

Regulatory T cells protect from type 1 diabetes following induction by coxsackievirus infection in the context of TGF- β .

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ABSTRACT

Objective: Coxsackievirus infections have long been associated with the induction of type 1 diabetes (T1D). Infection with coxsackievirus B4 (CB4) enhances T1D onset in non-obese diabetic (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 T1D onset in NOD mice. This defect may contribute to susceptibility to viral-induced T1D. We asked whether the immune response following CB4 infection could be manipulated in order to re-establish peripheral tolerance while maintaining the immune response to virus.

Research Design and Methods: NOD mice expressing 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 following CB4 infection in the presence of TGF- β prevented T1D. Interestingly, 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 T1D without altering the anti-viral response. Finally, when TGF- β was administered systemically to NOD mice post-infection the incidence of T1D 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 T1D without concomitant loss of the capacity to control viral infection.

ABBREVIATIONS: APC: antigen presenting cells, CB4: coxsackievirus B4, NOD: non-obese diabetic, NOR: non-obese resistant, NS: not significant, PFU: plaque forming units, PI: post-infection, PLN: pancreatic lymph node, T1D: type 1 diabetes, TGF- β : transforming growth factor beta, Treg: regulatory T cell

Coxsackieviral infections commonly precede the onset of type 1 diabetes (T1D) in patients (1) and in animal models coxsackievirus B4 (CB4) infection significantly accelerated diabetes onset (2; 3). In non-obese diabetic (NOD) mice, islet destruction and development of T1D are preceded by a period of non-invasive peri-insulinitis 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 T1D (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 (TGF)- β in the function and/or generation of Tregs in the periphery (6; 9-14). In particular, Chen and colleagues demonstrated that *in vitro* stimulation naïve T cell 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). Further, 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 was also demonstrated enhanced survival of transplanted islets 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 following viral infection to protect from T1D.

NOD mice expressing TGF- β specifically in the β cells of the pancreas (NODTGF β mice) (17) were infected with CB4 and despite meeting all the criteria for susceptibility to viral-induced diabetes these mice were protected from T1D induction. Protection from T1D 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 post-infection (PI) 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

Mice. NOD/ShiLtJ mice were obtained from The Jackson Laboratory (Bar Harbor, USA). NODTGF β 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, USA) (17). All mice were bred and maintained in our rodent facility and tested for diabetes prior to 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). 10-12 week old mice were infected intraperitoneally with sublethal doses of 100 PFU.

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, USA) while biotin conjugated antibodies directed against CD40 (clone 3/23), CD80 (clone 16-10A1) and,

CD86 (clone GL1) were purchased from BD Biosciences (Mississauga, Canada).

Immunohistochemical staining. Tissue sections were prepared as previously described (18). Staining was performed using standard procedures for hematoxylin and eosin (iCapture center, Vancouver, British Columbia). 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 NODTGF β mice and mechanically disrupted. Single cell suspensions were treated for 10 minutes at 37°C in a PBS solution containing 1mg/ml of collagenase. Recovered cells were stained for flow cytometry.

Regulatory T cell functional inactivation. CB4 infected NODTGF β mice received intravenous injection of 450 μ g anti-CD25 antibody (clone PC61) at day 3 and day 6 PI. Alternatively, mice were injected intraperitoneally with a single dose of 100 μ g of purified anti-CTLA-4 (clone UC10-4B9, eBioscience, San Diego, USA) at 24 hours PI.

Regulatory T cells adoptive transfer. PLNs were harvested from CB4 infected NODTGF β mice at day 7 PI. Tregs were purified using a Robosep automated cell separator (Stem cell technologies, Vancouver, Canada). CD4⁺ CD25⁺ T cells were sequentially purified using modified CD4 and CD25 enrichment kits (Stem cell technologies, Vancouver, Canada). 1 X 10⁵ purified Tregs were adoptively transferred intraperitoneally into NOD mice at 24 hours PI.

Intracellular cytokine staining. Splenocytes were restimulated with PMA (500ng/ml) and ionomycin (10ng/ml) in the presence of Golgi Plug (BD Biosciences, Mississauga, Canada) in IMDM containing 10%FBS. 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), IL-17 (clone TC11-18H10.1) and TNF α (clone MP6-XT22) were obtained from eBiosciences (San Diego, USA). Fluorescently conjugated antibodies to IL-4 (clone 11B11) and IFN γ (clone XMG1.2) were obtained from BD Biosciences (Mississauga, Canada).

Systemic TGF- β treatment. NOD mice were injected intraperitoneally with 100ng of recombinant human TGF- β 1 (Sigma-Aldrich, Oakville, Canada) 24 hours PI with CB4.

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

RESULTS

CB4 infection in the context of TGF- β protects from type 1 diabetes. As reported previously (2), infection of NOD mice with CB4 resulted in a significant acceleration of diabetes in more than 60% of infected mice as compared to uninfected age-matched controls (Supplemental Figure 1A). This occurs regardless of gender, as viral-induction of T1D does not follow the same gender bias observed for spontaneous disease. NOD mice harboring a transgene driving expression of TGF- β specifically in the pancreas (NODTGF β) were previously described (17) and they spontaneously develop diabetes, albeit at a reduced rate compared to non-transgenic NOD mice (Supplemental Figure 1B) (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 insulinitis with susceptibility to viral-induction of disease (2; 21). Islet inflammation in uninfected NODTGF β mice was not significantly different from their NOD counterparts with nearly 30% of islets

presenting with invasive insulinitis at the time of infection (10-12 weeks old) (Figure 1A and Supplemental Table 1), indicating the presence of activated β cell specific autoreactive T cells within the pancreas. As such, NODTGF β mice meet the criteria previously described (2; 21) for susceptibility to CB4-induced T1D. Strikingly, NODTGF β mice infected with CB4 did not develop T1D unlike their NOD counterparts (Figure 1B). Upon infection, no significant change in islet inflammation was observed in NODTGF β mice whereas by 7 days PI, significant increases in insulinitis were observed in NOD mice (Figure 1A and Supplemental Table 1). This was particularly marked in NOD mice that were diabetic by day 7 PI as more than 90% of islets in these mice presented with invasive insulinitis (n=5). Additionally, the percentage of islets free of insulinitis was not significantly decreased in NODTGF β mice following CB4 infection (Figure 1A and Supplemental Table 1) indicating that no new islets were being targeted following infection. This phenotype is reminiscent of both the BDC2.5 TCR transgenic model and the non-obese resistant (NOR) mouse where Tregs prevent T1D by precluding the progression of islet pathology from peri-insulinitis to invasive insulinitis (22; 23).

NODTGF β mice are polarized to a Th1 response following infection with CB4. Previously NODTGF β mice were found to be polarized towards a Th2 phenotype at steady state (17). Furthermore, it has recently been established that TGF- β acts as a co-factor 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 T1D following infection. Cytokine production from splenic T cells was analyzed *ex vivo* prior to infection and at 7 days PI. Prior to infection, very few T cells were observed to produce cytokines and slightly more T cells from NODTGF β mice compared to NOD mice were observed

to produce IL-4 although this difference was not statistically significant (Supplemental Figure 2). As predicted following a viral infection, T cells preferentially produced Th1 cytokines (IFN γ and TNF α) in both NODTGF β and NOD mice (Figure 2). Interestingly, despite previous reports of Th2 polarization prior to infection (17), T cells from NODTGF β mice did not abundantly produce IL-4 (Figure 2) clearly confirming a Th1 response following 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 NODTGF β mice compared to NOD mice (Figure 2). These data clearly indicate that NODTGF β mice mount a Th1 response similar to NOD mice following infection and that polarization towards a Th2 or Th17 phenotype was not involved in the protection from T1D.

CB4 infection of NODTGF β mice leads to a significant increase in the number of Tregs in the pancreatic lymph node 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 NODTGF β mice. Following CB4 infection, significantly increased levels of CD4⁺ Foxp3⁺ Tregs were found in PLNs (Figure 3A-D), but not the spleen (Supplemental Figure 3) of NODTGF β mice as compared to 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 to insulinitis severity and/or onset of spontaneous T1D (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 NODTGF β mice as compared to similarly infected NOD mice, uninfected NOD mice and uninfected NODTGF β mice (Figure 3E). As expected, no differences in activation were observed between T cells in the PLNs of NODTGF β or NOD mice following infection (Supplemental Figure 4). This data suggests 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 T1D.

Infection of β cells of the pancreas is required for induction of Tregs. It was previously demonstrated that CB4 infection induced T1D via presentation of pancreatic β cells and their self-antigens to the pre-existing population of diabetogenic T cells (27). In order to determine whether infection of β cells was also necessary to activate or generate functional Tregs, NODTGF β 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 (Figure 3 C-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.

Functional inactivation of Tregs re-establishes susceptibility of NODTGF β to type 1 diabetes. To confirm the functional role of these TGF- β -induced Tregs in the protection from diabetes, CB4 infected NODTGF β mice were treated with an anti-CD25 antibody that has been previously

demonstrated to functionally inactivate and/or deplete Tregs (28). Antibody treatment PI re-established susceptibility of NODTGF β mice to CB4-induced T1D as disease developed with the same kinetics and incidence to that observed for NOD mice following infection while mock-treated mice remained protected from disease (Figure 4A, 1B). These data confirm that T1D can be induced in NODTGF β mice and they are not simply impervious to the induction of disease following 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 NODTGF β mice protect CB4 infected NOD mice from T1D. To further demonstrate the functional role of TGF- β induced Tregs in the protection from T1D, Tregs were purified from the PLNs of NODTGF β mice at 7 days PI and adoptively transferred to NOD mice 24 hours post-CB4 infection. Following Treg transfer, recipient NOD mice that were adoptively transferred with donor Tregs from infected NODTGF β mice were protected from diabetes development for over 15 days PI while mock-treated mice still developed accelerated diabetes following infection (Figure 4B). However, the observed protection may only be transient as one of the adoptively transferred mice developed diabetes 17 days post-infection (Figure 4B). 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 T1D 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 NODTGF β mice with a neutralizing antibody directed against CTLA-4 at 24 hours PI. Antibody treated NODTGF β mice developed diabetes with increased incidence compared to mock-treated NODTGF β mice (Figure 4C). This suggests that Tregs maintain protection from T1D in a CTLA-4 dependent manner.

NODTGF β mice show reduced upregulation of costimulatory molecule following infection. TGF- β treated antigen presenting cells (APCs) have previously been shown to induce tolerance in a Treg dependent manner (31; 32). Compared to infected NOD mice, flow cytometry analysis revealed that macrophages isolated from the pancreas, PLNs and spleen of infected NODTGF β mice at day 7 PI have significantly reduced surface expression of the costimulatory molecule CD40 (Figure 5A-B). A similar trend is observed with the costimulatory molecules CD80 and CD86 (Figure 5 C-D). Their surface expression was significantly reduced on macrophages isolated from the spleen of infected NODTGF β mice, this reduction was also observed on macrophages isolated from the PLNs although this difference was not statistically significant (Figure 5C,D). Interestingly, this reduced upregulation was not observed on dendritic cells from the spleen (Supplemental Figure 5) or PLN (data not shown). It is interesting to note that NOD and NODTGF β macrophages express similar levels of costimulatory molecules prior to infection (Supplemental Figure 6) and that these molecules are upregulated to the same extent in both mice at day 3 PI (Supplemental Figure 7). Differences in surface expression of costimulatory molecules were not observed until day 7 (Figure 5). This time frame coincides with the kinetics of increases in the number of Tregs following infection in the

NODTGF β mice, suggesting that presentation of pancreatic self-antigen by these “semi-mature” macrophages may act in the generation or activation of these protective Tregs in the NODTGF β mice. Furthermore, we observed that functional inactivation of Tregs did not re-establish upregulation of costimulatory molecules on macrophages (data not shown) indicating that they are unlikely to be the targets of suppression in our model. Interestingly, viral clearance of both CB3 (18; 33) and CB4 (Figure 6A) was not affected in NODTGF- β mice when compared to 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 as delayed clearance typically results in a fatal outcome and no increase in death was observed in the NODTGF β mice following 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 T1D. 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 T1D. One day following 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 PI (Figure 7A). This TGF- β mediated protection correlated with increases in Tregs in both the PLNs (Figure 7B) and the spleen following infection (Figure 7C) compared to similarly treated mock-infected mice. Protection was transient, however, as disease induction was observed by day 28 PI (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 multi-dose 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 (Figure 6B), 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 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 pre-existing population of autoreactive T cells (2; 3). As viruses, such as coxsackievirus, are common human pathogens, this mechanism also likely operates to induce T1D in humans. This suggests that protective approaches identified in mouse models would likely translate into potential therapies. Our data builds on previous reports on the protective role of TGF- β in order to demonstrate that the immune system can be manipulated so 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 indicates 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 pre-existing population of autoreactive T cells has previously been established as the primary criteria for susceptibility to viral-induction of T1D (2; 3). Similar to NOD mice from which they were derived, NODTGF β 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). Since NODTGF β mice fulfill the criteria for susceptibility to viral-induced disease such as the development of autoreactive T cells and susceptibility to disease following functional inactivation of Tregs our results strongly suggest that NODTGF β mice are protected by mechanisms that are actively induced following infection in the context of TGF- β rather than simply being impervious to viral-induced T1D. We observed significant increases in the percentage of Tregs in the PLNs and, more importantly, the pancreas of NODTGF β mice. Interestingly, we do not observe increased presence of Tregs in NODTGF β mice prior to infection as compared to wild-type counterparts confirming the role of viral infection in the induction of Tregs in our model. This data is in contrast to observations where a pulse of TGF- β induced prior to the end of the priming phase of disease was sufficient to induce Tregs without any further manipulations and mediate 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 as adoptive transfer of Tregs purified from the PLNs was sufficient to protect NOD mice from CB4-induced T1D. 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-insulinitis to invasive insulinitis. Importantly, 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 in order to prevent disease. As such, the protection from disease following infection in NODTGF β mice can be explained by two non-exclusive mechanisms. First, protection may be maintained either by a re-establishment or an increase in the suppressive capacity of Tregs. Second, conversion of β cell-reactive T lymphocytes 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 following infection was sufficient to induce protection. In this regard, two separate reports have demonstrated that *in vitro* expanded antigen specific Tregs have greater T1D suppressive capacity than polyclonal Tregs in NOD mice (35; 36). In another model of T1D, it was demonstrated that adoptive transfer of as little as 2000 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 NODTGF β mice following infection should be amply sufficient to completely protect from the induction of T1D.

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 NODTGF β mice do not mature to the same extent as macrophages from NOD mice in response to infection. Since the timing of this defect corresponds with the increase in Tregs in our model, we speculate that antigen presentation by these “semi-mature” 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 “semi-mature” phenotype and were capable of inducing expansion of Tregs (40). These type II monocytes were further demonstrated to preferentially secrete immunosuppressive rather than pro-inflammatory 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 NODTGF β and NOD mice. Viral clearance is likely unaffected since 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, in our model. This is supported by previous observations in the BDC2.5 model where macrophages engulf islets in response to CB4 infection and are likely responsible for induction of T1D (41). Furthermore, since 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.

NODTGF β mice have previously been demonstrated to be polarized towards a Th2 phenotype at steady-state (17). As T1D 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, following infection, we observed that T cells from NODTGF β 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 T1D in CB4-infected NODTGF β mice. Further, while 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 post-infection and no increases in Th17 cells were observed in the NODTGF β 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 NODTGF β mice. It has been reported that IFN γ can inhibit development of Th17 cells (44) and this likely explains their absence following infection. Taken together, these results strongly indicate that suppression of T1D was not the result of polarization of T helper cells towards a Th2 phenotype.

Finally, our data supports a therapeutic role for TGF- β , as systemic treatment was sufficient to significantly reduce T1D incidence by day 15 PI. 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 anti-viral response

In conclusion, we provide evidence that cytokines like TGF- β can be used to manipulate the immune response to infection in order 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 anti-viral response. Our data builds on previous reports on the role of TGF- β and, in a clinically relevant model of viral induced autoimmunity, clearly demonstrates that Tregs can be generated/activated following viral infection in the context of TGF- β and protect from T1D. 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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FIGURE LEGENDS

Figure 1. CB4 infection of TGF- β expressing NOD mice does not induce T1D

A) Histological analysis of pancreata from NOD and NODTGF β mice 7 days PI with CB4 or mock-infection with 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 8 mice per group. B) Diabetes incidence of NOD (filled squares) and NODTGF β (open diamonds) mice following infection with CB4. Stars denote significant change in overall phenotype and change in mice presenting with insulinitis.

Figure 2. T cells from NODTGF β mice are polarized to a Th1 phenotype following CB4 infection.

Cytokine production from A) CD4 T cells and B) CD8 T cells from the spleen of NOD (black bars) and NODTGF β (white bars) mice was measured *ex vivo* by intracellular flow cytometry following restimulation with PMA and ionomycin. Data are presented as mean \pm s.e.m. and are representative of 4 mice per group from 2 separate experiments.

Figure 3. CB4 infection of NODTGF β mice leads to increases in Tregs in the pancreatic lymph node and in the pancreas.

Representative histograms of Foxp3 expression by CD4⁺ T cells in the PLN of NODTGF β mice 7 days following A) mock-infection with DMEM or infection with B) CB4 or C) CB3. Numbers shown on the histograms represent percentage of Foxp3 positive cells. Isotype controls are represented by shaded grey areas. D) Average percentage of Foxp3⁺ CD4⁺ T cells in the PLN of NODTGF β mice following mock-infection with DMEM (black bars, n=10) or infection with CB4 (white bars, n=13) or CB3 (grey bars, n=12) E) Average percentage of CD4⁺ cells expressing Foxp3 in the pancreas of NOD (black bars, uninfected: n=4, infected: n=14) or NODTGF β mice (white bars, uninfected: n=4, infected: n=7). Data are presented as mean \pm s.e.m. from at least 2 separate experiments.

Figure 4. TGF- β induced Tregs protect from T1D diabetes in a CTLA-4 dependent manner.

A) Diabetes incidence of CB4 infected NODTGF β mice treated with anti-CD25 (filled diamonds) antibodies or mock-treated with DMEM (open diamonds). B) Diabetes incidence of CB4 infected NOD mice adoptively transferred with NODTGF β Tregs (open diamonds) or mock-treated (filled squares) C) Diabetes incidence of CB4 infected NODTGF β mice treated with anti CTLA-4 antibodies (filled diamonds) or mock-treated with DMEM (open diamonds).

Figure 5. Pancreatic expression of TGF- β reduces upregulation of costimulatory molecules on macrophages following CB4 infection.

A) Representative histograms of CD40 expression on macrophages (CD11b⁺ CD11c⁻) from NOD (black line) or NODTGF β (grey line) mice 7 days PI with CB4. B) Average mean fluorescence intensity of B) CD40, C) CD80 and D) CD86 expression on macrophages (CD11b⁺ CD11c⁻) from NOD (black bars) or NODTGF β (white bars) mice. Data from the spleen, PLN and pancreas are presented as mean \pm s.e.m. and are representative of at least 4 mice per group from at least 2 separate experiments.

Figure 6. Clearance of viral infection is not affected in the presence of TGF- β .

Viral load in pancreas of A) NOD mice (black bars, n=5) and NODTGF β mice (white bars, n=6) or B) NOD mice (black bars, n=5) and NOD mice treated systemically with 100ng of

recombinant TGF- β (white bar, n=5) were measured post-CB4 infection. Data are presented as log 10 plaque forming units per gram of tissue and represent the average from duplicate values obtained from each mouse in the group. Data are representative of at least 2 separate experiments. Any samples not yielding any plaque forming units were assigned a value of 2 log 10 pfu/g representing the limit of detection of the assay.

Figure 7. Systemic TGF- β treatment transiently protects from diabetes in a Treg dependent manner. A) Diabetes incidence of CB4 infected NOD mice treated 24 hours PI with 100ng of recombinant human TGF- β (open diamonds) or mock-treated with DMEM (filled squares). Average percentage of Foxp3⁺ CD4⁺ T cells in the B) PLN or C) spleen in TGF- β treated NOD mice 7 days following infection with CB4 (white bars, n= 9) or mock-infection with DMEM (black bars, n=6). Data are presented as mean value \pm s.e.m. and are representative of at least 2 separate experiments.

NOTE: The figures for this article can be found using the link entitled “Figures”. (Available at <http://dx.doi.org/10.2337/db07-1460>.)