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Inducible Adeno-Associated Virus-Mediated IL-2 Gene Therapy Prevents Autoimmune Diabetes

Kevin S. Goudy,* Mark C. Johnson,* Alaina Garland,* Chengwen Li, † R. Jude Samulski, †,‡ Bo Wang,* and Roland Tisch*,*§

IL-2 and TGF-β1 play key roles in the immunobiology of Foxp3-expressing CD25+CD4+ T cells (Foxp3+Treg). Administration of these cytokines offers an appealing approach to manipulate the Foxp3+Treg pool and treat T cell-mediated autoimmunity such as type 1 diabetes. However, efficacy of cytokine treatment is dependent on the mode of application, and the potent pleiotropic effects of cytokines like IL-2 may lead to severe side effects. In the current study, we used a gene therapy-based approach to assess the efficacy of recombinant adeno-associated virus vectors expressing inducible IL-2 or TGF-β1 transgenes to suppress ongoing β cell autoimmunity in NOD mice. Intramuscular vaccination of recombinant adeno-associated virus to 10-wk-old NOD female mice and a subsequent 3 wk induction of IL-2 was sufficient to prevent diabetes and block the progression of insulitis. Protection correlated with an increased frequency of Foxp3+Treg in the periphery as well as in the draining pancreatic lymph nodes and islets. IL-2 induced a shift in the ratio favoring Foxp3+Treg versus IFN-γ-expressing T cells infiltrating the islets. Induction of IL-2 had no systemic effect on the frequency or activational status of T cells and NK cells. Induction of TGF-β1 had no effect on the Foxp3+Treg pool or the progression of β cell autoimmunity despite induced systemic levels of activated TGF-β1 that were comparable to IL-2. These results demonstrate that inducible IL-2 gene therapy is an effective and safe approach to manipulate Foxp3+Treg and suppress T cell-mediated autoimmunity and that under the conditions employed, IL-2 is more potent than TGF-β1. The Journal of Immunology, 2011, 186: 3779–3786.

Type 1 diabetes (T1D) is characterized by the T cell-mediated destruction of the insulin-producing β cells found in the islets of Langerhans (1–3). The breakdown of self-tolerance within the T cell compartment is attributed to genetic and environmental factors, which promote a functional imbalance favoring pathogenic type 1 immune regulatory β cell-specific T effectors. Accordingly, one strategy of immunotherapy for T1D and other T cell-mediated autoimmune diseases has been the induction and/or expansion of regulatory T cells (Treg) (4). In this regard, Foxp3-expressing CD4+ Treg (Foxp3+Treg) have garnered a great deal of interest.

Foxp3+Treg are highly potent immunoregulatory effectors that modulate T cell and APC responses via cell contact-dependent and -independent mechanisms (5–8). Foxp3+Treg have been subdivided into natural and adaptive effectors. Natural Foxp3+Treg differentiate in the thymus, whereas adaptive Foxp3+Treg are derived from conventional non-Foxp3+expressing CD4+ T cells in the periphery. Cytokines such as IL-2 and TGF-β1 play critical roles in the maintenance and/or differentiation of natural and adaptive Foxp3+Treg. For instance, IL-2 is required for expansion and survival of Foxp3+Treg in vivo (9–13). In addition, IL-2 and TGF-β1 are necessary for differentiation of conventional CD4+ T cells into Foxp3+Treg (14–16). The lack of Foxp3+Treg in mice results in systemic T cell-mediated autoimmunity (17, 18). Furthermore, in the NOD mouse, a spontaneous model of T1D, defects in the number and/or function of Foxp3+Treg have been associated with progression of the diabetogenic response (19–22). The latter is in part attributed to reduced expression of IL-2 by T cells in NOD mice, which is believed to limit intraislet survival of Foxp3+Treg (23–25). Dysregulation of Foxp3+Treg has also been reported in diabetic patients (26).

Administration of cytokines such as IL-2 and TGF-β1 has been used to manipulate T cell-mediated autoimmunity. A key factor determining the efficacy of the approach is the mode of cytokine delivery. For instance, continuous administration of rIL-2 alone was insufficient to block β cell autoimmunity in NOD mice (27). Lack of efficacy is likely due to the short 1/2 of IL-2 in serum coupled with limitations in dosing. For example, increased doses of IL-2 could lead to expansion of pathogenic T effectors, in addition to activation of nonautoimmune T and NK cells, leading to systemic complications (28). Low-dose administration of Ab complexes of IL-2 has overcome some of these issues and has been shown to induce Foxp3+Treg and prevent the development of diabetes in NOD mice (24). However, the IL-2–Ab complexes need to be administered continuously over a number of weeks (24). An alternative approach to cytokine-based immunotherapy is an inducible gene therapy strategy. The use of inducible genetic vaccines obviates the need for production, purification, and storage of...
recombinant proteins and permits regulation of expression of the transgene in vivo.

With this in mind, we assessed recombinant adeno-associated virus (rAAV)-based transfer of inducible genes encoding IL-2 and TGF-β1 to manipulate β cell autoimmunity in NOD mice. rAAV vectors are highly amenable for gene delivery because a wide variety of tissues can be transduced with minimal toxicity, leading to long-term transgene expression in vivo without significant immunogenicity (29, 30). Furthermore, upon transduction, rAAV largely persists as nonintegrating circular monomers or concatemers in the nucleus, and consequently, the risk of genomic insertion and insertional mutagenesis is low (29, 30). Clinical studies using rAAV-mediated gene transfer to treat various genetic disorders have generated promising results (31). Improved methods to engineer and produce packaged rAAV coupled with the availability of multiple serotypes to manipulate the immunogenicity of the recombinants further enhance clinical application of rAAV-based gene transfer (32).

In the current study, we demonstrate that i.m. injection of rAAV followed by a short pulse of IL-2 but not TGF-β1 is sufficient to prevent diabetes in NOD mice by increasing the frequency of Foxp3+Treg without nonspecifically activating and/or expanding T cells and other effector cells.

Materials and Methods

Mice

NOD/LtJ and NOD.CB17-Prkdcscid/J (NOD.scid) mice were bred and maintained under specific pathogen-free conditions in an American Association for Laboratory-accredited animal facility. All procedures were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

rAAV engineering, packaging, and vaccination

Full-length cDNA encoding murine Il2 (of the NOD genotype) and Tgfb1 were subcloned into an AAV-Tet-on vector plasmid (33). The AAV–Tet-on vector (a kind gift provided by Dr. Sihong Song, University of Florida) contains a bidirectional promoter with a tetracycline response element flanked by mini-CMV promoters that regulate expression of the Il2 or Tgfb1 genes and the reverse transcriptional transactivator gene (Fig. 1A). Transgene expression was verified by measuring via ELISA IL-2 and TGF-β1 secretion by HEK 293 cells transfected with AAV–Tet-on–IL-2/TGF-β1 plasmid DNAs.

AAV virus production was previously described (34). Briefly, HEK 293 cells were transfected via calcium phosphate with the adeno helper-encoding plasmid (pXX6-80), AAV1-encoding plasmid (pXR-1), and the Tet-on–IL-2/TGF-β1 plasmids to package AAV serotype 1 (AAV1) virus. Nuclear fractions were harvested and virus purified with an iodixonal (Sigma-Aldrich) gradient. The virus-containing fractions and titer were determined by Southern blot.

NOD female mice were vaccinated with 5 × 10¹⁰ viral particles (v.p.) of rAAV1–Tet-on–IL-2/TGF-β1 (AAV1–Tet–IL-2/TGF-β) in contralateral hind limb muscles using an insulin syringe. Twenty-four hours after rAAV injection, mice were fed ad libitum chow containing 200 mg/kg doxycycline (Dox); BioServ) for 3 wk.

ELISA

Serum was collected, diluted 1:3 in 1% BSA PBS, and levels of IL-2 or TGF-β1 at varying times postinjection were measured. The anti–IL-2 Ab set (JES6-1 and JES6-5; ebiscience) was used at 2 μg/ml on a high-binding ELISA plate (Costar). Active TGF-β1 was detected using a human TGF-β1 duo set (R&D Systems) according to the manufacturer’s recommendations.

T cell isolation

Single-cell suspensions prepared from the pancreatic lymph nodes (PLN) and spleen were filtered with a 70-μm strainer (Fisher Scientific). PBL were obtained via submandibular puncture using lancets (Golden Rod) and RBC lysed with ACK solution. Islet-infiltrating cells were isolated from purified, hand-picked islets. Briefly, pancreases were digested with 2.0 mg/ml collagenase P (Roche) for 20 min at 37°C, and islets purified on a Ficoll (Sigma-Aldrich) gradient. Lymphocytes infiltrating the islets were harvested by dissociating the islets using enzyme-free cell dissociation solution (Sigma-Aldrich).

FACS

Total cells from the respective tissues were stained with a variety of fluorochrome-conjugated mAbs specific for: CD3 (2C11), CD4 (L3T4), CD8 (Ly-2), CD25 (PC61.5), CD44 (IM7), CD62L (MEL14), DX5 (DX5), and Foxp3 (FJK.16) (ebiscience). Fc receptors were blocked with a 1/200 dilution of rat Ig prior to staining. Intracellular Ki67 (B56; BD Biosciences) staining was done using Cytofix/Cytoperm reagents (ebiscience) according to the manufacturer’s specifications. Data were acquired on a Cyan flow cytometer (DakoCytomation) and analyzed using Summit software (DakoCytomation).

Intracellular cytokine staining was performed on single-cell suspensions as previously described (35). Briefly, lymphocytes were stimulated with 10 ng/ml PMA (Sigma-Aldrich) and 150 ng/ml ionomycin (Sigma-Aldrich) in complete RPMI 1640 medium for 6 h at 37°C, 10 μg/ml brefeldin A (Sigma-Aldrich) was added for the final 4 h of incubation. Cells were stained for surface molecules, fixed, and permeabilized with Cytofix/Cytoperm reagents (ebiscience) and stained for intracellular IFN-γ (XM1g.1).2 (ebiscience).

FIGURE 1. Inducible expression of IL-2 and TGF-β1 in rAAV-vaccinated NOD mice. A, Organization of the Tet-on/Dox-inducible expression cassette. B and C, Groups of five NOD mice were injected i.m. with 5 × 10¹⁰ v.p. AAV1–Tet–IL-2 (IL-2), AAV1–Tet–TGF-β (TGF-β), and AAV1–Tet–IL-2 plus AAV1–Tet–TGF-β (IL-2+TGF-β) or left untreated (Untx) and serum levels of IL-2 (B) and active TGF-β1 (C) measured via ELISA after 1 wk of induction (shaded) and 1 wk postinduction (e.g., Week 4). D, Similarly, groups of five NOD mice were injected i.m. with 5 × 10¹⁰ v.p. AAV1–Tet–IL-2, fed Dox-containing chow (Induced) or not (Uninduced), and serum IL-2 measured. ***p < 0.001 (two-way ANOVA ± SEM): IL-2 alone and IL-2 plus TGF-β versus Untx and TGF-β alone (B); TGF-β alone and IL-2 plus TGF-β versus Untx and IL-2 alone (C); induced AAV1–Tet–IL-2 versus uninduced AAV1–Tet–IL-2 and Untx (D).
Cell adoptive transfers

Splenocytes from diabetic NOD mice ($5 \times 10^6$) were suspended in PBS and injected i.p. into 6–8 wk-old NOD.scid male mice alone or in combination with MACS (Miltenyi Biotec) isolated splenic CD4+ T cells ($3 \times 10^6$) from NOD mice treated with rAAV or left untreated. Purity of isolated CD4+ T cells was >95% and the frequency of Foxp3+CD25+CD4+ T cells determined as described above. Mice were monitored biweekly post-transfer for diabetes as described below.

Diabetes monitoring and insulitis scoring

Diabetes was diagnosed upon elevated urine glucose readings using Ketostix (Bayer).

Pancreases were harvested and fixed with formalin for 24 h. Serial sections 90 um apart were prepared and stained with H&E. More than 100 islets were scored per group, except in the case of AAV-Tet–TGF-β1 mice, in which only one animal was used to determine insulitis at the final time point.

Results

rAAV-encoded inducible IL-2 but not TGF-β1 is sufficient to increase Foxp3+Treg in vivo

It is well established that IL-2 and TGF-β1 play key roles in the immunobiology of Foxp3+Treg and that these cytokines can be exploited via genetic vaccination for the purpose of immunotherapy of T cell-mediated autoimmunity. Nevertheless, continued systemic expression of IL-2 and TGF-β1 may lead to unwanted complications due to the pleiotropic effects of these cytokines. In addition, the relative efficacy of IL-2 versus TGF-β1 in mediating a protective response is ill defined. Accordingly, rAAV vectors were established encoding murine IL-2 and TGF-β1, using a tetracycline/Dox-inducible (e.g., Tet-on) transcription cassette to regulate transgene expression in vivo (Fig. 1A). As an initial step, the in vivo inducibility of the respective rAAV recombinants was tested. NOD mice were vaccinated i.m. with $5 \times 10^{10}$ v.p. of AAV1-Tet–IL-2 or AAV1-Tet–TGF-β1 or left untreated and then fed Dox-containing chow for 3 wk only. One week after transgene induction, levels of IL-2 and active TGF-β1 were elevated in the serum of NOD mice receiving AAV1-Tet–IL-2 and AAV1-Tet–TGF-β1, respectively, compared with serum levels prior to Dox-induction and in untreated mice (Fig. 1B, 1C). Furthermore, 1 wk after removing the Dox chow and stopping transgene expression, serum levels of IL-2 and active TGF-β1 were reduced in the respective rAAV-treated NOD mice to levels detected prior to Dox feeding and that seen in untreated animals (Fig. 1B, 1C). Notably, no significant increase in serum levels of IL-2 was detected in NOD mice vaccinated with AAV1-Tet–IL-2 but not fed Dox-containing chow, indicating minimal leakiness of transgene expression in the absence of induction (Fig. 1D). Together, these findings demonstrate that IL-2 and TGF-β1 transgene expression is readily induced and tightly regulated.

The in vivo effect of IL-2 and TGF-β1 induction on Foxp3+Treg was assessed. Ten-week-old NOD female mice were vaccinated with AAV1-Tet–IL-2 and AAV1-Tet–TGF-β1 alone or in combination. Dox-containing chow was then given for 3 wk, after which animals received normal chow. A temporal analysis of PBL demonstrated a significant increase in the frequency of Foxp3+CD25+CD4+ T cells after 1 and 2 wk of Dox induction in NOD mice treated with AAV1-Tet–IL-2 alone or the combination of AAV1-Tet–IL-2 and AAV1-Tet–TGF-β1 compared with uninduced levels for the same animals (Fig. 2A, 2B). Despite coinduction of TGF-β1, the frequency of Foxp3+CD25+CD4+ T cells was similar between the two groups (Fig. 2A, 2B). In addition, a significant albeit transient increase in the expression level of CD25 (Fig. 2C) and Foxp3 (Fig. 2D) by Foxp3+CD25+CD4+ T cells was detected with Dox treatment in these experimental groups. Furthermore, the elevated frequency of Foxp3+Treg in PBL of Dox-fed, AAV1-Tet–IL-2–injected mice correlated with an increased frequency of proliferating Foxp3+CD25+CD4+ T cells as measured by Ki67 staining (Fig. 2E). Once Dox treatment was halted, the frequency of Foxp3+CD25+CD4+ T cells in PBL was reduced to levels similar to that prior to transgene induction (Fig. 2A, 2B). In contrast, no increase in Foxp3+CD25+CD4+ T cells or changes in the expression level of CD25 and Foxp3 (Fig. 2A–D) or proliferation (data not shown) were detected in NOD mice injected with AAV1-Tet–TGF-β1 alone and/or in uninduced animals injected with AAV1-Tet–IL-2 or mice left untreated.

In NOD mice vaccinated with AAV1-Tet–IL-2 and treated with Dox for 3 wk, a marked increase in the frequency of Foxp3+CD25+CD4+ T cells was also observed in the spleen, PLN, and popliteal lymph nodes but not in the thymus relative to AAV1-Tet–IL-2–injected animals left uninduced (Fig. 2F). Similar results were obtained in NOD mice treated with the combination of AAV1-Tet–IL-2 and AAV1-Tet–TGF-β1.

A potential concern regarding systemic expression of IL-2 and TGF-β1 is nonspecific effects on non-Foxp3–expressing T cells and other immune effectors (28, 36–39). Analysis of PBL of NOD mice vaccinated with AAV1-Tet–IL-2, AAV1-Tet–TGF-β1, or both and induced for 2 wk showed no significant increase in the frequency of CD25+CD4+ T cells and non-Foxp3–expressing CD25+CD4+ T cells (Fig. 3A) or changes in the frequency of CD8+ T cells (Fig. 3B) and NK cells (Fig. 3C). Furthermore, no difference was detected in the total cellularity of the spleen and PLN of AAV1-Tet–IL-2–treated animals fed Dox versus normal chow (Fig. 3D). In contrast, total cellularity was markedly reduced in the islets of the AAV1-Tet–IL-2–treated NOD mice in which IL-2 expression was induced (Fig. 3D). Notably, this reduction correlated with a decrease in the frequency of islet-infiltrating CD4+ and CD8+ T cells actively proliferating, as measured by Ki67 staining (Fig. 3E). Together, these results indicate that a short course of transgene induction in NOD mice vaccinated with AAV1-Tet–IL-2 or the combination of AAV1-Tet–IL-2 and AAV1-Tet–TGF-β1 but not AAV1-Tet–TGF-β1 alone results in an increased frequency of Foxp3+Treg in PBL and peripheral tissues. In addition, induction of IL-2 has no significant systemic effects on non-Foxp3–expressing CD4+ T cells, CD8+ T cells, NK cells, and overall cellularity.

rAAV-encoded inducible IL-2 but not TGF-β1 is sufficient to suppress ongoing β cell autoimmunity and prevent diabetes

Next, the efficacy of rAAV-mediated IL-2 and TGF-β1 transgene induction to suppress ongoing β cell autoimmunity and block the development of overt diabetes was tested. NOD female mice 10 wk of age were vaccinated with AAV1-Tet–IL-2, AAV1-Tet–TGF-β1, or both rAAV vectors or left untreated, fed Dox-containing chow for 3 wk, and then the onset of diabetes was monitored. At 10 wk of age, β cell autoimmunity is well established in NOD female mice. Consistent with the lack of an increase in Foxp3+ Treg detected above, NOD female mice vaccinated with AAV1-Tet–TGF-β1 developed diabetes at a similar time of onset and frequency (7 out of 10) as the untreated group of NOD mice (9 out of 10) (Fig. 4A). In contrast, a delayed onset and reduced frequency of diabetes was detected in NOD female mice vaccinated with either AAV1-Tet–IL-2 (4 out of 15) or AAV1-Tet–IL-2 plus AAV1-Tet–TGF-β1 (2 out of 5) (Fig. 4A). Furthermore, histological analyses of pancreases of nondiabetic 35-wk-old NOD female mice demonstrated that a significant frequency of islets remained free of insulitis in AAV1-Tet–IL-2 and AAV1-Tet–IL-2 plus AAV1-Tet–TGF-β1–injected animals relative to those vaccinated with AAV1-Tet–TGF-β1 alone or left untreated (Fig. 4B).
To further assess the mechanism of protection induced by IL-2 treatment, a coadoptive transfer experiment was carried out. Ten-week-old NOD female mice were vaccinated with AAV1-Tet–IL-2 and fed Dox-containing chow or not for 3 wk. CD4+ T cells were then isolated from the spleen of rAAV1-injected mice, mixed with splenocytes from diabetic NOD mice, and transferred into NOD.scid recipients. All of the NOD.scid mice receiving diabetogenic splenocytes only or the mix containing CD4+ T cells from uninduced AAV1-Tet–IL-2–injected NOD donors developed diabetes (Fig. 5A). In contrast, four out of five NOD.scid mice receiving diabetogenic splenocytes and CD4+ T cells from Dox-treated, AAV1-Tet–IL-2–injected NOD donors remained free of diabetes (Fig. 5A). The latter result was consistent with an increased frequency of Foxp3+CD25+CD4+ T cells detected in the pool of splenic CD4+ T cells isolated from induced versus uninduced NOD mice vaccinated with AAV1-Tet–IL-2 (Fig. 5B).

In sum, these results demonstrate that a short course of IL-2 induction is sufficient to effectively suppress ongoing β cell autoimmunity and prevent the onset of overt diabetes in NOD female mice. Furthermore, IL-2–mediated protection correlates with a CD4+ T cell pool with an increased capacity to suppress the adoptive transfer of diabetes in NOD.scid recipients. In contrast, induction of TGF-β1 has a minimal effect on the diabetogenic response under the conditions tested.

rAAV-encoded inducible IL-2 promotes increased Foxp3+Treg and reduced T effectors in islet infiltrates

The above insulitis data indicate that IL-2 induction results in significantly reduced islet infiltration that persists long-term (Fig.
With this in mind, the status of the islet-infiltrating T cells in NOD mice vaccinated with AA V1–Tet-IL-2 and fed Dox-containing chow for 3 wk was examined. Initially, proliferation of non-Foxp3–expressing CD25+CD4+ and CD8+ T cells was measured as determined by Ki67 staining. A significant reduction in the frequency of Ki67-positive Foxp32CD25+CD4+ T cells (Fig. 6A) and CD8+ T cells was detected in the islets of induced versus uninduced AA V1-Tet–IL-2–vaccinated NOD mice. Similarly, the frequency of IFN-γ–expressing CD4+ and CD8+ T effectors in the islets of induced versus uninduced NOD mice was markedly diminished (Fig. 6B). Furthermore, an increased frequency of Foxp33CD25+CD4+ T cells was detected in the islets of induced versus uninduced NOD mice (Fig. 6C), resulting in a reduced ratio of effector T cells to Foxp3+Treg (Fig. 6C).

4B). With this in mind, the status of the islet-infiltrating T cells in NOD mice vaccinated with AA V1–Tet-IL-2 and fed Dox-containing chow for 3 wk was examined. Initially, proliferation of non-Foxp3–expressing CD25+CD4+ and CD8+ T cells was measured as determined by Ki67 staining. A significant reduction in the frequency of Ki67-positive Foxp3+CD25+CD4+ T cells (Fig. 6A) and CD8+ T cells was detected in the islets of induced versus uninduced AA V1-Tet–IL-2–vaccinated NOD mice. Similarly, the frequency of IFN-γ–expressing CD4+ and CD8+ T effectors in the islets of induced versus uninduced NOD mice was markedly diminished (Fig. 6B). Furthermore, an increased frequency of Foxp33CD25+CD4+ T cells was detected in the islets of induced versus uninduced NOD mice (Fig. 6C), resulting in a reduced ratio of effector T cells to Foxp3+Treg (Fig. 6C).

FIGURE 3. AA V1-Tet–IL-2 preferentially targets Foxp3+Treg expansion but has no nonspecific systemic effects on CD4+ and CD8+ T cells and NK cells. A, PBL from groups of five NOD female mice left untreated (Untx) or vaccinated with AA V1-Tet–TGF-β (TGF-β), AA V1-Tet–IL-2 plus AA V1-Tet–TGF-β (IL-2 + TGF-β), or AA V1-Tet–IL-2 (IL-2 induced versus uninduced) 2 wk postinduction were assessed for the frequency of CD25+Foxp3+, CD25+Foxp3−, and CD25+Foxp3+ T cells. ***p < 0.001 was determined by comparing CD25+Foxp3+ frequency to each group (two-way ANOVA ± SEM). Similarly, the frequency of CD8+ T cells (B) and NK cells (C) was measured in PBL from the groups of AA V1-Tet–IL-2–vaccinated NOD mice that were either induced for 2 wk or left uninduced. Total cellularity in the spleen, PLN, and islets (D) and the frequency of islet infiltrating Ki67-staining T cells (E) were assessed in groups of four NOD female mice vaccinated with AA V1-Tet–IL-2 and induced for 3 wk or left uninduced. *p < 0.05, induced versus uninduced (two-way ANOVA ± SEM); **p < 10−3, induced versus uninduced (Student t test).
results demonstrate that IL-2–mediated protection correlates with an increased frequency of Foxp3+Treg and a concomitant decrease in the proliferative status and frequency of effector CD4+ and CD8+ T cells in the islet infiltrates.

Interestingly, a distinct profile for IFN-γ-expressing T effectors was detected in the PLN of induced AAV1–Tet–IL-2–treated NOD mice. The frequency of IFN-γ-expressing CD8+ T cells was significantly increased in the PLN (p = 0.008) but not the spleen or popliteal lymph nodes of induced versus uninduced AAV1–Tet–IL-2–vaccinated animals (Fig. 7B, 7D). A moderate increase in IFN-γ-expressing CD4+ T cells was also observed in the PLN, but this increase relative to uninduced NOD mice was not statistically significant (Fig. 7A, 7C). In addition, there was a trend toward an increased ratio of effector T cells to Foxp3+Treg relative to uninduced NOD mice, although this difference was not statistically significant (Fig. 7E). Taken together, these results demonstrate that systemic IL-2 has distinct effects on effector T cells depending on the tissue.

Discussion

IL-2 and TGF-β1 play key roles in the induction, maintenance, and/or function of Foxp3+Treg and have been used to prevent and treat T cell-mediated autoimmunity (24, 27, 40). Notably, the mode of administration has proven to be a key factor in determining the immunotherapeutic efficacy and safety of these cytokines. This is particularly true for IL-2, which has a short 1/2 life in vivo and for which the dose is critical in determining an anti-versus proinflammatory response (28, 39). Our results demonstrate that ongoing β cell autoimmunity is effectively blocked and diabetes prevented in AAV1–Tet–IL-2–treated animals after a short course of IL-2 expression in vivo. Somewhat surprisingly, TGF-β1 expression failed to suppress ongoing β cell autoimmunity, demonstrating that under the conditions used, IL-2 is more effective than TGF-β1 in suppressing ongoing autoimmunity.

Administration of high doses of rIL-2 protein has been used to enhance cellular immunity to viral or tumor Ags through expansion of activated proinflammatory CD4+ and CD8+ T cells, in addition to activating NK cells (for review, see Ref. 28). In our study, a brief period of IL-2 induction resulted in an elevated frequency of Foxp3+Treg in the periphery as well as in the islets and PLN. The effector T cell to Foxp3+Treg ratio was significantly reduced in the islets (Fig. 6C), consistent with a reduction in frequency and proliferative status of T cells in the islets (Fig. 3D, 3E) and the limited insulitis detected in AAV1-Tet–IL-2–treated NOD mice remaining diabetes free (Fig. 4B). Increased proliferation of Foxp3+CD25+CD4+ T cells (Fig. 2E) suggests that IL-2 expanded an established pool of Foxp3+Treg. CD62L+CD25+CD4+ T cells FACs sorted from the PLN of induced versus uninduced AAV1–Tet–IL-2–treated NOD mice exhibit equal suppressor activity in vitro (data not shown), suggesting that quantitative and not qualitative changes in the Foxp3+Treg pool account for protection. Furthermore, the lack of induction of β cell-specific IL-4, IL-10, or TGF-β1–secreting T cells in the PLN or spleen as measured by ELISPOT or ELISA (M. C. Johnson and R. Tisch, unpublished observations) argues that the protective effect following AAV1–Tet–IL-2 treatment is largely mediated via an increased Foxp3+ Treg pool. Indeed, coadptive transfer experiments demonstrated that an increased frequency of Foxp3+CD25+CD4+ T cells in the spleen of Dox–treated NOD mice injected with AAV1–Tet–IL-2 effectively suppressed diabetes in recipient animals (Fig. 5).

Importantly, the level and duration of rAAV-derived IL-2 expression was sufficient to affect Foxp3+Treg without systemic complications. For example, no increase was detected in the

FIGURE 6. Analyses of islet-infiltrating effector T cells and Foxp3+Treg in AAV1–Tet–IL-2–vaccinated NOD mice. Islets from groups of five NOD female mice vaccinated at 10 wk of age with AAV1–Tet–IL-2 and induced for 3 wk or left uninduced were compared for the frequency of Ki67+ T cells (A) (***p < 0.01 induced versus uninduced [Student t test ± SEM]), the frequency of Foxp3+CD25+CD4+ IFN-γ+CD8+ and IFN-γ+CD4+ T cells (B) (**p < 0.001 induced versus uninduced [Student t test ± SEM]), the ratio of IFN-γ+ T cells to Foxp3+CD25+CD4+ T cells (C) (**p < 0.001 [Student t test ± SEM]), and the mean fluorescence intensity of CD25 for Foxp3+CD25+CD4+ T cells (D).

[45x498]fluorescence intensity of CD25 for Foxp3+CD8+ T cells in the islet infiltrates.

[45x567]duced versus uninduced [Student t test ± SEM]), the frequency of Foxp3+CD25+CD4+ IFN-γ+CD8+ and IFN-γ+CD4+ T cells (B) (**p < 0.001 induced versus uninduced [Student t test ± SEM]), the ratio of IFN-γ+ T cells to Foxp3+CD25+CD4+ T cells (C) (**p < 0.001 [Student t test ± SEM]), and the mean fluorescence intensity of CD25 for Foxp3+CD25+CD4+ T cells (D).
frequency and/or proliferation of CD4+ and CD8+ T cells and NK cells in PBL and/or spleen, and total cellularity in the spleen and PLN was unaffected (Fig. 3). The preferential expansion of Foxp3+Treg under apparently limiting amounts of IL-2 is in agreement with a recent report by Abbas and colleagues (41). This group demonstrated that Foxp3+Treg respond more readily to IL-2 than conventional T cells and that primed conventional T cells respond to IL-2 only after a number of rounds of Ag stimulation. Interestingly, despite the reduced insulitis, an increased frequency of IFN-γ+CD8+ T cells and to a lesser extent IFN-γ+CD4+ T cells was detected in the PLN of AA V1-Tet–IL-2–treated NOD mice shortly after induction (Fig. 7). The latter may represent T effectors having undergone a number of rounds of Ag stimulation. Interestingly, despite the reduced insulitis, an increased frequency of IFN-γ+CD8+ T cells and to a lesser extent IFN-γ+CD4+ T cells was detected in the PLN of AA V1-Tet–IL-2–treated NOD mice shortly after induction (Fig. 7). The latter may represent T effectors having undergone a number of rounds of Ag stimulation and therefore more sensitive to the effects of IL-2. Nevertheless, our results demonstrate that systemic levels of IL-2 can be obtained that adequately balance the anti- versus proinflammatory properties of this cytokine. Notably, reducing the dose of AA V1-Tet–IL-2 or the time of Dox-induced IL-2 expression resulted in an increased ratio of islet-infiltrating effector T cells to Foxp3+Treg (Supplemental Fig. 1). This finding indicates that injection of 5 × 10^{10} v.p. of AA V1-Tet–IL-2 coupled with 3 wk of Dox feeding represents the minimal dose and time of induction, respectively, to effectively block ongoing β cell autoimmunity in the islets without unwanted systemic effects.

The lack of an effect on the Foxp3+Treg pool and/or progression of β cell autoimmunity by AA V1-Tet–TGF-β alone or in combination with AA V1-Tet–IL-2 was unexpected. For example, induction of adaptive Foxp3+Treg in vitro requires TCR stimulation in the presence of TGF-β and IL-2 (14–16). In addition, the Foxp3+Treg pool is impaired in mice in which T cells are deficient of TGF-βR expression (12, 42). Furthermore, Piccirillo and colleagues (40) reported that i.m. injection of plasmid DNA encoding TGF-β1 to ∼10-wk-old NOD female mice every second week prevented diabetes. Lack of efficacy seen for AA V1-Tet–TGF-β may reflect an insufficient induction period and/or levels of TGF-β1 expression. Notably, comparable levels of activated TGF-β1 and IL-2 were detected in Dox-fed NOD mice injected with AA V1-Tet–TGF-β and AA V1-Tet–IL-2, respectively (Fig. 1). This latter finding further demonstrates the increased potency of IL-2 versus TGF-β1 in influencing Foxp3+Treg and blocking β cell autoimmunity.

Intramuscular injection of rAAV vectors encoding cytokines and other immunomodulatory proteins has been used to successfully prevent diabetes in NOD mice (43–46). These studies, however,
employed rAAV vectors with constitutively active promoters, resulting in continuous transgene expression. Long-term expression of cytokines with pleiotropic effects would in all likelihood lead to unwanted complications. This is a particularly important issue with IL-2 for which in vivo availability can lead to opposing responses. The current study highlights the advantage of using an inducible promoter to regulate rAAV transgene expression. The duration of induction can be readily controlled, and the turning on and off of transgene expression at multiple times may be exploited for maintaining protection long term.

In summary, we show that a short course of IL-2 induction in AAV1-Tet–IL-2–injected NOD mice is sufficient to increase the frequency of CD4+ CD25+ T-cells in type 1 diabetes. Defects in IL-2R signaling contribute to diminished maintenance of FOXP3+CD25+ regulatory T-cells of type 1 diabetic subjects. Diabetes 59: 407–415.


