Cytotoxic Mechanisms Employed by Mouse T Cells to Destroy Pancreatic β-Cells

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Several cytotoxic mechanisms have been attributed to T cells participating in β-cell death in type 1 diabetes. However, sensitivity of β-cells to these mechanisms in vitro and in vivo is likely to be different. Moreover, CD4+ and CD8+ T cells may use distinct mechanisms to cause β-cell demise that possibly involve activation of third-party cytotoxic cells. We used the transfer of genetically modified diabeticogenic T cells into normal, mutant, and bone marrow chimeric recipients to test the contribution of major cytotoxic mechanisms in β-cell death. We found that 1) the killing of β-cells by CD4+ T cells required activation of the recipient's own cytotoxic cells via tumor necrosis factor-α (TNF-α); 2) CD8+ T-cell cytotoxic mechanisms destroying β-cells were limited to perforin and Fas ligand, as double knockouts of these molecules abrogated the ability of T cells to cause diabetes; and 3) individual CD8+ T-cell clones chose their cytotoxic weaponry by a yet unknown mechanism and destroyed their targets via either Fas-independent or Fas-dependent (~40% of clones) pathways. Fas-dependent destruction was assisted by TNF-α.

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The cooperation between cytotoxic mechanisms used by different cell types involved in type 1 diabetes is important to understand for the therapeutic interventions in the disease progression. In type 1 diabetes, pancreatic islets are destroyed by infiltrating T cells (1–3) and their proxies, such as activated macrophages (4). Both CD4+ and CD8+ T cells are critical for development of type 1 diabetes (1–3,5). Multiple death pathways were suggested to be critical in type 1 diabetes including perforin (Prf)/granzyme B, tumor necrosis factor (TNF) family receptors (Fas/CD95, TNF receptors [TNFRs], DR4), cytokines (interleukin [IL]-1 and interferon-γ) (6–11), and oxidative damage (12,13). However, controversies remain that most likely result from in vitro investigation of β-cell toxicity of activated cells or cytokines. In vitro experiments reveal a potential for cytokines to kill β-cells, but their real involvement and source in vivo are not always clear. Besides, pleiotropic factors (such as Fas or TNF-α) can be involved in functions other than destruction of β-cells. Moreover, the death pathways induced by CD4+ and CD8+ T cells are very likely to be different. Indeed, CD4+ T cells were not dependent on Fas to kill β-cells in a model that used an ectopic antigen expressed in the islets (14), whereas CD8+ T cells were shown to destroy β-cells via Fas and Prf (11,15).

Thus, we sought to use genetic approaches to investigate how diabeticogenic CD4+ and CD8+ T cells destroy β-cells in vivo. In addition, we addressed the question of why some diabeticogenic CD8+ T cell clones were reported to be heavily dependent on Fas for β-cell destruction, whereas others using the same T-cell receptor (TCR) were not (15,16).

We find that diabeticogenic CD4+ T cells do not directly transmit death signals to β-cells but activate third-party killer cells through secretion of TNF-α, that cytotoxic mechanisms used by CD8+ T cells are redundant and interchangeable, and that individual CD8+ T cells have a preference in the use of their cytotoxic mechanisms. The understanding of effector mechanisms involved in β-cell destruction is important because of the potential to prevent β-cell demise by therapeutic measures.

RESEARCH DESIGN AND METHODS

Mice. NOD/ShiLtJ (NOD), NOD.CB17-PrkdcsidJ (NOD.SCID), B6.MRL-Faslo/J, B6.Tg.Cg-Tg (TeraBDC.2, TeraBDC.2.5) 1D01/DoIj (BDC.2), B6.129Stf-raf1tm1Mor/J (B6.Rag-2), B6.NOD-121m1f1-D17Mit10/JLtJ (B6. Rag-2), C57BL/6 J (B6), C57BL/6-PyF1tm120/SgJ (B6.PyF1), C57BL/6-TgIns2a/J (B6.TNFα-), and B6Sn.C3-Faslo/CgO (B6.Faslpr) mice were from The Jackson Laboratory (Bar Harbor, ME). B6.Tg(129s)Tgfr21IgP2IgP2J (Tgfr21IgP2IgP2) Achi mice were produced by us and are available from The Jackson Laboratory.

Transgenic mice. B6.Kb transgenic mice were generated by microinjection of B6 oocytes with a 7-kb genomic fragment encoding Kb major histocompatibility complex (MHC) class I molecule (a gift from Dr. F. Lemonnier). B6.G9C8 transgenic mice express TCR from H-2Kb-restricted insulin-specific CD8+ T-cell clone G9CS (17). Genomic TCR constructs [a gift of Dr. F.S. Wong (18)] were injected into B6 oocytes. Transgenics were further crossed to B6.Kb mice and to various knockout (KO) mice. B6.Kb and B6. G9C8 transgenic mice were produced at The Jackson Laboratory transgenic facility. BDC2.5 mice were bred to B6.H2Z mice to obtain BDC2.5.H2Z mice, which were further crossed to Prf— and B6P0α mice.

All mice were housed in a specific pathogen-free research facility and used in accordance with institutional guidelines for animal welfare. The Biological Sciences Division Institutional Review Board at the University of Chicago approved all animal studies.

Activation of diabeticogenic T cells and adoptive cell transfer. Diabeticogenic CD8+ (G9CS clone or B6.KbG9C8) or CD4+ T-cell (BDC2.5) were activated in vitro by culturing transgenic splenocytes (2 × 10^6 cells/mL) with irradiated (20 Gy) NOD splenocytes (2 × 10^6 cells/mL) and the relevant peptide (Insulin B [InsB]15–23 for G9CS [LVLVGERG], 5 μg/mL; or BDC2.5 mimotope [AHPFPWAMD, 5 μg/mL]; Invitrogen) in Click’s medium (Irvine Scientific, Santa Ana, CA) supplemented with 5% FCS (Atlanta Biologicals, Lawrenceville, GA), 2 × 10^–7 mol/L β-mercaptoethanol (Bio-Rad Laboratories, Hercules, CA), 30 mmol/L penicillin-streptomycin mixture (Invitrogen), and 3 mg/mL L-glutamine (Invitrogen). Three days after activation, recombinant IL-2 was added to the medium at 5 U/mL. Two days later, cells were collected (>90% of collected live cells were activated T-cells), washed with PBS, and injected intravenously into retro-orbital plexus (10^7 cells/mouse) of recipient mice irradiated (7.25 Gy) to ensure more uniform induction of diabetes (17). Both male and female recipient mice (5–9 weeks of age) were used. To show that T cells from Fas ligand (FasL), Prf, and TNF triple-deficient mice were homing normally to the islets, activated T-cells were labeled with a fluorescent dye and injected into mice, and pancreata were fixed, frozen in OCT compound (Sakura Finetek USA, Torrance, CA), sectioned, and examined as described (17,19). Proliferation of T cells in vitro was tested by [3H]thymidine incorporation. Diabetes development was monitored by testing urine glucose (Diastix; Bayer, Leverkusen, Germany) for 21 days.

Single-cell cloning of transgenic CD8+ T cells. B6.KbG9C8 spleen cells were activated with 3 μg/mL InsB15–23 peptide for 3 days and rested in 5 U/mL.
IL-2 for 2 days. Following this, the cells were harvested and plated at 1 cell/well in a 96-well U-bottom plate and activated with 3 μg/ml InsB15–23 peptide in the presence of 3 × 10^5 irradiated NOD splenocytes per well. A total of 5 IU/ml IL-2 was added after 3 days of stimulation and replenished every 2 days. The cells were peptide restimulated with irradiated NOD splenocytes after every 10 days of culture. Isolated clones were further expanded in 96-well U-bottom plates before being assayed for in vitro cytotoxicity. Some experiments included G9C8 T cells from RAG-negative mice, but the results were similar to those obtained with clones from RAG-sufficient mice (not shown) and for that reason were combined.

In vitro 5- (and 6-)carboxyfluorescein diacetate succinimidyl ester-labeled target-killing assay. Splenocytes from control (B6) or target (NOD, B6.K, NOD.TNFR1/2−/− TNFR2−/−; TNR1/2 KO/2 KO), or B6.K (wild-type) mice were labeled in PBS with 0.5 [5- and 6-carboxyfluorescein diacetate succinimidyl ester] (CFSElow) and 5.0 μmol/L (CFSEhigh) CFSE (Molecular Probes, Invitrogen), respectively (20). Control and target cells were then resuspended in Click’s medium containing 5 μg/ml InsB15–23, mixed at a 1:1 ratio, and plated at 6 × 10^5 total cells/well in a 96-well U-bottom plate. Equal numbers of CD8+ T cells were added to the wells. After 16 h of culture, cells were stained with propidium iodide (Sigma-Aldrich, St. Louis, MO), and at least 3 × 10^5 events were acquired for their destruction. Previous reports using transfer experiments included G9C8 T cells from RAG-negative mice, but the results were similar to those obtained with clones from RAG-sufficient mice (not shown) and for that reason were combined.

RESULTS

CD4+ T-cell-mediated islet destruction is independent of FasL or Prf but depends on TNF-α. Because insulin-producing cells are negative for MHC class II molecules, direct contact of CD4+ T cells with β-cells is likely not required for their destruction. Previous reports using transfer of diabetogenic BDC2.5 CD4+ T cells supported this idea by showing indirect (4) and MHC class II-independent (22) destruction of the islets by the CD4+ T cells. However, the signaling pathways leading to activation of the cytotoxic intermediates and the actual cytotoxic mechanisms involved in islet destruction are not clearly defined. To address this issue, we tested several cytotoxic receptors for their involvement in killing of β-cells by activated CD4+ T cells or their proxies.

In the first experiment, Fas receptor-deficient B6, H2KbRag1−/− Rag2−/− Tcrb−/− and control B6.H2KbRag1−/− Tcrb−/− and NOD, Rag2−/− mice were injected with in vitro-activated BDC2.5 CD4+ T cells. Diabetes developed similarly in all groups (Fig. 1A). To make sure that complete Fas deficiency in the recipient did not produce an artificial enhancement of cytotoxic activity of the cells causing β-cell death, we injected BDC2.5 cells into NOD recipients lacking Fas specifically in β-cells due to the Cre-mediated deletion of floxed Fas (NOD.FasKrlip-Cre3) (23,24). Diabetes induction in these mice was no different from control recipients (Fig. 1B). Thus, Fas–FasL signaling is dispensable for both direct and indirect killing of β-cells by BDC2.5 CD4+ T cells.

Next, we used NOD.TNFR1−/− TNFR2−/− double-KO (NOD.TNFR1/2 KO) recipient mice. Upon transfer of pre-activated BDC2.5 CD4+ T cells, diabetes development was significantly delayed (Fig. 1C). Transfer of BDC2.5 Prf−/− and BDC2.5 Prf−/− gldgld (Prf- and FasL-deficient) CD4+ T cells did not significantly enhance the delay in diabetes induction in NOD.TNFR1/2 KO recipient mice (Fig. 1D). Thus, TNF-α was the major factor that participated in β-cell destruction by CD4+ T cells. TNF-α does not require direct contact of CD4+ T cell with β-cells in order to be produced and can be secreted subsequent to T cell recognition of a β-cell antigen presented by an antigen-presenting cell (APC). Thus, it was equally possible that TNF-α was directly cytotoxic to β-cells or worked as a mediator to induce another cytotoxic mechanism in a third-party cell.

TNF-α produced by CD4+ T cells is not directly cytotoxic to β-cells. To address the role of TNF-α in killing of β-cells by CD4+ T cells, we produced NOD→NOD.TNFR1/2 KO bone marrow chimeras and injected them with BDC2.5 CD4+ T cells. The bone marrow-derived TNF-sufficient recipient cells completely restored the ability of BDC2.5 CD4+ T cells to induce diabetes (Fig. 2A). Thus, it became clear that TNF-α is not involved in direct destruction of β-cells because recipient’s β-cells did not have receptors for it. Instead, TNF-α was required to activate other cell types (of bone marrow origin) that then killed β-cells. Because the two TNFRs differ in function (25,26), we tested which of the receptors was involved in β-cell destruction. Diabetes induction in NOD.TNFR1−/− or control mice was comparable upon transfer of activated BDC2.5 CD4+ T cells (Fig. 2B). A slight delay was observed in NOD.TNFR2−/− recipients compared with NOD mice, but this delay was not as strong as the delay observed in NOD.TNFR1/2 KO mice (Fig. 2). Thus, TNF-α-mediated signals involved in activation of intermediaries seem to be transduced via both TNFR1 and TNFR2. Because transfer of activated BDC2.5 CD4+ T cells into NOD.TNFR1/2 KO mice delayed but did not abolish type 1 diabetes development, a contribution of another yet unknown factor involved in β-cell destruction by CD4+ T cells can be predicted.

Cytotoxic pathways used by diabetogenic CD8+ T cells. CD8 T cells can kill their targets upon direct contact or produce cytotoxic cytokines. Previous reports (15,27–29) support the idea that cytotoxic mechanisms involving direct contact with the target cells (β-cells) are important. At the same time, cytokines, such as TNF-α, IL-1, and interferon-γ were cytotoxic to β-cells (9,10,29). Using transfer of genetically modified activated CD8+ T cells, we sought to establish the importance of the major cytotoxic mechanisms.

We took advantage of TCR-transgenic mice carrying a TCR from a CD8+ T-cell clone G9CS (17) specific for an insulin-derived peptide (InsB15–23) bound to a Kd MHC class I molecule. Transgenic mice were produced on the B6 background and crossed to B6 mice carrying Kd as a genomic transgene to allow proper selection and activation of the TCR. The microarray data have been deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus (21) and are accessible through Gene Expression Omnibus Series accession number GSE31726 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31726).

Statistical analysis. Statistical significance was determined using the Student t test, Mann-Whitney test, or Kaplan-Meier survival analysis using GraphPad Prism (version 4.0c for Macintosh; GraphPad). Error bars represent SE.
transferred into irradiated NOD recipients. None of the mice injected with triple-KO cells were diabetic (Fig. 3A). Control NOD mice injected with wild-type B6.KdG9C8 CD8+ T cells were diabetic by day 8. Furthermore, when activated B6.KdG9C8.Prf2/2TNF2/2 double-KO CD8+ T cells were transferred into B6.KdRag2/2lpr/lpr and control B6.KdRag2/2 mice, diabetes was induced in both sets of control mice by day 10 posttransfer, but not in the B6.KdRag2/2lpr/lpr mice (Fig. 3B). Together, these data indicated that Prf, FasL, and TNF-a composed the minimal group of effector cytokine pathways required for induction of diabetes by activated insulin-specific CD8+ T cells. The conclusion also implies that no other factors acting independently of the trio are by themselves capable of b-cell destruction.

These cytotoxic pathways could be redundant, or perform unique functions. Thus, we tested combinations of these three deficiencies. B6.KdG9C8.TNF2/2CD8+ T cells were capable of transferring diabetes into NOD.SCID or NOD.SCIDilepr mice, indicating that activated CD8+ T cells were able to destroy the islets via the Prf pathway in the absence of FasL or TNF-a signaling (Fig. 3C). We then transferred B6.KdG9C8.Prf2/TNF2/2 double-KO CD8+ T cells into NOD.TNFR1/2 KO and control TNFR-sufficient NOD mice. The double-TNFR KO recipients were used to eliminate the role in disease induction of other TNF family factors [lymphotoxins that can signal through the TNFRs (30)]. Diabetes was induced equivalently between the two groups of recipients, showing that the Fas pathway was capable of compensating for the absence of Prf (Fig. 3D) or both Prf- and TNF-a-mediated killing (Fig. 3E).

These results demonstrate that cytotoxic CD8+ T cells can use either Prf- or FasL-mediated killing to cause destruction of pancreatic islets, and both pathways can fully compensate for the absence of the other. Moreover, in this experimental setting, TNF-a seemed to be completely dispensable (Fig. 3C–E).

Cytotoxic pathways in individual clones of CD8+ T cells are not redundant. The results obtained with the diabetogenic G9C8 cells from TCR-transgenic animals contradicted our previously published results showing that the transfer of insulin-specific diabetogenic G9C8 CD8+ T-cell clone cells to NODlpr/lpr mice did not result in diabetes (7). The failure to induce type 1 diabetes was not due to enhanced expression of FasL on lymphocytes in lpr/lpr mice (31,32), as G9C8 clone cells also failed to induce type 1 diabetes in B6.KdRag2/2lpr/lpr, lacking lymphocytes (Fig. 4A). At the same time, activated polyclonal B6.KdG9C8 CD8+ T cells (monoclonal for the TCR, but otherwise polyclonal) efficiently induced diabetes in B6.KdRag2/2lpr/lpr, B6.Krag2/2, and NOD recipient mice (Fig. 4B).

Therefore, it became evident that in vivo cytotoxicity of the G9C8 clone was highly dependent on the Fas–FasL pathway, which was not the case for the functionally polyclonal G9C8-transgenic T cells. Because the clone and

FIG. 1. CD4+ T cells do not use FasL- and Prf-mediated cytotoxicity to induce diabetes. Total of 10⁷ activated BDC2.5 CD4+ T cells were transferred intravenously into B6.H2b Rag2/2 lpr/lpr (A) or NOD.FasKi-RIP-Cre3 (B) mice and indicated control mice. C: Total of 10⁷ activated BDC2.5 CD4+ T cells from wild-type, BDC2.5.Prf2/2, or BDC2.5.Prf2/2gld/gld mice were transferred into equal numbers of NOD (filled symbols) or NOD.TNFR1/2 KO mice (open symbols). Figures represent combined data from one to three independent experiments. P values were found using Kaplan-Meier statistics.
transgenic T cells shared the G9C8 TCR, it was evident that their antigen specificity was not relevant to the differences in their cytotoxic properties. These differences could be due to long-term in vitro cultivation of the clone or may be explained by intrinsic properties of individual clones. To address this, we used an in vitro cytotoxicity assay to simultaneously test multiple T-cell clones. Where-as the G9C8 clone failed to destroy Fas-negative targets (Fig. 4A), killing by G9C8 TCR-transgenic T cells was only slightly reduced (proportionally to E:T ratios tested). Importantly for data interpretation, the killing of B6.Kd and NOD targets was similar (Fig. 4D). We then generated multiple single-cell clones from activated B6.Kd/G9C8 T cells by limiting dilution. Most of the 71 clones tested displayed reduced killing of B6.Kd/prpr-prpr targets and an only marginally compromised killing of NOD.TNFR1/2 KO targets, indicating that Fasl and, to a significantly lesser extent, TNF-α were essential for the strong in vitro target lysis (Fig. 5A). Because the low maximal killing of the wild-type targets by some clones could affect quantitative assessments, we compared 27 clones with target killing of >40%. Fourteen out of 27 clones showed substantially reduced killing of B6.Kd/lpr/lpr targets (Fig. 5B). This pattern was very similar to that displayed by the G9C8 clone in a target killing assay (Fig. 4C). Thus, ~40% of T-cell clones that have been in culture for only a short period of time (compared with years in culture for the original G9C8 clone) demonstrated a similar phenotype: dependence on Fas. To test whether this phenotype is stable, we compared two clones. From what we have observed with the transfer of the BDC2.5 CD4+ T cells (Fig. 2B), in which the two receptors had redundant function. At the same time, in an in vitro cytotoxicity assay, G9C8 clone cells killed NOD.TNFR1/2 KO and NOD spleen cell targets with equal efficiency, indicating a lack of dependence on TNFR signaling in this context (Fig. 4C).

We then compared mRNA and protein expression of the cytotoxic molecules between G9C8 clone and G9C8 TCR-transgenic T cell populations. The G9C8 clone displayed a twofold higher level of TNF-α transcript and higher protein expression (Fig. 6B and C), whereas the levels of Fasl were only slightly higher, and no differences in Prf expression were found.

Taken together, these results suggest that TNF-α mediating signaling is required to facilitate Fasl-mediated destruction of β-cells in vivo and that chronically activated T cells acquire different physiological properties including killing mechanisms.
The genetic approach used in this study allowed us to make several important observations concerning the role of cytotoxic mechanisms involved in destruction of insulin-producing cells. CD4+ T cells play a crucial role in induction of diabetes as demonstrated by the absence of spontaneous disease in CD4-null NOD mice (33) and the requirement for both CD4+ and CD8+ T cells for induction of diabetes in a transfer model (5,34,35). Because CD4+ T cells, unlike CD8+ T cells, are not involved in direct contact with MHC class II-negative β-cells, they depend on recruitment of other cell types [such as macrophages (4)] to act as the final effectors in β-cell destruction. However, the precise signaling pathways required for efficient β-cell destruction and recruitment of the final effectors are not clear. Direct cytotoxic mechanisms (FasL and Prf) were not involved in β-cell destruction, as BDC2.5 CD4+ T cells recognizing islet

![Image](https://example.com/diabetes-fig3.png)

**FIG. 3.** Prf- and FasL-mediated death pathways are essential and interchangeable for diabetes induction by B6.KdG9C8-transgenic CD8+ T cells. A: Total of 10^7 activated B6.KdG9C8 or triple-KO (Prf^-/-;TNF^-/- gld/gld) G9C8 CD8+ T cells were transferred intravenously into irradiated NOD recipients to induce diabetes. B: Total of 10^7 activated B6.KdG9C8.Prf^-/-;TNF^-/- G9C8 CD8+ T cells were injected intravenously into indicated irradiated recipient mice and observed for diabetes induction. C: NOD.SCID or NOD.SCID lpr/lpr recipient mice were injected intravenously with 10^7 activated B6.KdG9C8.TNF^-/- CD8+ T cells. D: Activated B6.KdG9C8 Prf^-/-;CD8+ T cells (10^7) were transferred intravenously into B6.Kd Rag^-/- lpr/lpr and NOD and B6.KdRag^-/- control mice. E: Total of 10^7 activated B6.KdG9C8.Prf^-/-;TNF^-/- or B6.KdG9C8.Prf^-/- gld/gld CD8+ T cells were transferred intravenously into irradiated NOD or NOD.TNFR1/2 KO recipients. Numbers in parentheses indicate mice per group. Figures represent combined data from at least two independent experiments. P values for curves were found using Kaplan-Meier statistics and Mann-Whitney test for bar graphs. Numbers above bars represent diabetes incidence (diabetic/total number of mice).
antigen chromogranin A (36) induced type 1 diabetes in NOD mice lacking Fas in β-cells (either NOD.Rag-/- lpr/lpr mice or mice with β-cell–specific deletion of Fas, NOD.FasKI.RIP-Cre3). Similarly, β-cell–specific removal of Fas in a model with ectopic expression of a viral antigen in the islets failed to block β-cell destruction by CD4+ T cells (14). However, the lack of TNF-α signaling in the recipient significantly delayed the induction of type 1 diabetes (Fig. 1C), which was not further affected by the combined deletion of Prf and FasL (Fig. 1D). TNF-α is a cytokine with a range of activities important in diabetes development, including upregulation of cell adhesion by endothelial cells promoting homing of leukocytes (37–39), upregulation of MHC class I and II expression by islets (40,41), activation of APCs and T cells, and direct killing of β-cells in vitro has been shown (42). However, this cytokine produced by activated CD4+ T cells did not kill β-cells in vivo directly; type 1 diabetes development in TNFR-negative recipients was restored when bone marrow–derived cells were replaced with TNFR-sufficient cells (Fig. 2A). Taken together, the results of the studies of diabetogenic CD4+ T cells lead to three conclusions: 1) TNF-α works as an inducer of cytotoxic capabilities in a third-party cell; 2) activation of third-party cells through Fas, which can work as an activating molecule (43), does not happen or is not important; and 3) possible killing (through Fas) of islet APCs within the islets is not critical for the third-party cell–mediated destruction of β-cells.

Interestingly, TNFR1 and TNFR2 (to a slightly lesser degree) were important for CD4+ T cell-dependent induction of cytotoxicity (Fig. 2B). TNFR1 is considered to be proapoptotic and has been shown to be important for TNF-α–mediated destruction of islets in an allogenic islet transplant model (8). For the induction of cytotoxicity in a third-party cell, TNF-α provided activating signaling through both of its receptors. Macrophages (4) or other cell types such as dendritic cells and natural killer cells can be the third-party killers. All three cell types can be activated by TNF-α, and further investigation is required to put the responsibility for killing of β-cells on one (or all) of these subsets.

TNF-α produced by CD8+ T cells can be directly cytotoxic, although the importance of this was not supported by our experiments (Fig. 3C). It may also play a role (similar to CD4+ T cell–derived TNF-α) in the activation of third-party cytotoxic cells. This became apparent in experiments using the G9C8 clone. The clone used FasL to kill β-cells and in vitro killing of target cells was TNF-α-independent.
At the same time, the induction of type 1 diabetes was delayed in NOD.TNFR1/2 KO recipients (Fig. 6A). Interestingly, the delay in disease development was entirely dependent on TNFR1 and not on TNFR2 (Fig. 6A). This difference with CD4+ T cells could also mean that TNF-α is capable of facilitation of Fas expression by β-cells (which is inherently low on these cells without activation) (44,45) or of sensitivity of target cells to its engagement. TNFR1 has been implicated in the development of spontaneous type 1 diabetes (46), and in our hands, NOD.TNFR1/2 KO mice were also completely protected from spontaneous diabetes (incidence of zero out of nine) for the 30 weeks of observation.

Our report on the role of Fas in type 1 diabetes development (7) induced a long-lasting discussion with experimental evidence both pro (29,47) and against (32,48) participation of Fas in β-cell destruction. Involvement of other cytotoxic molecules in β-cell destruction has been suggested. Preferential use of Prf, for example, was linked to TCR affinity and the age of animals (49).

Thus, we addressed the controversial issues using a genetic approach. We have learned that Fas and Prf are both important, and each of them can kill β-cells on their own. Another important conclusion is that (at least in the transfer model, in which all other in vivo requirements for T cell activation are bypassed) the list of cytotoxic mechanisms used by CD8+ T cells can be reduced to just two, FasL- and Prf. Other mechanisms can be involved, but they must play an accessory role, whereas FasL or Prf is necessary.

One unresolved problem involving our initial work was that whereas G9C8 clone failed to cause diabetes in NODlpr/lpr mice (7), T cells derived from a transgenic mouse with the same TCR successfully induced the disease (Fig. 4B). TCR-transgenic T cells are monospecific, but polyclonal in all other aspects. Thus, the G9C8 clone could have been unique in losing its ability to kill via Prf, or individual T cells from the transgenic mouse varied in which weapon they chose. Among highly cytotoxic T-cell clones derived from G9C8-transgenic mice, ~40% failed to destroy Fas-negative targets. These clones were in culture for about 25 days (compared with the original G9C8 clone that had been in culture for many years), suggesting that the decision to forgo Prf-mediated killing was made rather early. Moreover, these clones were stable, as the secondary clones reproduced the phenotype of the original clones. These results argue that individual clones of T cells vary in their preferred cytotoxic mechanisms. Interestingly, expression of the Prf-encoding Prf1 gene was not different between the G9C8 clone and activated T cells from G9C8 TCR-transgenic (Fig. 6B). Thus, the regulation of cytotoxicity must be posttranslational. The choice of cytotoxic pathway used by a given T cell could be stochastic or inducible by external signals, which is an interesting issue warranting further investigation. Another diabetogenic CD8+ T-cell clone, 8.3, has been shown to

FIG. 5. Individual T-cell clones isolated from B6.KdG9C8 TCR-transgenic mice differ in ability to destroy Fas-negative targets. A: Killing of NOD, B6.Kd lpr/lpr, and NOD.TNFR1/2 KO by B6.KdG9C8 origin single-cell primary clones. B: Selected clones with high cytotoxic activity (>40% killing of NOD targets). C: Cytotoxic properties of secondary subclones from selected clones 23 and 19 (subclones are shown ranked by the strength of lysis of control splenocytes).
destroy β-cells via a FasL-mediated mechanism, although non-β-cell targets were killed by a different mechanism by the same cells (16). Thus, there may be other factors that aid destruction of specific cell types by different cytotoxic mechanisms.

These complementary mechanisms and the actual mechanism(s) of β-cell killing by secondary effectors need to be elucidated in future experiments because of their potential importance as targets for therapeutic intervention in type 1 diabetes.

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V.V. performed experiments and cowrote the manuscript. L.A. performed experiments and reviewed and edited the manuscript. D.M.S. performed experiments and reviewed and edited the manuscript. A.V.C. designed the study and wrote and edited the manuscript. V.V. and A.V.C. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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