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

Joan Verdaguer, Abdelaziz Amrani, Brad Anderson, Dennis Schmidt and Pere Santamaria

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

References
This article cites 58 articles, 28 of which you can access for free at:
http://www.jimmunol.org/content/162/8/4614.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Two Mechanisms for the Non-MHC-Linked Resistance to Spontaneous Autoimmunity

Joan Verdaguer, Abdelaziz Amrani, Brad Anderson, Dennis Schmidt, and Pere Santamaria

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 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⁺ T cells. The other segregates as a dominant trait and is mediated by IL-4- and TGF-β-dependent 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 cellulary complex autoimmune responses. The Journal of Immunology, 1999, 162: 4614–4626.

Insulin-dependent diabetes mellitus (IDDM) 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. 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.

The diabetes-prone nonobese diabetic (NOD) mouse, which spontaneously develops a form of diabetes closely resembling human IDDM, is homozygous for a unique MHC haplotype (H-2k) that encodes a single MHC class II molecule (I-Ak) with unique structure and biochemical behavior. Genetic studies have shown that, while necessary, I-Ak homozygosity is insufficient for development of diabetes, and that diabetesogenesis requires interactions between pathogenic MHC molecules and putative products encoded on as many as 17 different non-MHC-linked loci. 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. 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. 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 or are not highly pathogenic in genetically susceptible backgrounds.

Our previous studies with I-Ak-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. These studies, however, also revealed that positive selection of pathogenic TCRs in diabetes-resistant backgrounds...
expressing diabeticogenic 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 anti diabeticogenic genes. This was done by following the fate of our two β cell-specific TCRs (4.1, IAβ7 restricted; and 8.3, Kβ4 restricted) in NOR and (NOD × NOR)F1 mice, both of which are resistant to islet inflammation (insulinitis) and diabetes, despite deriving ~88% of their genome from the NOD mouse, including two copies of the H-2β7 haplotype (25, 26). These studies have resulted in the discovery of two mechanisms of non-MHC-linked control of diabeticogenic T cells. One of these mechanisms is recessive and induces a reduction in the peripheral frequency of diabeticogenic 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 diabeticogenic 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

NOD mouse, including two copies of the H-2g7 haplotype (25, 26). Mit11 (Idd11) and Idd31 loci. RAG-2 G2 mice were generated by backcrossing the RAG-2 mutation of RAG-2 C57BL/6J129 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-F3 and RAG-2 4.1-F1 mice were generated by intercrossing RAG-2 8.3-NOR or RAG-2 4.1-NOR mice were produced by backcrossing 8.3-TCRβ-transgenic NOD 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 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/6J129 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-F3 and RAG-2 4.1-F1 mice were generated by intercrossing RAG-2 8.3-NOR or RAG-2 4.1-NOR mice (24) with NOR 2 NOR or RAG-2 8.3-TCRβ-transgenic NOD mice were produced by backcrossing 8.3-TCRβ-transgenic NOD (NOR × NOR)F1 mice, 27) with NOR mice for up to nine generations. Fas + NOD.Ipr mice were generated by backcrossing the Fas + gene of B6.MRL-Fas + mice (from The Jackson Laboratory) onto the NOD background for up to seven generations. Fas + NOD.Ipr mice were generated by intercrossing 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 of a cell-reactive CD8+ T cell lines. Islet-derived CD8+ 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 mice 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 106 CD8+ or CD4+ T cell/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 × 105) in 96-well tissue culture plates for 3 days at 37°C in 5% CO2. Cultures of 4.1-CD41 T cells received 105 irradiated splenocytes from nontransgenic NOD mice, as feeders. Cultures were pulsed with 1 μCi of [3H]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 (105 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-Kβ2 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-Kβ2, cells were considered to contain β cell-reactive CD8+ T cells (24). To determine the frequency of β cell-reactive CD4+ T cells, four replicate cultures of 10-fold serial dilutions of CD8+ T cell-derived splenocytes (104–105 cells/well) were stimulated with 2.5 × 105 irradiated islet cells and 2 × 104 irradiated NOD splenocytes for 4 days, followed by rIL-2 for 10 days and a second restimulation with islet cells, splenocytes, and rIL-2 (24). By day 12, 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 in 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.

Diabetes

Diabetes was assessed by measuring urine glucose levels with Diasix strips (Miles, Ontario, Canada) twice weekly. Animals were considered diabetic after two consecutive readings ≥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-Kβ2 transfectants were provided by Dr. J. Yewdell (National Institutes of Health, Bethesda, MD). Hydromics secreting mAbs GK1.5 (anti-CD4) and 53-6.7 (anti-CD8) were obtained from the American Tissue Culture Collection (Manassas, VA). A hydromics secreting the Vβ8.1/8.2-specific mAb KJ16 was a gift from P. Marrack (National Jewish Center, Denver, CO). Anti-Ly-2 (CD69) phycoerythrin (53-6.7), anti-LT4+ FITC (IM7), anti-LT4+ 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-CD54+R FITC (23G2), anti-L-selectin biotin (CD62L) (Mel-14), anti-CD95 biotin (H1.2F3), anti-Vβ8.1/8.2 FITC (MR5-2), and anti-H-2Kβ FITC (SF1-1.1) were purchased from PharMingen (San Diego, CA). Anti-IL-2R FITC (CD25) (AM13) 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 antiserum and bisinylated anti-guinea pig IgG were obtained from Dako (Carpinteria, CA). Biotinylated swine anti-goat IgG was obtained from Cedarlane Laboratories. Thyml, 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).
Target cells (L1210-Fas<sup>+</sup>, L1210-Fas<sup>−</sup>, and single NOD or NOR islet cells) were incubated with <sup>51</sup>Cr sodium chromate (DuLab/NEN, Boston, MA) and seeded at 1 x 10<sup>5</sup> cells per 100 µL/well. Some experiments (those in Fig. 5F) employed islet cells derived from islets that had been preincubated overnight with supernatants from 3-day-old islet-derived CD8<sup>+</sup> T cell lines, or with IL-1α (10<sup>3</sup> U/L), to increase their susceptibility to Fas-mediated cytotoxicity (28). Effector cells (islet-infiltrating T cells, islet-derived serine esterase<sup>+</sup> CD8<sup>+</sup> T cell lines, or islet-derived serine esterase<sup>+</sup> CD8<sup>+</sup> 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 <sup>51</sup>Cr release (24).

Cloning and sequencing of TCRα mRNAs

The TCRα-chain cDNA molecules of islet-stimulated splenic CD8<sup>+</sup> T cells from 8.3-TCR<sup>+</sup>-transgenic NOD and 8.3-TCR<sup>+</sup>-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<sup>+</sup> T cell-depleted T cell lines was reverse transcribed using oligo(dT)<sub>12–18</sub> (Life Technologies) as a primer, and the resulting cDNAs were amplified by PCR (27).

In vitro and in vivo cytokine secretion

Splenic CD4<sup>+</sup> T cells (2 x 10<sup>5</sup>/well) were incubated with γ-irradiated NOD islet cells (10<sup>5</sup>/well) and splenocytes (10<sup>5</sup>/well) in 96-well plates for 48 h at 37°C in 5% CO<sub>2</sub>. 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 intrasel 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 anticytokerin (M), anti-HLA class I (clone L243, 1/400 dilution), rat Ig Abs and horseradish peroxidase (HRP)-streptavidin conjugate (Dianova, Mississauga, ON, Canada). The slides were developed with diaminobenzidine (DAB; Sigma).

To determine the percentages of granzyme B<sup>+</sup> 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 immunoperoxidase, 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<sup>2</sup> tests and by simple regression.

Results

IDDM resistance in 8.3-(NOD × NOR)<sub>F</sub><sub>1</sub> and 8.3-NOR mice

We have shown that 8.3-CD8<sup>+</sup> T cells are highly diabetogenic in 8.3-NOD mice (24). Since these cells require the assistance of endogenous CD4<sup>+</sup> 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<sup>+</sup> T cells, we first crossed 8.3-NOD mice with NOR/Lt mice to generate 8.3-(NOD × NOR)<sub>F</sub><sub>1</sub> (8.3-F<sub>1</sub>) mice. NOR/Lt mice are homozygous for the prodiabetogenic H-2<sup>k</sup> 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 anti-diabetogenic alleles at Idd5, Idd9/11, and Idd13 loci. The incidence of diabetes in 8.3-F<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> littermates that were followed developed diabetes (data not shown). We then backcrossed the 8.3-TCR of a diabetic 8.3-F<sub>1</sub> 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<sub>1</sub> (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<sub>1</sub> 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-F1 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-F1 (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-F1 mice (33 ± 9%) (p < 0.0005 and p < 0.0002, respectively).

alone or in combination, afford dominant protection from 8.3-CD8$^+$ T cell-induced diabetes in 8.3-F1 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 anti-diabetogenic 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-F1 mice

Three-color cytofluorometric studies of thymocytes revealed that the resistance of 8.3-F1 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. 2A). 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. 2A) and several differentiation markers, including CD5, MHC class I (Kk), CD24, CD44, and CD69 (data not shown).

The cytofluorometric profiles of the spleens of 8.3-F1 mice were also remarkably similar to those of 8.3-NOD mice (Fig. 2B); 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. 2B, 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 F1 mice, in 4 of 10 N2 mice, and in 11 of 13 N4-N5 mice (8.3-F1 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-NOR 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 (CD24 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 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-NOR mice to proliferate in response to islet stimulation (Fig. 2A). Subsequent limiting dilution (Fig. 2B) and proliferation assays (Fig. 2C) 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-NOR 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
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 TCR$\beta$-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-TCR$\alpha$-chain; the percentages of V$\beta$8.1/2 splenic T cells in NOD ($n = 3$), NOR ($n = 4$), 8.3-TCR$\beta$-transgenic NOD (N8; $n = 6$), and 8.3-TCR$\beta$-transgenic NOR (N9; $n = 2$) mice. Endogenous V$\alpha$ repertoire of islet-stimulated CD8$^{+}$ T cells from 8.3-TCR$\beta$-transgenic mice. CD4$^{+}$ T cell-depleted splenocytes from 8.3-TCR$\beta$-transgenic NOD ($n = 3$) and 8.3-TCR$\beta$-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 TCR$\alpha$ 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-TCR$\alpha$-chain are underlined.
focused on investigating this phenotype further. The dominant diabetes resistance observed in 8.3-F1 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. 3E, 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 Vβ8.1+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. 4A), that the insulitis lesions of these mice had CD4+CD8+ T cell ratios similar to those

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.
Cytofluorometric studies of 8.3-F1 mice indicated that their diabetic tolerance or ignorance of β cells incorporating BrdU was calculated on pancreata collected 12 h later. Data correspond to 4–8 islets/mouse. Demonstrated that 8.3-CD8 cells (note that IL-2 was barely detectable by RT-PCR). These data pg/mg), but undetectable levels of IL-4 and IL-2 (data not shown). Resistance could not be accounted for by deletion of 8.3-CD8 abundant CD8 pancreatic islets of these mice were severely infiltrated and contained T cells (Figs. 1 and 2). Our next set of experiments focused on attempting to elucidate the mechanism(s) underlying the genetic resistance to autoimmune diabetes in 8.3-NOR mice, and that they accumulate in the islets of nondiabetic mice at ages beyond which their chances of developing diabetes were <5% (>10 wk).

Diabetes resistance in 8.3-F1 mice in the absence of T cell tolerance or ignorance

Cytofluorometric studies of 8.3-F1 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-F1 mice undergo anergy in situ, we compared the percentage of proliferating cells (incorporating exogenous BrdU) in islets of nondiabetic 8.3-F1 and 8.3-NOR mice. As shown in Fig. 5A, 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 insulinic cells from nondiabetic 8.3-F1, and 8.3-NOD mice that expressed granzyme B (a marker of cytotoxic granule content), as detected by immunohistochemistry. As shown in Fig. 5B, 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. 5C, islet-derived CD8+ T cell clones from 8.3-F1 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-NOR mice using the same stimulation protocol expressed serine esterase activity (Fig. 5C). Subsequent cytotoxicity assays using serine esterase+ CD8+ CTL clones from 8.3-F1 and 8.3-NOD mice demonstrated that the diabetes resistance of 8.3-F1 mice could not be accounted for by an

FIGURE 5. Functional in vivo and in vitro activity of islet-associated CD8+ T cells from 8.3-NOD and 8.3-F1 mice. A. BrdU incorporation in vivo. Nondiabetic mice (n = 2–4/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-F1 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-F1 (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-F1 mice against NOD and NOR islet cells. E. Fas-based cytotoxicity of islet cell suspensions from 8.3-NOD and 8.3-F1 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 NOR. Ipr (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−/− mice.
FIGURE 6. Diabetogenesis and T cell development in 4.1-NOD, 4.1-NOR, and 4.1-F1 mice. A, Incidence of IDDM in female (4.1-NOD \( n = 57 \)), 4.1-F1 \( (n = 23) \), and 4.1-NOR \( (n = 26) \) and male (4.1-NOD \( n = 48 \), 4.1-F1 \( n = 13 \), 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-F1, 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 \( (n = 14; \text{age} 61 ± 25\text{ days}) \) and 4.1-NOR mice \( (n = 6; \text{age} 62 ± 15\text{ days}) \). Similar results were obtained with older mice (data not shown). C, Proliferative activity of splenic CD4\(^+\) T cells from 4.1-NOD and 4.1-NOR mice. CD4\(^+\) T cells \( (2 \times 10^5/well) \) were incubated, in triplicate, with \( 10^5 \) irradiated NOD islet cells and \( 10^5 \) irradiated NOD splenocytes for 3 days, pulsed with \( [\text{H}]\text{thymidine} \), harvested, and counted. D, Peripheral frequency of \( \beta \) 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) \( (15–30 \text{ islets/mouse}) \). 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.
inability of 8.3-F1-derived 8.3-CD8+ T cells to kill β cells, or by an inherent resistance of NOR β cells to perforin-based cytotoxicity (Fig. 5D); 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 insulin T cells of 8.3-F1 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-F1 mice might result from the relative inability of the insulin CD8+ T cells of 8.3-F1 vs 8.3-NOD mouse 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. 5E, cells from both types of mice killed 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 NOR, NOD, and NOD.lpr (Fas−) β cells, both in the absence and presence of cytokines that potentiate Fas-mediated cytotoxicity (28). As shown in Fig. 5F, 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-F1 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)F1 and 4.1-NOR mice

The results of these experiments suggested that the genetically dominant resistance of NO(F1)NOR β cells 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-Aβ7-restricted, β cell-specific TCR (4.1-TCR) (23) in (NOD × NOR)F1 (4.1-F1) and NOR (4.1-NOR) mice derived from a diabetic 4.1-NOD mouse. Interestingly, both 4.1-F1 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. 6A). 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-F1 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 F1 littermates that were followed was 0% at 32 wk (data not shown). Thus, like 8.3-F1 mice, 4.1-F1 mice also display dominant resistance to diabetes.

Absence of deletion, anergy, ignorance, and peripheral immune deviation in 4.1-F1 and 4.1-NOR mice

We then investigated whether the diabetes resistance of 4.1-F1 mice was also dissociated from known forms of T cell tolerance. Cytolymphometric, 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-F1 (not shown), 4.1-NOR, and 4.1-NOD mice matured similarly (Fig. 6B), proliferated equally well in response to NOD (and NOR, data not shown) islet cell stimulation in vitro (Fig. 6C), contained similar numbers of β cell-reactive CD4+ T cells (Fig. 6D), and had similar insulitogenic activity (Fig. 6, E and F). As in 8.3-F1 and 8.3-NOR mice, there was no evidence of immune deviation in the periphery of nondiabetic 4.1-F1 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 incidence of diabetes than 4.1-NOD mice, the splenic 4.1-CD4+ T cells of >11-wk-old 4.1-F1 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 cell stimulation (Table I).

The diabetes resistance of 4.1-F1 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 I. Cytokine profile of peripheral 4.1-CD4+ T cells in 4.1-NOD, 4.1-(NOD × NOR)F1, and 4.1-NOR mice
<table>
<thead>
<tr>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
</tr>
<tr>
<td>4.1-NOD</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>4.1-(NOD × NOR)F1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>NOD</td>
</tr>
<tr>
<td>2</td>
</tr>
</tbody>
</table>

* 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.
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-F1 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-F1 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-F1 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-F1 mice was associated with recruitment of TGF-β1-producing cells to islets. The levels of TGF-β1 in islets of seven different 4.1-F1 and 4.1-NOR mice, however, were barely detectable (1.8 ± 2 pg/mg) (data not shown). The mechanism that prevents diabetogenesis in F1 (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-F1 and 4.1-F1 mice

The remarkable similarity between the results of studies with 4.1-F1 and 8.3-F1 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-F1, RAG-2−/− 8.3-NOD, RAG-2−/− 4.1-F1, 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-F1, and RAG-2−/− 4.1-F1 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-F1 littermates was much lower: only 2 of 11 RAG-2−/− 4.1-F1 females (18%) and none of 8 RAG-2−/− 4.1-F1 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-F1 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-NOD mice (Fig. 1). It should be pointed out that direct comparison of the incidences of diabetes in RAG-2−/− and RAG-2−/− 8.3-F1 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-F1 and 4.1-F1 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 × NOR)F1 mice, which are genetically resistant to spontaneous IDDM despite being H-2k 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 celluarily 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-F1, 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 prodiaabetic 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 × NORF₂, mice segregates with Idd5, Idd9, and Idd13 (26). Although Idd5, linked to cte4 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-encoded susceptibility to cyclophosphamide-induced apoptosis are unrelated phenomena: 1) the putative gene within the Idd5 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 di-morphic β₂m (β₂-microglobulin) locus; NOD and NOR mice express different β₂m isofoms; and these isofoms account for conformational differences between the otherwise identical K⁺ and D⁺ MHC class I molecules of these mice (41–43). Since the Idd13-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⁺/β₂m⁺ 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 β₂m 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-F₁, male mice might also be due to this. However, we favor the alternative possibility that NOR/Lt mice also bear recessive prodiaabetic genes that in males, but not females, can override the antidiabetic function of the dominant protective element(s). Ongoing studies of Idd13⁺-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-F₁ mice are diabetes resistant, but do not delete transgenic 8.3-CD8⁺ T cells. Experiments with another highly diabetogenic, but I-Aβ²-restricted, β cell-specific TCR revealed that the diabetes resistance of F₁ 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, 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-F₁, male mice might also be due to this. However, we favor the alternative possibility that NOR/Lt mice also bear recessive prodiaabetic genes that in males, but not females, can override the antidiabetic function of the dominant protective element(s). Ongoing studies of Idd13⁺-congenic 8.3-NOD mice should answer some of these questions.
IDDM, despite not being able to delete 4.1 thymocytes (23). It would also be reasonable to expect that less powerful forms of some 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 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 diabetic 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. Usui for providing IL-2−/−, T. Delovitch for suggesting the use of pancreatic extracts to measure intrapancreatic cytokine content, K. Hirawa for advice on immunopathology, and D. Serreze and R. Tobias for advice on Id1 typing. We also thank S. Bou for excellent technical assistance, L. Bryant for flow cytometry, R. Dawson and L. Mork for animal care, H. Kominek for editorial assistance, and Judy Patterson for secretarial help.

References

The Journal of Immunology