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An Islet-Specific Pulse of TGF-β Abrogates CTL Function and Promotes β Cell Survival Independent of Foxp3+ T Cells

Maja Wållberg,* F. Susan Wong,† and E. Allison Green*,‡

Effective therapies that prevent chronic inflammation from developing into type 1 diabetes remain elusive. In this study, we show that expression of TGF-β for just 1 wk in inflamed islets of NOD mice significantly delays diabetes development. Time course studies demonstrated that the brief TGF-β pulse protects only if administered when extensive β cell destruction has occurred. Surprisingly, TGF-β-mediated protection is not linked to enhanced Foxp3+ regulatory T cell activity or to decreased intra-islet presentation of islet Ags. Instead, TGF-β disables the transition of primed autoreactive CD8+ T cells to cytotoxic effectors and decreases generation, or maintenance, of CD8+ memory T cells within the pancreas, significantly impairing their diabetogenic capacity. The Journal of Immunology, 2011, 186: 2543–2551.

Type 1 diabetes is an autoimmune disease resulting from immune-mediated destruction of the insulin-producing β cells in the islets of Langerhans of the pancreas. At the beginning of the last century, this disease was fatal, but the discovery of insulin (1) and subsequent improvements in the management of the disease have made it possible to live with type 1 diabetes. However, people living with the disease face many health problems, and as the incidence is rising by 5% per year in the developed world (2), it is important to discover how to delay disease progression to develop improved treatments.

Progression to diabetes is characterized by distinct phases, as follows: an initial priming phase in which T cells specific for islet Ags are activated in the pancreatic lymph node (PLN); a second regulatory phase in which β cell-specific T cell responses are suppressed within the islet, e.g., by T regulatory cell (Treg)–mediated mechanisms; and a third aggressive phase in which T cells are released from suppression and differentiate into effector and memory cell populations that ultimately destroy β cells (3). Studies in the NOD mouse (4), a mouse model for human diabetes, have identified several therapeutic strategies for preventing diabetes development if implemented at the priming phase of the disease. In contrast, intervention at later stages is less likely to succeed if inflammation is already established and β cell destruction has begun. As patients with type 1 diabetes are already in the aggressor phase at time of diagnosis, the ideal therapy would disable the autoaggressive phase and enable β cell regeneration to increase the β cell mass.

The immunosuppressive cytokine TGF-β controls proliferation and differentiation in many different cell types (5), and ablation of the cytokine or its signaling receptors results in multiorgan autoimmune inflammation and subsequently death a few weeks after birth (6–9). TGF-β has pleiotropic effects on most immune cells (reviewed in Ref. 5), and in part, TGF-β contributes to maintaining peripheral tolerance of the immune system by promoting the survival of naturally occurring CD4+Foxp3+ Tregs (6, 8), and inducing the differentiation of induced CD4+Foxp3+ Tregs (10–13). The potency of TGF-β to control autoaggressive immune cells in type 1 diabetes is highlighted by the finding that constitutive islet-specific expression of TGF-β from birth in NOD mice (14, 15) prevents diabetes development either through alteration of APCs (14) or induction of apoptosis in infiltrating lymphocytes (15). However, such constitutive expression of TGF-β results in massive fibrosis of pancreatic tissue due to the presence of TGF-β in the neonatal period and is detrimental to health. Furthermore, prolonged exposure of the immune system to TGF-β has been linked to cancer (16). These negative aspects of TGF-β suggest caution should be exercised in developing TGF-β–based immunotherapies. Nevertheless, recent studies documenting that anti-CD3 Ab therapy prevents diabetes progression in NOD mice and reverses diabetes in humans via an undefined TGF-β–mediated mechanism have reignited interest in the effects of TGF-β in therapies for type 1 diabetes (17). We recently developed a NOD mouse model in which the timing and duration of islet-specific TGF-β expression are controlled (18) by the tetracycline-regulated gene transcription system (16). We established that adult-specific expression of TGF-β from 5 wk of age, when islet infiltration with immune cells is initiated, but islets are yet to become chronically inflamed, protects NOD mice from diabetes, possibly through induction of CD25+ Treg. Although promising, these results were based on prolonged expression of TGF-β for several weeks in the islets of NOD mice. To identify a viable use of TGF-β, or any pathway activated by it, in controlling type 1 diabetes progression several key questions need to be answered, as follows: can brief, transient production of TGF-β control diabetes development in chronically inflamed islets; in which phase of the disease course is TGF-β most effective; and what are the mechanisms by which TGF-β disables the diabetogenic response?
Previously, we developed a NOD mouse model of chronic intraislet inflammation, the rat insulin promoter (RIP)–TNF NOD mouse (19). Such mice express the diabetes-relevant proinflammatory cytokine TNF constitutively in the islets under the control of the RIP on the NOD mouse genetic background. In RIP–TNF NOD mice, TNF is expressed from birth throughout the life of the mouse. Disease development is characterized by sequential infiltration of islets with dendritic cells (DCs), B cells, and T cells starting at 15 d of age. Between 4 and 6 wk of age, the islets of RIP–TNF NOD mice contain many Foxp3+ Tregs, and autoaggressive T cells are kept in check. However, between 8 and 10 wk of age, regulation of the autoimmune cells breaks down 10 wk of age, and disease progression is more rapid and synchronized (19). These mice have been invaluable in determining how chronic inflammation in the islets leads to β cell destruction (20). In this study, we use the RIP–TNF NOD mice to address the therapeutic potential of transient expression of TGF-β to control diabetes development in a chronically inflamed environment and provide unique insights into the mechanisms by which TGF-β modulates the diabetogenic response.

**Materials and Methods**

**Mice and diabetes detection**

RIP–TNF NOD (19), Tet–TGF NOD (18), Foxp3 KI–GFP NOD (21), G9C5 TCR transgenic NOD mice (22, 23), and NOD-SCID mice were bred and maintained under specific pathogen-free barrier conditions. In the Tet–TGF NOD mice, islet-specific TGF-β expression is controlled by the Tet on-off switch, as previously described (16). Briefly, tetracycline transactivator (tTA) is expressed in the islets using a RIP and the TGF-β gene is downstream of tetracycline-responsive elements (TRE). In the absence of doxycycline, tTA binds TRE and induces TGF-β expression from a minimal CMV promoter, whereas in the presence of doxycycline, tTA fails to bind TRE and gene transcription is silenced. The RIP–TNF NOD mice were bred to the Tet–TGF NOD to produce a double-transgenic mouse model (24). In the RIP–TNF NOD mice, TNF is expressed from birth throughout the life of the mouse. Disease development is characterized by sequential infiltration of islets with dendritic cells (DCs), B cells, and T cells starting at 15 d of age. Between 4 and 6 wk of age, the islets of RIP–TNF NOD mice contain many Foxp3+ Tregs, and autoaggressive T cells are kept in check. However, between 8 and 10 wk of age, regulation of the autoimmune cells breaks down and CD8+ T cells within the islets transform into aggressive CTL that cause β cell destruction, with overt diabetes occurring in 100% of the colony by 15 wk of age. Therefore, RIP–TNF NOD mice develop diabetes in a similar manner to NOD mice, but disease progression is more rapid and synchronized (19). These mice have been invaluable in determining how chronic inflammation in the islets leads to β cell destruction (20). In this study, we use the RIP–TNF NOD mice to address the therapeutic potential of transient expression of TGF-β to control diabetes development in a chronically inflamed environment and provide unique insights into the mechanisms by which TGF-β modulates the diabetogenic response.

**Abs, flow cytometry, and cell sorting**

Anti-granzyme B and mouse IgG1 Abs were purchased from Caltag Laboratories. Abs against CD4 (clone RM4-5) and CD8 (clone 53-6.7) were purchased from eBioscience, and rat anti-perforin Ab (clone CB5.4) from Alexis Biochemicals. Abs to detect intracellular Foxp3 (clone FJK-16s; eBioscience) were used according to the manufacturer’s instructions. All other Abs for flow cytometry were purchased from BD Biosciences. Flow cytometry was performed on a CyAn ADP (Dako), and data analyzed using FlowJo software (Tree Star). Cell sorting was carried out using a MoFlo cell sorter (Dako) using Summit software. For immunofluorescence, a rat anti-insulin Ab (R&D Systems) was used with an anti-rat Alexa 488 Ab as the secondary Ab (Molecular Probes).

**TGF-β sandwich ELISA**

TGF-β sandwich ELISA was performed using a purified anti–TGF-β Ab for capture and biotinylated anti–TGF-β for detection. The Abs were purchased from BD Biosciences and used according to the manufacturer’s instructions. Streptavidin-conjugated HRP and 3,3,5,5-tetramethylbenzidine (both from BD Biosciences) were used to detect binding.

**Isolation of β cells**

Pancreatic islets were isolated according to standard protocols (24). Briefly, the pancreas was infused with collagenase P (Sigma-Aldrich) solution via the bile duct and then extracted. Islets were handpicked following separation on a Histopaque (Sigma-Aldrich) gradient, and β cells were released by trypsin-EDTA (Sigma-Aldrich) treatment at 37°C for 10 min. β Cells were isolated using a modified protocol from Ppelpeers et al. (25). Briefly, cells were incubated with anti-CD45 Abs prior to sorting, and cells that were CD45 negative, with high forward scatter and high autofluorescence in the FL-1 channel, were isolated on a MoFlo cell sorter (Dako). To confirm that the isolated cells were β cells, mRNA levels for insulin were assessed by TaqMan PCR (Supplemental Fig. 1A), and cells were also placed on glass slides and stained for insulin (Supplemental Fig. 1B). The latter procedure established that the purity of the sorted β cells was >80%.

**Treg assay**

CD45+GFP+ (i.e., Foxp3+) cells or CD45+GFP+ (i.e., Foxp3-) mixed effector cells (comprising CD4+ and CD8+ T cells, B cells, etc.) from the pancreas, or pooled inguinal lymph nodes of Foxp3 KI–GFP NOD–TNF–TGF mice pulsed with TGF-β or nonpulsed Foxp3 KI–GFP NOD TNF littermates, were isolated by MoFlo cell sorting. CD45+GFP+ Foxp3+ mixed effector cells were labeled with 1 μM CFSE (Molecular Probes); resuspended in DMEM supplemented with 10% FCS, 50 μM 2-ME, 1-glutamine, sodium pyruvate, and 50 μM penicillin and streptomycin (Life Technologies); and seeded into the wells of a 96-well plate precoated with 5 μg/ml anti-CD3 Ab (clone 145-2C11; BD Biosciences) at a concentration of 106 cells/well. CD45+GFP+Foxp3+ Tregs were added to the ratios indicated in the figure legends, and after incubation for 72 h at 37°C, 5% CO2, the cells were stained with the indicated surface markers for analysis by flow cytometry. Intracellular staining of Foxp3 was performed to gate out the regulatory cells from the analysis, as GFP and CFSE are picked up in the same channel (FL-1) on the CyAn.

**Cell culture for detection of granzyme B, perforin, and intracellular cytokines**

Lymphocytes were extracted from the pancreas, as described previously (19), stained for CD45, and sorted on a MoFlo cell sorter (Dako) to achieve a pure lymphocyte population. U-bottom well plates were coated with 5 μg/ml anti-CD3 and 1 μg/ml anti-CD28. A total of 106 cells/well was resuspended in DMEM supplemented with 10% FCS, 50 μM 2-ME, 1-glutamine, sodium pyruvate, and 50 μM penicillin and streptomycin (Life Technologies). Following a 24-h stimulation at 37°C, with Golgistop and Golgiglup (both from BD Biosciences) added for the last 6 h of culture, the cells were fixed and intracellular molecules were detected by flow cytometry.

**Cytotoxicity assay**

Cytotoxicity of CD8+ T cells was examined with the CytoTox 96 Non-Radioactive Cytotoxicity Assay, according to the manufacturer’s instructions (Promega). For effector cells, CD8+ T cells specific for proinsulin

**FIGURE 1.** A 1-wk pulse of TGF-β impairs diabetes development in TNF mice. TNF–TGF mice and control TNF littermates were removed from doxycycline-supplemented food at 4 or 8 wk of age (termed 4w and 8w, respectively) and then placed back on doxycycline-supplemented diet 7 d later. This regimen resulted in islet-specific production of TGF-β for 1 wk, starting 2 d after removal of the animals from the doxycycline-supplemented diet. As an additional control, a group of TNF–TGF mice was kept on doxycycline-supplemented food continuously (termed TGF off). All groups of mice were assessed for diabetes development. The data are presented as a Kaplan–Meier survival curve, and statistical comparisons in disease development between TGF-β–expressing versus control TNF–TGF mice fed doxycycline-supplemented diet continuously were determined using the log-rank test. A p value for the TNF–TGF mice expressing TGF-β for 1 wk starting at 8 wk of age was <0.0001 compared with both the TNF control group and the TNF–TGF control groups with TGF-β permanently switched off, and was considered significant.
FIGURE 2. TGF-β does not manipulate CD4+ Tregs to control diabetes development in chronically inflamed pancreata. A, Flow cytometric analysis of CD4+ T cells isolated from the pancreas or PLN of TNF–TGF mice (n = 8) and control TNF littermates (n = 10) following expression of TGF-β for 1 wk starting at 8 wk of age. Left-hand side panels, Show the percentage of Foxp3+ within a CD45+CD3+CD4+ gate; right-hand side panels, show absolute numbers of CD4+Foxp3+ T cells. The data are representative of at least three independent experiments, and each dot represents one mouse examined. B and C, Tet–TGF and TNF NOD mice were crossed to Foxp3 KI–GFP NOD mice carrying a bicistronic GFP–Foxp3 reporter insert. GFP+Foxp3+ Tregs were isolated from the pancreas of Foxp3 KI–GFP TNF–TGF mice after expression of TGF-β for 1 wk starting at 8 wk of age, or Foxp3 KI–GFP TNF littermates. The GFP Foxp3+ Tregs were mixed with CFSE-labeled CD45+GFP+ effector cells, comprising CD4+ and CD8+ T cells as well as APCs, isolated from the inguinal lymph nodes of Foxp3 KI–GFP TNF mice at the ratios indicated in the figure. After 72 h of stimulation with plate-bound anti-CD3 Ab,
peptide were isolated from the spleen of G9CS TCR transgenic mice (23), and preactivated by culturing the cells for 3 d in the presence of 10 ng/ml B15–23 insulin peptide and 10 U/ml IL-2. For targets, either the cell line P815 pulsed with 1 μg/ml B15–23 insulin peptide or isolated β cells were used. A total of 105 target cells/ml was cultured with effector cells at the E/T ratio indicated in the figure legends. Following a 24-h incubation, supernatants were harvested and lactate dehydrogenase (LDH) release was measured. Specific lysis was calculated as ([LDH release − min]/[max − min]) × 100%, in which the minimal release (min) corresponded to the spontaneous release of LDH of both effectors and targets, and the maximal lysis corresponded to release after lysis induced by addition of Triton X-100 (max).

Immunoﬂuorescence

Pancreata were snap frozen in liquid nitrogen, and 6-μm sections were cut using the Leica CM1900 microtome (Leica) and placed onto polylysine-coated slides (Menzel Glazer). Sections were stained with the Abs indicated, and nuclei were visualized with DAPI (Molecular Probes). Following mounting with Vectashield (Vector Laboratories), the sections were viewed under the Axioskop 2 plus fluorescence microscope (Zeiss). Apoptosis was detected using a TUNEL staining kit (Roche), following the manufacturer’s instructions.

TagMan PCR

RNA was isolated from cells using a kit from Qagen, according to the manufacturer’s instructions, and cDNA was prepared (26). TaqMan PCR was performed using commercially available primer-probe sets labeled with FAM (Applied Biosystems) and analyzed on an ABI Prism (Applied Biosystems) using SDS software, according to the manufacturer’s instructions. A standard curve was generated by doing a serial 1:10 dilution of cDNA from activated CD8+ T cells, with the highest concentration given a value of 1, and used to translate the results into semiquantitative values. The amount of mRNA in each sample was calculated as the ratio between the target and the endogenous control, either CD8 or β2 microglobulin, as indicated.

Results

Transient expression of TGF-β in the islets abrogates diabetes development specifically during the aggressor phase

Expression of TGF-β in the islets of Tet–TGF NOD mice is tightly controlled, with no TGF-β detected as long as doxycycline is present in the diet (18). To establish the kinetics of TGF-β induction following removal of doxycycline from the diet, we isolated islets at different time points and measured TGF-β levels by ELISA. TGF-β protein was detected as early as 2 d postdoxycycline removal, with maximal levels recorded at day 7 (Supplemental Fig. 2). To determine the kinetics of TGF-β repression, Tet–TGF NOD mice were placed on doxycycline-supplemented diet, and TGF-β levels in isolated islets were measured as above. As shown in Supplemental Fig. 2, by 4 d postintroduction of doxycycline to the diet, no TGF-β was detectable.

To investigate the ability of transient TGF-β production in the islets to control diabetes progression under chronic inflammatory conditions, we crossed the RIP–TNF NOD mice (hereafter called TNF mice) to Tet–TGF NOD mice (18), creating double-transgenic TNF–TGF mice. We selected two major time points in the etiology of the disease in TNF mice, the postpriming regulatory phase (starting at 4 wk of age) and the early CTL-mediated β cell destruction phase (starting at 8 wk of age), to determine whether a 1-wk period of expression of TGF-β prevented diabetes development. Expression of TGF-β between 4 and 5 wk of age had no impact on disease progression as both TNF–TGF and control single-transgenic TNF littermates developed diabetes with similar kinetics and penetrance (Fig. 1). This was unexpected, as most successful therapeutic interventions in NOD mice act at the earliest regulatory phase of diabetes development (27). In contrast, expression of TGF-β between 8 and 9 wk of age resulted in significantly delayed kinetics of diabetes development ($p < 0.0001$). This ability to delay diabetes progression was specific to the transient TGF-β pulse, as control TNF–TGF mice that were continually fed doxycycline-supplemented food to prevent TGF-β expression rapidly developed diabetes by 12 wk of age, as expected.

$TGF-\beta$ does not enhance Foxp33 Treg function to prevent diabetes progression

Because expression of TGF-β between 8 and 9 wk of age significantly delayed diabetes progression, all future studies focused on this unique period to determine how TGF-β slows diabetes development. As extensive investigations have attributed a role for TGF-β in Foxp33 Treg induction (10, 13) and function (28, 29), we hypothesized that TGF-β delayed diabetes development at the early CTL-mediated β cell destruction phase through induction of Foxp33 Tregs. We investigated the presence of Foxp33 Tregs in the pancreata or draining PLN of mice that received the TGF-β pulse between 8 and 9 wk of age. In all cases, mice were examined at 10 wk of age, 1 wk after the reinstatement of doxycycline-supplemented diet when no TGF-β production was detectable from isolated islets (Supplemental Fig. 2). Flow cytometric analysis of TGF-β–pulsed TNF–TGF or nonpulsed TNF littermates revealed no changes in either the percentage of CD4+Foxp3+ T cells, their absolute numbers (Fig. 2A), or their expression of surface markers associated with their function (Supplemental Fig. 3), either in the pancreas or the PLN. To test whether TGF-β enhanced the suppressive function of Foxp33 Tregs in the pancreas, we crossed TNF–TGF mice to Foxp33 GATA-3 reporter NOD mice (21). GFP+Foxp33 Tregs isolated from the pancreas of TGF-β–pulsed TNF–TGF and nonpulsed TNF mice were equally capable of suppressing in vitro proliferation of anti-CD3-Ab-
stimulated CD4+ T cells (Fig. 2B, top and middle panels) and CD8+ T cells (Fig. 2C, top and middle panels). Interestingly, when GFP*Foxp3+ Tregs from the pooled inguinal lymph nodes were assessed for their ability to suppress proliferation of effector CD4+ and CD8+ T cells (Fig. 2B, lower panels), although no differences in the functionality of the GFP*Foxp3+ Tregs isolated from the two groups of mice were detected, the GFP*Foxp3+ Tregs isolated from the inflamed pancreas. This finding, in agreement with Tang et al. (30), emphasizes how an inflamed environment can dampen Treg function and TGF-β cannot rescue this effect. Nevertheless, in vivo adoptive transfer experiments demonstrated that GFP*Foxp3+ Tregs isolated from the pancreas of TNF-TGF mice, or TNF littermates, were both equally potent in preventing β cell destruction by autoaggressive immune cells isolated from the pancreas of TNF mice upon cotransfer into NOD–scid mice (Fig. 2D). Because CD4+ T cells are not necessary for diabetes progression in TNF mice (31), we performed total CD4+ T cell depletion studies to determine whether delayed disease in TNF–TGF mice was due to enhanced CD4+Foxp3+ Treg functionality (32). Depletion of CD4+ T cells had no effect on diabetes development in TNF–TGF mice or control TNF littermates in comparison with the isotype control Ab-treated groups (Fig. 2E). Thus, our results suggest that transient expression of TGF-β in inflamed islets controls autoimmunity via a CD4+ Treg-independent mechanism.
TGF-β does not increase sensitivity of intrapancreatic T cells to suppression

In light of our observations, we investigated potential Treg-independent mechanisms that could account for the TGF-β-mediated delay in diabetes development. We ruled out the possibility that TGF-β decreased expression of costimulatory molecules on DCs in the pancreas, a feature that could impair their Ag-presenting capabilities (33), as flow cytometric analysis revealed no differences in the cell surface expression of costimulatory molecules on DCs isolated from the TNF–TGF mice or TNF littermates (Supplemental Fig. 4). We also discounted the hypothesis that TGF-β attenuates (Supplemental Fig. 4). We also discounted the hypothesis that TGF-β impairs in situ presentation of islet Ag to T cells, as proliferation of CD4+ and CD8+ T cells, as measured by levels of Ki67 expression, was identical between TGF-β–pulsed TNF–TGF mice and nonpulsed TNF littermates (Supplemental Fig. 5). Finally, we demonstrated that TGF-β did not enhance the sensitivity of autoreactive T cells in the pancreas to suppression (34), as in vitro suppressor assays demonstrated that T cells from TGF-β–pulsed TNF–TGF mice and nonpulsed TNF littermates were equally responsive to suppression by Foxp3+ Tregs (Supplemental Fig. 6).

TGF-β decreases intrapancreatic CD8+ T cell transition to CTL and formation, or maintenance, of memory CD8+ T cells

CD8+ T cells are central to the destruction of β cells in TNF mice (31) and possibly in humans (35, 36). We, therefore, focused our studies to determine whether TGF-β modulated the activation and/or generation of CTL in chronically inflamed islets. We found that although CD8+ T cells isolated from the pancreas of TGF-β–pulsed TNF–TGF mice and nonpulsed TNF littermates had similar frequencies of granzyme B+, perforin+, and IFN-γ+ CTL when stimulated in vitro (Fig. 3A), mRNA levels for these molecules were reduced in freshly isolated pancreatic CD8+ T cells from TNF–TGF mice in comparison with control TNF littermates (Fig. 3B). Although these findings support reports that TGF-β downregulates effector molecules in CD8+ T cells in vivo and that this TGF-β–mediated effect is reversible in vitro (37), we decided to perform a more detailed investigation of the activation status and functionality of CD8+ T cells present in the islets of TNF–TGF mice in comparison with TNF littermates to determine whether TGF-β acts directly, or indirectly, on these cells to delay disease progression. Quantification of the absolute numbers of activated CD8+CD44highCD62Llow T cells and CD8+CD44high killer cell lectin-like receptor G1high T cells (Fig. 3C, left and middle panels) and memory CD8+CD44highCD62Lhigh (Fig. 3C, right panel) revealed significant reductions in all of these populations in the pancreas of TNF–TGF mice in comparison with TNF littermates. This reduction in CD8+ T cells with an activated/memory phenotype was not due to TGF-β–mediated apoptosis of CD8+ T cells (38), because absolute numbers of CD8+ T cells and dual immunofluorescence staining for annexin V and CD8 in the islets and PLN of TNF–TGF and TNF littermates were identical (data not included). To determine the physiological relevance of this TGF-β–mediated change in the CD8+ T cell population in the pancreas, we assessed the diabetogenic capacity of the lymphocytes. GFP+ Foxp3+ Treg-depleted CD45+ lymphocytes isolated from the pancreas of Foxp3 KI–GFP–TGF mice or Foxp3 KI–GFP TNF littermates were adoptively transferred into NOD–scid mice, and the development of diabetes was monitored. Treg-depleted lymphocytes from Foxp3 KI–GFP–TGF mice rapidly induced diabetes in recipient mice between 7 and 10 wk posttransfer, as expected. In contrast, significantly decreased kinetics and penetrance of diabetes development were evident following transfer of Treg-depleted lymphocytes from Foxp3 KI–GFP TNF–TGF mice (Fig. 3D). Thus, a brief pulse of TGF-β at the CTL transition stage of diabetes development rapidly halted the diabetogenic capacity of the CD8+ T cells, potentially by impairing both their activation and memory transformation or maintenance.

TGF-β inhibits insulin-specific CTL activity

We next tested whether TGF-β modulated the cytotoxic activity of activated G9C8 TcR transgenic CD8+ T cells that are specific for aa 15–23 of the insulin β-chain (B15–23) (22, 23). Incubation of preactivated G9C8 TcR transgenic CD8+ T cells with B15–23 peptide-pulsed targets in the presence of TGF-β resulted in significantly decreased target cell death compared with cultures containing no TGF-β (Fig. 4A). We next tested the capacity of preactivated G9C8 TcR transgenic CD8+ T cells to kill β cells from TGF-β–expressing versus nonexpressing mice. As shown in Fig. 4B, there was decreased killing of TGF-β–expressing β cells compared with non–TGF-β–expressing β cells. Consolidating these data, we hypothesized that transient expression of TGF-β would inhibit CTL killing of β cells and enhance β cell numbers in vivo. This theory was confirmed as the numbers of apoptotic β cells in pancreatic sections from TGF-β–pulsed TNF–TGF mice were significantly decreased in comparison with nonpulsed TNF littermates (Fig. 5A, 5B). Furthermore, absolute numbers of β cells, assessed by FACS according to the protocol developed by Pipeleers et al. (25), retrieved from the pancreas were significantly increased following the TGF-β pulse period (Fig. 5B, 5C). Thus, TGF-β decreases intrapancreatic CD8+ T cell transition to CTL and formation, or maintenance, of memory CD8+ T cells.
islets by impairing the functionality of anti-islet CTLs, thereby decreasing β cell destruction.

Discussion

We show that a brief pulse of TGF-β in the islets when inflammation and β cell destruction are already significantly delays diabetes development by decreasing both the cytotoxicity of islet-reactive T cells and the number of memory T cells.

In vitro activation of CD4+CD25− or CD4+Foxp3− T cells in the presence of TGF-β leads to upregulation of Foxp3 expression in naive cells (11) and the CD4+ T cells present in the islets of our mice are activated. Previously, Peng et al. (18) found that prolonged secretion of TGF-β in wild-type NOD islets prior to insulitis led to increased presence of CD4+CD25+ Tregs correlating with protection from diabetes development. We found no evidence to support TGF-β-mediated increase in Foxp3+ Tregs following either a short 1-wk pulse or a longer 3-wk pulse starting at 8 wk of age (Supplemental Fig. 7). Indeed, our data showing that depletion of CD4+ T cells did not impair the suppressive effect of TGF-β on diabetes development highlight an alternative role for this cytokine in controlling autoimmunity.

We hypothesize that TGF-β specifically targets activated T cells to decrease their functionality. This theory is supported by several key findings when comparing effector CD4+ and CD8+ T cells from the pancreas of TGF-β–pulsed TNF–TGF in comparison with nonpulsed TNF littermates; CD8+ T cells isolated from the pancreas of TNF–TGF mice had reduced mRNA transcripts for granzyme B, perforin, and IFN-γ, and decreased capacity to differentiate into memory cells, or to maintain their numbers, compared with CD8+ T cells isolated from nonpulsed TNF littermates. Furthermore, aggressor cells from the pancreas of TGF-β–pulsed mice had impaired ability to transfer diabetes to NOD–scid recipients in comparison with nonpulsed TNF littermates. Furthermore, activated insulin-specific G9C8 TCR transgenic CD8+ cells failed to lyse either B15–23 peptide-pulsed targets in the presence of TGF-β, or TGF-β-expressing β cells. This ability of TGF-β to decrease the efficacy of activated CTL to destroy their target cells is not unique for islet-specific CD8+ T cells; the ability of OVA-specific CD8+ TCR OT-I CTL to destroy OVA-pulsed targets was similarly impeded by addition of TGF-β to the cultures (data not shown). Our results support a previous report using a viral murine model of type 1 diabetes that also showed that TGF-β could impair anti-islet CTL responses (40). However, in contrast to this study, we found no evidence that TGF-β killed activated CD8+ T cells; absolute numbers of total CD8+ T cells isolated from the pancreas of TGF-β–pulsed TNF–TGF and nonpulsed TNF littermates were equal (data not shown).

It is not clear which differentiation stage of CD8+ T cells is targeted by TGF-β as both CTL and memory cell numbers are decreased. At 8 wk of age, CD8+ T cells in the pancreas of TNF-expressing NOD mice have an activated phenotype and produce perforin, granzyme B, and IFN-γ (31). Thus, for such CD8+ T cells to exhibit a decreased activation profile, and loss of CTL-associated cytokine transcription following a brief exposure to TGF-β, suggests that TGF-β can promote “de-differentiation” of CTLs back to a naive-like phenotype. Although an alternative hypothesis is that CTL activity in inflamed islets may be dynamic, requiring constant transition of naive T cells to effector cells, and that this event is impaired by TGF-β (40), the fact that we have found no evidence of naive T cells in chronically inflamed islets suggests that this hypothesis is unlikely. In addition, the fact that exposure to TGF-β at 4 wk of age, before the CTL transition in islets, had negligible impact on disease progression argues in favor of TGF-β targeting activated CD8+ T cells.

Previous studies have demonstrated that CD8+ T cells exposed to TGF-β during differentiation have reduced capacity to develop into memory cells (41). The observed decrease of memory T cells.
in our study may be a secondary effect of decreased effector function in CD8+ T cell, as a recent report described how memory CD8+ T cells arise from activated, granzyme B–expressing cells (38). It is possible that the reduced expression of effector molecules on CD8+ T cells in the pancreases of TGFβ–pulsed TNF–TGFβ mice results in their diminished conversion to memory.

In the TNF–NOD mouse, β cell destruction is a CD8+ T cell–dependent, CD4+ T cell–independent process, whereas diabetes progression in the wild-type NOD mouse requires both CD4+ and CD8+ T cell collaboration. Therefore, it is important to note that fewer memory CD4+ T cells were detectable in the pancreas of TNF–TGFβ mice compared with TNF littermates (data not shown). Thus, a transient pulse of TGFβ– within chronically inflamed islets creates an environment that is not conducive to memory T cell propagation and, as a consequence, a pool of cells incapable of transferring disease to NOD–scid recipients.

Accumulating evidence indicates that CD8+ T cells are integral to diabetes development in humans; islet-reactive CD8+ T cells have been cloned from the blood of patients with type 1 diabetes (35), and histochemical analysis of pancreata from diabetic patients has documented the presence of CD8+ T cells (36, 42). Our data demonstrate the ability of TGFβ– to impair the important features of the CD8+ T cell anti–β cell response, namely cytolytic function and formation, or maintenance, of immunological memory. Further studies into the mechanism(s) through which TGFβ– creates these effects may yield candidate targets for therapy and important knowledge about disease dynamics.

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Disclosures

The authors have no financial conflicts of interest.

References


An ISLET-SPECIFIC PULSE OF TGF-β STOPS CTL ACTION


