Regulatory T-Cells Protect From Type 1 Diabetes After Induction by Coxsackievirus Infection in the Context of Transforming Growth Factor-β

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OBJECTIVE—Coxsackievirus infections have long been associated with the induction of type 1 diabetes. Infection with coxsackievirus B4 (CB4) enhances type 1 diabetes onset in NOD mice by accelerating the presentation of β-cell antigen to autoreactive T-cells. It has been reported that a progressive defect in regulatory T-cell (Treg) function is, in part, responsible for type 1 diabetes onset in NOD mice. This defect may contribute to susceptibility to viral-induced type 1 diabetes. We asked whether the immune response after CB4 infection could be manipulated to reestablish peripheral tolerance while maintaining the immune response to virus.

RESEARCH DESIGN AND METHODS—NOD mice expressing transforming growth factor-β (TGF-β) specifically in the β-cells were infected with CB4, and the functional role of Tregs in disease protection was measured. Systemic treatments with TGF-β were used to assess its therapeutic potential.

RESULTS—Here, we report that Tregs induced after CB4 infection in the presence of TGF-β prevented type 1 diabetes. The capacity to directly infect pancreatic β-cells correlated with increased numbers of pancreatic Tregs, suggesting that presentation of β-cell antigen is integral to induction of diabetogenic protective Tregs. Furthermore, the presence of these viral induced Tregs correlated with protection from type 1 diabetes without altering the antiviral response. Finally, when TGF-β was administered systemically to NOD mice after infection, the incidence of type 1 diabetes was reduced, thereby signifying a potential therapeutic role for TGF-β.

CONCLUSIONS—We demonstrate manipulations of the immune response that result in Treg-mediated protection from type 1 diabetes without concomitant loss of the capacity to control viral infection. ***Diabetes*** **57:1302–1311, 2008**

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APC, antigen-presenting cell; CB4, coxsackievirus B4; CIHR, Canadian Institutes of Health Research; DMEM, Dulbecco's modified Eagle's medium; H-E, hematoxylin-eosin; IFN-γ, γ-interferon; IL, interleukin; MSFHR, Michael Smith Foundation for Health Research; PFU, plaque-forming unit; PLN, pancreatic lymph node; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; Treg, regulatory T-cell.

Coxsackieviral infections commonly precede the onset of type 1 diabetes in patients (1), and in animal models, coxsackievirus B4 (CB4) infection significantly accelerated diabetes onset (2,3). In NOD mice, islet destruction and development of type 1 diabetes are preceded by a period of noninvasive peri-insulitis, strongly suggesting that there is a window of time in which peripheral tolerance is partially maintained (4). Several groups have reported that, in the NOD mouse, regulatory T-cells (Tregs) gradually lose their capacity to suppress effector T-cell proliferation correlating with the spontaneous onset of type 1 diabetes (5–7). This loss of functional Tregs results in impaired peripheral tolerance to β-cell antigens and represents an important checkpoint in disease progression (8).

Several reports have ascribed a role for transforming growth factor-β (TGF-β) in the function and/or generation of Tregs in the periphery (6,9–14). In particular, Chen et al. (9) demonstrated that in vitro stimulation of naïve T-cells in the presence of TGF-β led to the expression of the Treg-specific transcription factor Foxp3 and functional suppression by these Foxp3-expressing cells (9). Furthermore, a short pulse of TGF-β in the islets of NOD mice suppressed spontaneous onset of diabetes through an expansion of $Foxp3^+$ Treg cells within the islets of the pancreas (15). A systemic TGF-β gene therapy approach also demonstrated enhanced survival of transplanted islet cells that correlated with increased Treg numbers in the pancreas (16). Although these reports highlight the potential importance of TGF-β in diabetes, it remains to be determined whether TGF-β can induce protective Tregs in a clinically relevant model of viral-induced diabetes.

In this report, we demonstrate that TGF-β–induced Tregs can be activated/generated after viral infection to protect against type 1 diabetes. NOD mice expressing TGF-β specifically in the β-cells of the pancreas (NODTGFβ mice) (17) were infected with CB4, and despite meeting all the criteria for susceptibility to viral-induced diabetes, these mice were protected from type 1 diabetes induction. Protection from type 1 diabetes was correlated with an increased presence of Tregs in the pancreatic lymph nodes (PLNs) and pancreas. Furthermore, we demonstrated that recombinant TGF-β administered systemically after infection could replace transgenic TGF-β and was sufficient to protect NOD mice from CB4-induced diabetes. Our data indicate that TGF-β induces Tregs to maintain self-tolerance to anti-islet autoimmunity without suppressing the response to the virus.

RESEARCH DESIGN AND METHODS

NOD/ShiLtJ mice were obtained from The Jackson Laboratories (Bar Harbor, ME). NODTGFβ transgenic mice expressing TGF-β under the control of the human insulin promoter were generated in the laboratory of Dr. N. Sarvetnick (The Scripps Research Institute, La Jolla, CA) (17). All mice were bred and maintained in our rodent facility and tested for diabetes before infection. All procedures performed followed the guidelines of the institutional animal care committee.

Virus. Stocks of CB4 Edwards strain 2 were prepared as described previously (18,19). Ten- to 12-week-old mice were infected intraperitoneally with sublethal doses of 100 plaque-forming units (PFUs).

Flow cytometry. Single-cell suspensions were stained for the appropriate markers and analyzed by flow cytometry. Fluorescently conjugated antibodies directed against CD11b (clone M1/70), CD11c (clone HL3), CD4 (clone L3T4), CD25 (clone PC61 or 7D4), and foxp3 (clone FJK-16s) were purchased from eBiosciences (San Diego, CA), whereas biotin-conjugated antibodies directed against CD40 (clone 3/23), CD80 (clone 16-10A1), and CD86 (clone GL1) were purchased from BD Biosciences (Missisauga, Canada).

Immunohistochemical staining. Tissue sections were prepared as previously described (18). Staining was performed using standard procedures for hematoxylin-eosin (H-E; iCapture Center, Vancouver, British Columbia, Canada). Serial sections of the pancreas were graded for islet pathology based on a three-tier scale.

Isolation of pancreatic infiltrating cells. Pancreata were isolated from infected NOD and NODTGFβ mice and mechanically disrupted. Single-cell suspensions were treated for 10 min at 37°C in a PBS solution containing 1 mg/ml collagenase. Recovered cells were stained for flow cytometry.

Treg functional inactivation. CB4-infected NODTGFβ mice received intravenous injection of 450 μg anti-CD25 antibody (clone PC61) at days 3 and 6 after infection. Alternatively, mice were injected intraperitoneally with a single dose of 100 μg purified anti–CTLA-4 (clone UC10-4B9; eBiosciences) at 24 h after infection.

Treg adoptive transfer. PLNs were harvested from CB4-infected NODTGFβ mice at day 7 after infection. Tregs were purified using a Robosep automated cell separator (Stem Cell Technologies, Vancouver, British Columbia, Canada). $CD4^+$ $CD25^+$ T-cells were sequentially purified using modified CD4 and CD25 enrichment kits (Stem Cell Technologies). Purified Tregs (1×10^5) were adoptively transferred intraperitoneally into NOD mice at 24 h after infection.

Intracellular cytokine staining. Splenocytes were restimulated with 500 ng/ml PMA and 10 ng/ml ionomycin in the presence of Golgi Plug (BD Biosciences) in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum. Cells were stained for surface markers, fixed, permeabilized, stained for cytokines, and analyzed by flow cytometry. Fluorescently conjugated antibodies to CD4 (clone L3T4), CD8 (clone 53-6.7), interleukin (IL)-17 (clone TC11-18H10.1), and tumor necrosis factor-α (TNF-α) (clone MP6-XT22) were obtained from eBiosciences. Fluorescently conjugated antibodies to IL-4 (clone 11B11) and γ-interferon (IFN-γ) (clone XMG1.2) were obtained from BD Biosciences.

Systemic TGF-β treatment. NOD mice were injected intraperitoneally with 100 ng recombinant human TGF-β1 (Sigma-Aldrich, Oakville, Canada) 24 h after infection with CB4.

Statistical analysis. The unpaired Student's *t* test (flow cytometry analysis) and the Mann-Whitney *U* test (diabetes incidence curves and insulitis index) were used. A P value of <0.05 was considered significant.

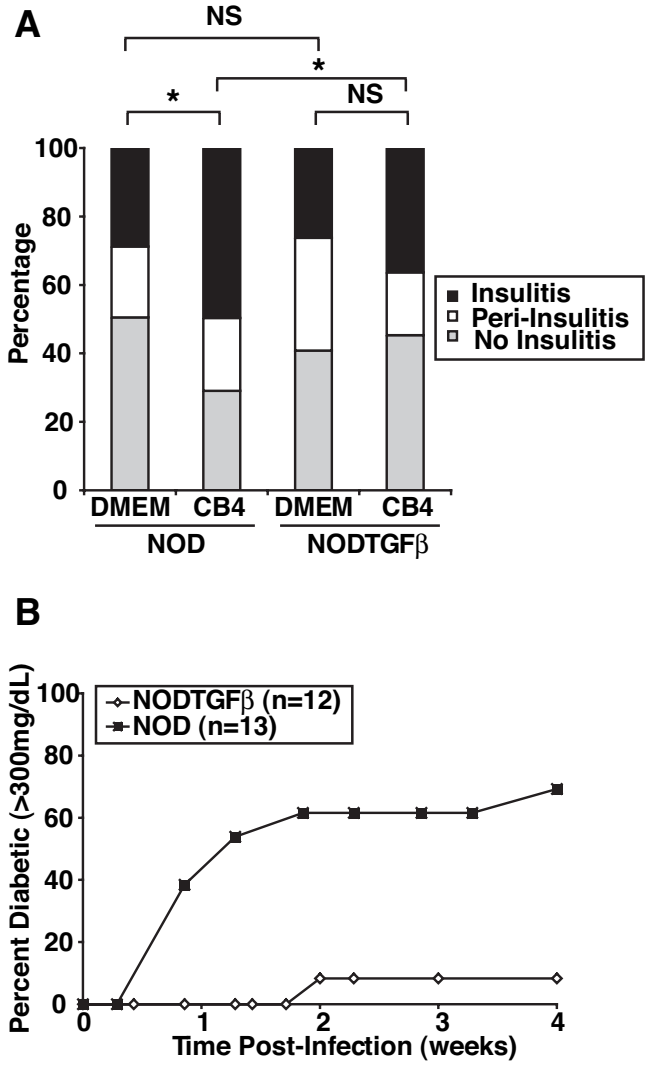

FIG. 1. CB4 infection of TGF-β–expressing NOD mice does not induce type 1 diabetes. *A*: Histological analysis of pancreata from NOD and NODTGFβ mice 7 days after infection with CB4 or mock infection with Dulbecco's modified Eagle's medium (DMEM). Consecutive pancreatic sections were stained with H-E and scored for islet pathology. Data are presented as percentages and were obtained from a minimum of 140 scored islets representing at least eight mice per group. *B*: Diabetes incidence of NOD (■) and NODTGFβ (◇) mice after infection with CB4. *Significant change in overall phenotype and change in mice presenting with insulitis.

RESULTS

CB4 infection in the context of TGF-β protects against type 1 diabetes. As reported previously (2), infection of NOD mice with CB4 resulted in a significant acceleration of diabetes in >60% of infected mice compared with uninfected age-matched controls (Supplemental Fig. 1*A*, available in an online appendix at http://dx.doi.org/10.2337/db07-1460). This occurs regardless of sex, because viral induction of type 1 diabetes does not follow the same sex bias observed for spontaneous disease. NOD mice harboring a transgene driving expression of TGF-β specifically in the pancreas (NODTGFβ) were previously described (17), and they spontaneously develop diabetes, albeit at a reduced rate compared with nontransgenic NOD mice (Supplemental Fig. 1*B*) (17). Furthermore, these mice develop autoreactive T-cells with diabetes transfer potential (17). Importantly, these mice present with relatively normal pancreatic organization as opposed to other described models (17,20). Previous reports have linked the presence of autoreactive T-cells and the degree of insulitis with susceptibility to viral induction of disease (2,21). Islet inflammation in uninfected NODTGFβ mice was not significantly different from that in their NOD counterparts, with ~30% of islets presenting with invasive insulitis at the time of infection (10–12 weeks old) (Fig. 1*A*; Supplemental Table 1), indicating the presence of activated β-cell–specific autoreactive T-cells within the pancreas. As such, NODTGFβ mice meet the criteria previously described (2,21) for susceptibility to CB4-induced type 1 diabetes. Strikingly, NODTGFβ mice infected with CB4 did not develop type 1 diabetes—unlike their NOD counterparts (Fig. 1*B*). On infection, no significant change in islet inflammation was observed in NODTGFβ mice, whereas by 7 days after infection, significant increases in insulitis were observed in NOD mice (Fig. 1*A*; Supplemental Table 1). This was particularly

marked in NOD mice that were diabetic by day 7 after infection because >90% of islets in these mice presented with invasive insulitis ($n = 5$). Additionally, the percentage of islets free of insulitis was not significantly decreased in NODTGFβ mice after CB4 infection (Fig. 1*A*; Supplemental Table 1), indicating that no new islets were being targeted after infection. This phenotype is reminiscent of both the BDC2.5 TCR transgenic model and the nonobese resistant mouse in which Tregs prevent type 1 diabetes by precluding the progression of islet pathology from periinsulitis to invasive insulitis (22,23).

NODTGFβ mice are polarized to a Th1 response after infection with CB4. Previously NODTGFβ mice were found to be polarized toward a Th2 phenotype at steady state (17). Furthermore, it has recently been established that TGF-β acts as a cofactor with IL-6 in the generation of Th17 cells (24–26). As such, we investigated whether changes in T-cell polarization were involved in the protection from type 1 diabetes after infection. Cytokine production from splenic T-cells was analyzed ex vivo before infection and at 7 days after infection. Before infection, very few T-cells were observed to produce cytokines, and slightly more T-cells from NODTGFβ mice compared with NOD mice were observed to produce IL-4 although this difference was not statistically significant (Supplemental Fig. 2). As predicted after a viral infection, T-cells preferentially produced Th1 cytokines (IFN-γ and TNF-α) in both NODTGFβ and NOD mice (Fig. 2). Despite previous reports of Th2 polarization before infection (17), T-cells from NODTGFβ mice did not abundantly produce IL-4 (Fig. 2), clearly confirming a Th1 response after viral infection. Finally, only a few CD4 T-cells were observed to produce IL-17 and, more importantly, there was no significant increase in the percentage of Th17 cells in NODTGFβ mice compared with NOD mice (Fig. 2). These data clearly indicate that NODTGFβ mice mount a Th1 response similar to NOD mice after infection and that polarization toward a Th2 or Th17 phenotype was not involved in the protection from type 1 diabetes.

CB4 infection of NODTGFβ mice leads to a significant increase in the number of Tregs in the PLN and pancreas. It has been demonstrated that stimulation of T-cells in the presence of TGF-β can induce the conversion of naïve T-cells to a Treg phenotype (9–12). Accordingly, we examined whether increases in Treg presence were responsible for the protection from diabetes observed in NODTGFβ mice. After CB4 infection, significantly increased levels of $CD4^+$ $Foxp3^+$ Tregs were found in PLNs (Fig. 3*A*–*D*) but not the spleen (Supplemental Fig. 3) of NODTGFβ mice compared with uninfected littermates. Infection of NOD mice also resulted in significant increases in Tregs in the PLNs (11.9% in uninfected mice, $n = 7$ vs. 16.8% in infected mice, $n = 10$), and this is analogous to a prior report in which similar increases were associated with insulitis severity and/or onset of spontaneous type 1 diabetes (7). This implies that measuring mere increases in the proportion of Tregs in the PLNs may not be directly predictive of Treg-mediated protection. A prior study reported that Tregs from BDC2.5 mice did not efficiently suppress activation of diabetogenic T-cells in the PLNs and inferred that Treg function may be limited to within the confines of the pancreas (22). In the pancreas, we observed a significantly greater percentage of $CD4^+$ $Foxp3^+$ Tregs in CB4-infected NODTGFβ mice compared with similarly infected NOD mice, uninfected NOD mice, and uninfected NODTGFβ mice (Fig. 3*E*). As expected, no differences in activation were observed between T-cells in the PLNs of NODTGFβ or NOD mice after infection (Supplemental Fig. 4). These data suggest that Tregs may primarily act directly in the pancreas rather than in the draining lymph node to suppress diabetogenic T-cells and prevent onset of type 1 diabetes.

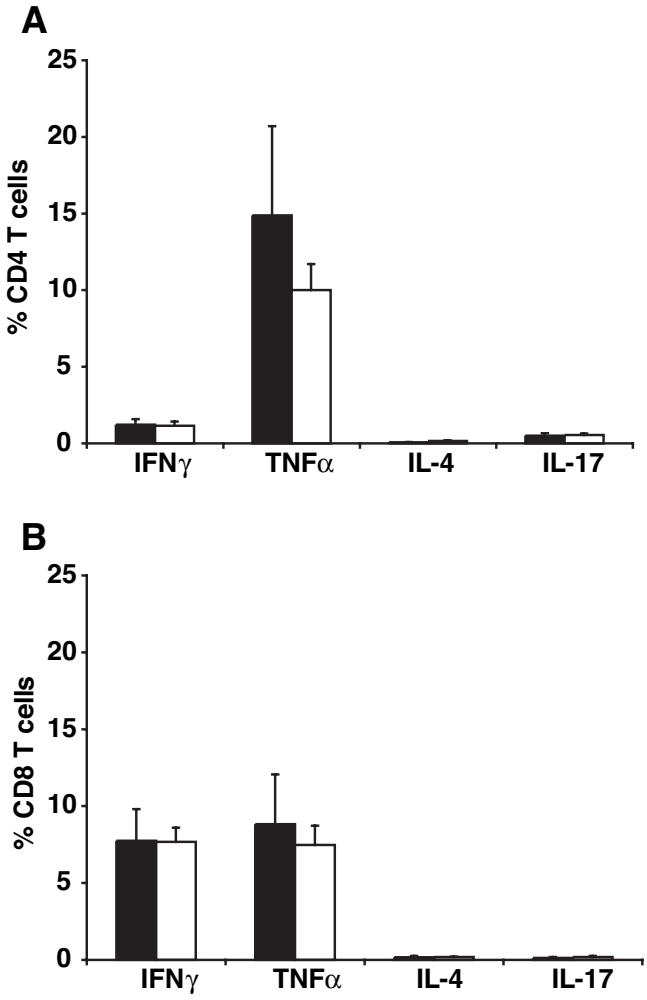

FIG. 2. T-cells from NODTGFβ mice are polarized to a Th1 phenotype after CB4 infection. Cytokine production from CD4 T-cells (*A*) and CD8 T-cells (*B*) from the spleen of NOD (■) and NODTGFβ (□) mice was measured ex vivo by intracellular flow cytometry after restimulation with PMA and ionomycin. Data are presented as means ± SE and are representative of four mice per group from two separate experiments.

Infection of β-cells of the pancreas is required for induction of Tregs. It was previously demonstrated that CB4 infection induced type 1 diabetes via presentation of pancreatic β-cells and their self-antigens to the preexisting population of diabetogenic T-cells (27). To determine whether infection of β-cells was also necessary to activate or generate functional Tregs, NODTGFβ mice were infected with a closely related virus, CB3. Both CB3 and CB4 infect the acinar tissue of the pancreas, causing considerable pathology and inflammation; however, only CB4 infects pancreatic β-cells (21). CB3 infection did not lead to any changes in the proportions of Tregs in the PLNs (Fig. 3*C* and *D*), suggesting that presentation of β-cell antigens is necessary to induce protective Tregs in this model and that mainly β-cell antigen-specific Tregs are activated or generated to protect against diabetes.

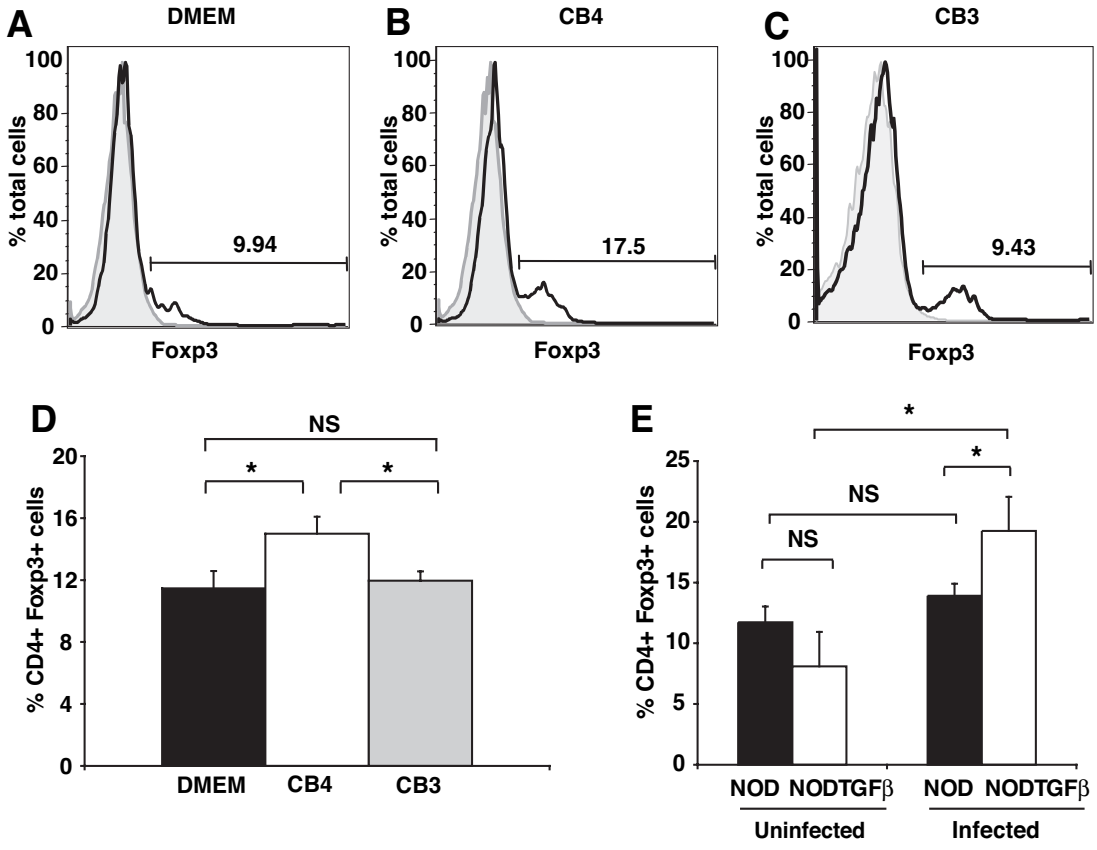

FIG. 3. CB4 infection of NODTGFβ mice leads to increases in Tregs in the PLN and in the pancreas. Representative histograms of Foxp3 expression by CD4$^+$ T-cells in the PLN of NODTGFβ mice 7 days after mock infection with DMEM (*A*) or infection with CB4 (*B*) or CB3 (*C*). Numbers shown on the histograms represent percentage of Foxp3$^+$ cells. Isotype controls are represented by shaded gray areas. *D*: Average percentage of Foxp3$^+$ CD4$^+$ T-cells in the PLN of NODTGFβ mice after mock infection with DMEM (■, $n = 10$) or infection with CB4 (□, $n = 13$) or CB3 (▨, $n = 12$). *E*: Average percentage of CD4$^+$ cells expressing Foxp3 in the pancreas of NOD (■, uninfected [$n = 4$]; infected [$n = 14$]) or NODTGFβ mice (□, uninfected [$n = 4$]; infected [$n = 7$]). Data are presented as means ± SE from at least two separate experiments.

Functional inactivation of Tregs reestablishes susceptibility of NODTGFβ to type 1 diabetes. To confirm the functional role of these TGF-β–induced Tregs in the protection from diabetes, CB4-infected NODTGFβ mice were treated with an anti-CD25 antibody that has been previously demonstrated to functionally inactivate and/or deplete Tregs (28). Antibody treatment after infection reestablished susceptibility of NODTGFβ mice to CB4-induced type 1 diabetes, as disease developed with the same kinetics and incidence as that observed for NOD mice after infection while mock-treated mice remained protected from disease (Figs. 4*A* and 1*B*). These data confirm that type 1 diabetes can be induced in NODTGFβ mice and that they are not simply impervious to the induction of disease after viral infection. Instead, suppression of diabetes is actively induced and maintained. By demonstrating loss of function through antibody-mediated functional inactivation, this experiment showed that TGF-β–induced Tregs are responsible for the protection from diabetes.

Adoptive transfer of Tregs from NODTGFβ mice protects CB4-infected NOD mice from type 1 diabetes. To further demonstrate the functional role of TGF-β–induced Tregs in the protection from type 1 diabetes, Tregs were purified from the PLNs of NODTGFβ mice at 7 days after infection and adoptively transferred to NOD mice 24 h after CB4 infection. After Treg transfer, recipient NOD mice that were adoptively transferred with donor Tregs from infected NODTGFβ mice were protected from diabetes development for >15 days after infection while mock-treated mice still developed accelerated diabetes after infection (Fig. 4*B*). However, the observed protection may only be transient because one of the adoptively transferred mice developed diabetes 17 days after infection (Fig. 4*B*). This may infer that a source of TGF-β is required to maintain protection. These data demonstrate gain of function, further confirming the role of Tregs in the protection observed in our model.

Tregs maintain protection from type 1 diabetes in a CTLA-4–dependent manner. The costimulatory molecule CTLA-4 is expressed at high levels on the surface of Tregs and has been demonstrated to play an important role in both the function (29) and the TGF-β–mediated conversion of Tregs in vitro (30). To investigate the functional requirement of CTLA-4 in TGF-β–induced Treg-mediated protection, we treated CB4-infected NODTGFβ mice with a neutralizing antibody directed against CTLA-4 at 24 h after infection. Antibody-treated NODTGFβ mice developed diabetes with increased incidence compared with mock-treated NODTGFβ mice (Fig. 4*C*). This suggests that Tregs maintain protection from type 1 diabetes in a CTLA-4–dependent manner.

NODTGFβ mice show reduced upregulation of costimulatory molecule after infection. TGF-β–treated antigen-presenting cells (APCs) have previously been shown to induce tolerance in a Treg-dependent manner (31,32). Compared with infected NOD mice, flow cytometry analysis revealed that macrophages isolated from the

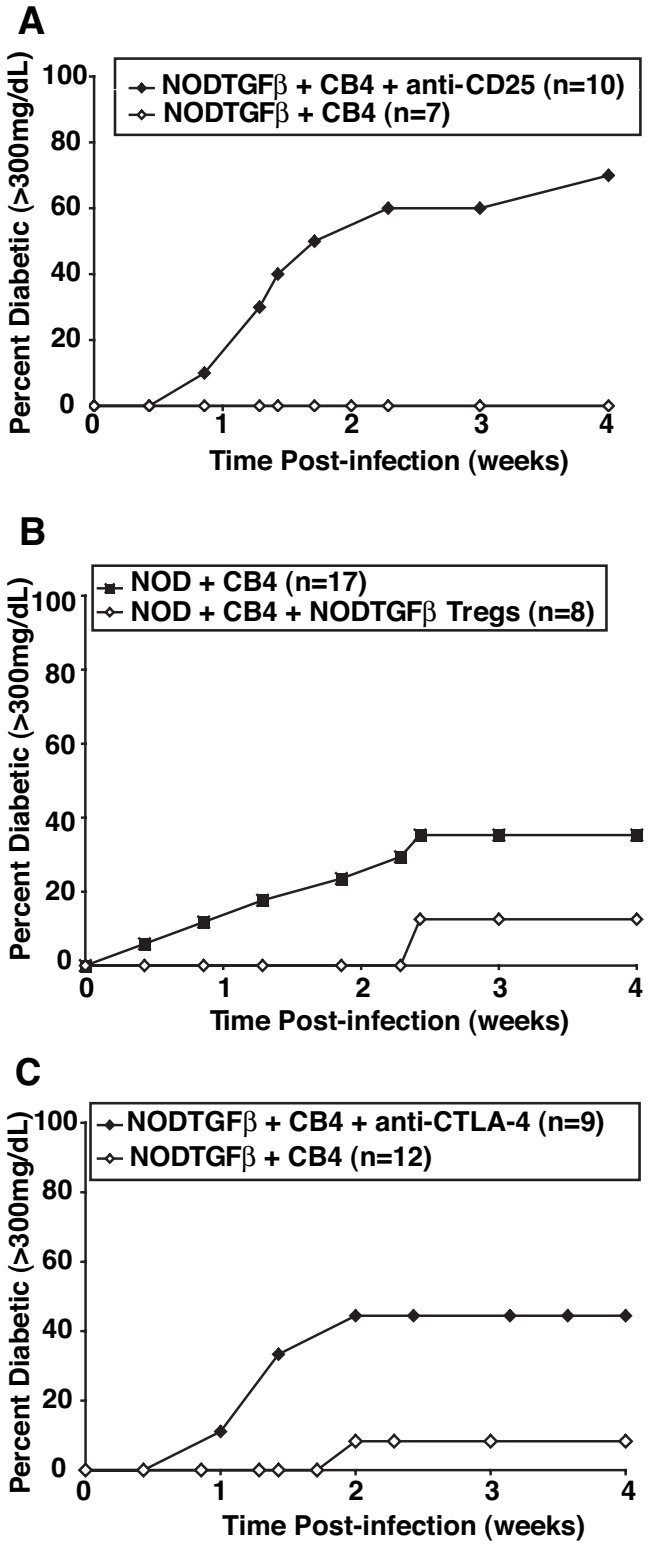

FIG. 4. TGF-β–induced Tregs protect from type 1 diabetes in a CTLA-4–dependent manner. *A*: Diabetes incidence of CB4-infected NODTGFβ mice treated with anti-CD25 (♦) antibodies or mock treated with DMEM (◇). *B*: Diabetes incidence of CB4-infected NOD mice adoptively transferred with NODTGFβ Tregs (◇) or mock treated (■). *C*: Diabetes incidence of CB4-infected NODTGFβ mice treated with anti–CTLA-4 antibodies (♦) or mock treated with DMEM (◇).

pancreas, PLNs, and spleen of infected NODTGFβ mice at day 7 after infection have significantly reduced surface expression of the costimulatory molecule CD40 (Fig. 5*A* and *B*). A similar trend is observed with the costimulatory molecules CD80 and CD86 (Fig. 5*C* and *D*). Their surface expression was significantly reduced on macrophages isolated from the spleen of infected NODTGFβ mice; this reduction was also observed on macrophages isolated from the PLNs, although this difference was not statistically significant (Fig. 5*C* and *D*). This reduced upregulation was not observed on dendritic cells from the spleen (Supplemental Fig. 5) or PLN (data not shown). It is interesting to note that NOD and NODTGFβ macrophages express similar levels of costimulatory molecules before infection (Supplemental Fig. 6) and that these molecules are upregulated to the same extent in both mice at day 3 after infection (Supplemental Fig. 7). Differences in surface expression of costimulatory molecules were not observed until day 7 (Fig. 5). This time frame coincides with the kinetics of increases in the number of Tregs after infection in the NODTGFβ mice, suggesting that presentation of pancreatic self-antigen by these semimature macrophages may act in the generation or activation of these protective Tregs in the NODTGFβ mice. Furthermore, we observed that functional inactivation of Tregs did not reestablish upregulation of costimulatory molecules on macrophages (data not shown), indicating that they are unlikely to be the targets of suppression in our model. Viral clearance of both CB3 (18,33) and CB4 (Fig. 6*A*) was not affected in NODTGF-β mice when compared with that in infected NOD mice, indicating that the influence of TGF-β does not negatively affect the protective immune response directed against the virus. This is further supported because delayed clearance typically results in a fatal outcome and because no increase in death was observed in the NODTGFβ mice after coxsackievirus infection. These data indicated that changes in costimulatory molecule expression on macrophages are more relevant to the induction of autoimmunity than to the immune response to viral infection.

Systemic TGF-β treatment protects NOD mice from type 1 diabetes. To assess the potential therapeutic role of TGF-β during viral-induced autoimmunity and to validate the biological relevance of our results in this transgenic model, we asked whether systemic TGF-β treatment would also be sufficient to protect from coxsackievirus-induced type 1 diabetes. One day after CB4 infection, NOD mice (10–12 weeks old) were treated with a single dose of recombinant TGF-β and monitored for induction of diabetes. We observed a significant reduction of diabetes incidence by day 15 after infection (Fig. 7*A*). This TGF-β–mediated protection correlated with increases in Tregs in both the PLNs (Fig. 7*B*) and the spleen after infection (Fig. 7*C*) compared with similarly treated mock-infected mice. Protection was transient, however, because disease induction was observed by day 28 after infection (data not shown). Mice were only given a single dose of TGF-β, and given the short half-life of TGF-β in vivo (34), it would not be expected to persist in the mice. This suggests that a multidose regimen would likely extend protection. Similar to what we observed for transgenic expression of TGF-β, systemic treatment with TGF-β did not affect clearance of the viral infection (Fig. 6*B*), suggesting that treatment does not reduce the capacity of the host to mount an immune response to the virus. Most notably, this indicates that TGF-β could be administered after exposure to virus and

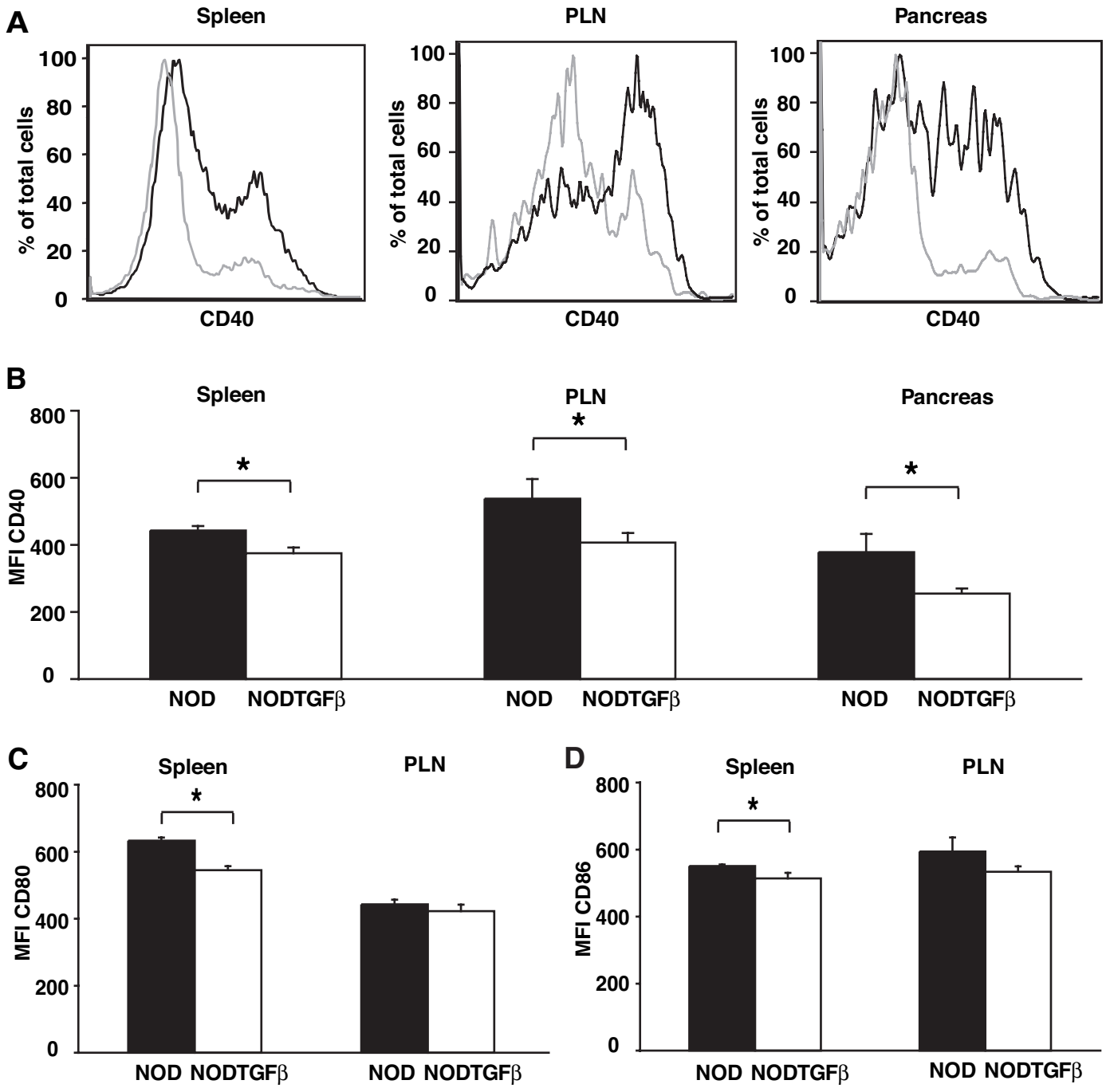

FIG. 5. Pancreatic expression of TGF-β reduces upregulation of costimulatory molecules on macrophages after CB4 infection. *A*: Representative histograms of CD40 expression on macrophages ($CD11b^+$ $CD11c^-$) from NOD (black line) or NODTGFβ (gray line) mice 7 days after infection with CB4. *B*: Average mean fluorescence intensity of CD40 (*B*), CD80 (*C*), and CD86 (*D*) expression on macrophages ($CD11b^+$ $CD11c^-$) from NOD (■) or NODTGFβ (□) mice. Data from the spleen, PLN, and pancreas are presented as means ± SE and are representative of at least four mice per group from at least two separate experiments.

act to modulate disease induction without adverse effects on the host.

DISCUSSION

Viral infections clearly represent the last step of disease progression in animal models and require a preexisting population of autoreactive T-cells (2,3). Because viruses, such as coxsackievirus, are common human pathogens, this mechanism also likely operates to induce type 1 diabetes in humans. This suggests that protective approaches identified in mouse models would likely translate into potential therapies. Our data build on previous reports on the protective role of TGF-β to demonstrate that the immune system can be manipulated such that infection with a virus normally associated with acceleration of disease, such as CB4, can actively lead to the induction of mechanisms of tolerance and ultimately lead to protection from diabetes. Importantly, our data indicate that changes in the cytokine milieu can lead to protection from diabetes without compromising the capacity of the immune response to control viral infection.

The presence of a preexisting population of autoreactive

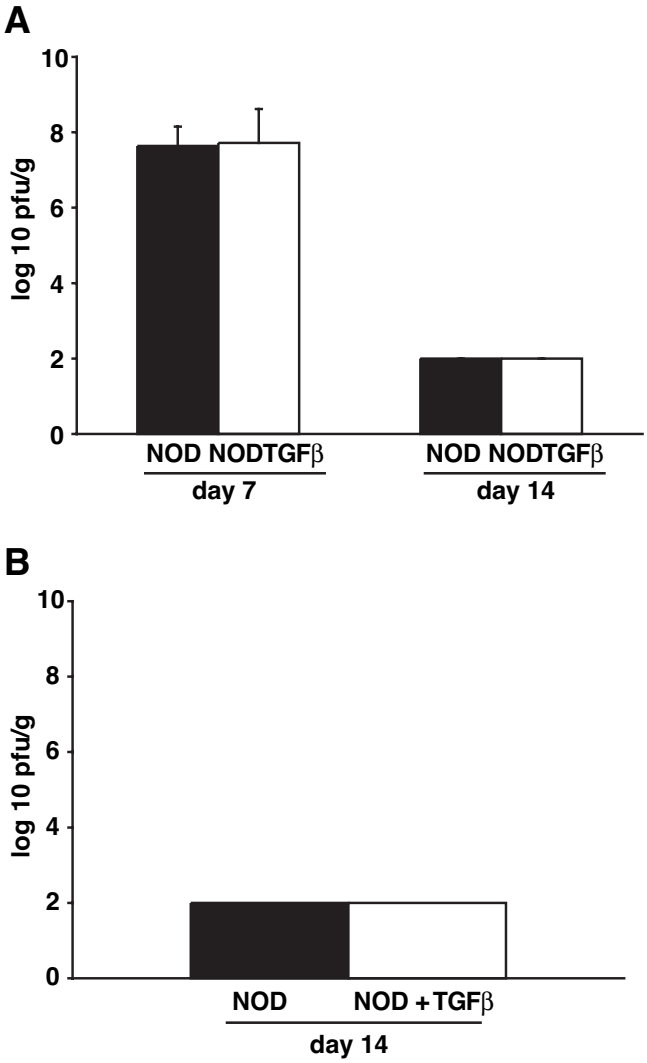

FIG. 6. Clearance of viral infection is not affected in the presence of TGF-β. Viral load in pancreas of NOD mice (■, $n = 5$) and NODTGFβ mice (□, $n = 6$) (*A*) or NOD mice (■, $n = 5$) and NOD mice treated systemically with 100 ng recombinant TGF-β (□, $n = 5$) (*B*) were measured after CB4 infection. Data are presented as log 10 PFU/g tissue and represent the average from duplicate values obtained from each mouse in the group. Data are representative of at least two separate experiments. Any samples not yielding any PFUs were assigned a value of 2 log 10 PFU/g representing the limit of detection of the assay.

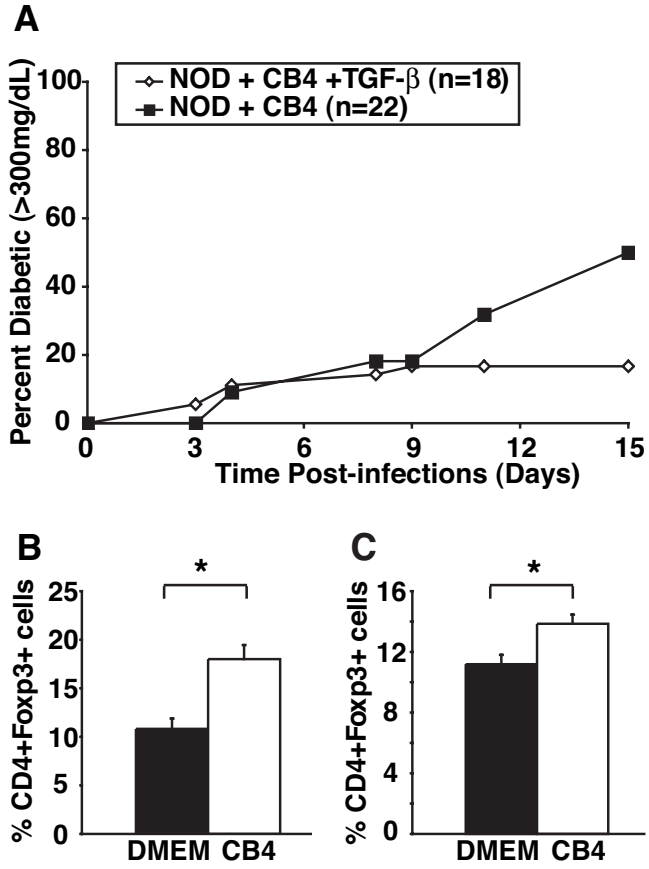

FIG. 7. Systemic TGF-β treatment transiently protects from diabetes in a Treg-dependent manner. *A*: Diabetes incidence of CB4-infected NOD mice treated 24 h after infection with 100 ng recombinant human TGF-β (◇) or mock treated with DMEM (■). Average percentage of $Foxp3^+$ $CD4^+$ T-cells in the PLN (*B*) or spleen (*C*) in TGF-β–treated NOD mice 7 days after infection with CB4 (□, $n = 9$) or mock infection with DMEM (■, $n = 6$). Data are presented as means ± SE and are representative of at least two separate experiments.

T-cells has previously been established as the primary criteria for susceptibility to viral induction of type 1 diabetes (2,3). Similar to NOD mice from which they were derived, NODTGFβ mice harbor diabetogenic T-cells capable of transferring disease and develop spontaneous diabetes (17). Importantly, the constitutive expression of TGF-β in this model did not result in any profound alterations of pancreatic architecture contrary to a similar model presented in a previous report (20). Because NODTGFβ mice fulfill the criteria for susceptibility to viral-induced disease, such as the development of autoreactive T-cells and susceptibility to disease after functional inactivation of Tregs, our results strongly suggest that NODTGFβ mice are protected by mechanisms that are actively induced after infection in the context of TGF-β rather than simply being impervious to viral-induced type 1 diabetes. We observed significant increases in the percentage of Tregs in the PLNs and, more importantly, the pancreas of NODTGFβ mice. We do not observe increased presence of Tregs in NODTGFβ mice before infection compared with wild-type counterparts confirming the role of viral infection in the induction of Tregs in our model. These data are in contrast to observations in which a pulse of TGF-β induced before the end of the priming phase of disease was sufficient to induce Tregs without any further manipulations and mediated protection from spontaneous diabetes (15). We speculate that these discrepancies may arise from differences in the levels or timing of TGF-β production. Previous studies have demonstrated that stimulation of naïve T-cells in the presence of TGF-β converts these cells into functional Tregs with suppressive capacity (9–12). To our knowledge, this represents the first report of a viral infection in the context of TGF-β actively inducing the generation/activation of Tregs directly in vivo and yielding protection from autoimmunity.

Our data clearly demonstrate that Tregs function to prevent diabetes directly within the pancreas. The activation or generation of Tregs may, however, still occur in the PLNs because adoptive transfer of Tregs purified from the PLNs was sufficient to protect NOD mice from CB4-induced type 1 diabetes. Similar to the BDC2.5 model, the presence of functional Tregs did not affect the activation of T-cells in the PLNs (22); instead, Tregs prevented the transition from peri-insulitis to invasive insulitis. Impor-

tantly, despite an ongoing autoimmune response at the time of infection, TGF-β–induced Tregs prevented new islets from becoming targets, thereby allowing for the maintenance of insulin production. We further demonstrated that infection of the islet cells themselves is an important requirement for the generation of Tregs. This strongly implies that self-reactive T-cells are converted to Tregs or that self-reactive Tregs are activated to prevent disease. As such, the protection from disease after infection in NODTGFβ mice can be explained by two nonexclusive mechanisms. First, protection may be maintained by either a reestablishment or an increase in the suppressive capacity of Tregs. Second, conversion of β-cell–reactive T-cells into Tregs may be responsible for decreasing the available pool of activated autoreactive T-cells. The antigenic specificity of the generated Tregs may also explain why a small increase in Treg percentage after infection was sufficient to induce protection. In this regard, two separate reports have demonstrated that in vitro expanded antigen-specific Tregs have greater type 1 diabetes–suppressive capacity than polyclonal Tregs in NOD mice (35,36). In another model of type 1 diabetes, it was demonstrated that adoptive transfer of as little as 2,000 Tregs was sufficient to prevent disease, further illustrating the potent suppressive capacity of fully functional Tregs (37). This implies that increases in the percentage of antigen-specific Tregs in the pancreas observed in NODTGFβ mice after infection should be amply sufficient to completely protect from the induction of type 1 diabetes.

Several groups have investigated the interaction between Treg and APCs (reviewed in 38). It has been suggested that immature APCs may lead to the generation of Tregs (31,32) and that, in turn, Tregs may maintain tolerance by acting directly on APCs (39). Here, we demonstrate that macrophages from the pancreas, PLN, and spleen of NODTGFβ mice do not mature to the same extent as macrophages from NOD mice in response to infection. Because the timing of this defect corresponds with the increase in Tregs in our model, we speculate that antigen presentation by these semimature macrophages is responsible for Treg generation. In support of this hypothesis, it was recently demonstrated that monocytes isolated from glatiramer acetate–treated mice presented with a similar semimature phenotype and were capable of inducing expansion of Tregs (40). These type II monocytes were further demonstrated to preferentially secrete immunosuppressive rather than proinflammatory cytokines (40). We are currently investigating whether type II monocytes are also involved in the protection observed in our model. Importantly, we saw no differences in viral clearance between NODTGFβ and NOD mice. Viral clearance is likely unaffected because dendritic cells mature normally in response to infection despite the expression of TGF-β. Overall, these data indicated that macrophages might be more involved in the induction of autoimmunity than in the response to CB4 infection. This is supported by previous observations in the BDC2.5 model in which macrophages engulf islets in response to CB4 infection and are likely responsible for induction of type 1 diabetes (41). Furthermore, because viral clearance remains unaffected, these data also suggest that the Tregs generated in our model act to suppress only self-reactive lymphocytes and do not affect viral-specific lymphocytes. Taken together, this indicated that the presence of TGF-β at the site of infection has a profound effect on the induction of autoimmunity without affecting the response to pathogen infection, suggesting that a TGF-β–based therapeutic approach would not run the risk of fatal side effects.

NODTGFβ mice have previously been demonstrated to be polarized toward a Th2 phenotype at steady-state (17). Because type 1 diabetes has been well described as a Th1-driven disease (reviewed in 42), this change in polarization could well have explained the protection observed in our model. However, after infection, we observed that T-cells from NODTGFβ mice responded similarly to NOD mice by producing IFN-γ and TNF-α preferentially over IL-4, demonstrating a strong Th1 response. As such, polarization to a Th2 phenotype is not responsible for the observed protection from type 1 diabetes in CB4-infected NODTGFβ mice. Furthermore, although it is well established that TGF-β along with other cofactors such as IL-6 can induce pathogenic Th17 cells (reviewed in 43), a very limited number of Th17 cells were observed after infection, and no increases in Th17 cells were observed in the NODTGFβ mouse. Despite the continuous presence of TGF-β in the transgenic mice and the induction of IL-6 typically associated with viral infection, no differences were observed between infected NOD and NODTGFβ mice. It has been reported that IFN-γ can inhibit development of Th17 cells (44), and this likely explains their absence after infection. Taken together, these results strongly indicate that suppression of type 1 diabetes was not the result of polarization of helper T-cells toward a Th2 phenotype.

Finally, our data support a therapeutic role for TGF-β because systemic treatment was sufficient to significantly reduce type 1 diabetes incidence by day 15 after infection. Protection was transient, indicating that treatments maintaining more prolonged exposure to TGF-β will be necessary to achieve long-term protection from diabetes. More specifically, TGF-β may need to be present continuously throughout the course of viral infection to ensure that the cytokine is present at the time of self-antigen presentation. To this effect, it was recently determined that continuous TGF-β exposure is necessary to maintain Foxp3 expression and suppressive capacity of Tregs converted in vitro (45). Alternatively, treatments using TGF-β agonists or delivery methods that maintain longer expression of TGF-β may help maintain tolerance, although these approaches would need to be fully tested to ensure that the side effects of treatment do not outweigh the benefits. Besides validating our studies in the transgenic mouse, these data signify an important short-term therapeutic role for this cytokine against viral-induced autoimmunity without striking side effects in terms of the antiviral response.

In conclusion, we provide evidence that cytokines like TGF-β can be used to manipulate the immune response to infection to maintain tolerance to self-antigens while still allowing for proper control of infections. By changing the cytokine milieu in the pancreas, coxsackievirus infection results in the induction of suppression as opposed to activation of autoimmunity without concomitant loss of the antiviral response. Our data build on previous reports on the role of TGF-β and, in a clinically relevant model of viral induced autoimmunity, clearly demonstrate that Tregs can be generated/activated after viral infection in the context of TGF-β and protect from type 1 diabetes. Taken together, our results further attest to a potential role for TGF-β in therapies directed at preventing viral-induced autoimmune diseases.

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