Cytotoxic T-Cells From T-Cell Receptor Transgenic NOD8.3 Mice Destroy β-Cells via the Perforin and Fas Pathways

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Cytotoxic T-cells are the major mediators of β-cell destruction in type 1 diabetes, but the molecular mechanisms are not definitively established. We have examined the contribution of perforin and Fas ligand to β-cell destruction using islet-specific $CD8^+$ T-cells from T-cell receptor transgenic NOD8.3 mice. NOD8.3 T-cells killed Fas-deficient islets in vitro and in vivo. Perforin-deficient NOD8.3 T-cells were able to destroy wild-type but not Fas-deficient islets in vitro. These results imply that NOD8.3 T-cells use both pathways and that Fas is required for β-cell killing only when perforin is missing. Consistent with this theory, transgenic NOD8.3 mice with β-cells that do not respond to Fas ligation were not protected from diabetes. We next investigated the mechanism of protection provided by overexpression of suppressor of cytokine signaling-1 (SOCS-1) in β-cells of NOD8.3 mice. SOCS-1 islets remained intact when grafted into NOD8.3 mice and were less efficiently killed in vitro. However, addition of exogenous peptide rendered SOCS-1 islets susceptible to 8.3 T-cell–mediated lysis. Therefore, NOD8.3 T-cells use both perforin and Fas pathways to kill β-cells and the surprising blockade of NOD8.3 T-cell–mediated β-cell death by SOCS-1 overexpression may be due in part to reduced target cell recognition. *Diabetes* 55:2412–2418, 2006

Type 1 diabetes results from the selective destruction of insulin-secreting β-cells within the pancreas. Autoreactive T-cells play a major role in β-cell death both in humans and in the NOD mouse model of type 1 diabetes. Although efficient disease progression requires both $CD4^+$ and $CD8^+$ T-cells (1,2), the relative contribution of effector mechanisms used by these activated T-cells is unclear. Evidence from the NOD mouse implicates perforin, a major component of the lytic granules of cytotoxic T-cells (CTLs) and natural killer cells, as an important mediator of β-cell death. Although genetic deficiency of perforin significantly reduces the incidence of diabetes in NOD mice, a small percentage of knockout animals still develop disease (3). Proinflammatory cytokines such as interferon (IFN)-γ, tumor necrosis factor-α, and interleukin-1 are important candidates in non-perforin–dependent killing. Combinations of these cytokines are toxic to β-cells in vitro (4) and may enhance recognition of β-cells by upregulation of major histocompatibility complex (MHC) class I molecules and cell death receptors including Fas.

We have studied the effector mechanisms utilized by a population of $CD8^+$ T-cells recognizing an epitope within the islet-specific glucose 6-phosphatase catalytic subunit–related protein (IGRP). In wild-type NOD mice, up to 40% of $CD8^+$ T-cells infiltrating the islets recognize IGRP before the onset of disease (5–8). Quantitation of IGRP-specific T-cells in the peripheral blood using MHC class I tetramers can be used to predict which NOD mice will develop diabetes (9), suggesting that this is an important T-cell specificity in the NOD model. The expression of an IGRP-specific T-cell receptor (TCR) in NOD mice (NOD8.3) accelerates the onset and increases the incidence of spontaneous disease (10). Such TCR transgenic mice provide simplified models of diabetes in which the contribution of a single population of $CD4^+$ or $CD8^+$ T-cells can be studied in isolation. These mice develop diabetes with high disease penetrance and at an accelerated rate but provide a basis for understanding the mechanisms involved in nontransgenic mice and possibly in humans.

We have previously reported that overexpression of suppressor of cytokine signaling-1 (SOCS-1) in β-cells of NOD8.3 mice completely prevents diabetes, although insulitis and T-cell activation appear to proceed normally (11). We have now analyzed the effector mechanisms used by 8.3 T-cells to understand which of these can be blocked by SOCS-1 in vivo. The surprising result is that 8.3 T-cells clearly use both the perforin-granzyme and Fas pathways to kill β-cells, indicating that both of these pathways are blocked by overexpression of SOCS-1.

RESEARCH DESIGN AND METHODS

NOD mice expressing a SOCS-1 transgene under the control of the rat insulin promoter (RIP-SOCS-1) (11) and NOD8.3 mice expressing the TCRαβ rearrangements of the $H\text{-}2K^d$–restricted, β-cell–reactive, $CD8^+$ T-cell clone NY8.3 have been described (12). Perforin knockout NOD mice were obtained from The Jackson Laboratories type 1 diabetes repository and crossed to NOD8.3 transgenic mice ($NOD8.3.PO^{o/o}$). NOD*lpr* (13), RIP-B7-NOD.*scid* (14), RIP-

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CTL, cytotoxic T-cell; dn, dominant-negative; FasL, Fas ligand; FADD, Fas-associated death domain; IGRP, islet-specific glucose 6-phosphatase catalytic subunit–related protein; IFN, interferon; MHC, major histocompatibility complex; PLN, pancreatic lymph node; RIP, rat insulin promotor; SOCS-1, suppressor of cytokine signaling-1; TCR, T-cell receptor.

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β2m/NODβ2m^{null} (15), and NOD mice expressing a dominant-negative (dn) Fas-associated death domain (FADD) molecule in β-cells (RIP-dnFADD [16]) have been described. Animal studies were carried out at St. Vincent's Institute (Fitzroy, Victoria, Australia) in accordance with accepted standards of humane animal care and were approved by the institutional animal ethics committee.

Isolation of islets. Islets of Langerhans were isolated as described previously (17). Islets were handpicked and cultured at 37°C in 5% CO_2 in CMRL medium-1066 (Life Technologies, Gaithersburg, MD), containing antibiotics, 2 mmol/l glutamine, and 10% FCS.

Generation of spleen-derived CD8$^+$ T-cell lines. Spleen cells from NOD8.3 TCR transgenic mice were stimulated with irradiated NOD splenocytes pulsed with IGRP$_{206–214}$ (VYLKTNVFL) or the peptide mimic NRP (KYNKANWFL). Splenocytes were cultured for 2 days in RPMI-1640 (Life Technologies) supplemented with antibiotics, 2 mmol/l glutamine, nonessential amino acids, 50 μmol/l mercaptoethanol, and 10% FCS (complete RPMI-1640) before addition of 10 units/ml of recombinant human interleukin-2. Activated cells were further expanded for 6 days.

^{51}Cr release assay. Whole islets or cells of the H-2^d mastocytoma line P815 were loaded with 150 μCi of [^{51}Cr]sodium chromate (Amersham Pharmacia Biotech, Piscataway, NJ) for 90 min and resuspended in complete RPMI-1640. P815 cells were incubated with IGRP$_{206–214}$ or an irrelevant K^d binding peptide (LLO$_{91–99}$ or TUM). P815 cells (5×10^3/well) or islets (10/well, uniform shape and size) were incubated with T-cells in triplicate for 4–16 h at 37°C in 96-well plates. Medium alone or 2% Triton X-100 was added to a set of targets for determination of spontaneous and total cell lysis, respectively. The radioactivity of harvested supernatant was measured in a Cobra gamma counter (Perkin-Elmer). Specific ^{51}Cr release was calculated: percent lysis = (test counts per minute − spontaneous counts per minute)/(total counts per minute − spontaneous counts per minute) × 100. For perforin inhibition, T-cells were preincubated with 100 ng/ml concanamycin A (Sigma Chemical, St. Louis, MO) for 90 min. For blocking Fas ligand (FasL)-mediated killing, 10 μg/ml of the anti-FasL antibody Kay10 (Pharmingen, San Diego, CA) was added to wells before effectors.

Islet grafts, histology, and immunohistochemistry. Recipient mice were grafted with 500 islets under the kidney capsule at 5–7 weeks of age. Mice were monitored for the development of diabetes by measurement of urinary glucose levels using Diastix (Bayer Diagnostics, Bridgend, U.K.). Suspected hyperglycemia was confirmed by measurement of blood glucose levels using Advantage II Glucose Strips (Roche). Mice with blood glucose >18 mmol/l or with two readings of 13–18 mmol/l on consecutive days were considered diabetic. Histological analysis of paraffin-embedded tissues was performed as described previously (18). Grafts were histologically scored as the following: 0 = no infiltrate; 1 = peri-islet infiltrate; 2 = intraislet infiltrate, <50% destruction; 3 = intraislet infiltrate, >50% destruction; 4 = total β-cell loss, glucagon staining without insulin. For staining of CD4$^+$ and CD8$^+$ T-cells, tissues were frozen in OCT (Sakura Finetechnical, Tokyo, Japan). Sections were stained with biotinylated anti-CD4 or anti-CD8 (Pharmingen) using the avidin-biotin system for immunoperoxidase staining (Vector Laboratories, Burlingame, CA). Staining was developed with diaminobenzidine (Sigma), and sections were counterstained with hematoxylin.

Adoptive T-cell transfer. Islets isolated from NOD8.3 or RIP-SOCS-1.NOD8.3 mice were dispersed into single cells using 0.2% trypsin (Calbiochem, San Diego, CA)/10 mmol/l EDTA in Hanks' balanced salt solution. Lymphocytes were purified using fluorescence-activated cell sorting (FACSAria; Becton Dickinson, Franklin Lakes, NJ) and injected into the tail veins of RIP-B7-NOD.*scid* mice (3×10^5 cells/mouse). Recipients were monitored for development of diabetes.

Caspase-3 assay. Islets were dispersed and cultured with recombinant perforin and granzyme B (a gift from Phil Bird, Monash University, Melbourne, Victoria, Australia) for 2 h in Hanks' balanced salt solution containing 1 mmol/l $CaCl_2$, 0.4% BSA, and 25 mmol/l HEPES. Caspase-3 activity was measured as described previously (19).

Granzyme B real-time RT-PCR. In vitro activated 8.3 CTLs were expanded for 7 days and then incubated with NOD or SOCS-1 islets for 16 h (1×10^6 T-cells, 100 islets/well). Total RNA was isolated using TRIzol reagent (Life Technologies). RNA was also isolated from naïve splenocytes to determine basal levels of granzyme B. Real time RT-PCR analysis was performed with primer and probe Assay-on-demand sets (Applied Biosystems) for granzyme B and β-actin as a housekeeping reference gene. Analysis was performed on a Rotor-Gene RG-3000 cycler (Corbett Research, Sydney, Australia). Results represent means ± SE of duplicates from three independent samples.

Statistical analysis. Analyses of data were performed using GraphPad Prism (GraphPad Prism Software, San Diego, CA). Data are represented as means ± SE. Cytotoxicity assays were analyzed by paired *t* tests and caspase activity by one-way ANOVA. Survival curves were analyzed using the log-rank test.

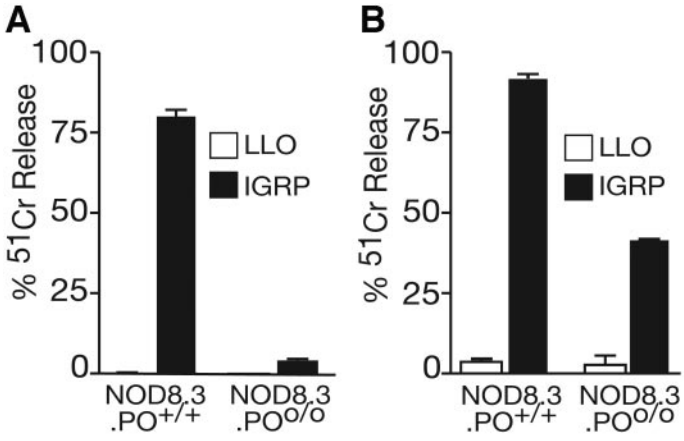

FIG. 1. 8.3 CTLs kill cell line targets through both perforin/granzyme and Fas/FasL pathways in vitro. Activated CTLs from NOD8.3.PO$^{+/+}$ and NOD8.3.PO$^{o/o}$ mice were used to kill P815 target cells pulsed with LLO$_{91–99}$ (irrelevant control) or IGRP$_{206–214}$ peptide at an effector-to-target ratio of 20:1 over 4 h (*A*) or 16 h (*B*). Data are means ± SE representative of at least three experiments.

RESULTS

IGRP-specific T-cells from NOD8.3 mice kill using perforin and FasL. We first examined the ability of activated 8.3 T-cells from perforin-deficient NOD8.3.PO$^{o/o}$ mice and NOD8.3.PO$^{+/+}$ littermate controls to lyse both non-β and β-cell targets in vitro. CTLs from NOD8.3.PO$^{+/+}$ mice killed IGRP peptide-pulsed P815 cells in a 4-h chromium release assay (Fig. 1*A*). Perforin-deficient NOD8.3.PO$^{o/o}$ T-cells were unable to kill peptide-pulsed targets in this short assay period, suggesting that cytotoxicity was largely perforin mediated. Pretreatment of CTLs from NOD8.3.PO$^{+/+}$ mice with the perforin inhibitor concanamycin A gave results concordant with using NOD8.3.PO$^{o/o}$ effectors (not shown). NOD8.3.PO$^{o/o}$ CTLs specifically killed peptide-pulsed targets over 16 h (Fig. 1*B*). As P815 cells express low levels of Fas, engagement by FasL on activated T-cells is likely to account for the killing observed in the absence of perforin as previously reported for NOD8.3.PO$^{o/o}$ T-cells (20).

To test whether the effector mechanism used to kill a cell line target differs from that used to kill a β-cell, CTLs from NOD8.3.PO$^{+/+}$ and NOD8.3.PO$^{o/o}$ mice were tested for cytotoxicity against whole islets isolated from NOD and Fas-deficient NOD*lpr* mice (Fig. 2*A*). T-cells from NOD8.3.PO$^{+/+}$ mice killed NOD islets in a perforin-dependent manner as shown by a reduction in killing after concanamycin A treatment (specific cytotoxicity 36.37 ± 2.77 vs. 8.93 ± 2.47% [mean ± SE], $P < 0.0001$). Fas was not required for cytotoxicity in vitro as NOD8.3.PO$^{+/+}$ T-cells also killed NOD*lpr* islets. Although inclusion of an anti-FasL antibody did not protect NOD islet targets (Fig. 2*B*), the residual killing observed using concanamycin A–treated NOD8.3.PO$^{+/+}$ CTLs could be further reduced by blocking Fas-FasL interactions. This result suggests that NOD8.3.PO$^{+/+}$ CTLs efficiently kill β-cells in vitro in a perforin-dependent manner but are also able to utilize FasL. Perforin-deficient NOD8.3.PO$^{o/o}$ T-cells could not kill NOD*lpr* islets (Fig. 2*A*) ($P < 0.005$ compared with wild-type islet targets) but killed wild-type targets irrespective of concanamycin A treatment. Thus, although 8.3 T-cells kill β-cell targets in vitro predominantly through the use of perforin, in the absence of perforin the effect of the Fas/FasL pathway utilized by 8.3 T-cells becomes more apparent.

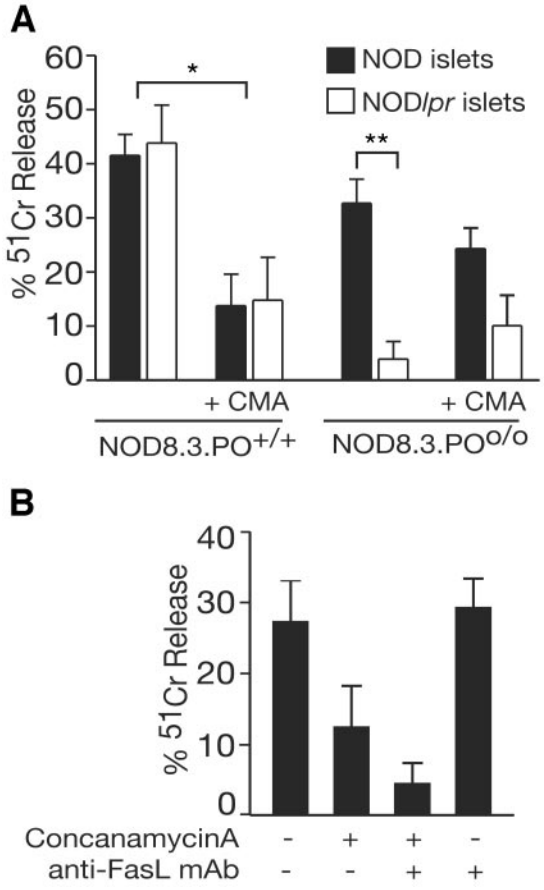

FIG. 2. 8.3 CTLs kill β-cells through both perforin/granzyme and Fas/FasL pathways in vitro. *A*: Activated CTLs from NOD8.3.PO$^{+/+}$ and NOD8.3.PO$^{o/o}$ mice were tested in cytotoxicity assays against whole islets from NOD or NOD*lpr* mice over 16 h. Each islet contains ~1,000 cells, such that the final effector-to-target ratio is 20:1. Data shown are means ± SE representative of at least three experiments. *P < 0.0001; **P < 0.005. *B*: Concanamycin A (CMA) or anti-FasL monoclonal antibody (mAb) was included to inhibit perforin or FasL-mediated lysis, respectively, by NOD8.3PO$^{+/+}$ CTLs.

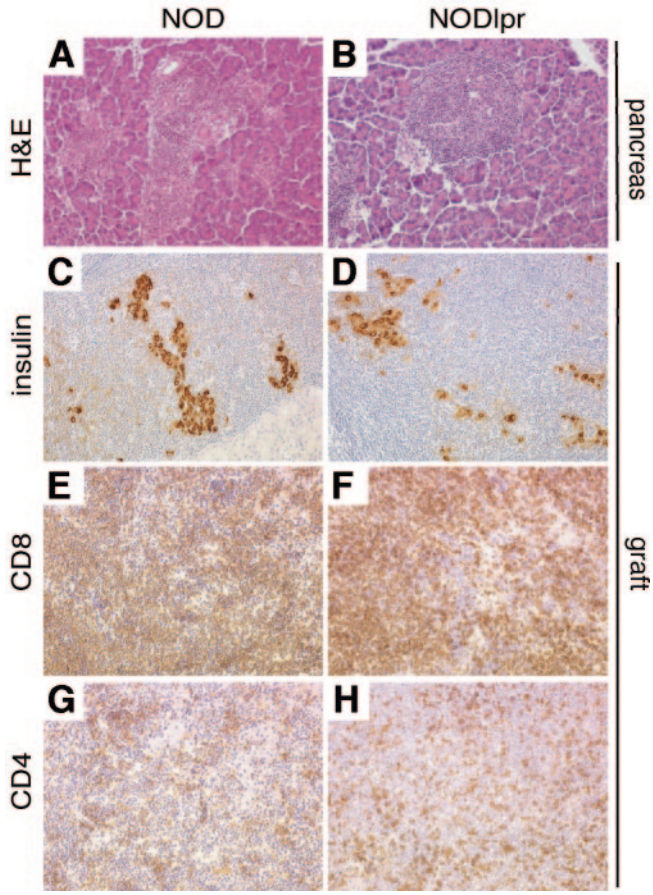

FIG. 3. Fas deficiency does not protect islet grafts from destruction in NOD8.3 mice. Islets isolated from NOD or NOD*lpr* mice were grafted under the kidney capsule of NOD8.3 recipients. Pancreas and grafts were harvested and sections were stained with hematoxylin and eosin (H&E) (*A* and *B*), insulin (*C* and *D*), anti-CD8 (*E* and *F*), or anti-CD4 (*G* and *H*). Representative sections from diabetic recipients of wild type or NOD*lpr* are shown. Original magnification ×200.

β-Cells are destroyed in the absence of Fas in NOD8.3 mice. To extend the observation that perforin-sufficient 8.3 T-cells are not dependent on FasL for cytotoxicity to an in vivo model, islets from wild-type or NOD*lpr* donors were grafted under the kidney capsule of NOD8.3 mice. Deficiency of Fas did not protect islet grafts from autoimmune attack as recipients of either wild-type or NOD*lpr* islets became diabetic (six of seven or five of six, respectively). Histological examination revealed infiltration and destruction of insulin-producing β-cells in the pancreas (Fig. 3*A* and *B*) and the graft (Fig. 3*C* and *D*) from both groups of diabetic mice (graft score for NOD and NOD*lpr* grafts 2.61 ± 0.5 [n = 4] and 3.09 ± 0.6 [n = 5], respectively). Wild-type and NOD*lpr* grafts contained large numbers of infiltrating CD8$^+$ T-cells (Fig. 3*E* and *F*) and a smaller proportion of CD4$^+$ T-cells (Fig. 3*G* and *H*). Graft rejection was MHC class I-dependent as islets from β2m-null mice grafted into NOD8.3 recipients remained intact (not shown). The destruction of Fas-deficient islets in NOD8.3 mice in vivo provides further evidence that 8.3 T-cells do not require Fas/FasL interactions to mediate β-cell death.

Spontaneous diabetes develops in NOD8.3 mice expressing a β-cell specific dnFADD protein. Models in which Fas-mediated killing of β-cells is prevented suggest a modest role for Fas/FasL interactions in NOD mice. For example, the expression of a dnFADD adaptor in β-cells of NOD mice blocks Fas receptor signaling and reduces the incidence of diabetes in homozygous NOD RIP-dnFADD mice from 70 to 45% (16). If 8.3 T-cells killed primarily by FasL, the dnFADD molecule would be expected to protect in this model. We mated NOD RIP-dnFADD mice to NOD8.3 mice and followed the littermates for diabetes incidence. Unlike non-TCR transgenic NOD mice, dnFADD did not provide protection in RIP-dnFADD.NOD8.3 mice, and in fact a slight increase in diabetes incidence was observed (Fig. 4). Infiltration scores were similar for NOD8.3 and RIP-dnFADD.NOD8.3 mice (not shown). The ability of dnFADD to afford some protection in NOD and not NOD8.3 mice may reflect the dominance of a single T-cell specificity and the rapid onset of disease in NOD8.3 mice. Nevertheless, the data support the ability of 8.3 T-cells to mediate disease in the absence of Fas/FasL interactions.

SOCS-1 protects β-cells from CD8-mediated destruction in vivo. We have previously reported that overexpression of SOCS-1 in β-cells of NOD8.3 mice prevents diabetes without affecting pancreatic infiltration (11). T-cell activation in these mice appears normal on the basis of the cell surface phenotype and infiltrating lymphocytes isolated from the islets of NOD8.3 or RIP-SOCS-1.NOD8.3

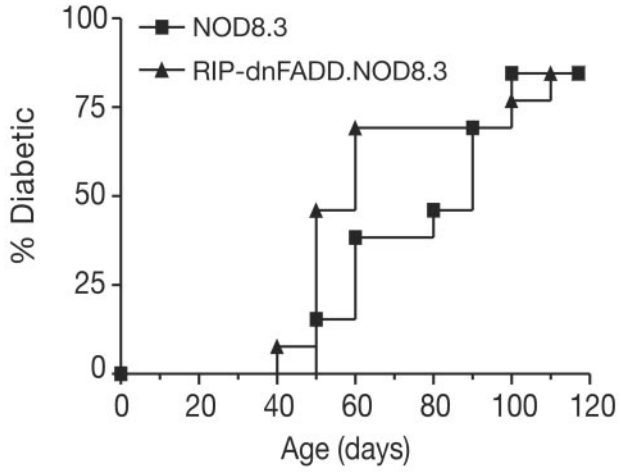

FIG. 4. Incidence of spontaneous diabetes in NOD.8.3 TCR transgenic mice expressing dnFADD in β-cells. NOD.8.3 mice were mated to NOD.RIP-dnFADD mice and analyzed for diabetes incidence. The diabetes incidence curves include both male and female mice. RIP-dnFADD.NOD8.3 (n = 13); NOD.8.3 (n = 12).

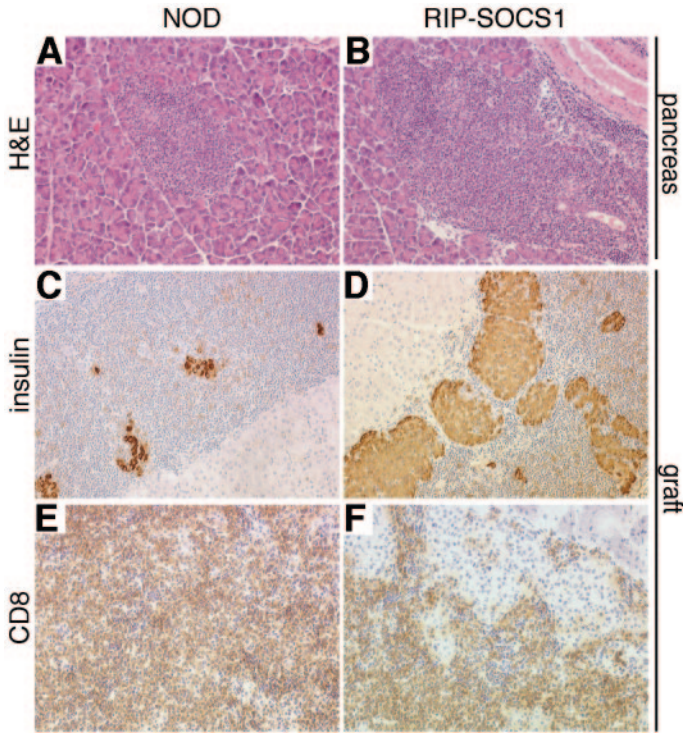

FIG. 5. Islet grafts overexpressing SOCS-1 in β-cells are protected in NOD8.3 recipients. Islets isolated from NOD or RIP-SOCS-1 mice were grafted under the kidney capsule of NOD8.3 recipients. Pancreas and grafts were harvested and sections stained with hematoxylin and eosin (H&E) (*A* and *B*), insulin (*C* and *D*), or anti-CD8 (*E* and *F*). Representative sections from recipients of wild-type or RIP-SOCS-1 islets are shown. Magnification ×200.

mice transfer disease equivalently in RIP-B7-NOD.*scid* recipients (Table 1). To confirm that protection in RIP-SOCS-1.NOD8.3 was not a result of compromised T-cell development and priming, islets from RIP-SOCS-1 mice were grafted into wild-type NOD8.3 recipients. Recipients of wild-type islets showed extensive β-cell destruction in the pancreas (Fig. 5*A*) and graft (Fig. 5*C*, graft score 2.61 ± 0.5 [n = 4]) accompanied by $CD8^+$ T-cell infiltration (Fig. 5*E*). The pancreata of NOD8.3 mice receiving RIP-SOCS-1 islets were also destroyed (Fig. 5*B*). RIP-SOCS-1 grafts, however, were well preserved as indicated by extensive insulin staining and intact islet structure (Fig. 5*D*) (graft score 1.3 ± 0.7 [n = 4], $P < 0.05$ compared with wild-type grafts) despite heavy infiltration of $CD8^+$ T-cells (Fig. 5*F*). After removal from the recipient, T-cells expanded out of RIP-SOCS-1 grafts were able to kill NOD islets in vitro (not shown). This finding suggests that in the presence of normal T-cell activation and expansion, SOCS-1 is able to block the effector mechanisms utilized by 8.3 T-cells.

SOCS-1 protects β-cells from CD8-mediated destruction in vitro. Fluorescence-activated cell sorter analysis of β-cells from NOD8.3 mice has shown that both MHC class I and Fas levels are markedly increased in this accelerated model (21). SOCS-1 is a negative regulator of the Janus-family tyrosine kinase (Jak)-signal transducer and activator of transcription (STAT) cytokine signaling pathway and inhibits the action of multiple cytokines including IFN-γ (reviewed in ref. 22). Overexpression of SOCS-1 in β-cells inhibits the effects of IFN-γ signaling such that Fas and MHC class I expression on β-cells are detectable only at basal levels in RIP-SOCS-1.NOD8.3 mice (11). We have previously proposed that the inability to induce Fas expression on RIP-SOCS-1 β-cells is a potential mechanism for protection in RIP-SOCS-1.NOD8.3 mice; however, we now find that 8.3 T-cells can kill in the absence of Fas/FasL interactions. To further examine how SOCS-1 overexpression protects β-cells from CTL-mediated destruction, we assessed the ability of 8.3 CTLs to kill RIP-SOCS-1 islets in vitro. Activated CTLs from NOD8.3 mice were consistently less efficient at killing islets isolated from RIP-SOCS-1 mice compared with wild-type islets over 16 h (Fig. 6*A*). Specific lysis of RIP-SOCS-1 islets was greatly reduced compared with wild-type targets especially when a lower effector-to-target ratio of 10:1 was used (Fig. 6*C*). As expected, the absence of Fas did not affect cytotoxicity because NOD*lpr* islets were killed by 8.3 CTLs. CTLs from NOD8.3.$PO^{o/o}$ mice were unable to kill RIP-SOCS-1 islets (Fig. 6*B*).

To eliminate the possibility that SOCS-1 islets were inherently resistant to the action of perforin, we assessed caspase-3 activity after exposure of islets to a sublytic dose of recombinant perforin together with granzyme B. Caspase-3 activity was equivalent in islet-cells from RIP-SOCS-1 and NOD mice following treatment with perforin and granzyme B (Fig. 6*D*). This suggests that caspase-dependent apoptosis induced by the action of perforin in conjunction with granzyme B remains unaffected by the overexpression of SOCS-1.

Decreased antigen recognition contributes to SOCS-1 mediated protection. We examined the expression of granzyme B mRNA as a surrogate marker for TCR engagement relevant to cytotoxic function. In vitro activated 8.3 CTLs were expanded for 7 days and then cultured with intact NOD or RIP-SOCS-1 islets for 16 h. At

TABLE 1
Diabetogenicity of infiltrating lymphocytes isolated from NOD8.3 mice is unaffected by β-cell–specific expression of SOCS-1

Donor	Diabetic	Age at onset (days)*
NOD8.3	2/2	24, 40
RIP-SOCS-1.NOD8.3	4/4	24, 24, 26, 26

Islet infiltrating lymphocytes were purified and injected into 12-week-old RIP-B7.NOD.*scid* mice (3×10^5 cells/mouse). *Days after transfer.

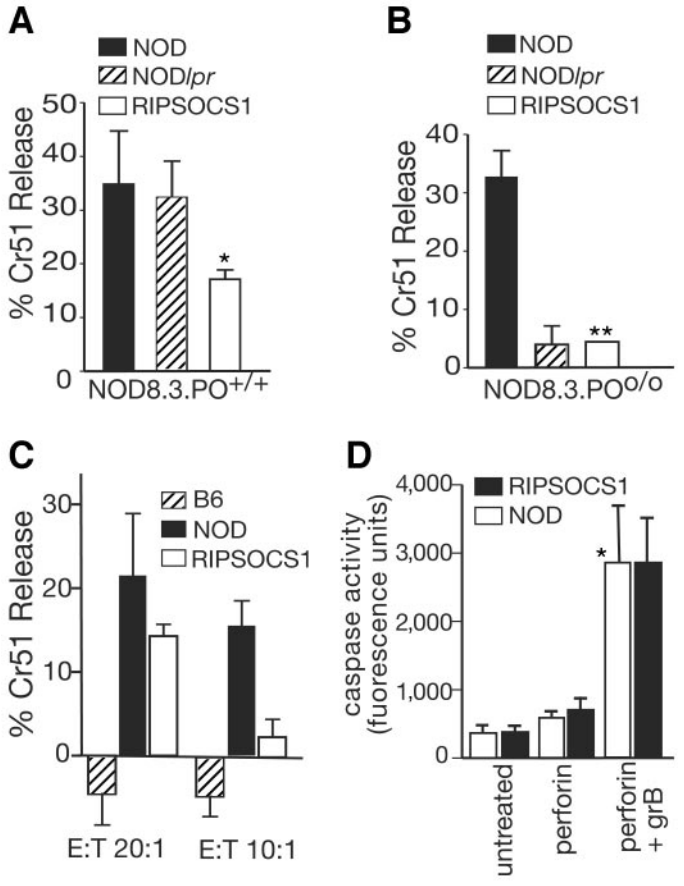

FIG. 6. SOCS-1 protects β-cells from CTL-mediated lysis in vitro. *A* and *B*: Activated CTLs from NOD8.3PO$^{+/+}$ or NOD8.3PO$^{o/o}$ mice were used at a 20:1 effector-to-target (E:T) ratio to kill NOD, NOD*lpr*, or RIP-SOCS-1 islets over 16 h. **P* < 0.0002, ***P* < 0.0001. *C*: Cytotoxicity of CTLs from NOD8.3PO$^{+/+}$ mice was tested at a lower E:T ratio of 10:1 with the inclusion of C57BL/6 islets as a specificity control. Data are means ± SE representative of three experiments. *D*: SOCS-1 does not protect islets against recombinant perforin and granzyme B. Islet cells from NOD or RIP-SOCS-1 mice were incubated with recombinant perforin (188 ng/ml) and granzyme B (grB) (8 μg/ml) for 2 h followed by measurement of caspase-3 activity. **P* < 0.01, one-way ANOVA.

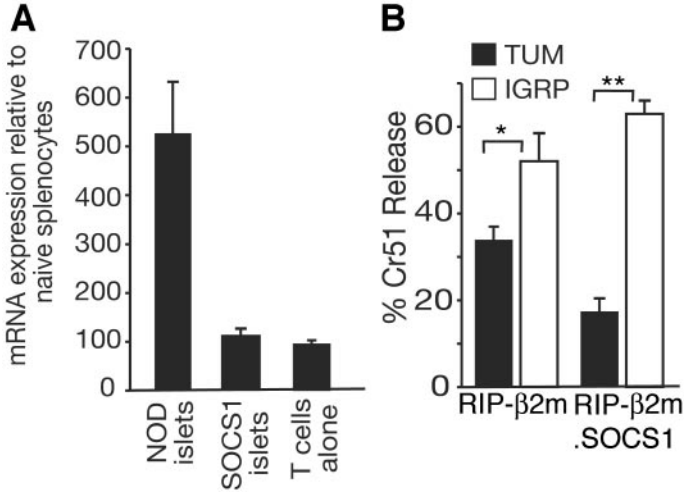

FIG. 7. SOCS-1 mediates protection through decreased antigen recognition. *A*: RNA was isolated from naïve NOD8.3 splenocytes (day 0) and in vitro activated NOD8.3 CTLs (day 7) after exposure to NOD or SOCS-1 islets for 16 h (10:1 effector-to-target ratio). Real-time RT-PCR analysis was performed for granzyme B using β-actin as a housekeeping reference gene. Results are expressed relative to naïve splenocytes and represent duplicates from three independent samples. *B*: Increased ligand density renders SOCS-1 islets susceptible to CTL-mediated lysis in vitro. Activated CTLs from NOD8.3 mice were used to kill RIP-β2m or RIP-β2m.RIP-SOCS-1 islets (10:1 effector-to-target ratio) exogenously loaded with IGRP or control TUM peptide (1 μmol/l). The mean ± SE of two experiments is shown. **P* < 0.04; ***P* < 0.01.

the end of the culture, granzyme B mRNA levels were determined and expressed relative to naïve splenocytes (Fig. 7*A*). A fivefold increase in granzyme B mRNA was measured in 8.3 CTLs after exposure to wild-type islets compared with T-cells alone. Granzyme B mRNA was not upregulated in 8.3 CTLs cultured with RIP-SOCS-1 islets, suggesting that overexpression of SOCS-1 in islets interferes with T-cell recognition and upregulation of cytotoxic mechanisms.

We next investigated whether reduced antigen presentation was responsible for the protective effect of SOCS-1, as we have found that overexpression of SOCS-1 in a β-cell line (NIT-1) prevents the IFN-γ–induced upregulation of proteins involved in antigen processing and presentation such as tapasin and the proteasomal subunit LMP-2 (not shown). To examine the effect of increased ligand density, cytotoxicity was assessed after addition of exogenous IGRP peptide. As islets contain a number of class I positive non–β-cells, the addition of exogenous peptide may render non–β-cells susceptible to killing by IGRP-specific CTLs. We therefore made use of islets isolated from RIP-β2m/NODβ2m^{null} (RIP-β2m) mice. These mice, maintained on a β2m-null background, have high levels of expression of a β2m transgene under the control of the rat insulin promoter. Consequently, RIP-β2m mice express class I on β-cells only. Class I MHC can still be upregulated in islets expressing the RIP-β2m transgene through upregulation of the class I heavy chain. As expected, islets from double transgenic RIP-β2m.RIP-SOCS-1 mice were more resistant to 8.3-mediated cytotoxicity than islets from RIP-β2m mice (Fig. 7*B*). To increase antigen presentation by RIP-β2m.RIP-SOCS-1 islets, exogenous IGRP peptide was added. The addition of IGRP peptide not only increased the specific lysis of islets from RIP-β2m mice, but also rendered RIP-β2m.RIP-SOCS-1 islets highly susceptible to CTL-mediated killing.

DISCUSSION

We have previously shown that overexpression of SOCS-1 in the islets of NOD8.3 mice prevents the development of diabetes. We have now examined the killing mechanisms utilized by 8.3 T-cells to better understand the basis of this protection. We have demonstrated in vitro and in vivo that 8.3 T-cells are able to kill β-cells using either perforin or FasL. Activated CTLs from NOD8.3 mice efficiently kill Fas-deficient islets in vitro, and NOD*lpr* islets are destroyed when grafted into NOD8.3 mice. Furthermore, NOD8.3 mice expressing a dnFADD adaptor to block Fas receptor signaling in β-cells still develop diabetes. Although NOD8.3 T-cells are not dependent on Fas-mediated lysis, they are still able to kill via engagement of Fas on target cells in vitro. In the absence of perforin, NOD8.3PO$^{o/o}$ CTLs retain their cytotoxicity by utilizing the Fas/FasL pathway.

Our data are at odds with previously published studies suggesting that CTL from NOD8.3 mice kill by Fas and not perforin (20,23). Much of the data showing that NOD8.3 T-cells kill via Fas has come from in vitro studies using calcium chelation with EGTA as a test of perforin dependence. We believe our data with genetic deficiency of

perforin and Fas in addition to chemical inhibition (with concanamycin A) are more robust. Additionally, our studies have mainly been carried out using primary islets as targets rather than cell lines, and we have obtained complementary in vivo data. We agree with the previous report that perforin deficiency does not prevent NOD8.3 mice from developing diabetes but have also found that Fas deficiency or Fas-signaling defects do not prevent diabetes. We interpret these results as showing that both mechanisms are in operation and can fully compensate for each other when one is removed in NOD8.3 mice.

The impact of Fas deficiency on β-cell destruction in models of diabetes has been studied in an increasingly specific way. After initial studies in *lpr* mice were questioned because of features including widespread upregulation of FasL and lymphocyte homeostasis, abnormalities unconnected with β-cell destruction, studies with Fas deficiency or Fas-signaling deficiency only on the β-cell have been devised. These have included transplantation of Fas-deficient β-cells into NOD mice (24,25), transgenic overexpression of dominant-negative Fas (26) or FADD (16) in β-cells, and β-cell–specific genetic deletion of Fas (27) and have shown at most a minor impact of β-cell Fas deficiency on spontaneous diabetes in NOD mice. Our current data increase the consistency of the emerging picture by showing, as in other models of autoimmune diabetes including $CD8^+$ (28) and $CD4^+$ T-cell–dependent TCR transgenic models (25,27; our unpublished data), that Fas is not exclusively utilized by pathogenic T-cells in NOD8.3 mice.

Our current studies suggest that overexpression of SOCS-1 in β-cells protects islets from both perforin- and Fas-mediated death in the NOD8.3 model. Protection from diabetes in RIP-SOCS-1.NOD8.3 mice may be mediated by SOCS-1 blocking the impact of T-cell effector mechanisms on the β-cell or by reducing the activation of autoreactive T-cells and reducing the expression or release of effector molecules. In the absence of β-cell death, cross-presentation in the pancreatic lymph nodes (PLNs) of NOD8.3 mice still occurs (29), although the magnitude of T-cell priming in the PLN increases after streptozotocin-induced β-cell damage (30). Consistent with this observation, insulitis and expression of activation markers in the PLNs of RIP-SOCS-1.NOD8.3 mice did not differ from those in NOD8.3 littermates. Islet-infiltrating lymphocytes from RIP-SOCS-1.NOD8.3 mice were able to mediate β-cell damage with no differences noted in the efficiency of disease transfer using infiltrate isolated from double or single transgenic NOD8.3 mice grafted into mice without β-cell SOCS-1 expression. Conversely, SOCS-1 islets grafted under the kidney capsule of NOD8.3 mice were heavily infiltrated but retained insulin staining. These data indicate that in the presence of normal T-cell activation, SOCS-1 provides protection, but they do not clarify whether this is due to reduced target recognition or protection from effector molecules.

SOCS-1 is a negative regulator of multiple cytokines including IFN-γ. Endogenous levels of SOCS-1 in β-cells are insufficient to terminate IFN-γ signaling (31). However overexpression of SOCS-1 by transfection or transgenesis prevents IFN-γ–induced upregulation of multiple genes including Fas and MHC class I. This is evident in RIP-SOCS-1.NOD8.3 mice, which express basal levels of class I and undetectable levels of Fas. We have previously hypothesized that decreased Fas expression on β-cells from RIP-SOCS-1 mice provides protection from NOD8.3 CTLs. However, it is now clear that 8.3 T-cells remain cytotoxic in the absence of Fas/FasL signaling, and Fas-deficiency cannot fully explain the protection of RIP-SOCS-1 β-cells from NOD8.3 T-cells. We believe our new data indicate that reduced antigen presentation is likely to be significant. Reduced antigen density when SOCS-1 is overexpressed in β-cells and MHC class I protein expression fixed at basal levels resulted in reduced in vitro transcription of effector molecules such as granzyme B in NOD8.3 T-cells. Activated 8.3 CTLs required a higher effector-to-target ratio to kill RIP-SOCS-1 islets in vitro and pretreatment of nontransgenic islets with IFN-γ increased the amount of killing of NOD (or NOD*lpr*) islets (not shown). These data are consistent with the marked sensitivity of 8.3 T-cells to antigen density. Increasing the ligand density specifically on β-cells through addition of exogenous peptide rendered SOCS-1 islets highly susceptible to 8.3 CTL-mediated cytotoxicity. This finding indicates that endogenous IGRP presentation in SOCS-1 islets is limiting and that cytokine-induced upregulation of antigen presentation is critical for 8.3 cytotoxicity. Even when CTL activation in the draining node has been normal, restimulation during the interaction with the target cell appears to be important for normal cytolysis.

The phenotype of RIP-SOCS1.NOD8.3 mice is also highly reminiscent of that of mice with reduced antigen presentation by β-cells. These include "β-bald" mice, in which β_2-microglobulin has been deleted by conditional gene targeting from the NOD β-cells (32), and RIP-E19 NOD mice, which are unable to transport class I MHC heavy chains to the cell surface (29). In all three models, class I MHC expression on β-cells is reduced, insulitis is not prevented in NOD mice, but diabetes is substantially (but not entirely) diminished. Overexpression of E19 or SOCS-1 completely prevents diabetes in NOD8.3 mice. This phenotype may be less marked in NOD than in NOD8.3 mice because of the dominance of a single $CD8^+$ T-cell specificity in the latter model as well as the role for $CD4^+$ T-cells in the former. Failure to develop diabetes despite islet infiltration has also been demonstrated in the RIP–lymphocytic choriomeningitis virus model, in which islet destruction can be initiated by Toll-like receptor triggering of IFN-α production and subsequent upregulation of MHC class I on β-cells (33). Our data are therefore consistent with the idea that upregulation of class I MHC on β-cells is a crucial step in triggering progression of diabetes mediated by $CD8^+$ T-cells.

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