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Cross-Priming of Diabetogenic T Cells Dissociated from CTL-Induced Shedding of β Cell Autoantigens

Jun Yamanouchi, Joan Verdaguer, Bingye Han, Abdelaziz Amrani, Pau Serra, and Pere Santamaria

Cross-presentation of self Ags by APCs is key to the initiation of organ-specific autoimmunity. As MHC class I molecules are essential for the initiation of diabetes in nonobese diabetic (NOD) mice, we sought to determine whether the initial insult that allows cross-presentation of β cell autoantigens in diabetes is caused by cognate interactions between naïve CD8+ T cells and β cells. Naïve splenic CD8+ T cells from transgenic NOD mice expressing a diabetogenic TCR killed peptide-pulsed targets in the absence of APCs. To ascertain the role of CD8+ T cell-induced β cell lysis in the initiation of diabetes, we expressed a rat insulin promoter (RIP)-driven adenovirus E19 transgene in NOD mice. RIP-E19 expression inhibited MHC class I transport exclusively in β cells and rendered these cells resistant to lysis by CD8+ (but not CD4+) T cells, both in vitro and in vivo. Surprisingly, RIP-E19 expression impaired the accumulation of CD8+ T cells in islets and delayed the onset of islet inflammation, without affecting the timing or magnitude of T cell cross-priming in the pancreatic lymph nodes, which is the earliest known event in diabetogenesis. These results suggest that access of β cell autoantigens to the cross-presentation pathway in diabetes is T cell independent, and reveal a previously unrecognized function of MHC class I molecules on target cells in autoimmunity: local retention of disease-initiating clonotypes. The Journal of Immunology, 2003, 171: 6900–6909.

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Studies with transgenic mice expressing low levels of OVA in pancreatic β cells have suggested that access of autoantigens to the cross-presentation pathway requires damage of Ag-expressing cells by CD8+ CTLs (10, 11). Although these studies used in vitro activated CTL, rather than naïve T cells, they suggested that CD8+ T cell-dependent, organ-specific autoimmune diseases might be initiated by CTL-induced tissue damage (1). This hypothesis is attractive because, unlike CD4+ T cells, CD8+ T cells can directly recognize peptide/MHC complexes on the surface of most cell types, and can differentiate into cytolytic effectors without the assistance of cross-priming APCs (12). Furthermore, as both apoptotic (13) and necrotic cells (14) can induce DC maturation, CTL-induced damage of parenchymal cells by autoreactive CD8+ CTL has the potential to amplify limited autoimmune responses into full-blown, polyclonal autoimmune diseases.

Type 1 diabetes (T1D) in humans and nonobese diabetic (NOD) mice is a prototypic CD8+ T cell-dependent autoimmune disease that is specifically directed against the pancreatic β cells (15). We and others have proposed that the initial β cell insult that triggers the shedding of β cell autoantigens, their loading onto DCs, and the activation of autoreactive CD8+ T cells is mediated by CD8+ CTLs (16–19). This view is compatible with two major observations: that β cells cannot normally present Ags to CD4+ T cells (20–22), and that both MHC class I- and CD8+ T cell-deficient NOD mice develop neither diabetes nor insulitis (23–25). Although the nature of the CD8+ T cell subpopulation that contributes to the initiation of T1D remains to be determined, several lines of evidence suggest that this subpopulation is dominated by clonotypes expressing Vα17-Jα24 TCRα chains. These clonotypes are Kd restricted, recognize the same peptide ligands (NRP-A7 and some of its mimics (26, 27)), are present at a very high frequency in the peripheral blood of young NOD mice (28), and constitute a large fraction of the CD8+ cells that can be propagated from the earliest insulitic lesions of NOD mice (19). Moreover, they are highly diabetogenic in TCR-transgenic mice (17, 18) and undergo an avidity maturation process that contributes to the progression of
insulitis to overt disease in wild-type NOD mice (29). Because these clonotypes are primed in the pancreatic lymph nodes (PLN) before the onset of insulin antibodies, and because the magnitude of this priming event increases linearly with the extent of β cell death within islets (30), they are useful probes with which to test the above hypothesis, namely, that cross-priming of these and other autoreactive T cells in the PLN is preceded by CTL-induced shedding of β cell autoantigens.

This hypothesis implies that naive CD8+ T cells must be able to cause β cell damage before undergoing APC-driven activation (i.e., in the absence of cross-priming), and that initiation of TID requires cognate interactions between naive autoreactive CD8+ T cells and β cells. To test these hypotheses, we sought to produce NOD mice lacking MHC class I molecules exclusively on β cells. As this could not be accomplished by targeting null mutations of the β2-microglobulin (β2m) or Kd and Dd genes (the NOD MHC class I genes) to β cells of NOD mice (see Discussion), we produced NOD mice expressing a rat-insulin promoter-driven adenovirus E19 (RIP-E19) transgene. The E19 protein, which is encoded by one of several immunoregulatory genes in the adenovirus early region 3 (31), binds with high affinity to Kd and Dd molecules in transfected cells and impair their transport from the endoplasmic reticulum to the surface (32, 33).

The work described in this study shows that naive CD8+ T cells expressing a representative, Vα17-Jα42+ TCR can kill peptide-pulsed targets without undergoing APC-driven activation, and that they do so by delivering Fas ligand (FasL) to the immunological synapse shortly after engaging cognate peptide/MHC class I complexes on target cells. We also show that β cells from preinsulitic, 3-wk-old RIP-E19-NOD mice are highly resistant to the cytotoxic activity of diabetogenic CD8+ T cells in vivo, and that both the initiation of insulitis and its progression to overt disease are significantly inhibited by transgene expression. Surprisingly, down-regulation of MHC class I transport in β cells impaired the accumulation of autoreactive CD8+ T cells in situ without affecting the age at onset or magnitude of β cell autoantigen cross-presentation in the PLN. When taken together, these observations demonstrate a critical role for cognate β cell:CD8+ T cell interactions in the retention of autoreactive CD8+ T cells during the earliest stage of diabetogenesis. They also challenge the view that the initial shedding of β cell autoantigens in T1D is triggered by CD8+ T cells, and strongly support the alternative hypothesis that activation of autoreactive T cells in the regional lymph nodes is a consequence of an earlier, T cell-independent event that is developmentally regulated.

Materials and Methods

Mice

The 8.3-NOD and 4.1-NOD mice have been described (18, 34). To produce RIP-E19-transgenic mice, we amplified the E19 gene from a vaccinia-E19 construct (32) (from J. Yewdell, National Institutes of Health, Bethesda, MD) by PCR. The PCR product was then placed under the control of the RIP1 (from D. Hanahan, University of California, San Francisco, CA) in a construct that included the SV40 small t intron and polyadenylation and transcription terminal signals. The RIP-E19 transgene was released from the vector and microinjected into (C57BL/6 × SJL) F1 eggs. The RIP-E19 transgenes were backcrossed onto the NOD background for 9–10 generations. Mice were typed for microsatellite polymorphisms linked to Ikd2, Ikd3, Ikd4, Ikd5, Ikd7, Ikd8/12, Ikd9, Ikd11, and Ikd15, to confirm absence of non-NOD-derived diabetes-protective alleles, as described (35). All mice were housed in specific pathogen-free conditions.

Diabetes

Diabetes was monitored by measuring urine glucose with Diastix (Miles, Ontario, Canada). Animals were considered diabetic after two readings ≥3 mmol/L.

Antibodies

Anti-H-2Kd PE or biotin, anti-CD8 FITC or PE, anti-CD4 PerCP, anti-Vα17.8/1.2 FITC, and hamster anti-CD3 mAbs were from BD PharMingen (San Diego, CA). Guinea pig anti-swine insulin Abs were from DAKO (Carpentaria, CA). Anti-FasL PE mAb (KAY-10) and rabbit anti-FasL polyclonal Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-guinea pig Alexa 488, anti-rabbit IgG Alexa 546, and streptavidin Alexa 488 were from Molecular Probes (Eugene, OR). Anti-hamster IgG biotin and streptavidin Cy3 were from Jackson ImmunoResearch Laboratories (West Grove, PA). CFSE was from Molecular Probes.

Purification of splenic B cells, DCs, and islet cells and Kd staining

Splenocytes were purified using anti-CD11c- and anti-B220-coated microbeads (Miltenyi Biotec, Auburn, CA), as described (17, 36). Purity was >85% CD11c or >95% B220+ cells. Islets were purified, as described (17), and dispersed into single cells before staining. Cell preparations were stained with anti-Kd PE mAb and analyzed by flow cytometry. Contaminating mononuclear cells in the islet cell prep were gated out on the forward/side scatter plot.

Peptides, tetramers, tetramer-coated beads, and tetramer-binding kinetics

The peptides TUM and NRP-A7 (26) were prepared using FMOCS chemistry, purified by reverse-phase HPLC to >80% purity, and sequenced by ion spray mass spectrometry (Chiron Technologies, Clayton, Australia). Tetramers were prepared, as described (29). Tetramer-coated beads were prepared by coating magnetic beads (Dynal, Great Neck, NY) with FITC-conjugated peptide/Kd tetramers, as per the manufacturer’s instructions. Kinetic analyses of tetramer binding were conducted, as described (29).

51Cr release assays

Splenic CD8+ T cells purified from 8.3-NOD mice using anti-CD8-coated microbeads (Miltenyi Biotec) (2 × 106 cells/well) were stimulated with NRP-A7-pulsed irradiated NOD splenocytes (105 cells/well) for 2–3 days, and expanded in 0.5 U/ml of IL-2 (Takeda, Osaka, Japan) for 7–10 days. These in vitro differentiated CTL were used as effectors in 51Cr release assays using NOD or RIP-E19-NOD islet cell targets (17). The cytolytic activity of naive 8.3-CD8+ T cells was measured over 8 or 20 h, using NRP-A7- or TUM-pulsed (1 μM) RMA-SKd cells.

Proliferation and cytokine assays

Splenic CD8+ T cells (2 × 106/well) were incubated with peptide-pulsed (1 μM) APCs (105 irradiated splenocytes/well) for 2 or 3 days (for cytokine and proliferation assays, respectively) at 37°C in 5% CO2. Cytokines in the supernatants were measured by ELISA. The 3-day cultures were pulsed with 1 μCi [3H]thymidine during the last 18 h and harvested.

Confocal microscopy

All incubations with Abs were done for 1–2 h at room temperature, unless indicated otherwise. Isolated and dispersed islet cells were incubated with anti-Kd biotin, fixed with 3% paraformaldehyde (PFA), and permeabilized with 0.2% Triton-X. Cells were incubated with anti-Kd biotin and guinea pig anti-insulin, and then with streptavidin Cy3 and anti-guinea pig IgG Alexa 488. To examine the membrane distribution of FasL, we incubated naive 8.3-CD8+ T cells with anti-CD3 mAb for 30 min at 4°C, and then with biotinylated goat anti-hamster IgG for 30 min at 37°C in the presence of PE-conjugated anti-FasL mAb. Receptor capping was stopped with 0.2% sodium azide. The cells were immediately fixed with 3% PFA, and then incubated with streptavidin Alexa 488. In other experiments, splenic 8.3-CD8+ T cells were incubated with FITC-conjugated NRP-A7/Kd tetramer-coated beads for 30 min at 37°C and then with anti-FasL PE mAb, transferred onto coverslides, and fixed with 3% PFA. Alternatively, the T cells were incubated with NRP-A7-pulsed (1 μM) RMA-SKd cells at 1:1 ratio for 30 min at 37°C, fixed with 3% PFA, and incubated with rabbit anti-FasL Abs. The conjugates were incubated with anti-rabbit IgG Alexa 546 and anti-CD3 FITC. Cells in all experiments were mounted with Vectashield with 4',6'-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) and analyzed using Leica or Olympus confocal microscopes. Image stacks were acquired serially at 0.2 μm, and were digitally deconvoluted using DeltaVision software (Applied Precision, Issaquah, WA). Between 20 and 40 synapses were analyzed in each experiment, and each experiment was repeated two or three times.
RT-PCR
RNA was purified from freshly isolated islets of 3- to 5-wk-old mice by using RNeasy kit (Qiagen, Mississauga, Ontario, Canada). Real-time RT-PCR was performed in an ABI Prism 7000 using Taqman one-step RT-PCR mastermix (Applied Biosystems, Foster City, CA) using the primers E19S (AAGTTTGGCCCCCAACA) and E19AS (CAAAGCGACGACT G TAATTAGCA), and the probe E19P (6FAM-AAAACACTGGCACCTT TCTGCTGACTG-TAMRA). Taqman rodent GAPDH control primers (Applied Biosystems) were used to measure GAPDH mRNA levels.

CFSE labeling and adoptive transfer experiments
Splenic 8.3-CD8⁺ cells were labeled with CFSE and transfused into 4- or 9-wk-old hosts (10⁷ cells). Hosts were sacrificed 6 days later, and their PLN and mesenteric lymph nodes (MLN) were examined for presence of CFSE⁺ cells. In other experiments, 3 × 10⁷ in vitro differentiated 8.3-CTL were injected i.p. into 3-wk-old hosts.

Histology and immunohistochemistry
Scoring of insulitis lesions and immunohistochemistry were done, as described previously (17).

Statistical analyses
Data were compared using linear regression and variance analysis, Mann-Whitney U test, or χ².

Results
Naive autoreactive CD7⁺ T cells deliver FasL to the immunological synapse and kill target cells in the absence of APC-induced activation
We first determined whether β cell-autoreactive CD8⁺ T cells need to undergo APC-driven activation to acquire cytolytic activity. This was done by investigating whether naive splenic CD8⁺ T cells purified from transgenic mice expressing a representative, Vα17-Jα42 TCR (8.3-NOD mice (18)), could kill peptide-pulsed RMA-SKd cells in vitro. Naive splenic 8.3-CD8⁺ T cells derived from 3-wk-old 8.3-NOD mice (that is, before initiation of cross-priming and insulitis) killed RMA-SKd cells pulsed with NRP-A7, but not the negative control peptide TUM, even at very low E:T ratios (Fig. 1A). As expected, no obvious differences in cytolytic activity were noted between the splenic CD8⁺ T cells of 3- vs 13-wk-old 8.3-NOD mice. Because Vα17-Jα42 TCRα⁺ T cells kill β cells exclusively via Fas (37), we next asked whether naive 8.3-CD8⁺ T cells might kill their targets by delivering FasL to the T cell surface upon TCR engagement. We first analyzed the membrane distribution of FasL on naive 8.3-CD8⁺ T cells in response to TCR ligation. Naive 8.3-CD8⁺ T cells were incubated with anti-CD3 mAb, biotinylated goat anti-hamster IgG (to cross-link...
the primary Ab), streptavidin Alexa 488 (to detect CD3 clustering), and a PE-labeled anti-FasL mAb (to determine the surface distribution of FasL). FasL was found to be exclusively associated with CD3 caps on the T cell surface (95–100% of total caps/experiment) (Fig. 1B). To confirm that this was also true for T cells that had been triggered by cognate peptide:MHC class I complexes, we repeated the above experiment, but using magnetic beads coated with FITC-labeled NRP-A7/K\textsuperscript{d} tetramers as TCR ligands. Staining of the cells with a PE-labeled anti-FasL mAb showed the presence of FasL exclusively at the bead:cell interface (Fig. 1C; 68.6–72.7% of all interfaces). These observations were further confirmed by analyzing the distribution of FasL on naive 8.3-CD8\textsuperscript{+} T cells engaging NRP-A7-pulsed RMA-SK\textsuperscript{d} cells. FasL was exclusively found in immune synapses (Fig. 1D; 80–88.9% of all synapses). FasL was absent on the surface of naive T cells cultured in the absence of ligand (data not shown). We therefore conclude that naive autoreactive CD8\textsuperscript{+} T cells from NOD mice can quickly deliver FasL to the immune synapse upon TCR ligation in the absence of APC-driven activation, allowing directional killing of targets. These observations indicated that naive V\textalpha{17-Jr}\textalpha{42} TCR\textalpha{+} CD8\textsuperscript{+} T cells have the potential to trigger the initial insult that allows cross-presentation of β cell autoantigens to other autoreactive T cells in the regional lymph nodes.

**RIP-E19-transgenic NOD (RIP-E19-NOD) mice**

To investigate the role of cognate CD8\textsuperscript{+} T cell:β cell interactions in diabetogenesis, we produced transgenic mice expressing a RIP-driven E19 transgene in β cells. Pulse-chase experiments of newly synthesized K\textsuperscript{d} molecules in NOD-derived insulinoma cells (NIT-1) upon infection with a vaccinia virus encoding the E19 protein had previously confirmed that E19 can inhibit MHC class I transport in NOD β cells, as it does in fibroblasts (data not shown) (32, 33). Of the 13 founders that were obtained, 12 transmitted the transgene to offspring. Mice from 7 of these 13 lines consistently transcribed E19 mRNA in islet cells before the age at onset of insulitis (the transgene was clearly expressed at least by 3 wk of age). Although the RIP can sometimes drive expression of transgenes in the thymus (38), RIP-E19-transgenic mice exclusively transcribed E19 mRNA in β cells (data not shown). Four of these 7 lines were backcrossed with NOD mice for 9–10 generations and then screened for homozygosity at NOD-derived Idd regions, to ensure that the transgene was not linked to antidiabeticogenic loci (39). The levels of islet E19 mRNA in different lines, as determined by real-time PCR, were inversely correlated with the levels of K\textsuperscript{d} on islet cells, as measured by flow cytometry (p < 0.01) (Fig. 2, A, B, and D). As expected, the levels of K\textsuperscript{d} on DCs and B lymphocytes were unaffected by transgene expression (Fig. 2C). Confocal microscopy studies confirmed that the β cells of preinsulitic transgenic mice (3–5 wk old) contained significant levels of intracellular K\textsuperscript{d} (Fig. 2E). In nontransgenic littermates, in contrast, virtually all K\textsuperscript{d} molecules were on the β cell surface (Fig. 2E). Therefore, the RIP-E19 transgene effectively impairs MHC class I transport in β cells in vivo, before the onset of insulitis.
Impaired progression of insulitis to overt diabetes in RIP-E19-NOD mice

We next followed the natural history of diabetes in RIP-E19-NOD lines expressing different levels of the RIP-E19 transgene. As shown in Fig. 3A, these lines displayed variable degrees of diabetes resistance when compared with wild-type NOD mice. Diabetes resistance was proportional to the levels of E19 mRNA in β cells (Fig. 2B), and inversely correlated with the levels of Kd on islet

**FIGURE 3.** RIP-E19 expression protects /H9252 cells from CD8/H11001 CTL-induced /H9252 cell damage in vitro and in vivo. A, Cumulative incidence of diabetes in RIP-E19-NOD vs NOD mice. The incidence of diabetes was computed for four individual lines. NOD, 114 females and 59 males; RIP-E19-NOD lines 10 and 23 (pooled because they displayed similar kinetics of disease), 53 females and 30 males; RIP-E19-NOD line 49, 34 females and 24 males; and RIP-E19-NOD line 41, 71 females and 38 males. B, Insulitis scores in nondiabetic 20-wk-old NOD and RIP-NOD-E19 (line 41) mice (3 and 8 females, respectively). C, Phenotype of insulitis in 20-wk-old NOD and RIP-E19-NOD mice. Pancreas sections were stained with H&E or with anti-CD4-, anti-CD8-, and HRP-labeled anti-rat IgG. Images are representative of at least three mice/strain. D, NRP-A7/Kd tetramer reactivity of CD8/H11001 T cells derived from islets of 20-wk-old NOD and RIP-E19-NOD mice. Pancreas sections were stained with H&E or with anti-CD4-, anti-CD8-, and HRP-labeled anti-rat IgG. Images are representative of at least three mice/strain. D, NRP-A7/Kd tetramer reactivity of CD8/H11001 T cells derived from islets of 20-wk-old NOD and RIP-E19-NOD mice (representative staining patterns). E, Average percentage of tetramer-reactive CD8/H11001 T cells per strain (left; background staining, as determined with TUM/Kd tetramers, was subtracted) and avidity of NRP-A7/Kd tetramer binding (right). Data correspond to islet-derived CD8/H11001 T cells from 20-wk-old females (n = 7 NOD and 10 RIP-E19-NOD mice). F, Cytotoxic activity of in vitro differentiated 8.3-CTL against NOD or RIP-E19-NOD (line 41) islet cells (i.c.) in vitro, at different E:T ratios. G, Diabetogenic activity of 8.3-CTL in 3-wk-old hosts. Mice were transfused with 3 × 10⁷ cells and followed for diabetes development. Data correspond to seven to eight females per group.
cells (p < 0.015) (Fig. 2D). It is worth noting in this work that the diabetes resistance of the highest expressors was significantly higher than that just reported by Pierce et al. (40) in E3 region-transgenic NOD mice, possibly owing to differences in the levels of transgene expression and β cell MHC class I transport blockade. At 20 wk of age, nondiabetic RIP-E19-NOD mice displayed insulitic lesions that were comparable in both magnitude (Fig. 3B) and cellular composition (Fig. 3C) to those seen in age-matched NOD mice. Importantly, both types of mice contained similar percentages of high avidity NRP-A7-reactive, Vα17-Iα42+ CD8+ T cells in these lesions, as determined by tetramer staining (Fig. 3, D and E). The reasons behind the significantly higher avidity of the islet-associated CTL of RIP-E19 vs wild-type NOD mice are unclear, but might be due to lower rates of reactivation-induced death of high avidity clonotypes in mice in which β cells cannot elicit CTL activation. Whatever the mechanism, the above results suggested that β cell MHC class I molecules play a critical role in the progression of full-blown insulitis to overt diabetes by allowing the lysis of β cells by MHC class I-restricted autoreactive CTL. In agreement with this interpretation of the data, purified islet cells from 3- to 5-wk-old RIP-E19-NOD mice were resistant to the cytolytic activity of differentiated 8.3-CD8+ CTL in vitro, as compared with islet cells from wild-type NOD mice (Fig. 3F). Adoptive transfer experiments of differentiated 8.3-CTL into 3-wk-old RIP-E19-NOD and NOD hosts (that is, before the onset of insulitis) indicated that this was also true in vivo. Whereas seven of the eight NOD hosts that were transfused with 8.3-CTL developed diabetes within 7–11 days after transfer, all seven 8.3-CTL-transfused RIP-E19-NOD hosts remained diabetes free for at least 7 wk after transfer (p < 0.0001) (Fig. 3G).

**Impaired initiation of insulitis in RIP-E19-NOD mice**

To formally exclude a role for MHC class I molecules on β cells in the initiation or progression of insulitis, we also compared the severity of insulitis in young NOD and RIP-E19-NOD mice. Whereas female NOD mice already displayed mild insulitic lesions by 5 wk of age, pancreata from female RIP-E19-NOD mice were completely free of insulitis at this age (p < 0.015) (Fig. 4A). Comparison of the T cell subset composition (i.e., CD4+ vs CD8+ T cells) of the moderate islet infiltrates of 12-wk-old RIP-E19-NOD mice with those of younger NOD mice displaying comparable insulitis scores did not reveal obvious differences (data not shown). In these studies, however, the few islets that displayed islet inflammation had significant insulitis scores, precluding a detailed analysis of the T cell composition of infiltrates in islets that have just begun to become infiltrated. Nevertheless, because the early insulitic lesions of NOD mice contain macrophages and DCs (our unpublished observations) and both CD4+ and CD8+ T cells (data not shown), the unexpected absence of mononuclear cells in islets of 5-wk-old RIP-E19-NOD mice suggests that MHC class I molecules on β cells somehow contribute to their recruitment.

**Unopposed initiation of Ag cross-presentation in the PLN of RIP-E19-NOD mice**

We have previously shown that priming of naive autoreactive CD8+ T cells in the PLN of wild-type NOD mice begins shortly before the onset of insulitis (30). Cross-priming of T cells is critical for the initiation of diabetes, as it does not occur in insulitis-resistant hosts (i.e., H-2o7-congenic C57BL/10 mice) (our unpublished observations). Furthermore, this response is preceded by β cell apoptosis, as its age at onset, kinetics, and magnitude are highly sensitive to small variations in the number of apoptotic β cells contained in pancreatic islets at the time of analysis (30). Because apoptotic cell death allows access of autoantigens to the cross-presentation pathway, the delayed onset of insulitis and the resistance of β cells to CTL damage in RIP-E19-NOD mice afforded a unique opportunity to investigate whether the initiation of cross-priming in T1D is precipitated by CD8+ T cell-induced β cell apoptosis. Naive splenic CD8+ T cells from 8.3-NOD mice were labeled with CFSE and then transfused into NOD and RIP-E19-NOD hosts. Analyses of the PLN- and MLN-associated T cells of 4- and 9-wk-old hosts 6 days after transfer confirmed that β cell-autoreactive CD8+ T cells proliferate exclusively in the PLNs (Fig. 4B). Surprisingly, the magnitude of this in vivo proliferative response was not affected by transgene expression (Fig. 4C). This indicated that access of β cell autoantigens to the cross-presentation pathway in the earliest stages of diabetogenesis is dissociated from CTL-induced β cell death. Thus, RIP-E19 expression delays the onset of insulitis without interfering with the priming of autoreactive CD8+ T cells in the regional lymph nodes.

**Impaired early accumulation of autoreactive CD8+ T cells, but not CD4+ T cells, in TCR-transgenic RIP-E19-NOD mice**

To investigate whether MHC class I molecules on β cells contribute to initiation of insulitis by interfering with the accumulation of autoreactive CD8+ T cells within islets, we introduced the RIP-E19 transgene into 8.3-NOD mice. We then compared the fate and function of the TCR-transgenic T cells in the presence vs absence...
of the RIP-E19 transgene. As expected, the flow cytometric profiles of thymocytes and splenocytes from RIP-E19/8.3-NOD mice were similar to those seen in 8.3-NOD mice, suggesting normal development of autoreactive CD8\(^+\) T cells in the presence of the RIP-E19 transgene (Fig. 5A). In agreement with this, the splenic CD8\(^+\) T cells of RIP-E19/8.3-NOD mice proliferated as well, and produced as much IFN-\(\gamma\) as those derived from 8.3-NOD mice, in response to antigenic stimulation in vitro (Fig. 5B). Notwithstanding the normal development and function of 8.3-CD8\(^+\) T cells, initiation of insulitis and progression of insulitis to overt diabetes in the double transgenic animals were clearly impaired. Whereas prediabetic 10-wk-old 8.3-NOD mice displayed severe insulitis scores, age-matched RIP-E19/8.3-NOD mice did not (Fig. 5C). As was the case with non-TCR-transgenic RIP-E19-NOD mice (see above), the cellular composition of the sparse islet infiltrates of 10-wk-old RIP-E19/8.3-NOD mice was similar to that seen in the severely infiltrated islets of age-matched 8.3-NOD mice (data not shown). Comparison of the levels of CD44 and CD69 on the PLN CD8\(^+\) T cells of 5-wk-old 8.3-NOD and RIP-E19/8.3-NOD mice confirmed that RIP-E19 expression does not inhibit diabetes by blunting the cross-priming of autoreactive CD8\(^+\) T cells in the regional lymph nodes (Fig. 5E).

We next investigated whether MHC class I molecules on \(\beta\) cells contribute to initiation of insulitis by selectively interfering with the accumulation of autoreactive CD8\(^+\) T cells in islets, rather than with the accumulation of all T cells regardless of MHC restriction.

**FIGURE 5.** RIP-E19 expression delays initiation of insulitis by inhibiting the accumulation of autoreactive CD8\(^+\) T cells within islets. A, CD4, CD8, and V\(\beta\)8.1/8.2 profiles of thymocytes and splenocytes from 8.3-NOD (\(n = 25\)) and 8.3/RIP-E19-NOD mice (\(n = 3\)). Numerical values correspond to percentages \(\pm\) SD of cells within each gate. B, Proliferation of, and secretion of IFN-\(\gamma\), by naive 8.3-CD8\(^+\) T cells in response to peptide-pulsed (1 \(\mu\)M) NOD splenocytes. C, Insulitis scores in 8.3-NOD vs RIP-E19/8.3-NOD mice (\(n = 3\) each). D, Incidence of diabetes in 8.3-NOD (\(n = 214\)) vs RIP-E19/8.3-NOD female mice (\(n = 34\)). E, Expression of CD69 and CD44 on PLN and MLN CD8\(^+\) T cells from 5-wk-old 8.3-NOD (\(n = 3\)) and RIP-E19/8.3-NOD female mice (\(n = 2\)).
For example, retention of MHC class I in the endoplasmic reticulum might impair the production of chemokines or other secreted factors important for the recruitment or retention of pathogenic T cells. If that were the case, the RIP-E19 transgene would be expected to impair the recruitment of both MHC class I- and non-MHC class I-restricted T cells. To investigate this possibility, we explored the influence of RIP-E19 expression on the natural history of diabetes in recombination-activating gene 2 (RAG-2)-deficient NOD mice expressing the 8.3-TCR or the diabetogenic, MHC class II (I-A<sup>dp</sup>)-restricted 4.1-TCR (18). As reported previously, these mice exclusively bear CD8<sup>+</sup> or CD4<sup>+</sup> T cells expressing the transgenic TCRs (18). Whereas RIP-E19/8.3-NO-D.RAG-2<sup>−/+</sup> mice developed a significantly reduced incidence and delayed onset of diabetes when compared with 8.3-NO-D.RAG-2<sup>−/+</sup> and 4.1-NO-D.RAG-2<sup>−/+</sup> mice were too low and variable (from mouse to mouse) (1.1 ± 1.1 (n = 4) vs 0.98 ± 1.1 (n = 3), respectively) to allow us to formally establish a role for β cell MHC class I molecules in the retention of 8.3-CD8<sup>+</sup> T cells in islets. This is because, as we have previously shown, the rate-limiting factor of diabetogenesis in 8.3-NOD.RAG-2<sup>−/+</sup> mice is the ability of these mice to develop insulitis, such that the only animals that develop significant insulitis in this model are those that are destined to become diabetic (18). Nevertheless, when taken together, these data suggest that MHC class I molecules on β cells contribute to the initiation of insulitis by promoting the retention of autoreactive CD8<sup>+</sup> T cells within islets, which might then be able to orchestrate rapid recruitment of APCs and other effectors to the site. An alternative, albeit not exclusive, possibility is that lack of MHC class I molecules on β cells prevents the reactivation of autoreactive CD8<sup>+</sup> T cells within islets.

**Discussion**

To investigate whether initiation and/or progression of T1D require cognate interactions between autoreactive CD8<sup>+</sup> T cells and β cells, we sought to produce NOD mice lacking MHC class I molecules exclusively on β cells. We reasoned that this could not be accomplished by targeting null mutations of the β<sub>2m</sub> or K<sub>d</sub> and D<sub>b</sub> genes to β cells of NOD mice for several reasons. Circulating β<sub>2m</sub> molecules in mice expressing β<sub>2m</sub>-molecules in all cell types, except β cells, would most likely be able to restore MHC class I expression on β<sub>2m</sub>-deficient β cells and therefore obscure any possible phenotypes induced by the former. Furthermore, the K and D genes carried by available embryonic stem cells are in tight linkage disequilibrium with I-E and/or I-A alleles that dominantly protect NOD mice against both insulitis and diabetes (41). As a result, NOD mice bearing K or D gene-targeted β cells would always be diabetes resistant, regardless of whether these mice expressed class I molecules on β cells or not. We therefore chose to address the role of β cell MHC class I-CD8<sup>+</sup> T cell interactions in diabetogenesis by inhibiting β cell MHC class I transport in vivo. The data have shown that down-regulation of MHC class I expression on β cells inhibits the progression of insulitis to overt disease, by rendering β cells resistant to CD8<sup>+</sup> CTL. Surprisingly, the onset of cross-priming in the PLN, which precedes the initiation of insulitis, but obviously requires the shedding of β cell autoantigens, was unaffected by transgene expression. Because the CTLs that were used as readouts in these experiments are representative of the CTLs that can be propagated from the earliest insulitic lesions of NOD mice (17, 19), these results imply that the events leading to cross-priming of autoreactive T cells in the regional lymph nodes are dissociated from CD8<sup>+</sup> CTL-induced β cell apoptosis. Most importantly, the data have shown that MHC class I molecules on β cells contribute to the initiation (albeit not amplification) of islet inflammation, by promoting the early retention of cross-primed autoreactive CD8<sup>+</sup> T cells within islets.

Although 8.3-CTL can kill peptide-pulsed non-β cell targets via perforin, they kill β cells exclusively via Fas (37). We reasoned that if CD8<sup>+</sup> T cells were truly responsible for effecting the initial insult in T1D (i.e., before access of β cell autoantigens to the cross-presentation pathway), they should be able to up-regulate their lytic machinery in a target-dependent manner, before undergoing APC-driven activation. Experiments using naïve splenic 8.3-CD8<sup>+</sup> T cells as effectors and peptide-pulsed RMA-SK<sub>6</sub> cells as targets clearly showed that these T cells do not need to engage Ag on APCs to acquire cytolytic activity. Cytolysis of peptide-pulsed targets by these T cells was not due to presence of in vivo activated CTLs in the splenocyte preparations, because splenic 8.3-CD8<sup>+</sup> T cells do not express memory or activation markers (18). Furthermore, T cells from 3- and 13-week-old mice (that is, collected before and long after the onset of cross-priming in the PLN) had similar cytotoxic activity (Fig. 1A). In addition, cytosis occurred at very low E:T ratios and required relatively long incubation times, compatible with Fas (as opposed to perforin)-mediated cytotoxicity (42). In support of this view, FasL cosegregated with CD3 caps, TCR clusters, and immune synapses in response to CD3 cross-linking or Ag-induced TCR ligation. Therefore, naïve autoreactive CD8<sup>+</sup> T cells have the potential to cause the initial insult that allows access of β cell autoantigens to the cross-presentation pathway.

Surprisingly, however, the earliest detectable cross-priming of Vα17-Jα42<sup>+</sup> CD8<sup>+</sup> T cells in the PLN of RIP-E19-NOD mice...
was not affected by the delayed onset of insulin or by the exquisitely sensitive resistance of their β cells to CD8\(^+\) CTL damage. Because the priming of naive 8.3-CD8\(^+\) T cells in RIP-E19-NOD begins weeks before the earliest detectable evidence of insulin, our observations strongly support the idea that initial access of β cell autoantigens to the cross-presentation pathway is CD8\(^+\) T cell independent. It is important to note in this study that the antigenic epitope that these T cells target originates in an endoplasmic reticulum–resident protein (43) that, presumably, can only access the cross-presentation pathway if β cells die. Conceivably, cross-priming of 8.3-CD8\(^+\) T cells in the PLN might occur at a lower threshold of β cell death than that required for the initiation of insulin (that is, after lysis of some of the RIP-E19 β cells that express reduced levels of MHC class I). However, we think that this is unlikely because the kinetics and magnitude of this event are highly sensitive to variations in the number of apoptotic β cells within islets (30). It would be inappropriate to use T cell-deficient or NOD.scid mice as hosts to further document these observations because T cells undergo generalized homeostatic proliferation in these mice (unpublished data). Interestingly, the timing of this early priming event is subsequent to a natural increase in the incidence of apoptotic β cells in the pancreas that peaks at \(\sim 2\) wk of age and that is magnified in diabetes-prone rodents (44, 45). Therefore, the observation that priming of CD8\(^+\) T cells proceeds unpimmed in mice whose β cells are resistant to CTL-induced damage supports the idea that developmental remodeling of the target tissue precedes the priming of T cells in T1D. In this regard, we note that apoptotic cell death cannot only enhance the availability of self Ag to the immune system (8), but can also promote DC maturation (13), and even initiate autoimmune responses (46).

Most importantly, RIP-E19-induced down-regulation of MHC class I molecules on β cells delayed the development of diabetes by impairing the retention of autoreactive CD8\(^+\) T cells within islets. RIP-E19 expression delayed the onset of insulin in RIP-E19/8.3-NOD mice, and protected RIP-E19/8.3-NOD.RAG-2\(^{-/-}\) mice from 8.3-CD8\(^+\) T cell-induced diabetes, but did not delay the onset or progression of diabetes in monoclonal T cell NOD mice expressing another diabeticogenic, but MHC class II-restricted TCR. These results support the idea that the initial stages of islet cell destruction are regulated by cognate interactions between MHC class I-restricted CD8\(^+\) T cells and β cells. In addition to promoting the local retention of the first autoreactive CD8\(^+\) T cells that reach the site, these interactions may elicit the activation of these T cells in situ and, as a result, promote the recruitment of other T cells and APCs to the site via inflammatory mediators, such as chemokines. This hypothesis is particularly attractive in light of recent observations indicating that CD8\(^+\) T cells can undergo activation by directly recognizing peptide/MHC complexes on the surface of nonhemopoietic cell types (12). The fact that RIP-E19-transgenic NOD mice do eventually develop full-blown insulin is not incompatible with this interpretation of the data, as MHC class I transport in the β cells of these mice is not completely blocked. Eventual recruitment of professional APCs to the site most likely overrides the initial need for β cell MHC class I molecules in islet inflammation.

In sum, the results of this study support a model in which initiation of diabetes is precipitated by a T cell-independent event that is capable of promoting access of β cell autoantigens to the cross-presentation pathway. Cross-priming of autoreactive CD8\(^+\) T cells in the regional lymph nodes would then promote the migration of disease-initiating CD8\(^+\) T cells into pancreatic islets, followed by retention of these T cells in situ by β cell MHC class I molecules. This would lead to recruitment of professional APCs and Th CD4\(^+\) T cells to the site, and to amplification of the inflammatory response in a β cell MHC class I-independent manner. MHC class I-restricted lysis of β cells by islet-associated CTL would contribute to disease progression by promoting the shuffling of β cell autoantigens to the cross-presentation pathway and the continuous recruitment of diabetogenic CTL to the site.

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