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

Vineeth Varanasi, Lia Avanesyan, Desiree M. Schumann, and Alexander V. Chervonsky

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 diabetogenic 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).

From the Department of Pathology, University of Chicago, Chicago, Illinois.
Corresponding author: Alexander V. Chervonsky, achervon@bsd.uchicago.edu.
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Thus, we sought to use genetic approaches to investigate how diabetogenic $CD4^+$ and $CD8^+$ T cells destroy β-cells in vivo. In addition, we addressed the question of why some diabetogenic $CD8^+$ 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 diabetogenic $CD4^+$ 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-Prkdcscid/J (NOD.SCID), B6.MRL-*Fas*^{lpr}/J, B6g7.Cg-Tg (*TcraBDC2.5*, *TcrbBDC2.5*) 1Doi/DoiJ (BDC2.5), B6.129S7-*Rag1*^{tm1Mom}/J (B6.Rag$^{-/-}$), B6.NOD- (D17Mit21-D17Mit10)/LtJ (B6.H2^{g7}), C57BL/6 J (B6), C57BL/6-*Prf1*^{tm1Sdz}/J (B6.Prf$^{-/-}$), B6.129S-*Tnf*^{tm1Gkl}/J (B6.TNFα$^{-/-}$), and B6Smn.C3-*Fasl*^{gld}/J (B6$^{gld/gld}$) mice were from The Jackson Laboratory (Bar Harbor, ME). NOD.B6(129S)*Tnfrsf1a*^{tm1Imx}*Tnfrsf1b*^{tm1Imx}/AchJ mice were produced by us and are available from The Jackson Laboratory.
Transgenic mice. B6.K^d transgenic mice were generated by microinjection of B6 oocytes with a 7-kb genomic fragment encoding K^d major histocompatibility complex (MHC) class I molecule (a gift from Dr. F. Lemonnier).

B6.G9C8 transgenic mice express TCR from H-2K^d–restricted insulin-specific $CD8^+$ T-cell clone G9C8 (17). Genomic TCR constructs [a gift of Dr. F.S. Wong (18)] were injected into B6 oocytes. Transgenics were further crossed to B6.K^d mice and to various knockout (KO) mice. B6.K^d and B6.G9C8 transgenic mice were produced at The Jackson Laboratory transgenic facility. BDC2.5 mice were bred to B6.H2^{g7} mice to obtain BDC2.5.H2^{g7} mice, which were further crossed to Prf$^{-/-}$ and B6$^{gld/gld}$ 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 diabetogenic T cells and adoptive cell transfer. Diabetogenic $CD8^+$ (G9C8 clone or B6.K^{d}G9C8) or $CD4^+$ T cells (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 G9C8 [LYLVCGERG, 5 μg/mL] or BDC2.5 mimotope [AHHPIWARMDA, 5 μg/mL]; Invitrogen) in Click's medium (Irvine Scientific, Santa Ana, CA) supplemented with 5% FCS (Atlanta Biologicals, Lawrenceville, GA), 2×10^{-5} mol/L β2-mercaptoethanol (Bio-Rad Laboratories, Hercules, CA), 20 mmol/L penicillin-streptomycin mixture (Invitrogen), and 3 mg/mL L-glutamine (Invitrogen). After 3 days of activation, recombinant IL-2 was added to the medium at 5 U/mL. Two days later, cells were collected (>99% 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 [^{3}H]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.K^{d}G9C8 spleen cells were activated with 3 μg/mL InsB$^{15-23}$ peptide for 3 days and rested in 5 IU/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 $InsB^{15-23}$ 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 using 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^d, NOD.TNFR1$^{-/-}$TNFR2$^{-/-}$ [TNFR1/2 KO], or B6.$K^{d\ lpr/lpr}$) mice were labeled in PBS with 0.5 [5- (and 6-)carboxyfluorescein diacetate succinimidyl ester] [$CFSE^{low}$]) and 5.0 μmol/L ($CFSE^{high}$) CFSE (Molecular Probes, Invitrogen), respectively (20). Control and target cells were then resuspended in Click's medium containing 5 μg/mL $InsB^{15-23}$, 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 3×10^3 $CFSE^{high}$ target cells were analyzed by flow cytometry (BD-FACS Canto; BD Biosciences, San Jose, CA) to estimate the frequency of live CFSE-labeled populations. Data were analyzed using FlowJo v8.8.6 (Tree Star, San Carlos, CA). Specific lysis was determined by estimating the killing of target cells in test wells with added T cells compared with control wells with no T cells, using the formula: percent specific killing = 1 − ([ratio in control wells]/[ratio in test wells]) × 100, where ratio in control wells or test wells = percent $CFSE^{low}$ cells/percent $CFSE^{high}$ cells. For the high-throughput testing, the effector to target cell (E:T) ratios were not determined for every clone, but they were similar.

Microarray analysis. RNA isolated from three samples of peptide-activated, IL-2–rested G9C8 clone and transgenic male mice (on NOD background to avoid drastic allelic differences in gene expression) were hybridized to Affymetrix Mouse 430v2.0 GeneChips (Affymetrix). Affymetrix GCOS software (Affymetrix) was used to run the comparison analysis method using default parameters, with signal scaling (target value = 150) to normalize signal from each chip. Resulting files were subjected to further analysis using GeneSpring GX7.3 (Agilent Technologies) and Ingenuity Pathway Analysis software. 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.

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.$H2^{g7}Rag^{-/-\ lpr/lpr}$ and control B6.$H2^{g7}Rag^{-/-\ lpr/+}$ and NOD.$Rag^{-/-}$ mice were injected with in vitro-activated BDC2.5 $CD4^+$ T cells. Diabetes developed similarly in all groups (Fig. 1*A*). 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.FasKi.Rip-Cre3) (23,24). Diabetes induction in these mice was no different from control recipients (Fig. 1*B*). 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 preactivated BDC2.5 $CD4^+$ T cells, diabetes development was significantly delayed (Fig. 1*C*). Transfer of BDC2.5 Prf$^{-/-}$ and BDC2.5 Prf$^{-/-\ gld/gld}$ (Prf- and FasL-deficient) $CD4^+$ T cells did not significantly enhance the delay in diabetes induction in NOD.TNFR1/2 KO recipient mice (Fig. 1*D*). 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 TNFR-sufficient cells completely restored the ability of BDC2.5 $CD4^+$ T cells to induce diabetes (Fig. 2*A*). 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. 2*B*). 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 G9C8 (17) specific for an insulin-derived peptide ($InsB^{15-23}$) bound to a K^d MHC class I molecule. Transgenic mice were produced on the B6 background and crossed to B6 mice carrying K^d as a genomic transgene to allow proper selection and activation with the cognate peptide. B6.K^dG9C8 mice were further crossed to B6.Prf$^{-/-}$, B6.TNF$^{-/-}$, and B6$^{gld/gld}$ mice to produce triple-KO (Prf/TNF-α/FasL-deficient) mice. To examine their ability to induce diabetes, triple-KO $CD8^+$ T cells activated in vitro with the cognate peptide were

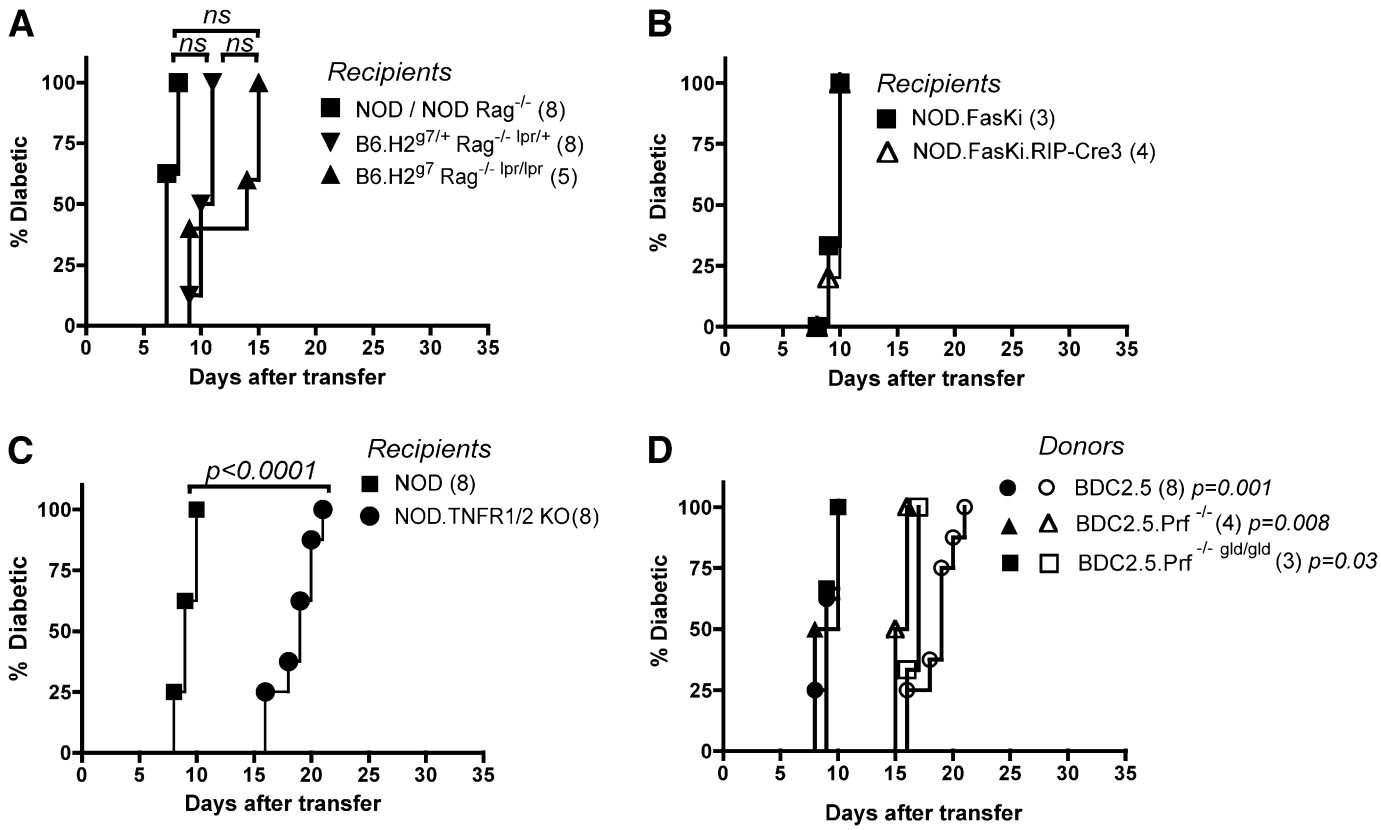

FIG. 1. CD4$^+$ T cells do not use FasL- and Prf-mediated cytotoxicity to induce diabetes. Total of 10^7 activated BDC2.5 CD4$^+$ T cells were transferred intravenously into B6.H2^{g7} Rag$^{-/-\ lpr/lpr}$ (*A*) or NOD.FasKi-RIP-Cre3 (*B*) mice and indicated control mice. *C*: Total of 10^7 activated BDC2.5 CD4$^+$ T cells was transferred intravenously into NOD or NOD.TNFR1$^{-/-}$TNFR2$^{-/-}$ (NOD.TNFR1/2 KO) mice. In *A–C*, numbers in parentheses indicate mice per group. *D*: Total of 10^7 activated BDC2.5 CD4$^+$ T cells from wild-type, BDC2.5.Prf$^{-/-}$, or BDC2.5.Prf$^{-/-\ gld/gld}$ 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.

transferred into irradiated NOD recipients. None of the mice injected with triple-KO cells were diabetic (Fig. 3*A*). Control NOD mice injected with wild-type B6.K^{d}G9C8 CD8$^+$ T cells were diabetic by day 8. Furthermore, when activated B6.K^{d}G9C8.Prf$^{-/-}$TNF$^{-/-}$ double-KO CD8$^+$ T cells were transferred into B6.K^{d}Rag$^{-/-\ lpr/lpr}$ and control B6.K^{d}Rag$^{-/-}$ or NOD recipient mice, diabetes was induced in both sets of control mice by day 10 posttransfer, but not in the B6.K^{d}Rag$^{-/-\ lpr/lpr}$ mice (Fig. 3*B*). Together, these data indicated that Prf, FasL, and TNF-α 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 β-cell destruction.

These cytotoxic pathways could be redundant, or perform unique functions. Thus, we tested combinations of these three deficiencies. B6.K^{d}G9C8.TNF$^{-/-}$ CD8$^+$ T cells were capable of transferring diabetes into NOD.SCID or NOD.SCID$^{lpr/lpr}$ recipients, indicating that activated CD8$^+$ T cells were able to destroy the islets via the Prf pathway in the absence of FasL or TNF-α signaling (Fig. 3*C*). We then transferred B6.K^{d}G9C8.Prf$^{-/-}$TNF$^{-/-}$ 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. 3*D*) or both Prf- and TNF-α–mediated killing (Fig. 3*E*).

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-α seemed to be completely dispensable (Fig. 3*C–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 NOD$^{lpr/lpr}$ 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.K^{d}Rag$^{-/-\ lpr/lpr}$, lacking lymphocytes (Fig. 4*A*). At the same time, activated polyclonal B6.K^{d}G9C8 CD8$^+$ T cells (monoclonal for the TCR, but otherwise polyclonal) efficiently induced diabetes in B6.K^{d}Rag$^{-/-\ lpr/lpr}$, B6.K^{d}Rag$^{-/-}$, and NOD recipient mice (Fig. 4*B*).

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

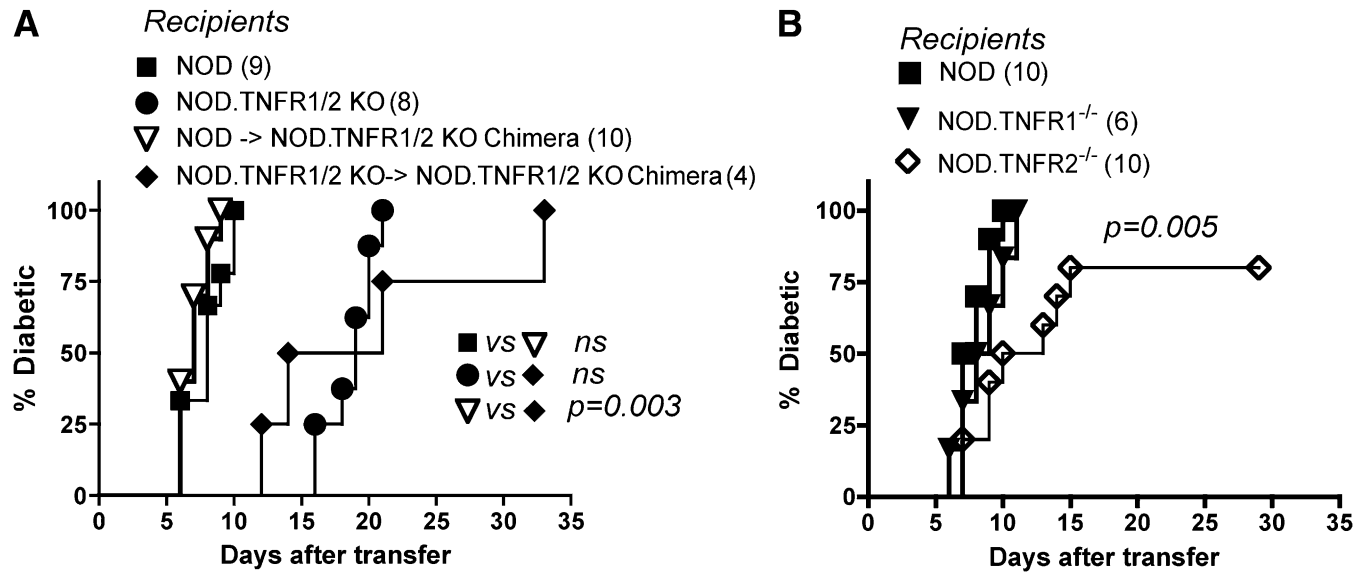

FIG. 2. Diabetes induced by CD4$^+$ T cells requires expression of TNFRs by bone marrow-derived cells. *A*: Total of 10^7 bone marrow cells from NOD or NOD.TNFR1/2 KO mice were injected intravenously into lethally irradiated NOD.TNFR1/2 KO recipients to establish bone marrow chimeras. Eight weeks later, chimeric mice and similarly irradiated control mice were injected intravenously with 10^7 activated BDC2.5 CD4$^+$ T cells to induce diabetes. *B*: Total of 10^7 activated BDC2.5 CD4$^+$ T cells was transferred intravenously into irradiated NOD.TNFR1$^{-/-}$, NOD.TNFR2$^{-/-}$, or control recipient mice. Numbers in parentheses indicate mice per group. Figure represents combined data from at least two 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. Whereas the G9C8 clone failed to destroy Fas-negative targets (Fig. 4*C*), 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.K^d and NOD targets was similar (Fig. 4*D*). We then generated multiple single-cell clones from activated B6.K^{d}G9C8 T cells by limiting dilution. Most of the 71 clones tested displayed reduced killing of B6.K$^{d\ lpr/lpr}$ 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. 5*A*). 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.K$^{d\ lpr/lpr}$ targets (Fig. 5*B*). This pattern was very similar to that displayed by the G9C8 clone in a target killing assay (Fig. 4*C*). 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, several original clones were subcloned and their cytotoxic properties determined. The subclones were more homogenous with fewer variations in their killing efficiency. Subclones of clone 23 and clone 19 displayed markedly lower killing of B6.K$^{d\ lpr/lpr}$ as compared with NOD.TNFR1/2 KO or NOD targets, consistent with the properties of parental clones (Fig. 5*C*). Subclones of another clone (13, not shown) displayed two types of reactivity (one with high lysis and another with low lysis of *lpr/lpr* targets); however, we cannot exclude the possibility that the original culture contained two clones.

Contribution of TNF-α to type 1 diabetes induction by the CD8$^+$ T cells. Although TNF-α seemed to be dispensable for killing of β-cells by G9C8 TCR-transgenic T cells, the G9C8 clone exclusively depended on FasL-mediated killing. We sought to determine whether under such conditions TNF-α signaling could play a role. When G9C8 clone cells were transferred into NOD.TNFR1/2 KO recipients, diabetes development was significantly delayed, and only 75% of the mice eventually developed the disease, compared with wild-type controls, all of which succumbed by day 8 posttransfer (Fig. 6*A*). The transfer of the G9C8 clone cells into NOD.TNFR2$^{-/-}$ single-KO recipients induced diabetes with kinetics similar to that of control TNFR-sufficient recipient mice (Fig. 6*A*), suggesting that signaling through this receptor was not important. In contrast, NOD.TNFR1$^{-/-}$ mice were far less sensitive to G9C8 clone (Fig. 6*A*). This is different from what we have observed with the transfer of the BDC2.5 CD4$^+$ T cells (Fig. 2*B*), 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. 4*C*).

We then compared mRNA and protein expression of the cytotoxic molecules between G9C8 clone and G9C8 TCR-transgenic T cells activated under similar conditions. The G9C8 clone displayed a twofold higher level of TNF-α transcript and higher protein expression (Fig. 6*B* 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—mediated 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.

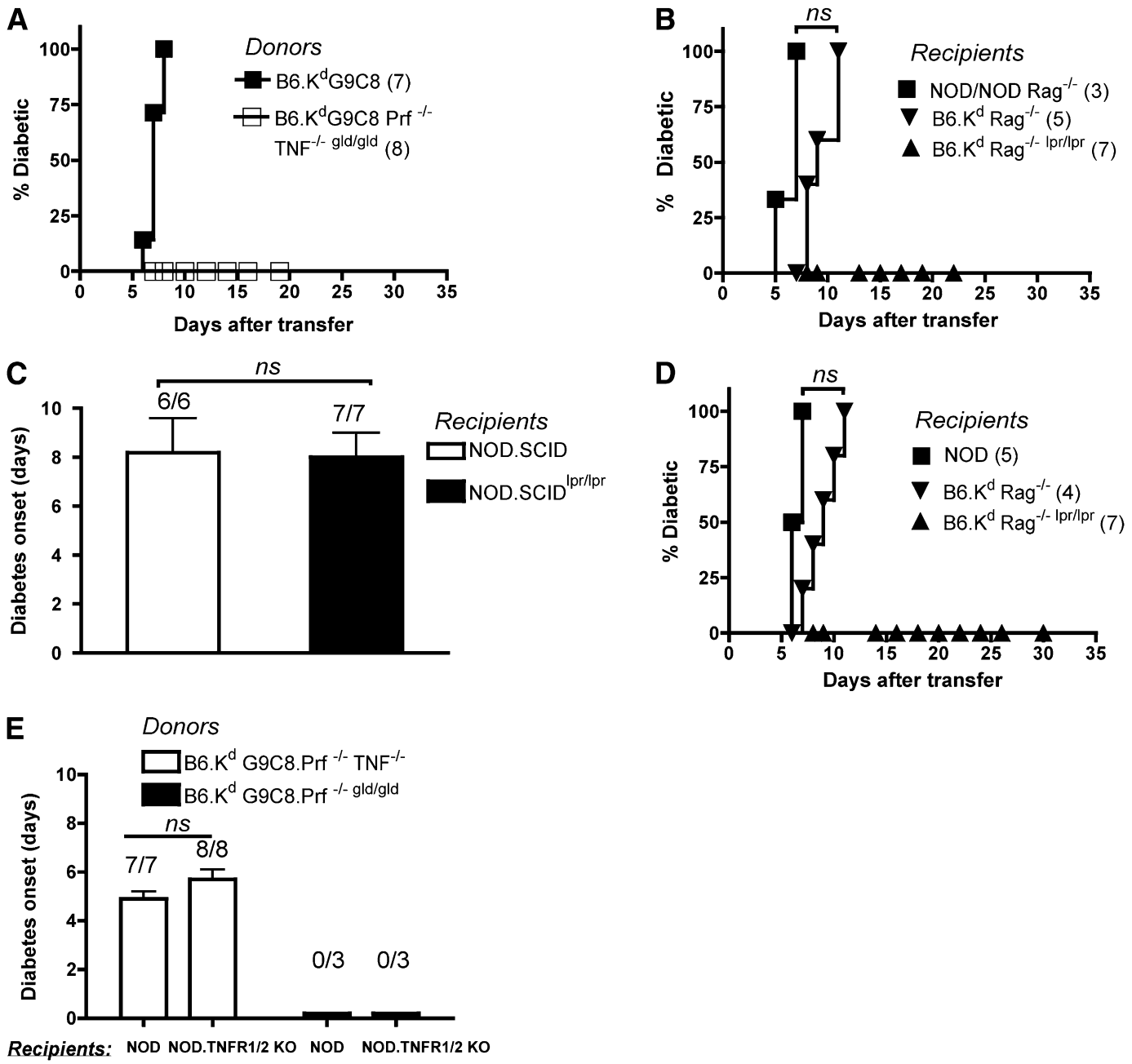

FIG. 3. Prf- and FasL-mediated death pathways are essential and interchangeable for diabetes induction by B6.K^{d}G9C8-transgenic CD8$^+$ T cells. *A*: Total of 10^7 activated B6.K^{d}G9C8 or triple-KO (Prf$^{-/-}$TNF$^{-/-\ gld/gld}$) G9C8 CD8$^+$ T cells were transferred intravenously into irradiated NOD recipients to induce diabetes. *B*: Total of 10^7 activated B6.K^{d}G9C8.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.K^{d}G9C8 TNF$^{-/-}$ CD8$^+$ T cells. *D*: Activated B6.K^{d}G9C8 Prf$^{-/-}$ CD8$^+$ T cells (10^7) were transferred intravenously into B6.K^d Rag$^{-/-\ lpr/lpr}$ and NOD and B6.K^{d}Rag$^{-/-}$ control mice. *E*: Total of 10^7 activated B6.K^{d}G9C8.Prf$^{-/-}$TNF$^{-/-}$ or B6.K^{d}G9C8.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).

DISCUSSION

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

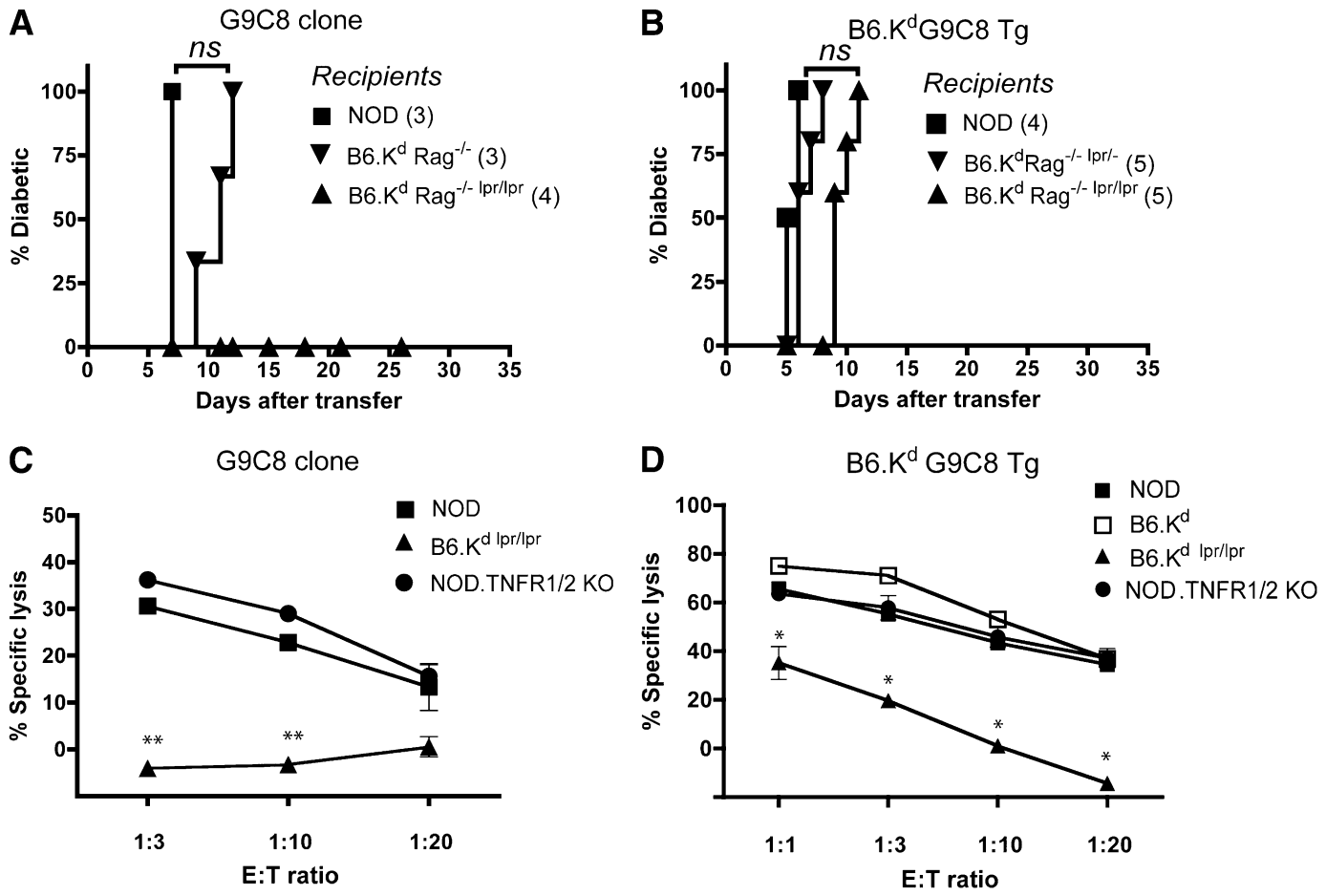

FIG. 4. Diabetes induction by G9C8 clone but not by CD8$^+$ T cells from G9C8 TCR-transgenic mice is dependent on Fas pathway. Total of 10^7 activated G9C8 clone cells (*A*) or B6.K^{d}G9C8 CD8$^+$ T cells (*B*) were transferred into indicated irradiated recipient mice and observed for diabetes development. Numbers in parentheses indicate mice per group. Activated G9C8 clone cells (*C*) or B6.K^{d}G9C8 cells (*D*) were cultured with CFSE-labeled splenocytes from NOD, B6.K^d, B6.K$^{d\ lpr/lpr}$, and NOD.TNFR1/2 KO mice mixed 1:1 with differentially labeled B6 control cells in the presence of 5 μg/mL of InsB$^{15-23}$ peptide and used at indicated E:T ratios. Sixteen hours later, cells were isolated, and survival of specific targets relative to survival of the control B6 cells was estimated to determine specific lysis. Data represent mean + SEM of three replicate samples at each E:T ratio. *P* values for curves were found using Kaplan-Meier statistics. **P* < 0.05, ***P* < 0.005.

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. 1*D*). 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. 2*A*). 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 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. 2*B*). 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. 3*C*). 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

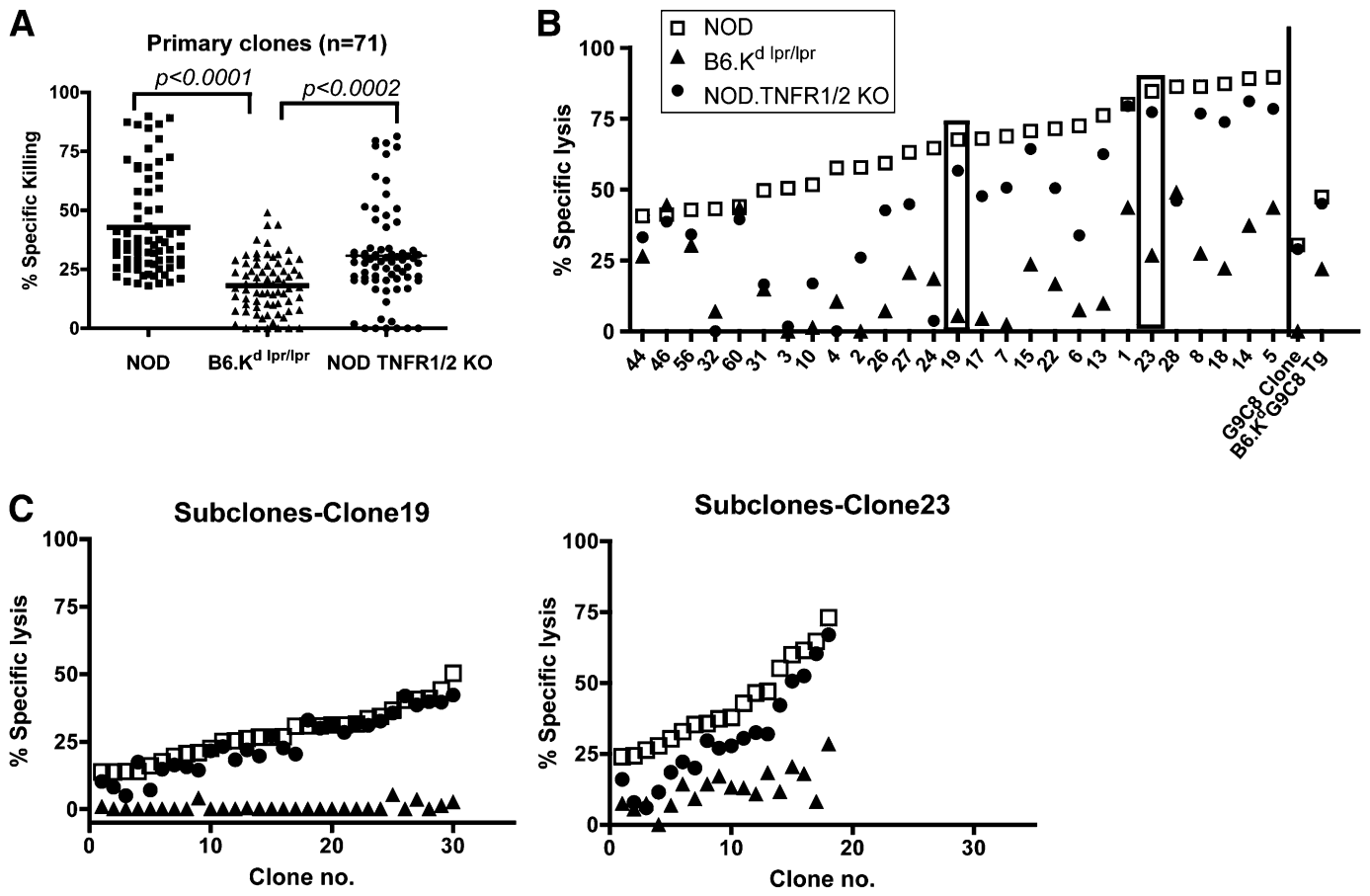

FIG. 5. Individual T-cell clones isolated from B6.K^{d}G9C8 TCR-transgenic mice differ in ability to destroy Fas-negative targets. *A*: Killing of NOD, B6.K$^{d\ lpr/lpr}$, and NOD.TNFR1/2 KO by B6.K^{d}G9C8 origin single-cell primary clones. *B*: Selected clones with high cytotoxic activity (>40% killing 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).

(Fig. 4*C*). At the same time, the induction of type 1 diabetes was delayed in NOD.TNFR1/2 KO recipients (Fig. 6*A*). Interestingly, the delay in disease development was entirely dependent on TNFR1 and not on TNFR2 (Fig. 6*A*). 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 NOD$^{lpr/lpr}$ mice (7), T cells derived from a transgenic mouse with the same TCR successfully induced the disease (Fig. 4*B*). 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. 6*B*). 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

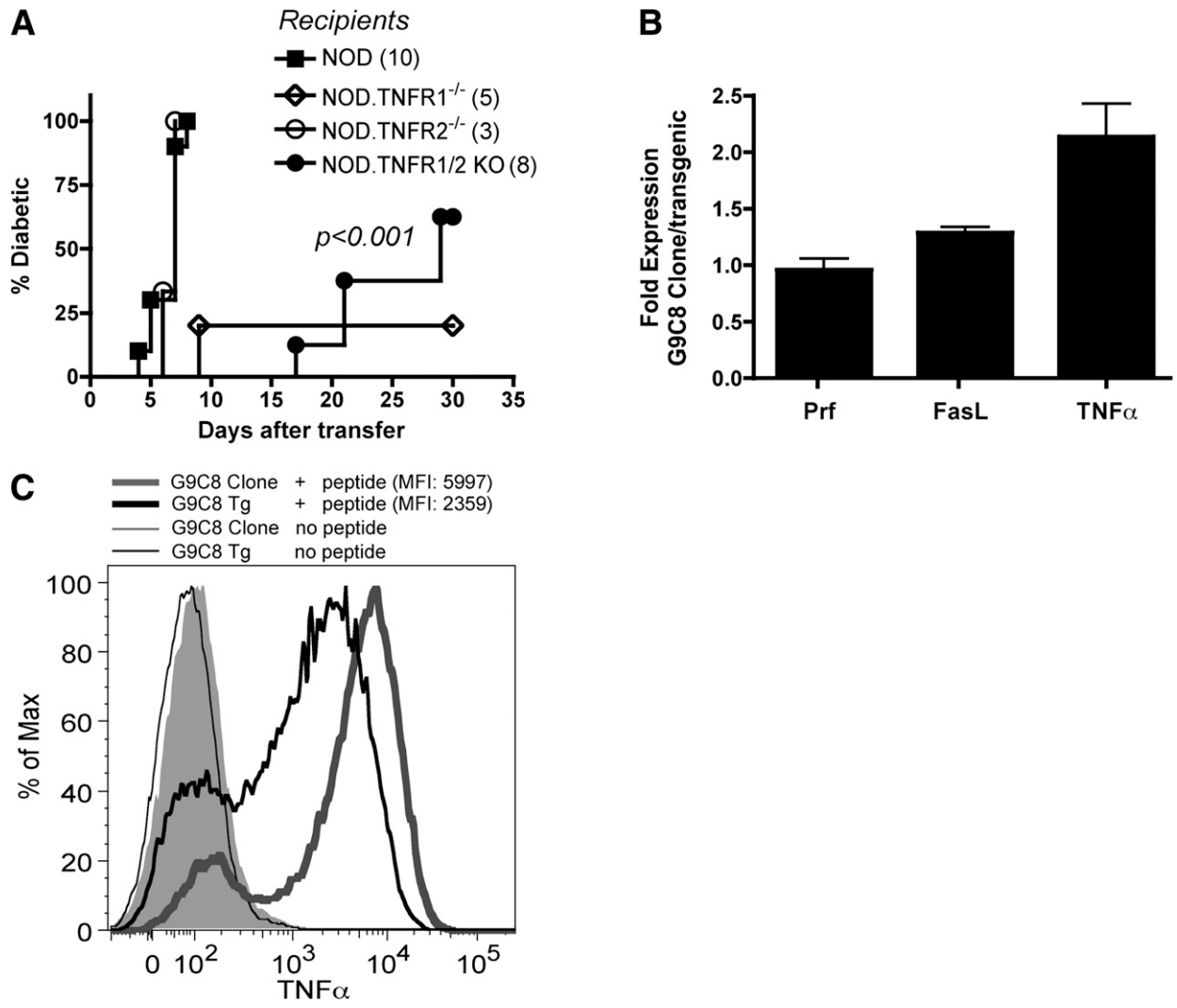

FIG. 6. TNF-α signaling facilitates type 1 diabetes induction by the G9C8 clone. *A*: Irradiated NOD or NOD.TNFR1/2 KO mice were injected intravenously with 10^7 G9C8 clone cells, and diabetes incidence and the day of onset were recorded. Numbers in parentheses indicate mice per group. Combined data from several experiments. *P* values for curves were found using Kaplan-Meier statistics. *B*: G9C8 clone cells and NOD.G9C8 TCR-transgenic spleen cells were similarly activated with $InsB^{15-23}$ peptide and subjected to a microarray RNA expression analysis. Data are expressed as fold overexpression in G9C8 clone compared with G9C8-transgenic $CD8^+$ T cells. *C*: Similarly activated rested G9C8 clone (shadowed histogram and thick gray line) and B6.K^dG9C8-transgenic cells (thin and thick black lines) were stimulated in vitro for 5 h with or without 5 μg/mL $InsB^{15-23}$ peptide in the presence of brefeldin A and stained for intracellular TNF-α. Histogram overlays show TNF-α levels in gated $CD8^+$ T cells. Numbers in parenthesis represent mean fluorescent intensity (MFI).

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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