Progression of spontaneous autoimmune diabetes is associated with a switch in the killing mechanism used by autoreactive CTL

Huilian Qin1, Jacqueline D. Trudeau1, Gregor S. D. Reid2, I-Fang Lee1, Jan P. Dutz3, Pere Santamaria4, C. Bruce Ver cherche1 and Rusung Tan1

1Department of Pathology and Laboratory Medicine, 2Department of Pediatrics and 3Department of Medicine, University of British Columbia, BC’s Children’s Hospital, 4480 Oak Street, Vancouver, British Columbia, V6H 3V4, Canada
4Department of Microbiology and Infectious Diseases and Julia McFarlane Diabetes Research Centre, Faculty of Medicine, University of Calgary, Health Sciences Centre, 3330 Hospital Drive NW, Calgary, Alberta, T2N 4N1, Canada

Keywords: avidity maturation, CTL, Fas, non-obese diabetic, perforin

Abstract
Autoimmune (type 1) diabetes mellitus results from the destruction of insulin-producing pancreatic β-cells by T lymphocytes. β-cell death that is induced by autoreactive CTL in diabetes involves both Fas/Fas ligand (FasL)- and perforin/granzyme-mediated pathways, although their relative contributions during the progression of the disease remain unknown. We demonstrate here that despite the preferential use of the Fas/FasL pathway for cytolysis of β-cell targets, transgenic β-cell-specific CTL were able to kill targets via the perforin pathway when triggered by a higher affinity stimulus. In addition, we show that the killing mechanism used by islet-associated CD8+ T cells from non-obese diabetic mice changed as the mice aged and correspondingly, with the stage of diabetes. These results provide direct evidence for age-related changes in the cytotoxic pathways used by diabetogenic T cells during the progression of autoimmune diabetes.

Introduction
Type 1 diabetes is a chronic autoimmune disease characterized by the selective destruction of pancreatic β-cells that leads to insulin deficiency and hyperglycemia. Studies in both humans and non-obese diabetic (NOD) mice have shown that β-cell destruction is mediated by T lymphocytes (1–3), in particular CD8+ CTL (4–6). The importance of autoreactive CTL in the development of diabetes is underscored by studies using β2-microglobulin-deficient and CD8+ T cell-depleted NOD mice, none of which develops islet inflammation (insulitis) or diabetes (4, 5, 7, 8). However, despite numerous studies investigating T cell activity during diabetes progression, the mechanism by which autoreactive CTL destroy pancreatic β-cells remains unclear.

CTL induce apoptosis of target cells primarily through the exocytosis of granules containing perforin and granzyme B, or by the interaction of Fas ligand (FasL) with Fas on the target cell surface (9). Although cytokines such as tumor necrosis factor-α have been shown to play a role in β-cell death, the granule exocytosis and FasL pathways can account for virtually all the cytotoxic effects of CTL. Nevertheless, the conditions under which CTL preferentially use one or the other mechanism are not completely known. For example, CTL clones that recognize endogenous antigens appear to kill preferentially via Fas (10), whereas the CTL response to non-cytopathic viruses (11) or to some altered peptide ligands (12) is perforin mediated. During the progression of diabetes in the NOD mouse, both perforin- and Fas-mediated mechanisms of killing have been observed. NOD mice lacking either Fas (NOD-lpr/lpr) (13) or FasL (NOD-gld/gld) (14) do not develop diabetes, and the destruction of β-cells in transgenic mice expressing a diabetogenic CD8+ TCR is mediated entirely through Fas (15). Conversely, perforin-deficient NOD mice develop diabetes at a significantly reduced rate despite the presence of insulitis (6). In addition, islets derived from NOD-lpr/lpr mice are destroyed by diabetogenic CTL when transplanted into NOD recipients, despite the absence of Fas (16). Taken together, these data suggest that autoreactive CTL from NOD mice use both Fas and perforin to induce β-cell death, but the mechanism that drives the use of one pathway or the other remains unresolved.
As NOD mice age, diabetes development is associated with progression from a prolonged period of insulitis to CTL-induced destruction of β-cells and overt diabetes (17). We have shown previously that the change from benign insulitis to overt diabetes is associated with an increase in the avidity of autoreactive CTL for a β-cell epitope (18). As NOD mice age, there is a selective expansion of islet-associated CTL with high-affinity TCRs for a family of β-cell peptide mimotopes that include the peptide designated NRP-A7. In the current study, we speculated that the preferential expansion of either Fas or perforin by autoreactive CTL was dependent on the avidity of the TCR-MHC–peptide interaction, and therefore dependent upon the age of the animal. Using CTL derived from the transgenic 8.3-TCR NOD mouse, whose CD8+ T cells recognize the NRP-A7 peptide, we show that the mechanism of TCR ligation affects whether the Fas or perforin pathway is used for target cell lysis. In addition, we show that the use of the Fas or perforin pathway by CTL obtained from islets of non-transgenic NOD mice is dependent on the age of mice from which the CTL were derived. Together, these data imply that disease progression in the NOD mouse is associated with an age-dependent switch in the killing mechanism employed by autoreactive CTL, and provide a possible explanation for the conflicting results obtained in previous studies.

**Methods**

**Mice**

8.3-TCRβ transgenic NOD (8.3-TCR NOD) mice have been described previously (19). NOD/Ltj and C57BL/6 (B6) mice were bred from stocks originally purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All animal experiments were performed in accordance with the rules of the Animal Care Committee, University of British Columbia.

**Cell lines and reagents**

NIT-1, βTC-3 and P815 cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). The peptides NRP-A7 (KYQAVTTTL) NRP-V7 (KYKANVFL) and TUM (KYQAVTTTL) were prepared by FMOC chemistry and purified by reverse-phase HPLC (>90% purity) at the University of British Columbia.

**Cell culture**

To obtain CTL lines, spleen cells from 8- to 7-week old 8.3-TCR NOD mice were re-suspended in complete medium [10^6 cells ml⁻¹ in RPMI 1640 culture media containing 10% fetal bovine serum, 50 U ml⁻¹ penicillin, 50 μg ml⁻¹ streptomycin and 50 μM 2-mercaptoethanol (2-ME)] containing 1 μg ml⁻¹ A7 peptide. On day 3, 10 U ml⁻¹ recombinant human IL-2 (Pharmingen, San Diego, CA, USA) was added, and the cells were incubated for an additional 7–10 days. For CTL from non-transgenic NOD mice, pancreatic islets from 9- or 15-week old female NOD mice were isolated by collagenase digestion of the pancreas (Type V, Sigma, Oakville, Canada) and separated on a dextran gradient. Islets were cultured in complete medium with 10 U ml⁻¹ recombinant IL-2 for 2 days, and then dispersed into single cells and filtered through a cell strainer.

**Cytotoxicity assays**

To up-regulate MHC class I expression, NIT-1 cells were incubated with 100 U ml⁻¹ IFN-γ at 37°C overnight. A total of 10⁶ target cells (NIT-1 or P815) were labeled with 100 μCi of ⁵¹Cr for 90 min at 37°C, washed three times and then incubated with 1 μg ml⁻¹ peptide at 37°C for 1 h. Target cells were washed two times and seeded at 10⁶ cells per well. Effectors were added to each well, in triplicate, at various effector : target (E : T) ratios. The plates were centrifuged at 500 rpm for 2 min, and incubated at 37°C for 4 h. After incubation, supernatants were collected for determination of ⁵¹Cr release [%lysis = 100 × (test cpm – spontaneous cpm)/(total cpm – spontaneous cpm)]. For the indirect killing assay, target cells were incubated with 5 μg ml⁻¹ anti-CD3 antibody (clone 145-2C11, Pharmingen) prior to incubation with effectors. Where indicated, 5 mM EGTA was added to chelate extracellular calcium (20).

**Results**

**8.3-TCR NOD CTL lyse target cells in an MHC-restricted and epitope-specific fashion**

To establish the mechanism used by diabetogenic CTL from NOD mice to lyse β-cell targets, we first generated CTL lines from 8.3-TCR NOD spleen cells by culture with NRP-A7 peptide and IL-2. 8.3-TCR CTL recognize the peptide mimotope NRP-A7 in the context of H-2Kd (21). Using these CTL lines, we observed that 8.3-TCR CD8+ T cells effectively killed transformed β-cells (NIT-1) derived from NOD mice (Fig. 1A). In contrast, these CTL lines did not lyse the β-cell line βTC-3, derived from H-2Kb-expressing C57BL/6 mice, or P815 cells (a non-β-cell line expressing H-2Kb). Killing of NIT-1 cells was unaffected by pre-incubation of the target cells with NRP-A7, but was reduced by incubation with the negative control peptide TUM (Fig. 1B). This reduction was likely a result of the displacement of endogenous β-cell peptides from MHC class I molecules on the NIT-1 cell surface. Lysis of P815 cells by 8.3-TCR CTL was dependent on prior incubation of the target cells with NRP-A7 peptide, whereas pre-loading of P815 cells with TUM did not have any effect (Fig. 1C). In agreement with our previous findings (21), these results indicate that 8.3-TCR CTL recognize a β-cell-derived peptide antigen presented in the context of H-2Kd, and that this specificity can be mimicked by the NRP-A7 peptide in complex with H-2Kd.

**Killing by 8.3-TCR CTL following stimulation by anti-CD3 is perforin dependent**

Killing of β-cells by 8.3-TCR CTL has been shown previously to be mediated by Fas, although under specific experimental conditions, we have been able to induce perforin-mediated killing using Fas-deficient target cells (15). To confirm that β-cell death induced by 8.3-TCR CTL is perforin independent, chromium-release assays were performed in the presence or absence of the calcium chelator EGTA. As the action of perforin is dependent on extracellular calcium, the addition of EGTA inhibits lysis by perforin-dependent CTL (20). We first confirmed that EGTA was an effective inhibitor of perforin-mediated
Cytolysis by preventing the lysis of ovalbumin-expressing target cells by ovalbumin-specific CTL upon addition of EGTA (data not shown). However, the peptide-specific killing of P815 cells by 8.3-TCR CTL was unaffected by the addition of EGTA (Fig. 2A), indicating that none of the 8.3-TCR CTL-mediated cytotoxicity was perforin dependent. Similarly, lysis of NIT-1 β-cells by 8.3-TCR CTL was also perforin independent (Fig. 2B). We wanted next to determine whether these transgenic CTL were capable of killing via a perforin-mediated pathway when ligated by a strong stimulus. To do this, we established an antibody-dependent lysis assay in which 8.3-TCR CTL were triggered by anti-CD3 antibody (instead of by MHC–peptide) (22, 23). In these assays, P815 target cells were pre-incubated

![Graph A](image1.png)

**Fig. 1.** 8.3-TCR CTL cytolysis of target cells is MHC restricted and epitope specific. A. CTL lines generated from 8.3-TCR NOD mice were co-incubated with chromium-labeled NIT-1 (NOD-derived β-cell line), BTC-3 (C57BL/6-derived β-cell line) or P815 (H-2d murine mastocytoma) cells at the indicated effector : target (E : T) ratios. B. 8.3-TCR CTL lines were co-incubated with chromium-labeled NIT-1 target cells or NIT-1 cells that had been pre-incubated with NRP-A7 or TUM (negative control) peptides. C. 8.3-TCR CTL lines were co-incubated with chromium-labeled P815 cells alone or P815 cells that had been pre-incubated with NRP-A7 or TUM peptides. All experiments were performed on at least three occasions with similar results.

![Graph B](image2.png)

**Fig. 2.** 8.3-TCR CTL usage of FasL or perforin is dependent upon differential TCR signaling. CTL lines generated from 8.3-TCR NOD mice were tested for lytic activity in standard chromium-release assays against P815 cells (A) or NIT-1 cells (B) alone or after pre-incubation with NRP-A7 or TUM peptides in the presence (EGTA) or absence (medium) of 5 mM EGTA. (C) 8.3-TCR CTL lysis is perforin dependent when triggered by anti-TCR antibody. Chromium-labeled P815 target cells were left unincubated (No Ab) or pre-incubated with an isotype control antibody, with anti-CD3 antibody then co-incubated with 8.3-TCR CTL in medium alone (Anti-CD3) or with 5 mM EGTA (EGTA). Data are represented as the mean (±standard error of the mean) of five separate experiments. For all assays shown, the effector : target ratio was 5 : 1.
with anti-CD3 antibody, which was bound by cell-surface FcR. Upon incubation of anti-CD3-coated P815 cells with 8.3-TCR CTL, the CTL were activated by the high-affinity CD3 antibody–TCR complex interaction, rather than by MHC–TCR interaction. Thus, the assay was peptide independent, and contingent only on antibody–TCR complex interaction. Under these conditions, efficient killing of P815 cells was achieved without pre-incubation with peptide and the killing was significantly inhibited by the addition of EGTA (Fig. 2C). Thus, by activating the TCR complex with antibody, 8.3-TCR CTL were induced to kill in a perforin-dependent manner.

The mechanism of CTL cytotoxicity in NOD mice is age dependent

We have previously shown that islet-associated autoreactive CTL from NOD mice undergo avidity maturation, resulting in the selective clonal expansion of CTL with higher avidity receptors for the family of NRP peptides (18). Our findings in the present study suggested that whether 8.3-TCR CTL kill via FasL or perforin may be determined by the affinity of the CTL–target cell interaction, and the subsequent signaling pathways that are activated. These two observations led us to hypothesize that the avidity maturation of diabetogenic CTL in NOD mice as they age is associated with a shift from killing that is predominantly FasL mediated, to killing that is primarily perforin mediated. To address this hypothesis, pancreatic islets were obtained from NOD mice at ages that corresponded to early (9 weeks old) or late (15 weeks old) stages of insulitis during the pre-diabetic period. The pooled islets from each group were cultured in IL-2 for 48 h (without antigenic stimulus), and then used as effector cells in chromium-release assays against peptide-loaded P815 target cells. As was seen with CTL lines derived from 8.3-TCR NOD transgenic mice, killing of target cells by CTL obtained from 9-week-old NOD mice was unaffected by EGTA, indicating that the mechanism of CTL lysis in younger NOD mice is perforin independent (Fig. 3). In contrast, islet-derived CTL obtained from 15-week-old mice killed in a perforin-dependent manner, as shown by the nearly complete inhibition of killing by EGTA ($P < 0.01$ versus medium). These results indicate that as NOD mice age, their islet-associated CTL switch from a perforin-independent to a perforin-dependent killing mechanism. As the target cells in each assay were the same (peptide-coated $K^d$-expressing targets), the results indicate that the switch from FasL- to perforin-mediated killing was due to changes in the CTL, most likely in the avidity of the TCR.

**Discussion**

In this report, we provide the first direct evidence for age-related changes in the killing pathways used by autoreactive CTL in NOD mice. The development of clinical diabetes in the NOD mouse is preceded by a prolonged period of islet inflammation and the switch to hyperglycemia occurs only after a sufficient number of $\beta$-cells have been destroyed, and insufficient insulin is secreted to maintain normal blood glucose levels. This switch from active islet inflammation to overt diabetes is associated with the avidity maturation of a population of diabetogenic CTL (18). Here, we show that as diabetes progresses from the early stages of insulitis to later stages of $\beta$-cell destruction, a population of diabetogenic CTL switch from killing $\beta$-cells using a mechanism that is primarily perforin independent, to one that is perforin dependent (Fig. 3). Perforin-independent killing is likely to be mediated through Fas/FasL interaction, as has been shown previously for killing
of β-cells in NOD mice (13, 15, 16, 24). Moreover, we show that by altering the mode of interaction between 8.3-TCR CTL and target cell by ligating the TCR with antibody rather than MHC-peptide, a switch from Fas–FasL-mediated to perforin-mediated killing could be induced (Fig. 2C). Since the process of avidity maturation of diabetogenic CTL and the age-related switch to perforin-dependent killing by diabetogenic CTL appear to occur in parallel, we speculate that the switch in killing mechanism to perforin-mediated cytotoxicity in older NOD mice is related to an increased affinity of the CTL for the β-cell target. A second possibility is that the level of MHC class I or co-stimulatory receptor expression on islet β-cells may change with age. Whatever the mechanism, the switch to perforin-mediated cytotoxicity may be an important final step in the progression toward complete β-cell destruction.

Interestingly, the autoreactive CTL in 8.3-TCR NOD mice were originally cloned from islet-infiltrating CD8+ T cells obtained from diabetic NOD mice (25). Our model would predict that these cells, present in the early stages of insulitis, would kill β-cells by a FasL-independent mechanism. However, these cells primarily kill in a Fas-dependent manner (Fig. 2A and B) suggesting that the clone isolated from islets was not of highest avidity. However, an age-dependent switch in the killing mechanism utilized by islet-associated CTL is a model that is supported by other published, yet seemingly contradictory, findings. According to our model, if the early autoreactive CD8+ T cells in the islets of NOD mice exclusively utilize the Fas/FasL pathway, then the Fas and FasL deficiencies present in the /pr/lpr and gld/gld mice, respectively, would eliminate early β-cell killing. In the absence of this early Fas-mediated killing there would be no insulitis, no avidity maturation of CTL and thus no perforin-dependent disease progression. In support of this hypothesis, neither the NOD-/pr/lpr nor the NOD-gld/gld mouse develops disease (13, 14). Also consistent with this model is the observation that an anti-FasL antibody affects the development of diabetes most profoundly when administered to very young (beginning at 2 weeks of age) NOD mice (26). In the case of perforin-deficient mice, while these animals develop insulitis, diabetes occurs at a greatly reduced rate (6), suggesting that perforin is not an absolute requirement for β-cell destruction, but greatly accelerates the process. Interestingly, transgenic mice that express lymphocytic choriomeningitis virus (LCMV) antigens in pancreatic β-cells developed insulitis, but did not develop diabetes following the adoptive transfer of perforin-deficient LCMV-specific CTL (24). These findings are also consistent with our model, since early insulitis is predicted to be FasL dependent and in the absence of perforin, the cytotoxic activity of the avidity-matured CTL population would be significantly decreased. Therefore, β-cell destruction is limited by Fas expression and delayed by loss of perforin. Finally, the destruction of Fas-deficient islets by effector cells from NOD mice (16, 26) and the inability of anti-FasL antibody to prevent diabetes in a CD8+-adoptive transfer model (24, 27) are consistent with our demonstration of the perforin dependence of high-avidity CTL present late in disease. In these studies, the CTL responsible for causing β-cell death are likely the populations that have already undergone avidity maturation, suggesting that they have an increased affinity for β-cells, and are thus able to kill via the perforin pathway.

In conclusion, these data provide the first experimental evidence to support a model of type 1 diabetes in which early insulitis is dependent on Fas/FasL-mediated killing, but rapid progression of disease is dependent on both avidity maturation of autoreactive CTL and their switch to a perforin-dependent mechanism of killing. Our findings also provide a unifying model for seemingly discordant findings reported previously regarding the relative importance of Fas/FasL and perforin in CTL killing of β-cells in NOD mice.

Acknowledgements

This work was supported by a joint grant from the Juvenile Diabetes Research Foundation and The Canadian Institutes for Health Research to the β-Cell Apoptosis Network and by a grant from the Canadian Diabetes Association in honor of the late Violet D. Mulcahy. We are indebted to Galina Soukatcheva and the University of British Columbia Pathology Islet Isolation Core for the provision of mouse islets and to Hideo Yagita for provision of reagents. J.D.T. is supported by a fellowship from the Michael Smith Foundation for Health Research. G.S.D.R. is the recipient of a post-doctoral fellowship from Candlelighters Canada/MRC. C.B.V. is a New Investigator of the Canadian Institutes of Health Research. P.S. is a Scientist of the Alberta Heritage Foundation for Medical Research.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
<td>Lympohocytic choriomeningitis virus</td>
</tr>
<tr>
<td>LCMV</td>
<td>Lympohocytic choriomeningitis virus</td>
<td></td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic</td>
<td></td>
</tr>
</tbody>
</table>

References


