALGRLHFGAGTQLIVIP
	Vα10-Jα40	1/12	CA	MED		GNEKITFGAGTKLTIKP
	Vα3-Jα26	1/12	CA	VSWTTATTD		LLWGSGTKLLIKP
	Vα5-Jα27	1/12	CA	VLT	S	NTDKVVFGTGTRLQVLP
	Vα10-Jα41	1/12	CA	M	NT	GYQNFYFGKGTSLTVIP
	Vα12-Jα23	1/12	CV	TGD		TNAYKVIFGKGTHLHVLP
N6	Vα4-Jα32	14/15	CV	LG		NNAGAKLTFGGGTRLTVRPP
	Vα2-Jα30	1/15	CV	TGD		TNAYKVIPGKGTHLHVLP
N7	Vαx-Jα30	4/6	CA	A	SS	GSWQLIFGSGTQLTVMP
	Vα4-Jα32	1/6	CV	LG		NNAGAKLTFGGGTRLTVRP
	Vα8-Jα30	1/6	CA	LSDRITQ		GKLIFGQGTKLSIKP
		0/49				

FIGURE 3. Reduction in the peripheral frequency of 8.3-CD8$^+$ T cells in 8.3-NOR mice is not associated with anergy and selectively targets cells bearing the 8.3-TCRα-chain. *A*, Proliferation of splenic CD8$^+$ T cells from 8.3-NOD and 8.3-NOR mice to islet cells. A total of 2×10^4 CD8$^+$ T cells was incubated with irradiated islet cells for 3 days, pulsed with [^{3}H]thymidine, harvested, and counted. Bars show the SEMs. *B*, Peripheral frequency of β cell-reactive CD8$^+$ T cells in 8.3-NOD and 8.3-NOR mice (results from two mice are shown), as determined by LDA. Cultures that secreted TNF-α in response to NIT-1, but not L929-K^d cells were considered to contain β cell-reactive T cells. The figure shows values for three of the four dilutions tested. *C*, General proliferative activity of splenic CD8$^+$ T cells of 8.3-NOD and 8.3-NOR mice. A total of 10^4 CD8$^+$ T cells was cultured in wells coated with dilutions of KJ16-containing ascites for 3 days, pulsed with [^{3}H]thymidine, harvested, and counted. *D*, Percentage of CD8$^+$/Vβ8.1-2$^+$ splenic T cells in NOD ($n = 3$), NOR ($n = 4$), 8.3-TCRβ-transgenic NOD (N8; $n = 6$), and 8.3-TCRβ-transgenic NOR (N9; $n = 2$) mice. *E*, Endogenous VJα repertoire of islet-stimulated CD8$^+$ T cells from 8.3-TCRβ-transgenic mice. CD4$^+$ T cell-depleted splenocytes from 8.3-TCRβ-transgenic NOD ($n = 3$) and 8.3-TCRβ-transgenic NOR mice ($n = 4$) were stimulated with irradiated NOD islets for 3 to 4 days, expanded with rIL-2, and restimulated twice with NOD islets and rIL-2. The TCRα cDNAs of each line were amplified by anchor PCR, cloned, and sequenced. N is the ratio between each cDNA's copy number/number of cDNAs sequenced. N-terminal residues homologous to those of the 8.3-TCRα-chain are underlined.

cell-reactive CD8$^+$ T cells in 8.3-NOR mice might increase with age, and that it would have to reach a certain threshold to be able to afford diabetes protection. Time course studies confirmed that deletion of 8.3-CD8$^+$ T cells in 8.3-NOR mice was indeed an age-dependent phenomenon: contrary to what happened in 8.3-NOD mice, in which the percentage of splenic CD8$^+$ T cells increased with age ($r = 0.682$, $p < 0.0001$), the percentage of splenic CD8$^+$ T cells in 8.3-NOR mice did not increase as the mice grew older ($r = 0.266$). Studies with nontransgenic and TCRβ-transgenic NOR mice confirmed that the deleting phenotype of 8.3-NOR mice was not mediated by an endogenous superantigen binding to the 8.3-TCRβ-chain; the percentages of Vβ8.1/8.2$^+$CD8$^+$ T cells in the spleens of NOR and 8.3-TCRβ-transgenic NOR mice were similar to those seen in the spleens of NOD and 8.3-TCRβ-transgenic NOD mice, respectively (Fig. 3*D*). Taken together, these results suggested that: 1) 8.3-CD8$^+$ T cells undergo partial deletion in 8.3-NOR mice; and 2) this phenotype segregates as a recessive trait. Our next set of experiments thus

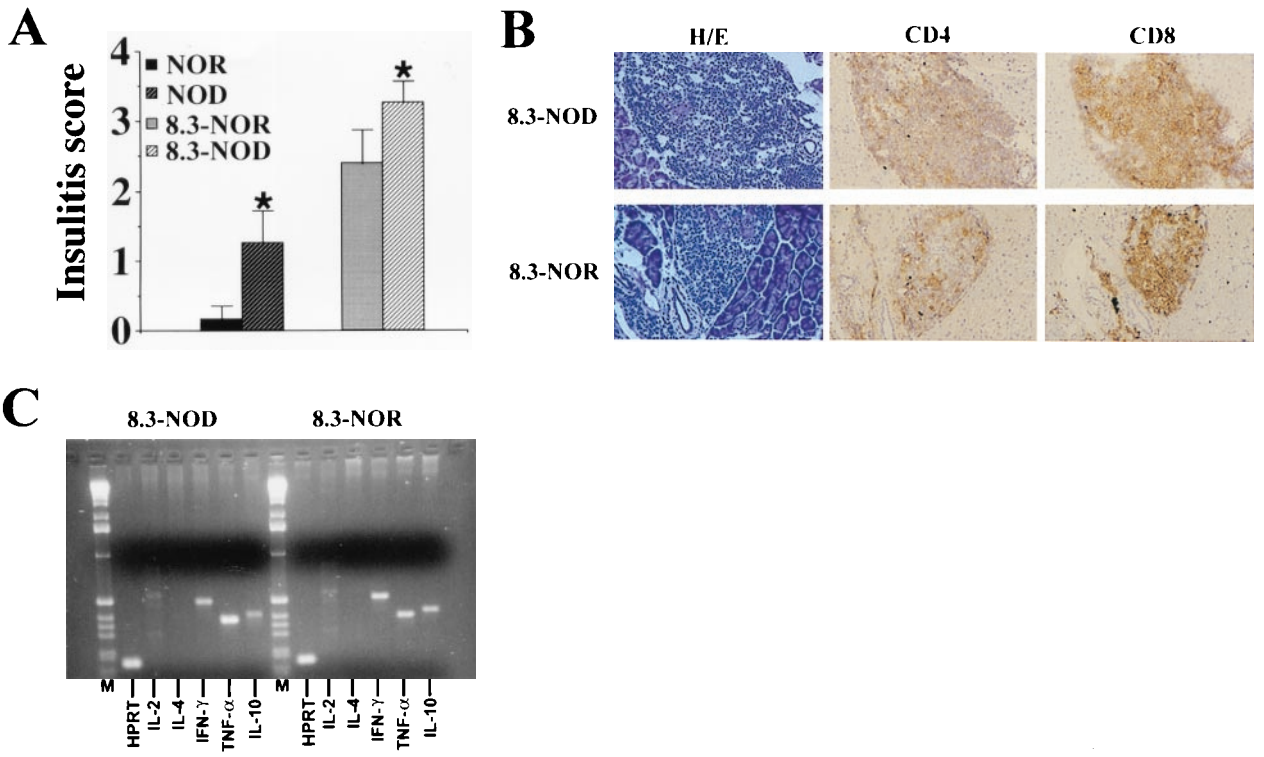


FIGURE 4. Diabetes resistance in 8.3-NOR mice is not associated with insulitis resistance or immune deviation. *A*, Insulitis scores of nondiabetic NOD, NOR, 8.3-NOD, and 8.3-NOR (N3) mice (n = 5–8/group; 9–11 wk old) (15–30 islets/mouse). All 8.3-NOR mice analyzed (nondiabetic and at an age in which the risk of developing diabetes is <2.5%) had moderate-to-severe insulitis. Bars show the SD of the means. *, $p < 0.006$ (*left*) and $p < 0.01$ (*right*). *B*, Phenotype of islet-infiltrating T cells. Pancreas sections (4–6 infiltrated islets/mouse; 6 mice/group) were stained with anti-CD8 (53.6-7) or anti-CD4 (GK1.5) mAbs, biotinylated anti-rat IgG, and HRP-streptavidin, and the slides were developed with DAB. Magnification, ×200. The insulitis $CD4^+/CD8^+$ T cell ratios of 8.3-NOD and 8.3-NOR mice were 0.6 ± 0.3 and 0.6 ± 0.2, respectively. *C*, Cytokine RT-PCR of islet-derived $CD8^+$ T cell lines. Lymphocytes grown from islets cultured in the presence of rIL-2 for 4 to 5 days were depleted of $CD4^+$ T cells and used as a source of mRNA for RT-PCR.

focused on investigating this phenotype further. The dominant diabetes resistance observed in 8.3-F_1 mice, which does not involve T cell deletion, will be addressed further below.

$CD8^+$ T cells expressing endogenously derived 8.3-TCRα-chains are undetectable in 8.3-TCRβ-transgenic NOR mice

To confirm that the reduction in the peripheral frequency of 8.3-$CD8^+$ T cells in 8.3-NOR mice was not an artifact of the high peripheral frequency of clonotypic 8.3-$CD8^+$ T cells, we investigated whether this phenotype also occurred in single-chain 8.3-TCRβ-transgenic NOR mice. We chose to use these mice to address this issue for several reasons: 1) unlike 8.3-TCRβ-transgenic NOD mice, 8.3-TCRβ-transgenic NOR mice do not develop diabetes (0% incidence, unpublished observations); 2) when compared with 8.3-NOD mice, 8.3-TCRβ-transgenic NOD mice only have a minor increase in the peripheral frequency of β cell-reactive $CD8^+$ CTL (27); and 3) the splenic $CD8^+$ T cells of 8.3-TCRβ-transgenic NOD mice display a highly heterogeneous TCRα repertoire, yet most of the islet-associated $CD8^+$ T cells in acutely diabetic animals bear endogenously derived TCRα-chains identical to the 8.3-TCRα-chain, indicating that expression of the 8.3-TCRβ transgene fosters the maturation of some 8.3-$CD8^+$ T cells (27). To investigate whether 8.3-TCRβ-transgenic NOR mice also have a reduction in the peripheral frequency of 8.3-$CD8^+$ T cells when compared with 8.3-TCRβ-transgenic NOD mice, we compared the endogenous TCRα repertoire of β cell-reactive $CD8^+$ T cells in 8.3-TCRβ-transgenic NOR and 8.3-TCRβ-transgenic NOD mice. To do this, we stimulated splenic $CD8^+$ T cells from several 8.3-TCRβ-transgenic NOD and 8.3-TCRβ-transgenic NOR mice with NOD islets in the presence of rIL-2 and sequenced multiple anchor PCR-generated TCRα cDNAs from each line. As shown in Fig. 3*E*, the 8.3-TCRα sequence was the predominant TCRα sequence within each of the three different 8.3-TCRβ-transgenic NOD lines studied (46/60 cDNAs). In contrast, while some of the 49 TCRα cDNAs derived from four 8.3-TCRβ-transgenic NOR lines encoded homologous junctional (CDR3) amino acid sequences, none of them encoded the 8.3-TCRα-chain or the 8.3-TCRα-CDR3 region sequence. These results therefore demonstrated that the reduction in the peripheral frequency of 8.3-TCR-bearing $CD8^+$ T cells in 8.3-NOR mice was not an artifact of transgenesis. Furthermore, these data suggested that the putative $CD8^+$ T cell-deleting element(s) of NOR mice preferentially target(s) β cell-reactive $V\beta8.1^+CD8^+$ T cells bearing the highly diabetogenic 8.3-TCRα-chain, as opposed to all β cell-reactive $CD8^+$ T cells regardless of TCRα usage.

Diabetes resistance in 8.3-NOR mice is not associated with insulitis resistance or immune deviation

To investigate whether the diabetes resistance of 8.3-NOR mice was associated with resistance to insulitis and/or local immune deviation, we determined whether nondiabetic 8.3-NOR mice (at least 1 SD older than the average age at onset of diabetes in these mice; <2.5% chances of ever becoming diabetic) developed insulitis, and whether the insulitis lesions of these mice were quantitatively and/or qualitatively similar to those of nondiabetic 8.3-NOD mice. Histopathologic and RT-PCR studies revealed that 8.3-NOR mice had severe insulitis (Fig. 4*A*), that the insulitis lesions of these mice had $CD4^+$:$CD8^+$ T cell ratios similar to those

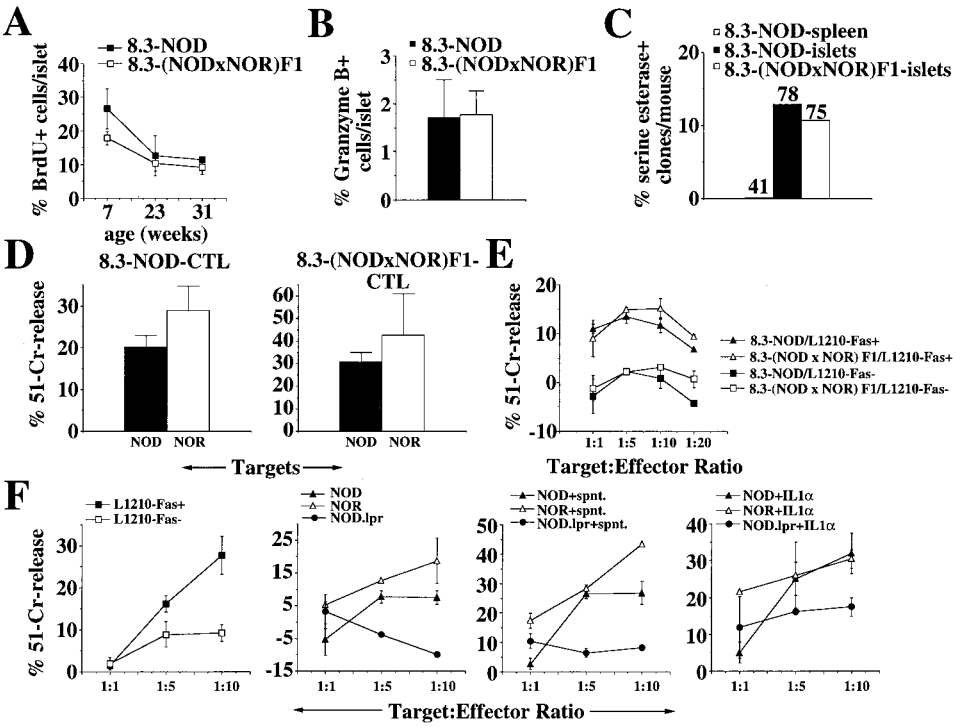


FIGURE 5. Functional in vivo and in vitro activity of islet-associated $CD8^+$ T cells from 8.3-NOD and 8.3-F_1 mice. *A*, BrdU incorporation in vivo. Nondiabetic mice ($n = 2–4$/age group) were given two i.v. injections of 200 μl of a 4 mg/ml solution of BrdU 4 h apart. The percentage of islet-infiltrating cells incorporating BrdU was calculated on pancreata collected 12 h later. Data correspond to 4–8 islets/mouse. *B*, Percentage of granzyme B^+ cells in islets. Data correspond to 9–10-wk-old 8.3-NOD ($n = 3$) and 8.3-F_1 mice ($n = 6$). *C*, Percentage of $CD8^+$ T cell clones derived from islets of 8–14-wk-old 8.3-NOD ($n = 3$) and 8.3-F_1 ($n = 3$) mice containing serine esterase activity. Spleen-derived $CD8^+$ T cell clones generated from two 8.3-NOD mice upon a single stimulation with islets in the presence of rIL-2 were used as negative controls. Numbers above the bars indicate number of clones tested. *D*, Cytotoxic activity of serine esterase$^+$ 8.3-$CD8^+$ T cell clones of 8.3-NOD and 8.3-F_1 mice against NOD and NOR islet cells. *E*, Fas-based cytotoxicity of islet cell suspensions from 8.3-NOD and 8.3-F_1 mice ($n = 4$/group, 8–10 wk old). *F*, Cytotoxic activity of islet-derived, serine esterase$^-$ $CD8^+$ T cell lines from two nondiabetic 8.3-NOD mice against L1210-Fas$^+$ and L1210-Fas$^-$ cells, and NOD, NOR, and NOD.*lpr* (Fas$^-$) islet cells cultured overnight in CM, or CM supplemented with $CD8^+$ T cell supernatants (spnt) or IL-1α (10^3 U/ml). Target NOD islet cells were from insulitis-free *RAG-2*$^-$ NOD mice.

seen in 8.3-NOD mice (Fig. 4*B*), and that the $CD8^+$ T cells derived from these lesions displayed cytokine profiles similar to those derived from the insulitis lesions of 8.3-NOD mice (Fig. 4*C*). These cytokine profiles were confirmed by measuring the intraislet content of IL-2, IL-4, and IFN-γ in 8.3-NOD and 8.3-NOR mice: the insulitis lesions of both types of mice contained IFN-γ (120–1200 pg/mg), but undetectable levels of IL-4 and IL-2 (data not shown) (note that IL-2 was barely detectable by RT-PCR). These data demonstrated that 8.3-$CD8^+$ T cells do not undergo immune deviation in 8.3-NOR mice, and that they accumulate in the islets of these mice quite efficiently.

Diabetes resistance in 8.3-F_1 mice in the absence of T cell tolerance or ignorance

Cytofluorometric studies of 8.3-F_1 mice indicated that their diabetes resistance could not be accounted for by deletion of 8.3-$CD8^+$ T cells (Figs. 1 and 2). Our next set of experiments focused on attempting to elucidate the mechanism(s) underlying the genetically dominant diabetes resistance of these mice. Since the pancreatic islets of these mice were severely infiltrated and contained abundant $CD8^+$ T cells (data not shown), we considered three possible mechanisms of diabetes resistance in these mice: 1) 8.3-$CD8^+$ T cells undergo anergy in situ; 2) 8.3-$CD8^+$ T cells fail to differentiate into β cell-cytotoxic T cells (CTLs); and/or 3) the local 8.3-$CD8^+$ CTLs are unable to kill NOR β cells because these cells do not express the target autoantigen, or are resistant to CTL-induced apoptosis. These questions were investigated by studying nondiabetic mice at ages beyond which their chances of developing diabetes were <5% (>10 wk).

To determine whether 8.3-$CD8^+$ T cells of 8.3-F_1 mice undergo anergy in situ, we compared the percentage of proliferating cells (incorporating exogenous BrdU) in islets of nondiabetic 8.3-F_1 and 8.3-NOD mice. As shown in Fig. 5*A*, no significant differences were noted between these mice at any of three different age points. To determine whether these cells differentiated into CTLs in situ, we compared the percentages of insulitic cells from nondiabetic 8.3-F_1 and 8.3-NOD mice that expressed granzyme B (a marker of cytotoxic granule content), as detected by immunohistochemistry. As shown in Fig. 5*B*, no differences were observed in these studies. The ability of 8.3-NOR $CD8^+$ T cells to differentiate into CTL in situ was confirmed by comparing the percentages of $CD8^+$ T cell clones containing serine esterase activity that could be isolated from islets of these two types of mice. As shown in Fig. 5*C*, islet-derived $CD8^+$ T cell lines from 8.3-F_1 mice contained as many serine esterase$^+$ clones as lines derived from 8.3-NOD mice. Differentiation of these islet-derived clones into CTL was not an artifact of in vitro stimulation, because none of 41 clones derived from splenic $CD8^+$ T cells of 8.3-NOD mice using the same stimulation protocol expressed serine esterase activity (Fig. 5*C*). Subsequent cytotoxicity assays using serine esterase$^+$$CD8^+$ CTL clones from 8.3-F_1 and 8.3-NOD mice demonstrated that the diabetes resistance of 8.3-F_1 mice could not be accounted for by an

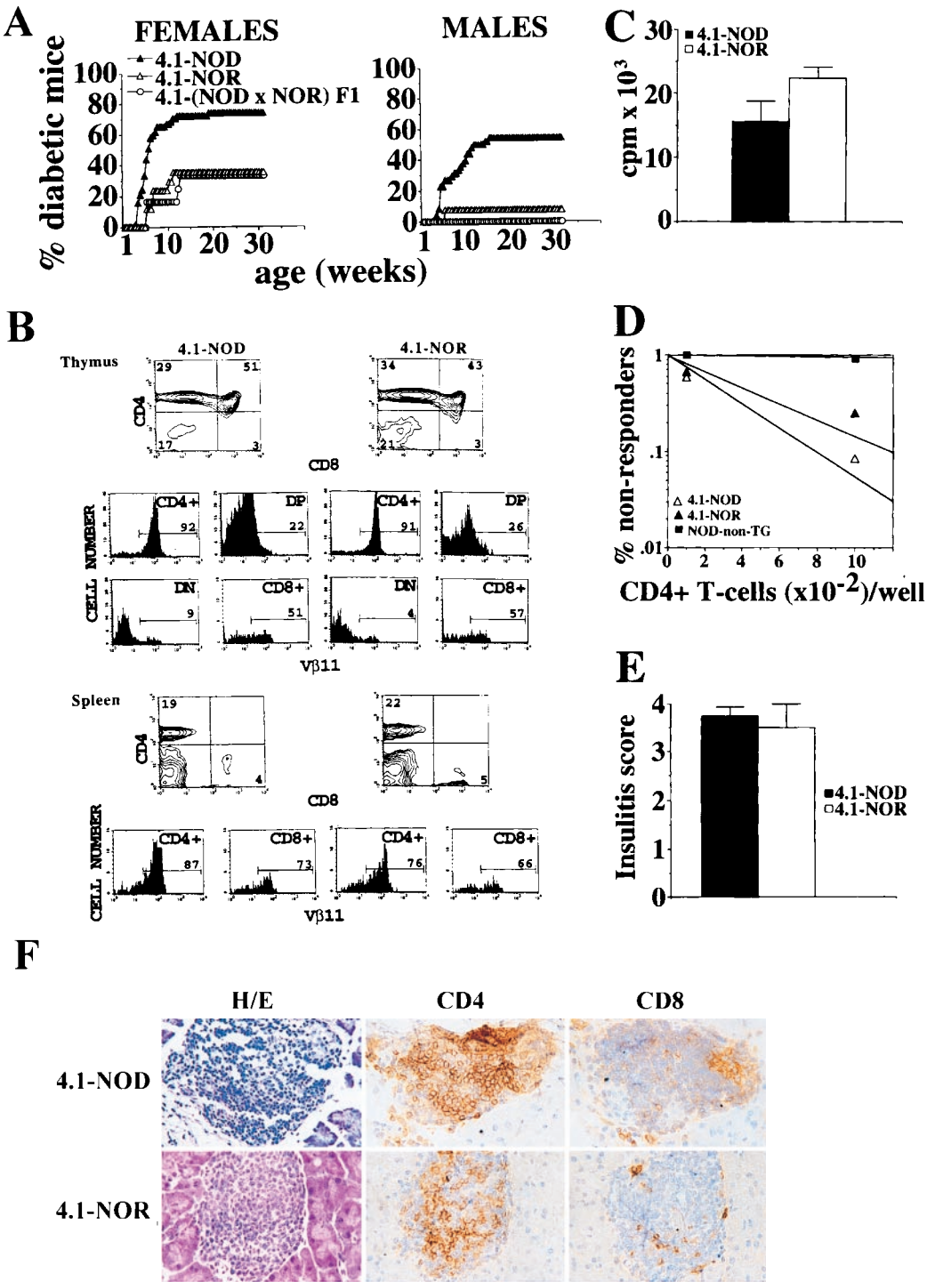


FIGURE 6. Diabetogenesis and T cell development in 4.1-NOD, 4.1-NOR, and 4.1-F_1 mice. *A*, Incidence of IDDM in female (4.1-NOD ($n = 57$), 4.1-F_1 ($n = 23$), and 4.1-NOR ($n = 26$)) and male (4.1-NOD ($n = 48$), 4.1-F_1 ($n = 13$), and 4.1-NOR ($n = 20$)) mice. Since the incidence and age at onset of diabetes in 4.1-NOR N5-N7 mice were similar to those seen in N2-N4 mice (see text), the figure shows values for N2-N7 mice. The average age at onset of diabetes in 4.1-F_1, 4.1-NOR, and 4.1-NOD females was: 60 ± 26 vs 76 ± 49 vs 47 ± 21 days. *B*, Flow-cytometry profiles of thymocytes and splenocytes from 4.1-NOD ($n = 14$; age 61 ± 25 days) and 4.1-NOR mice ($n = 6$; age 62 ± 15 days). Similar results were obtained with older mice (data nor shown). *C*, Proliferative activity of splenic $CD4^+$ T cells from 4.1-NOD and 4.1-NOR mice. $CD4^+$ T cells (2×10^4/well) were incubated, in triplicate, with 10^5 irradiated NOD islet cells and 10^5 irradiated NOD splenocytes for 3 days, pulsed with [^{3}H]thymidine, harvested, and counted. *D*, Peripheral frequency of β cell-reactive $CD4^+$ T cells in nondiabetic NOD, 4.1-NOD, and 4.1-NOR mice, as determined by limiting dilution assay. The figure shows values for two of the four dilutions tested. *E*, Insulitis scores of nondiabetic 4.1-NOD and 4.1-NOR mice ($n = 3$–6 mice/group; 7–12 wk old) (15–30 islets/mouse). Bars show the SD of the means. All mice had severe insulitis. *F*, Phenotype of insulitis T cells in 4.1-NOD and 4.1-NOR mice, as determined by immunopathology.

Table I. *Cytokine profile of peripheral 4.1-CD4$^+$ T cells in 4.1-NOD, 4.1-(NOD × NOR)F$_1$, and 4.1-NOR mice*[a]

Mouse	Expt.	IL-2 (pg/ml)	IL-4 (pg/ml)	IFN-γ (pg/ml)
4.1-NOD	1	75	<10	285
	2	50	0	53
	3	58	0	0
	4	57	17	69
4.1-(NOD × NOR)F$_1$	1	55	<5	50
	2	70	<5	73
4.1-NOR	1	<5	0	19
	2	22	0	22
NOD	1	<5	<5	<5
	2	22	0	13

[a] Splenocyes from nondiabetic mice (>11 wk old) were depleted of CD8$^+$ T cells using anti-CD8 mAb (53-6.7)-coated magnetic beads, adjusted to 2×10^4 cells/100 μl and cultured for 48 h in the presence of 1×10^5 NOD islet cells. The cytokine contents of the culture supernatants were determined in duplicate by ELISA.

inability of 8.3-F$_1$-derived 8.3-CD8$^+$ T cells to kill β cells, or by an inherent resistance of NOR β cells to perforin-based cytotoxicity (Fig. 5*D*); NOR β cells were at least as, if not more, susceptible to CTL-induced lysis as NOD β cells.

Since only a small percentage of the insulitic T cells of 8.3-F$_1$ and 8.3-NOD mice contained cytotoxic granules, we reasoned that 8.3-CD8$^+$ T cells might primarily kill β cells via the Fas pathway, as proposed for other CD8$^+$ T cells (30). In that case, the diabetes resistance of 8.3-F$_1$ mice might result from the relative inability of the insulitic CD8$^+$ T cells of 8.3-F$_1$ vs 8.3-NOD mice to effect Fas-dependent cytotoxicity and/or from resistance of NOR β cells to Fas-induced apoptosis. To investigate this, we first determined whether 8.3-CD8$^+$ T cells within islet cell suspensions from both types of mice could kill Fas$^+$ targets. As shown in Fig. 5*E*, cells from both types of mice killed Fas$^+$ (but not Fas$^-$) fibroblasts with similar efficiency. We then tested the ability of 4–5-day-old serine esterase$^-$ islet-derived T cell lines from 8.3-NOD mice to kill L1210-Fas$^+$ and L1210-Fas$^-$ fibroblasts, and NOD, NOR, and NOD.*lpr* (Fas$^-$) β cells, both in the absence and presence of cytokines that potentiate Fas-mediated cytotoxicity (28). As shown in Fig. 5*F*, these lines efficiently killed L1210-Fas$^+$ cells, Fas$^+$NOD islet cells, and Fas$^+$NOR islet cells, but not L1210-Fas$^-$ fibroblasts or Fas$^-$ NOD β cells; thus, 8.3-F$_1$ CD8$^+$ CTLs can kill Fas-expressing targets, and NOR and NOD β cells are equally susceptible to Fas-mediated cytotoxicity.

Diabetes resistance in 4.1-(NOD × NOR)F$_1$ and 4.1-NOR mice

The results of these experiments suggested that the genetically dominant resistance of (NOD × NOR)F$_1$ mice to diabetes was mediated by a novel mechanism. This prompted us to ask one fundamental question: does this mechanism selectively target 8.3-CD8$^+$ T cells, or does it also target other highly diabetogenic T cells regardless of phenotype or antigenic specificity? To address this question, we followed the fate of another highly pathogenic, but I-A^{g7}-restricted, β cell-specific TCR (4.1-TCR) (23) in (NOD × NOR)F$_1$ (4.1-F$_1$) and NOR (4.1-NOR) mice derived from a diabetic 4.1-NOD mouse. Interestingly, both 4.1-F$_1$ and 4.1-NOR mice (N2-N7 generations) displayed much lower incidences of diabetes than 4.1-NOD mice ($p < 0.0001$ for both females and males) (Fig. 6*A*). No obvious differences in disease incidence were noted between 4.1-TCR-transgenic mice of the N2-N4 and N5-N7 backcrosses (the latter typed as homozygous for NOR alleles at *Idd5*, *Idd9/11*, and *Idd13* loci) (females, 4/13 and 2/11, respectively; males, 1/10 and 0/7, respectively). The 4.1-F$_1$ and 4.1-NOR mice that developed diabetes did so slightly later than 4.1-NOD mice (i.e., 60 ± 26 vs 76 ± 49 vs 47 ± 21 days, respectively). As expected, the incidence of diabetes in the nontransgenic F$_1$ littermates that were followed was 0% at 32 wk (data not shown). Thus, like 8.3-F$_1$ mice, 4.1-F$_1$ mice also display dominant resistance to diabetes.

Absence of deletion, anergy, ignorance, and peripheral immune deviation in 4.1-F$_1$ and 4.1-NOR mice

We then investigated whether the diabetes resistance of 4.1-F$_1$ mice was also dissociated from known forms of T cell tolerance. Cytofluorometric, functional, and pathologic studies of nondiabetic mice (studied at ages beyond which the chances of developing diabetes were <9%; see Fig. 6) demonstrated that their diabetes resistance was not due to deletion, anergy, or ignorance; the 4.1-CD4$^+$ T cells of 4.1-F$_1$ (not shown), 4.1-NOR, and 4.1-NOD mice matured similarly (Fig. 6*B*), proliferated equally well in response to NOD (and NOR, data not shown) islet cell stimulation in vitro (Fig. 6*C*), contained similar numbers of β cell-reactive CD4$^+$ T cells (Fig. 6*D*), and had similar insulitogenic activity (Fig. 6, *E* and *F*). As in 8.3-F$_1$ and 8.3-NOR mice, there was no evidence of immune deviation in the periphery of nondiabetic 4.1-F$_1$ or 4.1-NOR mice (i.e., differentiation of 4.1-CD4$^+$ T cells into nonpathogenic Th2 cells): although there was considerable variation in the levels of IFN-γ secreted by CD4$^+$ T cells from different 4.1-NOD mice, the splenic 4.1-CD4$^+$ T cells of >11-wk-old 4.1-F$_1$ and 4.1-NOR mice (<4% chances of ever becoming diabetic) consistently secreted IL-2 and/or IFN-γ, but not IL-4, in response to islet stimulation (Table I).

The diabetes resistance of 4.1-F$_1$ and 4.1-NOR mice is not due to local immune deviation or to recruitment of IL-4- or TGF-β1-secreting cells to islets

These results did not rule out the possibility that 4.1-CD4$^+$ T cells undergo immune deviation in situ. Alternatively, since induction of Th2 responses against β cell autoantigens can inhibit diabetes

Table II. *Intra-islet content of cytokines in 4.1-NOD, 4.1-(NOD × NOR)F$_1$, and 4.1-NOR mice*[a]

Mouse	Expt.	IL-2 (pg of cytokines/mg of protein)	IL-4 (pg of cytokines/mg of protein)	IFN-γ (pg of cytokines/mg of protein)
4.1-NOD	1	50	125	137
	2	0	75	262
4.1-(NOD × NOR)F$_1$	1	0	0	25
	2	0	0	50
4.1-NOR	1	0	0	75
	2	25	0	25
NOD	1	0	25	0

[a] Purified islets from nondiabetic mice (>11 wk old) were homogenized in PBS containing protease inhibitors and centrifuged at 10,000 rpm to remove debris. The supernatants were adjusted at 200 μg of protein/ml, and 100 μl of each sample was used in duplicate to measure the cytokine content by ELISA.

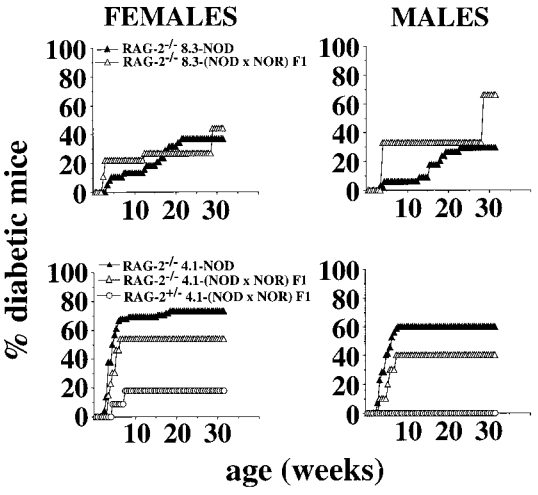


FIGURE 7. Cumulative incidence of diabetes in *RAG-2*$^-$ 8.3-F_1 and *RAG-2*$^-$ 4.1-F_1 mice vs *RAG-2*$^-$ 8.3-NOD and *RAG-2*$^-$ 4.1-NOD mice. Data correspond to 9 *RAG-2*$^-$ 8.3-F_1 females, 3 *RAG-2*$^-$ 8.3-F_1 males, 38 *RAG-2*$^-$ 8.3-NOD females, 34 *RAG-2*$^-$ 8.3-NOD males, 13 *RAG-2*$^-$ 4.1-F_1 females, 10 *RAG-2*$^-$ 4.1-F_1 males, 74 *RAG-2*$^-$ 4.1-NOD females, and 70 *RAG-2*$^-$ 4.1-NOD males.

progression in prediabetic NOD mice and disease recurrence in islet-grafted NOD mice (31), and diabetes does not develop in IL-4-treated mice (32), it was also possible that the genetic resistance of 4.1-F_1 or 4.1-NOR mice was due to recruitment of IL-4-secreting cells to islets. To address these possibilities, we quantitated the IL-2, IL-4, and IFN-γ content of islet extracts from nondiabetic mice of all three mouse strains by ELISA. These experiments revealed that while the islets of nondiabetic 4.1-NOD mice contained significant levels of both IL-4 and IFN-γ, the islets of nondiabetic 4.1-F_1 and 4.1-NOR mice (>11 wk of age; <4% chances of developing diabetes) contained IL-2 and/or IFN-γ, but undetectable levels of IL-4 (Table II). Islets from 4.1-F_1 and 4.1-NOR mice contained lower levels of IFN-γ than islets from 4.1-NOD mice, but the significance of these differences is unclear (Table II). Next, since TGF-β1 has been shown to have suppressive effects on the action of Th1 and Tc1 cells in vitro and in vivo, and can suppress the development of experimental autoimmunity in mice (33–35), it was also important that we determined whether the diabetes resistance of 4.1-F_1 mice was associated with recruitment of TGF-β1-producing cells to islets. The levels of TGF-β1 in islets of seven different 4.1-F_1 and 4.1-NOR mice, however, were barely detectable (1.8 $\pm$ 2 pg/mg) (data not shown). The mechanism that prevents diabetogenesis in F_1 (and NOR) mice does not therefore promote local immune deviation and does not involve the recruitment of IL-4- or TGF-β1-producing cells to islets.

Spontaneous diabetes in monoclonal 8.3-F_1 and 4.1-F_1 mice

The remarkable similarity between the results of studies with 4.1-F_1 and 8.3-F_1 mice prompted us to investigate whether the diabetes resistance of these mice was due to a form of immune suppression involving suppressor lymphocytes other than Th2 (IL-4-producing) or Th3 (TGF-β1-producing) cells. Since the diabetogenic potential of 4.1-CD4$^+$ and 8.3-CD8$^+$ T cells in NOD mice is not negatively affected by the presence of endogenous (nontransgenic) lymphocytes able to compete with the transgenic T cells for Ag recognition (24), this possibility could be tested by comparing the natural history of diabetes in *RAG-2*$^-$ 8.3-F_1, *RAG-2*$^-$ 8.3-NOD, *RAG-2*$^-$ 4.1-F_1, and *RAG-2*$^-$ 4.1-NOD mice; these mice cannot rearrange Ig or endogenous TCR genes and thus express monoclonal TCR repertoires (24). Interestingly, *RAG-2*$^-$ 8.3-F_1 and *RAG-2*$^-$ 4.1-F_1 mice developed diabetes almost as frequently, and as early as *RAG-2*$^-$ 8.3-NOD and *RAG-2*$^-$ 4.1-NOD mice, respectively (Fig. 7). As expected, the incidence of diabetes in *RAG-2*$^{+/-}$ 4.1-F_1 littermates was much lower: only 2 of 11 *RAG-2*$^{+/-}$ 4.1-F_1 females (18%) and none of 8 *RAG-2*$^{+/-}$ 4.1-F_1 males (0%) developed diabetes ($p < 0.015$ vs *RAG-2*$^-$ 4.1-NOD females, and $p < 0.004$ vs *RAG-2*$^-$ 4.1-NOD males) (Fig. 7). The same trend was noted in *RAG-2*$^{+/-}$ 8.3-F_1 mice; only 1 of 3 females, 33%, and none of 4 males, 0%, developed diabetes, incidences comparable with those seen in *RAG-2*$^{+/+}$ 8.3-F_1 mice (Fig. 1). It should be pointed out that direct comparison of the incidences of diabetes in *RAG-2*$^{+/-}$ and *RAG-2*$^{-/-}$ 8.3-F_1 mice is inappropriate in the context of this study, since the diabetogenic potential of 8.3-CD8$^+$ T cells is dramatically reduced in the absence of CD4$^+$ T cells bearing endogenous TCRs (24). Taken together, these findings demonstrated that the diabetes resistance of 8.3-F_1 and 4.1-F_1 mice is, at least in part, the result of a form of lymphocyte-mediated suppression not involving IL-4 or TGF-β1 that targets diabetogenic CD8$^+$ and CD4$^+$ T cells regardless of their fine antigenic specificity and MHC restriction.

Discussion

Genetic susceptibility and resistance to most spontaneous autoimmune disorders, including IDDM, are associated with highly polymorphic genes of the MHC and with multiple non-MHC-linked genes via unknown mechanisms. In this study, we have followed the fate of two highly diabetogenic TCRs in NOR and (NOD $\times$ NOR)F_1 mice, which are genetically resistant to spontaneous IDDM despite being *H-2*^{g7} homozygous (25, 26). These studies have revealed the existence of at least two simultaneous, but independent, mechanisms of non-MHC-linked genetic control of diabetogenic T cells. One segregates as a recessive trait and is associated with a marked reduction in the peripheral frequency of diabetogenic CD8$^+$ T cells. The second mechanism segregates as a dominant trait and is mediated by immunoregulatory functions provided by mature lymphocytes that interfere with the diabetogenicity of β cell-reactive CD4$^+$ and CD8$^+$ T cells, without causing their deletion, anergy, immune deviation, or ignorance, and without involving the recruitment of protective IL-4- or TGF-β1-producing cells to the pancreas. Although the existence of peripheral deletion and immune regulation as mechanisms of immune homeostasis is well established, our findings provide evidence for an association between a breakdown in these two specific forms of tolerance and the non-MHC-linked control of autoimmunity. They also demonstrate that protective non-MHC-linked genes may selectively target specific T cell types, rather than all autoreactive T cells, in cellularly complex autoimmune disorders.

The reduction in the peripheral frequency of 8.3-CD8$^+$ T cells that was observed in 8.3-NOR, but not 8.3-F_1, mice increased with age and was not mediated by endogenous superantigens binding to the transgenic TCRβ-chain. Studies of 8.3-TCRβ-transgenic NOR mice revealed that this phenotype was not an artifact of the high peripheral frequency of 8.3-CD8$^+$ T cells in 8.3-TCR-transgenic mice when compared with nontransgenic animals, and that it preferentially targeted CD8$^+$ T cells expressing TCRα-chains with CDR3 sequences identical to those of the CTL clone donating the 8.3-TCRβ transgene, rather than all autoreactive CD8$^+$ T cells regardless of TCR usage. While the underlying mechanisms remain unclear, we suspect that this reduction in the peripheral frequency of 8.3-CD8$^+$ T cells in 8.3-NOR mice is caused by deletion of 8.3-CD8$^+$ T cells in the periphery. Since initiation of diabetogenesis in nontransgenic NOD mice requires CD8$^+$ T cells

(36–38), and the 8.3-TCR uses a TCRα-CDR3 sequence that is highly homologous to TCRα-CDR3 sequences used by many NOD islet-derived β cell-cytotoxic $CD8^+$ T cells (27, 39), it is possible that some of the diabetes resistance of nontransgenic NOR mice results from their ability to delete the most pathogenic of all autoreactive $CD8^+$ T cells, rather than all autoreactive $CD8^+$ T cells regardless of their pathogenicity. The incomplete penetrance of 8.3-$CD8^+$ T cell deletion and diabetes resistance in 8.3-NOR mice does not argue against this view; the high frequency of 8.3-$CD8^+$ T cells in 8.3-NOR mice may overwhelm the mice's deleting machinery, particularly since NOR mice also appear to bear recessive prodiabetogenic genes. The complete absence of 8.3-TCRα sequences in TCRα cDNA libraries generated from islet-reactive T cell lines of 8.3-TCRβ-transgenic NOR mice, which have a much lower frequency of β cell-reactive $CD8^+$ T cells and do not develop diabetes, supports this interpretation of the data. Whatever the relative role of this phenomenon in the genetic resistance of NOR/Lt mice to spontaneous IDDM, these results demonstrate the existence of non-MHC-linked genetic elements other than endogenous superantigens that can control the fate of pathogenic autoreactive $CD8^+$ T cells in the periphery. Importantly, these elements target diabetogenic $CD8^+$ T cells while sparing diabetogenic $CD4^+$ T cells.

While we do not yet know the nature of these genetic elements, interpretation of our findings vis-à-vis the results of previous genetic studies provides some clues. It has been shown that IDDM resistance in (NOD × NOR)F_2 mice segregates with *Idd5*, *Idd9*, and *Idd13* (26). Although *Idd5*^{r}, linked to *ctla4* and *cd28* in chromosome 1, is associated with increased susceptibility of T cells to cyclophosphamide-induced apoptosis in vivo (40), two lines of evidence suggest that this reduction in the peripheral frequency of 8.3-$CD8^+$ T cells in 8.3-NOR mice and the *Idd5*^{r}-encoded susceptibility to cyclophosphamide-induced apoptosis are unrelated phenomena: 1) the putative gene within the *Idd5*^{r} region of NOR mice that controls apoptosis susceptibility/resistance is of NOD origin (40); and 2) 8.3-$CD8^+$ T cells from 8.3-NOR and 8.3-NOD mice show equal susceptibility to reactivation-induced apoptosis in response to several different stimuli, including islet cells and anti-TCR or anti-Fas mAbs (our unpublished observations). Although a contribution of *Idd5* to the "deleting" phenotype of 8.3-NOR mice cannot be ruled out, the fact that deletion of diabetogenic T cells in NOR mice selectively targets MHC class I-restricted $CD8^+$ T cells, but not MHC class II-restricted $CD4^+$ T cells, makes *Idd13* a more attractive candidate: *Idd13* contains the dimorphic $\beta_2 m$ (β_2-microglobulin) locus; NOD and NOR mice express different β_2m isoforms; and these isoforms account for conformational differences between the otherwise identical K^d and D^b MHC class I molecules of these mice (41–43). Since the *Idd13*^{r}-controlled diabetes resistance of NOR mice resides in a radioresistant nonhemopoietic cell type, perhaps the β cell itself (44), and since NOR β cells display a somewhat greater susceptibility to 8.3-CTL-induced lysis than NOD β cells (see Fig. 5), it would be reasonable to speculate that the reduction in the peripheral frequency of 8.3-$CD8^+$ T cells in 8.3-NOR mice results from engagement of target K^d/β_2m^b complexes on β cells or APCs with an affinity/avidity that surpasses a deleting threshold. This view is compatible with two observations: 1) "deletion" segregates as a recessive trait (i.e., two copies of the β_2m^b molecule would increase the avidity of the TCR-MHC class I interaction and hence the chances of reaching the deleting threshold); and 2) in 8.3-TCRβ-transgenic NOR mice, "deletion" does not systematically target all β cell-reactive $CD8^+$ T cells, but rather only those bearing the pathogenic 8.3-TCRα-CDR3 sequence. This interpretation would also provide an explanation for the paradoxical acceleration of IDDM in the few 8.3-NOR mice that developed IDDM: in young 8.3-NOR mice, in which the reduction in the peripheral frequency of 8.3-$CD8^+$ T cells is minimal, such an increased affinity would actually promote IDDM. The reasons behind the increased incidence (and accelerated onset) of diabetes in 8.3-NOR vs 8.3-F_1 male mice might also be due to this. However, we favor the alternative possibility that NOR/Lt mice also bear recessive prodiabetogenic genes that in males, but not females, can override the antidiabetogenic function of the dominant protective element(s). Ongoing studies of *Idd13*^{r}-congenic 8.3-NOD mice should answer some of these questions.

Peripheral deletion of diabetogenic $CD8^+$ T cells, however, is clearly not the only mechanism of diabetes resistance operating in NOR mice; 8.3-F_1 mice are diabetes resistant, but do not delete transgenic 8.3-$CD8^+$ T cells. Experiments with another highly diabetogenic, but I-A^{g7}-restricted, β cell-specific TCR revealed that the diabetes resistance of F_1 mice is primarily determined by one (or more) dominant protective element(s) that interfere(s) with the pathogenic activity of diabetogenic $CD8^+$ and $CD4^+$ T cells, regardless of their MHC restriction and antigenic specificity. This genetic element does not function by causing the deletion, anergy, or immune deviation of the transgenic T cells, by interfering with their insulitogenic activity, or by blocking their differentiation into CTLs in situ, but instead by promoting a form of immune suppression that is affected by mature B cells or endogenous T cells. The nature of the lymphocyte type and the specific mechanisms through which it prevents 4.1-$CD4^+$ and 8.3-$CD8^+$-induced β cell death are not yet known, but there are several possibilities. B cells constitute a substantial fraction of islet-infiltrating lymphocytes in our TCR-transgenic mice (24), and thus may be able to mediate this local immunosuppressive effect. Although B lymphocytes have a greater capacity than other APC types to preferentially activate Th2 cytokine responses (45), the absence of local immune deviation in TCR-transgenic NOR mice suggests that this is not the mechanism by which these cells might function. Alternatively, since anti-idiotypic and anti-TCR-V region Abs can dampen T cell-induced autoimmune responses (46, 47), B cells might afford diabetes protection by mounting powerful local anti-idiotypic responses in NOR (but not NOD) mice (i.e., against the diabetogenic TCRs). The fact that B cell-deficient NOD mice are resistant to diabetes (48), however, argues against a role for B cells in this immunosuppressive response, as this would imply that B cells have qualitatively opposed functions in NOD vs NOR mice. Rather, we favor the alternative, but not exclusive, possibility that this protective function is affected by T cells; T cells capable of suppressing autoimmune diabetes have been described, and some of these cells have actually been isolated from NOD mice (49–54). Since disease suppression in TCR-transgenic F_1 mice is dissociated from local production of IL-4 and TGF-β1, these suppressor T cells would more likely be related to a recently described type of antidiabetogenic $CD4^+$ Th1 cells (54) or to anti-idiotypic T cells (55–57), than to Th2, Th3, or IL-4-producing $TCR^+CD4^-CD8^-$ cells (58). It is noteworthy that this protective mechanism is not very efficient at suppressing $CD8^+$ T cell-induced diabetes in male 8.3-NOR mice, suggesting that NOR/Lt mice also bear recessive genes that potentiate the diabetogenic activity of pathogenic $CD8^+$ T cells in males.

Too little is known about the antidiabetogenic loci of NOR mice, to speculate as to whether this form of genetically dominant resistance to diabetes is encoded on one of these loci, on a combination of them, or on other unknown loci. Nonetheless, since the NOR mouse derives some of its genetic material from C57BL/6 and DBA/2 mice (25, 26), it is likely that these elements are also present in other genetic backgrounds. This mechanism may account, in part, for the diabetes resistance of 4.1-(NOD × C57BL/6.*I-A*β^{b-})F_1 mice, which were completely resistant to spontaneous

IDDM, despite not being able to delete 4.1 thymocytes (23). It would also be reasonable to expect that less powerful forms of these protective elements also exist in mice that are susceptible to autoimmunity. These variants, which would be able to suppress some autoreactive T cells, but not the most pathogenic ones (i.e., 8.3-CD8$^+$ and 4.1-CD4$^+$ T cells), might account for the dramatic acceleration (and increased incidence) of diabetes in BDC-2.5-NOD.*scid* vs BDC-2.5-NOD mice, which express another, less diabetogenic, β cell-reactive TCR (22). They may also account for dramatic differences in the incidence of experimental autoimmune encephalomyelitis in RAG-1$^+$ vs RAG-1$^-$ mice expressing a myelin basic protein-specific TCR (21).

In summary, this study has uncovered the existence of two mechanisms of non-MHC-linked genetic control of diabetogenic T cells. The fact that one of these mechanisms targets both CD4$^+$ and CD8$^+$ T cells, regardless of their molecular make-up and fine antigenic specificity, suggests that its failure may account for the clustering of multiple autoimmune disorders in affected individuals or their relatives (4, 59). Reductionist approaches such as the one used in this study should help define the mechanisms of action of specific non-MHC-linked chromosomal regions associated with spontaneous autoimmunity.

Acknowledgments

We thank Drs. F. Alt for providing RAG-2$^-$ mice, P. Goldstein for L1210-Fas$^+$ and L1210-Fas$^-$ cell lines, P. Marrack for providing the KJ16 hybridoma, T. Utsugi for providing rIL-2, T. Delovitch for suggesting the use of pancreatic extracts to measure intrapancreatic cytokine content, K. Hirasawa for advice on immunopathology, and D. Serreze and R. Tobias for advice on Idd typing. We also thank S. Bou for excellent technical assistance, L. Bryant for flow cytometry, R. Dawson and L. Morck for animal care, H. Kominek for editorial assistance, and Judy Patterson for secretarial help.

References

1. Delovitch, T. L., and B. Singh. 1997. The nonobese diabetic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD. *Immunity 7:727.*
2. Serreze, D. V., and E. H. Leiter. 1994. Genetic and pathogenic basis of autoimmune diabetes in NOD mice. *Curr. Opin. Immunol. 6:900.*
3. Wicker, L., E. Leiter, J. Todd, R. Renjilian, E. Peterson, P. Fischer, P. Podolin, M. Zijlstra, R. Jaenisch, and L. Peterson. 1994. β_2-microglobulin-deficient NOD mice do not develop insulitis or diabetes. *Diabetes 43:500.*
4. Vyse, T. J., and J. A. Todd. 1996. Genetic analysis of autoimmune disease. *Cell 85:311.*
5. Acha-Orbea, H., and H. O. McDevitt. 1987. The first external domain of the non-obese diabetic mouse class II I-Aβ chain is unique. *Proc. Natl. Acad. Sci. USA 84:2435.*
6. Carrasco-Marin, E., J. Shimizu, O. Kanagawa, and E. R. Unanue. 1996. The class II MHC I-A^{g7} molecules from non-obese diabetic mice are poor peptide binders. *J. Immunol. 156:450.*
7. Kaufman, D., M. Clare-Salzler, J. Tian, T. Forsthuber, G. Ting, P. Robinson, M. Atkinson, E. Sercarz, A. Tobin, and P. Lehmann. 1993. Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes mellitus. *Nature 366:69.*
8. Tisch, R., X. D. Yang, S. Singer, R. Liblau, L. Fugger, and H. McDevitt. 1993. Immune response to glutamic acid decarboxylase correlates with insulitis in nonobese diabetic mice. *Nature 366:72.*
9. Elliott, J., H. Quin, S. Bhatti, D. Smith, R. Singh, T. Dillon, J. Lauzon, and B. Singh. 1994. Immunization with the larger form of glutamic acid decarboxylase (GAD) prevents diabetes in NOD mice. *Diabetes 43:1494.*
10. Ridgway, W., M. Fasso, A. Lanctot, C. Garvey, and G. Fathman. 1996. Breaking self-tolerance in nonobese diabetic mice. *J. Exp. Med. 183:1657.*
11. Miller, J. F. A. P., and A. Basten. 1996. Mechanisms of tolerance to self. *Curr. Opin. Immunol. 8:815.*
12. Ohashi, P., S. Oehen, K. Burki, H. Pircher, C. Ohashi, B. Odermatt, B. Malissen, R. Zinkernagel, and H. Hengartner. 1991. Ablation of tolerance and induction of diabetes by virus infection in viral antigen transgenic mice. *Cell 65:305.*
13. Oldstone, M. B. A., M. Nerenberg, P. Southern, J. Price, and H. Lewicki. 1991. Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. *Cell 65:319.*
14. Ferber, I., G. Schönrich, J. Schenkel, A. Mellor, G. Hammerling, and B. Arnold. 1994. Levels of peripheral T cell tolerance induced by different doses of tolerogen. *Science 263:674.*
15. Scott, B., R. Liblau, S. Degermann, L. Marconi, L. Ogata, A. Caton, H. McDevitt, and D. Lo. 1994. A role for non-MHC genetic polymorphism in susceptibility to spontaneous autoimmunity. *Immunity 1:73.*
16. Heath, W., F. Karamalis, J. Donoghue, and J. F. A. P. Miller. 1995. Autoimmunity caused by ignorant CD8$^+$ T-cells is transient and depends on avidity. *J. Immunol. 155:2339.*
17. Von Herrath, M., S. Guerder, H. Lewicki, R. Flavell, and M. Oldstone. 1995. Coexpression of B7-1 and viral ("self") transgenes in pancreatic β cells can break peripheral ignorance and lead to spontaneous autoimmune diabetes. *Immunity 3:727.*
18. Förster, I., R. Hirose, J. Arbeit, B. Clausen, and D. Hanahan. 1995. Limited capacity for tolerization of CD4$^+$ T cells specific for a pancreatic neoantigen. *Immunity 2:573.*
19. Bertolino, P., W. Heath, C. Hardy, G. Morahan, and J. F. A. P. Miller. 1995. Peripheral deletion of autoreactive CD8$^+$ T cells in transgenic mice expressing H-2K^d in the liver. *Eur. J. Immunol. 25:1932.*
20. Katz, J. D., B. Wang, K. Haskins, C. Benoist, and D. Mathis. 1993. Following a diabetogenic T-cell from genesis through pathogenesis. *Cell 74:1089.*
21. Lafaille, J., K. Nagashima, M. Katsuki, and S. Tonegawa. 1994. High incidence of spontaneous autoimmune encephalomyelitis in immunodeficient anti-myelin basic protein T cell receptor transgenic mice. *Cell 78:399.*
22. Kurrer, M. O., S. M. Pakala, H. Hanson, and J. D. Katz. 1997. β cell apoptosis in T-cell mediated autoimmune diabetes. *Proc. Natl. Acad. Sci. USA 94:213.*
23. Schmidt, D., J. Verdaguer, N. Averill, and P. Santamaria. 1997. A mechanism for the major histocompatibility complex-linked resistance to autoimmunity. *J. Exp. Med. 186:1059.*
24. Verdaguer, J., D. Schmidt, A. Amrani, B. Anderson, N. Averill, and P. Santamaria. 1997. Spontaneous autoimmune diabetes in monoclonal T cell nonobese diabetic mice. *J. Exp. Med. 186:1663.*
25. Prochazka, M., D. Serreze, W. Franke, and E. Leiter. 1992. NOR/Lt mice: MHC-matched diabetes-resistant control strain for NOD mice. *Diabetes 41:98.*
26. Serreze, D. V., M. Prochazka, P. C. Reifsnyder, M. M. Bridgett, and E. H. Leiter. 1994. Use of recombinant congenic and congenic strains of NOD mice to identify a new insulin-dependent diabetes resistance gene. *J. Exp. Med. 180:1553.*
27. Verdaguer, J., J.-W. Yoon, B. Anderson, N. Averill, T. Utsugi, B.-J. Park, and P. Santamaria. 1996. Acceleration of spontaneous diabetes in TCRβ-transgenic nonobese diabetic mice by β cell-cytotoxic CD8$^+$ T-cells expressing identical endogenous TCRα chains. *J. Immunol. 157:4726.*
28. Yamada, K., N. Takane-Gyotoku, F. Ichikawa, C. Inada, and K. Nokada. 1996. Mouse islet cell lysis mediated by interleukin-1-induced Fas. *Diabetologia 39: 1306.*
29. Cameron, M. J., G. A. Arreaza, P. Zucker, S. W. Chensue, R. M. Strieter, S. Chakrabarti, and T. L. Delovitch. 1997. IL-4 prevents insulitis and insulin-dependent diabetes mellitus in nonobese diabetic mice by potentiation of regulatory T helper-2 cell function. *J. Immunol. 159:4686.*
30. Chervonsky, A., Y. Wang, F. Wong, I. Visintin, R. Flavell, C. A. Janeway, Jr., and L. Matis. 1997. The role of Fas in autoimmune diabetes. *Cell 89:17.*
31. Tian, J., M. Atkinson, M. Clare-Salzler, A. Herschenfeld, T. Forsthuber, P. Lehmann, and D. Kaufman. 1996. Nasal administration of glutamate decarboxylase (GAD65) peptides induces Th2 responses and prevents murine insulin-dependent diabetes. *J. Exp. Med. 183:1561.*
32. Rapoport, M., A. Jaramillo, D. Zipris, A. Lazarus, D. Serreze, E. Leiter, P. Cyopick, J. Danska, and T. Delovitch. 1993. Interleukin 4 reverses T cell proliferative unresponsiveness and prevents the onset of diabetes in nonobese diabetic mice. *J. Exp. Med. 178:87.*
33. Johns, L., K. Flanders, R. Ranges, and S. Sriram. 1991. Successful treatment of experimental allergic encephalomyelitis with transforming growth factor-β1. *J. Immunol. 147:1792.*
34. Abbas, A., K. Murphy, and A. Sher. 1996. Functional diversity of helper T lymphocytes. *Nature 383:787.*
35. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J. D. Vries, and M. Roncarolo. 1997. A CD4$^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature 389:737.*
36. Katz, J., C. Benoist, and D. Mathis. 1993. Major histocompatibility complex class I molecules are required for the generation of insulitis in non-obese diabetic mice. *Eur. J. Immunol. 23:3358.*
37. Wicker, L., J. Todd, and L. Peterson. 1995. Genetic control of autoimmune diabetes in the NOD mouse. *Annu. Rev. Immunol. 13:179.*
38. Serreze, D. V., E. H. Leiter, G. J. Christianson, D. Greiner, and D. C. Roopenian. 1994. Major histocompatibility complex class I-deficient NOD-β_2m^{null} mice are diabetes and insulitis resistant. *Diabetes 43:505.*
39. Santamaria, P., T. Utsugi, B.-J. Park, N. Averill, and J.-W. Yoon. 1995. β cell-cytotoxic CD8$^+$ T-cells from nonobese diabetic mice use highly homologous T-cell receptor α-chain CDR3 sequences. *J. Immunol. 154:2494.*
40. Colucci, F., M. Bergman, C. Penha-Goncalves, C. Cilio, and D. Holmberg. 1997. Apoptosis resistance of nonobese diabetic peripheral lymphocytes linked to the *Idd5* diabetes susceptibility region. *Proc. Natl. Acad. Sci. USA 94:8670.*
41. Ferrier, P., J. C. Fontecilla-Camps, D. Bucchini, D. H. Caillol, B. R. Jordon, and F. A. Lemonnier. 1985. Altered structure of HLA class I heavy chains associated with mouse β-2 microglobulin. *Immunogenetics 21:321.*
42. Nieto, M., E. S. Song, D. McKinney, M. McMillan, and R. S. Goodenow. 1989. The association of H-2L^d with human β-2 microglobulin induces localized conformational changes in the α-1 and -2 superdomain. *Immunogenetics 30:361.*
43. Danliczyk, U. G., and T. L. Delovitch. 1994. β-2-microglobulin induces a conformational change in the MHC class I H chain that occurs intracellularly and is maintained at the cell surface. *J. Immunol. 153:3533.*

44. Serreze, D. V., M. Bridgett, H. D. Chapman, E. Chen, S. Richard, and E. H. Leiter. 1998. Subcongenic analysis of the *Idd13* locus in NOD/Lt mice: evidence for several susceptibility genes including a possible diabetogenic role for β_2-microglobulin. *J. Immunol. 160:1472.*
45. Liblau, R., S. Singer, and H. McDevitt. 1995. Th1 and Th2 $CD4^+$ T cells in the pathogenesis of organ-specific autoimmune diseases. *Immunol. Today 16:34.*
46. Alboran, I. D., J. A. Gonzalo, G. Kroemer, E. Leonardo, M. A. R. Marcos, and C. A. Martinez. 1992. Attenuation of autoimmune disease and lymphocyte accumulation in MRL/*lpr* mice by treatment with anti-Vβ8 antibodies. *Eur. J. Immunol. 22:2153.*
47. Zhou, S. R., and J. N. Whitaker. 1993. Specific modulation of T cells and murine experimental allergic encephalomyelitis by monoclonal anti-idiotypic antibodies. *J. Immunol. 150:1629.*
48. Serreze, D., H. Chapman, D. Varnum, M. Hanson, P. Reifsnyder, S. Richard, S. Fleming, E. Leiter, and L. Shultz. 1996. B lymphocytes are essential for the initiation of T cell-mediated autoimmune diabetes: analysis of a new "speed congenic" stock of NOD.*Igm^{null}* mice. *J. Exp. Med. 184:2049.*
49. Boitard, C., R. Yasunami, M. Dardenne, and J.-F. Bach. 1989. T cell-mediated inhibition of the transfer of autoimmune diabetes in NOD mice. *J. Exp. Med. 169:1669.*
50. Reich, E. P., D. Scaringe, J. Yagi, R. S. Sherwin, and C. A. Janeway, Jr. 1989. Prevention of diabetes in NOD mice by injection of autoreactive T-lymphocytes. *Diabetes 38:1647.*
51. Pankewycz, O., J. Guan, and J. Benedict. 1992. A protective NOD islet-infiltrating $CD8^+$ T cell clone, I. S. 2.15, has in vitro immunosuppressive properties. *Eur. J. Immunol. 22:2017.*
52. Chosich, N., and L. Harrison. 1993. Suppression of diabetes mellitus in the nonobese diabetic mouse by an autoreactive (anti-I-A^{g7}) islet derived $CD4^+$ T cell line. *Diabetologia 36:716.*
53. Utsugi, T., M. Nagata, T. Kawamura, and J.-W. Yoon. 1994. Prevention of recurrent diabetes in syngeneic islet-transplanted NOD mice by transfusion of autoreactive T lymphocytes. *Transplantation 57:1799.*
54. Akhtar, I., J. Gold, L.-Y. Pan, J. Ferrara, X.-D. Yang, J. Kim, and K.-N. Tan. 1995. $CD4^+$ β islet cell-reactive T cell clones that suppress autoimmune diabetes in nonobese diabetic mice. *J. Exp. Med. 182:87.*
55. Lider, O., T. Reshef, E. Beraud, A. Ben-Nun, and I. R. Cohen. 1988. Anti-idiotypic network induced by T cell vaccination against experimental autoimmune encephalomyelitis. *Science 239:181.*
56. Lohse, A. W., F. Mor, N. Karin, and I. R. Cohen. 1989. Control of experimental autoimmune encephalomyelitis by T cells responding to activated T cells. *Science 244:820.*
57. Vandenbark, A. A., G. Hashim, and H. Offner. 1989. Immunization with a synthetic T cell receptor V-region peptide protects against experimental autoimmune encephalomyelitis. *Nature 341:541.*
58. Wilson, S., S. Kent, K. Patton, T. Orban, R. Jackson, M. Exley, S. Porcelli, D. Schatz, M. Atkinson, S. Balk, J. Strominger, and D. Hafler. 1998. Extreme Th1 bias of invariant Vα24JαQT cells in type 1 diabetes. *Nature 391:177.*
59. Santamaria, P., J. Barbosa, A. Lindstrom, T. Lemke, F. Goetz, and S. Rich. 1994. HLA-DQB1-associated susceptibility that distinguishes Hashimoto's thyroiditis from Graves' disease in type I diabetic patients. *J. Clin. Endocrinol. Metab. 78:878.*