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Systemic Overexpression of IL-10 Induces CD4⁺CD25⁺ Cell Populations In Vivo and Ameliorates Type 1 Diabetes in Nonobese Diabetic Mice in a Dose-Dependent Fashion

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Early systemic treatment of nonobese diabetic mice with high doses of recombinant adeno-associated virus (rAAV) vector expressing murine IL-10 prevents type 1 diabetes. To determine the therapeutic parameters and immunological mechanisms underlying this observation, female nonobese diabetic mice at 4, 8, and 12 wk of age were given a single i.m. injection of rAAV-murine IL-10 (10⁴, 10⁵, 10⁶, and 10⁹ infectious units (IU)), rAAV-vector expressing truncated murine IL-10 fragment (10⁹ IU), or saline. Transduction with rAAV-IL-10 at 10⁹ IU completely prevented diabetes in all animals injected at all time points, including, surprisingly, 12-wk-old animals. Treatment with 10⁸ IU provided no protection in the 12-wk-old injected mice, partial prevention in 8-wk-old mice, and full protection in all animals injected at 4 wk of age. All other treatment groups developed diabetes at a similar rate. The rAAV-IL-10 therapy attenuated pancreatic insulitis, decreased MHC II expression on CD11b⁺ cells, increased the population of CD11b⁺ cells, and modulated insulin autoantibody production. Interestingly, rAAV-IL-10 therapy dramatically increased the percentage of CD4⁺CD25⁺ regulatory T cells. Adoptive transfer studies suggest that rAAV-IL-10 treatment alters the capacity of splenocytes to impart type 1 diabetes in recipient animals. This study indicates the potential for immunomodulatory gene therapy to prevent autoimmune diseases, including type 1 diabetes, and implicates IL-10 as a molecule capable of increasing the percentages of regulatory cells in vivo. The Journal of Immunology, 2003, 171: 2270–2278.

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possess Tr properties are classified by their constitutive expression of the CD4+CD25+ phenotype and rely heavily on IL-10 for their development (12). Data suggesting that CD4+CD25+ populations represent Tr cells capable of reversing/preventing autoimmune bowel disease as well as associate with the prevention of diabetes development in nonobese diabetic (NOD) mice (13, 14). Although it is not clear how these Tr cells are induced, whether it is by DC or thymus selection, IL-10 has been shown to play an important role in their development (15).

The effect IL-10 administration has on the formation of type 1 diabetes in NOD mice is remarkably dependent on time and mode (i.e., early vs late, systemic vs local), to the point of being described as paradoxical (16). For example, early systemic treatment with exogenous murine IL-10 can inhibit type 1 diabetes in NOD mice, while, in contrast, local expression (intraislet) accelerates the onset of disease (17–22). In addition, aberrations in IL-10 levels or function have been associated with type 1 diabetes in both humans and NOD mice (22, 23). If one were to envision therapeutic cytokine delivery as a means for the prevention of type 1 diabetes, the relatively short half-life of IL-10 and the practicality of using a cytokine for initiation of immune deviation would be limited, at least in part, due to the need for repeated administration. We and others have previously demonstrated that injection of skeletal muscle with a nonpathogenic recombinant adeno-associated virus (rAAV) vector results in a robust, long term systemic production of a transgene (24–27). Furthermore, our studies demonstrated that complete prevention of diabetes was achievable with a gene therapy approach using a rAAV-IL-10 construct (26). However, studies to date have not addressed the issue of the effectiveness of the time and dose of systemic IL-10 administration (including that via gene therapy). In addition, the potential mechanisms associated with type 1 diabetes prevention have yet to undergo extensive examination in the context of long term systemic production of murine IL-10 in NOD mice. Both voids form the basis for this report.

Materials and Methods

Mice

NOD mice, purchased from Taconic Farms (NOD.MttTac; Germantown, NY) were housed in specific pathogen-free facilities at the University of Florida. The institution animal care and use committees at the University of Florida approved all animal manipulations. Female NOD mice were obtained at 4–8 wk of age and monitored twice weekly for hyperglycemia until they became diabetic, as defined by two consecutive nonfasting blood glucose levels >240 mg/dl. Female NOD.Rag and C57BL/6 mice were purchased from the University of Florida mouse facility (Department of Pathology, College of Medicine).

Plasmid construction, rAAV vector generation, and therapeutic administration

Murine IL-10 cDNA (a gift from Nora Sarvetnick, Scripps Institute, La Jolla, CA) was cloned into the p3.2 plasmid, with expression driven by a fused CMV-chicken β-actin promoter (CB). ΔIL-10 was created by site-directed mutagenesis, creating a truncated IL-10 peptide by introducing a stop codon 33 bp downstream of the start codon (6). Recombinant AAV serotype 2 production, titer determination, and infectivity were performed as previously described (26). Four-, 8-, and 12-wk-old female NOD mice were injected into the caudal muscle of the pelvic limb. These injections used 100 μl of saline containing 1 × 106, 1 × 107, 1 × 108, and 1 × 109 infectious units (IU) of rAAV-IL-10, or saline alone.

Histopathology and immunohistochemistry

Pancreas, kidney, liver, leg muscle, lung, ovary, jejunum, salivary gland, spleen, and lymph nodes were fixed in periodate-lysine-paraformaldehyde buffer, embedded in paraffin, and sectioned at 4 μm. All sections were stained with H&E for histological assessment.

Serum cytokine measurement

Mouse serum was analyzed for the presence of murine IL-10 (mIL-10) using the Luminex LabMAP system with IL-10 detection beads (Bio-source, Camarillo, CA). The manufacturer’s protocol was followed with the incorporation of a MultiScreen MABVN 1.2-μm, 96-well filter plate and MultiScreen Vacuum Manifold (Millipore, Bedford, MA). Samples were read using the Luminex 100 (Upstate Biotechnology, Lake Placid, NY), with IL-10 concentrations interpolated using the Softmax program against the linear range on the standard curve (26–19,500 pg/ml).

RNA extraction and RT-PCR for rAAV-IL10-specific transgene expression

Total RNA from muscles or cells was extracted with TRizol reagent and treated with RNase-free DNase (Life Technologies). RT and the first PCR were performed with primers P1 and P2 using AccessQuick RT-PCR system (Retro-Script) for 35 cycles. The second PCR was performed with primers P3 and P4 using Platinum PCR Supermix (Life Technologies) for 35 cycles. Primer sequences were: P1, 5'-AGTCGCTGCGAGCTGCTGC-3'; P2, 5'-CTGCTCCACTGCCTTGCTCT-3'; P3, 5'-GGCTCTGACTGACCGTGTA-3'; and P4, 5'-GCAAGCTTAGAGAGCATGGG-3'.

Flow cytometry of splenocytes

Splenocytes from all surviving 8-wk-old treated mice sacrificed at 32 wk were stained for CD4, CD11b, CD11c, CD25, CD45RB, CD45R/b220, and Thy1.2 (BD Pharmingen, San Diego, CA). All data were analyzed on FCS express (De Novo Software, Thornhill, Ontario, Canada).

Insulin autoantibodies (IAA) analysis

IAA were measured by RIA using radiolabeled insulin (Amersham Pharmacia Biotech, Indianapolis, IN) and protein A-Sepharose (Sigma-Aldrich, St. Louis, MO) (26). An index was calculated as [(unknown cpm − negative control cpm)/positive control cpm – negative control cpm] × 100. The cutoff of 10.1 was chosen based on the mean index + 3 SD of C57BL/6 mice.

Splenocyte studies

Splenocytes were cultured at 2 × 104 cells/well in 200 μl of RPMI 1640 medium (10% FBS) in 96-well, round-bottom plates. Supernatants were collected at 24 and 48 h for cytokine analysis in response to Con A (1 μg/ml) or media alone and were tested for cytokine production. IL-2, IL-4, IL-10, IL-12, and TNF-α were measured using the Luminex Multibead assay (Upstate Biotechnology) for mouse cytokines on the Luminex 100 following the manufacturer’s suggested protocol. For studies of in vivo activity, 8-wk-old female NOD.Rag mice were injected i.p. with splenic lymphocytes (2 × 107) obtained from 20-wk-old, newly diagnosed diabetic NOD mice or 32-wk-old rAAV-IL10 1 × 107 treated NOD mice under conditions of adoptive transfer (26).

OVA responses

Mice surviving past the age of 30 wk of age were administered 100 μg of OVA peptide linked to a carrier (mject kit; Pierce, Rockford, IL) emulsified in aluminum hydroxide. Intraperitoneal injections of 100 μl were administered at 30 wk of age, followed by a booster shot of the same concentration administered 2 wk later. Total serum levels for anti-OVA Ig Abs were determined at the time of sacrifice. Mouse serum was analyzed for the presence of OVA Abs using ELISA techniques against OVA Ag, and data were read in OD units.

Statistical analysis

Statistical analysis was performed with life-table analysis for comparison of diabetes frequencies, one-way ANOVA, and correlation analysis performed with Pearson test. All data are presented as the mean ± SD. Statistically significance is defined as p < 0.05.

Results

Effects of time and dose on rAAV-IL-10 therapy for type 1 diabetes prevention

Previously, we have shown that a single injection of rAAV-IL-10 into NOD mice at an early time point (4 wk of age) lead to the complete abrogation of diabetes (26). To evaluate the therapeutic parameters of rAAV-IL-10 therapy on the prevention of type 1 diabetes, we sought to study the effects systemic IL-10 production
would have on diabetes development at different time points (4-, 8-, and 12-wk-old female NOD mice; n = 5–12/group per time point) and with different doses of rAAV-IL-10 (1 × 10^9, 1 × 10^8, and 1 × 10^6 IU). With respect to time, it was of interest to determine the effects this treatment would have on mice treated at later time points in the effector phase of prediabetes.

The experimental plan involved the use of the same rAAV constructs, mode of injection, injection site, and colony of animals as in our previous experiments (26). Recombinant AAV-IL-10 (Fig. 1A) was serially diluted to proper doses with saline (volume, 100 μl/injection) and was injected i.m. into the hind limb caudal muscle. For controls, mice were injected with saline or 1 × 10^10 IU of rAAV2-CMV-ΔIL-10 (a truncated form of mIL-10 producing inactive protein; Fig. 1B) at the same time points. Surprisingly, mice receiving the highest-dose of IL-10 (1 × 10^10 IU) in the 12-wk-old injected group completely abrogated the development of diabetes (five of five; 0% incidence at 32 wk of age; p < 0.003 vs saline or Δ-IL-10; p < 0.009 vs 1 × 10^4 IU rAAV-IL-10; p < 0.04 vs 1 × 10^5 IU rAAV-IL-10; Fig. 2A). While 12-wk-old injected animals receiving a dose of 1 × 10^6 IU did develop diabetes, the onsets of their diseases were also significantly delayed compared with those of saline-treated controls (p < 0.05). Mice receiving the highest dose treatment (1 × 10^10 IU) in the 8-wk-old injected group also failed to develop diabetes (six of six; 0% incidence at 32 wk of age; p < 0.03 vs saline controls; p < 0.006 vs Δ-IL-10), while a trend indicative of a delay (albeit, not statistically significant) in disease kinetics was witnessed in the 1 × 10^9 IU-treated animals (Fig. 2B). No mice receiving a dose of 1 × 10^5 IU at 4 wk of age (10 of 10; 0% incidence; p < 0.0001 vs saline or rAAV-Δ-IL-10) developed diabetes by 32 wk (Fig. 2C). Both saline- and rAAV-Δ-IL-10-treated groups developed diabetes at similar rates (p = NS; 60–90% incidence at all injection times), and low dose treatment groups demonstrated an increased incidence of diabetes compared with higher dose treatment animals (Fig. 2C; p < 0.01, 1 × 10^5 vs 1 × 10^6). Results from these experiments suggest that protection from disease onset varied in accordance with the time and dose of rAAV-IL-10 administration.

To assess the IL-10 gene expression in myocytes, RNA was extracted from the hind limbs of all treatment groups, including saline-treated mice, and was tested for transgene product using nested RT-PCR techniques with specific primers. However, RT-PCR reactions using RNA from low dose-injected animals failed to produce this band (data not shown). Analysis of secreted IL-10 production was performed using Beadlyte technology to test sera for the presence of mIL-10. These studies confirmed that of all the animals sacrificed at 14 wk of age (n = 2/group), IL-10 was only detectable in animals receiving 1 × 10^5 IU. Interestingly, no IL-10 was detectable in the 1 × 10^6 IU-injected, 4-wk-old animals even though they showed no signs of disease development (data not shown).

**Effect of rAAV-IL-10 on the insulitis lesion**

Insulitis is a key feature in the development of type 1 diabetes in NOD mice. In contrast to a normal islet (stage 0 insulitis; Fig. 3A), the mildest form of inflammation is infiltration with inflammatory cells (e.g., DC, macrophages and T and B cells) around the perivascular duct and peri-islet regions of the islets of Langerhans. The peri-insulitis process in NOD mice normally begins when the animals are 4–6 wk of age (stage 0; Fig. 1). Stage 2 insulitis follows with an increase in number of infiltrating cells (Fig. 3A) in the islet that is graded based upon <50% of the islet having infiltration, as well as affecting more islets. As insulitis progresses to stage 3, the quantity of intraislet inflammatory cell accumulation increases to >50% (stage 3; Fig. 3A), with stage 4 representing complete inflammation of the islet (stage 4; Fig. 3A).

![FIGURE 1. The rAAV-CB-IL-10 and rAAV-CMV-ΔIL-10 constructs.](http://www.jimmunol.org/)

A. AAV serotype 2 vector cassette map, where ITR is the rAAV inverted terminal repeat, CMVp is the CMV immediate early promoter, and PA is the SV40 poly(A) signal. The circle following the gene is the SV40 poly(A) signal. B. CBp is the CMV enhancer and chicken-β actin promoter with a hybrid chicken-rabbit β-globin intron; ΔIL-10, a point mutation, was introduced, creating a frame shift that resulted in the production of the first 33 aa of mIL-10. Plasmids containing these constructs were cotransfected into HEK 293 cells with plasmids containing the proteins coding for Rep, Cap, E2, and E4 necessary for rAAV2 virus production. Cells were harvested, counted on a particle scale, and tested for infectious center assays on HEK 293 cells to determine the particle-to-infectivity ratio (1:100).

**FIGURE 2. IL-10 prevents type 1 diabetes in a dose- and time-dependent fashion.** Life-table analyses demonstrate the percentage of mice (n = 5–12/group) remaining normoglycemic after injection with saline ( ), Δ-IL-10 ( ), 1 × 10^5 IU rAAV-CB-IL-10 ( ), 1 × 10^6 IU rAAV-CB-IL-10 ( ), 1 × 10^7 IU rAAV-CB-IL-10 ( ), or 1 × 10^9 IU rAAV-CB-IL-10 ( ). Virus was thawed once and serially diluted in sterile saline buffer in a volume of 100 μl, and 50 μl was contralaterally injected i.m. in the caudal muscle. A. NOD mice injected at 4 wk of age. B. NOD mice injected at 8 wk of age. C. NOD mice injected at 12 wk of age. The arrow indicates the time of intervention. All mice were treated with saline, rAAV-ΔIL-10-control virus, or rAAV-IL-10 virus at the above-mentioned doses and followed for diabetes onset by blood glucose determinations. No 1 × 10^9 IU rAAV-CB-IL-10-treated group was tested in the 4-wk-old group. All animals were tested twice per week for blood glucose levels.
To examine the anti-inflammatory effects rAAV-IL-10 has on the insulitis lesion, we monitored insulitis in nondiabetic animals from the 4-, 8-, and 12-wk injected groups harvested at 32 wk of age. Insulitis was scored as described above and determined in percentages based upon a weighted average of 7–12 animals/group. The insulitic stage of the islet was noted, with the overall number of islets counted and averaged per group (Fig. 3, B, D, and F). The quantity of islets varied from 5–12/animal, and this number was independent of the dose and time of administration of rAAV-IL-10 or control preparation. However, the number of qualitative (stage 0) islets was dependent on time and dose of rAAV-IL-10, as the high dose rAAV-IL-10 groups demonstrated more stage 0 islets, especially at the earlier time points. These data were converted to percentages of the stage of islets compared with the overall number of islets (Fig. 3, C, E, and G). The time dependency of this treatment is more apparent when comparing the percentage of the overall number of stage 0 islets in mice receiving $1 \times 10^{8}$ IU (the only dose comparable across all three times of injection). Mice injected at 4 wk of age with this dose (Fig. 3G) averaged 38% vs animals treated at 8 wk (Fig. 3E) scoring 16% or 12 wk (Fig. 3C) with only 6% ($p < 0.01$). Aside from pancreatic evaluation, nine additional organs were subjected to histological evaluation and apart from a localized myositis at the site of injection (data not shown; observed in our previous work (26)), no additional histological abnormalities were observed. Overall, these data suggest that intervening early with rAAV-IL-10 therapy prevents disease progression by preserving islets as a result of reduced inflammation. In addition, it suggests that early intervention can reduce the requirements of rAAV-IL-10 therapy, but even more importantly, high dose treatment can halt disease onset and maintain the integrity of islets days, weeks, or months before time of overt diabetes would be anticipated. Pancreases from diabetic animals in all treatment groups were also subject to histologic evaluation and were indicative of inflammation and absence of islet cells associated with overt disease (data not shown).

**Effects of rAAV-IL-10 treatment on IAA production**

Similar to humans with type 1 diabetes, NOD mice develop IAA. Although IAA are not thought to harbor islet-destructive capabilities, IAA are an excellent predictor of future development of type 1 diabetes in NOD mice, in that a majority of animals that are positive for this marker by 8 wk of age develop the disease. Indeed, in the controls in this study there was a strong association for those animals who were measured as IAA positive and the subsequent development of diabetes (Fig. 4). However, after administration of rAAV-IL-10 this rule no longer applied to this model, especially to the animals receiving the $10^{8}$ and $10^{9}$ IU treatments or treated at 4 wk of age. For example, eight to 10 mice (4 wk treated) that were IAA positive before injection with $10^{8}$ IU of rAAV-IL-10 were effectively prevented from developing diabetes, but continued to test positive for IAA (Fig. 4C). Thus, IAA are predictive of type 1 diabetes development in NOD mice, but rAAV-IL-10 treatment alters their predictability as a result of the significant effect of the intervention on IAA production.

**Mechanisms afforded by rAAV-IL-10 in the prevention of type 1 diabetes**

Given the introductory discussion on the importance of Tr cells, we performed flow cytometric analysis of splenocytes to examine...
the roles that CD4+CD25+ Tr cells and APCs play in the prevention of diabetes in rAAV-IL-10 conditions. Splenocytes from all 8-wk-old injected mice surviving until 32 wk of age were harvested and stained with specific Abs. The CD4+CD25+ cell populations showed a dose effect consistent with the treatment group (Fig. 5, A–C). Specifically, the 10^9 IU injected group showed the highest percentage of the total population of CD4+CD25+ cells of total CD4+ cells, showing a 2-fold increase over saline controls (p < 0.01; Fig. 5C). We then examined the expression of CD45RB on the CD4+CD25+ cells for high and low expression (Fig. 5D). The percentage of CD45RBlow expression on the CD4+CD25+ cells remained comparable among all the groups (representative dot lots shown). Thus, the generation of the CD4+CD25+ cell population under rAAV-IL-10 conditions expressed CD45RBlow.

The generation of Tr cells and the induction of anergic T cell populations showed a dose effect consistent with the treatment group (Fig. 6A). The percentage of CD45RBlow expression on the CD4+CD25+ cells remained comparable among all the groups (representative dot lots shown). Thus, the generation of the CD4+CD25+ cell population under rAAV-IL-10 conditions expressed CD45RBlow.

Recombinant AAV-IL-10 effects on the humoral and cellular immune response

To learn what effect rAAV-IL-10 had on the cytokine profile of splenocytes, we analyzed the levels of IL-2, IL-4, IL-10, TNF-α, and IFN-γ. Splenocytes from the 8-wk-old treated mice were cultured with mitogenic stimulation using Con A at a concentration of 1 μg/ml, with cytokine release measured (media) after 24- and 48-h stimulation periods. In comparison with the controls (51 ± 14 pg/ml) and low dose (10^5 rAAV-IL10; 48 ± 3 pg/ml) animals,
The levels of TNF-α were not affected by rAAV-IL-10 treatment, these reductions did not reach the level of statistical significance (data not shown).

In the presence of IL-10, iDC lose their ability to mount strong Ag-specific immune responses. To determine whether this phenomenon held true in our model we injected surviving 4-wk-old treated mice with doses of OVA Ag conjugated to a carrier complex with adjuvant 14 days apart, with sacrifice 21 days after the primary injection. Using serum Ab measurements against OVA Ag, we measured the Ag-specific responses from all treatment groups by ELISA (Fig. 7). OVA-specific Abs were observed in all treatment groups, while serum drawn before Ag stimulation showed no OVA Ab production (data not shown). The levels of OVA-specific Abs were not significantly attenuated (p = NS) in the highest dose (10⁸ IU) group in the 4-wk-old injected (Fig. 7) animals in comparison with the other groups.

II-10 therapy modulates ability to adoptively transfer diabetes

Finally, to learn whether rAAV-IL-10 therapy modulates type 1 diabetes by altering the β-cell destructive capacity of lymphocytes, we performed adoptive transfer experiments. Young female NOD.Rag-scid animals results in the rapid development of diabetes. These experiments not only implicated CD4⁺CD25⁺ cells as being imperative for autoimmune regulation in NOD mice, but they suggest that such cell populations can regulate already activated effector cells that are present in the late stages of disease progression. Interestingly, no animals developed diabetes when receiving cells from rAAV-IL-10-treated animals.

Discussion

While multiple reviews have been penned regarding the potential for gene therapy to prevent or reverse type 1 diabetes, few reports have actually been published demonstrating such effectiveness in actual application. To this limited body, we have now added evidence for rAAV-IL-10-mediated gene therapy to serve as an effective treatment for the prevention of type 1 diabetes. Specifically, these studies furthered our previous work (26) in elucidating key molecular mechanisms that systemic rAAV-IL-10 production confers. In addition, this work added to the important and growing body of immunology literature examining the mechanisms regulating the formation of CD4⁺CD25⁺ Tr cells.

Regulatory cells have been implicated in inducing tolerance and regulating diabetes development in NOD mice when cotransferred with diabetogenic T lymphocytes (28). These studies also demonstrated that transfer of CD25-negative populations from 10-wk-old nondiabetic NOD mice into NOD.scid animals results in the rapid development of diabetes. These experiments not only implicate CD4⁺CD25⁺ cells as being imperative for autoimmune regulation in NOD mice, but they suggest that such cell populations can regulate already activated effector cells that are present in the late stages of disease progression.
stages of insulitis. The regulatory properties of the CD4+CD25+ cells are thought to be conferred in a cell contact-dependent and/or -independent fashion. Singh et al. (29) have described a regulatory cell that has the CD4+CD25+CD45RB↓ phenotype and resolves colitis in a contact-independent fashion. We observed increased numbers of cells with this phenotype in animals treated with high doses of IL-10 in a model in which autoimmune diabetes was interrupted. We would contend that the elevated systemic IL-10 environment reduces IL-2 production by T cells in vivo, thus arresting expanding T cells and preventing naive T cell expansion. However, this model would not clearly explain how the already primed effector cells are controlled. Future experiments will be directed at answering this important question.

**NOD mice** are known to have attenuated regulatory cell development, possibly as a result of reduced thymic development. Typically, 5–10% of peripheral CD4+ T cells in NOD mice are suppressor cells (13, 28). In our study, populations of CD4+CD25+ regulatory cells were at their highest levels in the highest dose–treated animals. In this experiment, high dose rAAV-IL-10 animals had CD4+CD25+ cell populations reaching 20% of the total CD4+ T cell population, while the control animals only demonstrated 7–9%, in concordance with previous studies (13). Indeed, in our studies the quantity of rAAV-IL-10 administered demonstrated a dose effect on the population of regulatory cells; the greater the IL-10 concentration, the larger the population in vivo. This is supported by in vitro experiments showing that IL-10 and IFN-α increased the CD4+CD25+ Tr population (30). Further studies are needed to investigate, on a longitudinal basis, both the natural history of these IL-10-induced Tr population shifts and the relationship to thymic development.

One key question is whether the increase in this cell population really controls the formation of type 1 diabetes? Previous studies have suggested that the pathology resulting from autoimmune diseases is regulated by the ratio of CD4+ T cells to non-CD4+ Tr cells (28). Specifically, low ratios of regulatory cells to other cells conferred higher rates of autoimmune disease in normal strains of mice, while high ratios of these cells protected against autoimmune disease. Our adoptive transfer studies showed the inability of splenocytes from rAAV-IL-10-treated animals to transfer diabetes. Caution must also be exercised in interpretation of these adoptive transfer studies, as while splenocytes from rAAV-IL-10-treated mice failed to transfer disease, our investigations did not compare...
studies have indicated that a subset of the CD4 lack of Tr cells that control their activities. Furthermore, recent appears that their escape from peripheral tolerance is a result of the deletion has been speculated, but remains unclear. However, it eliminate these cells that eventually lead to islet-speci
central tolerance process in the thymus, type 1 diabetics fail to most healthy individuals who clear autoreactive cells during the history of disease need to be performed in a fashion similar to that used by Yang et al. (27).

A more destructive facet of autoimmunity is the development of autoreactive T cells targeted against self-Ags and tissues. Unlike most healthy individuals who clear autoreactive cells during the central tolerance process in the thymus, type 1 diabetics fail to eliminate these cells that eventually lead to islet-specific reactivity and β-cell destruction. Why these cells escape central tolerance deletion has been speculated, but remains unclear. However, it appears that their escape from peripheral tolerance is a result of the lack of Tr cells that control their activities. Furthermore, recent studies have indicated that a subset of the CD4+ T cells that constitutively express the activation molecule CD25 have a regulatory effect on disease development in the NOD mouse.

The immunosuppressive effect of IL-10 observed in these animals appears to represent a cocontributor to disease prevention. Results from our flow cytometric analysis showed that monocytes and macrophages in the highest rAAV-IL-10-treated groups had significantly less expression of MHC II molecules compared with the control groups. Lymphocytes require sufficient interaction with this molecule (and other costimulatory molecules) to be primed against Ag, or they become anergic. The low levels of MHC II expression we observed suggest that the suppression in part arises from anergic T cells. However, since the same molecule is used by B cells to initiate Ag-specific Ab production, we were able to measure OVA Ab levels to determine whether the state of immunosuppression affected such cells. These studies suggest that IL-10 did not inhibit OVA Ab-specific production. As a result, the role of IL-10 in diabetes prevention appears to result from more than just its immunosuppressive powers.

APCs are a group of cells that function to present foreign and self Ags to T cells through the MHC molecule (TCR:MHC, signal 1) and costimulatory molecules (signal 2). Of these APCs, the DC cell is a 10–100 times more potent stimulator of responding T cells. In its immature state the DC expresses low levels of MHC II and costimulatory molecules, but with activation stimuli, the surface expression of these molecules is greatly increased. There has been growing evidence that iDC are cells that possess potent tolerogenic capabilities by induction of anergic and regulatory cells. Thus, the suppressed expression of the MHC II complex on IL-10-treated DC most likely associates with reduced costimulatory expression, leading to an APC that poorly primes T cells.

Several reports have shown that IL-10 (immunosuppressive) treatment of DC generate Tr cells in vitro (30, 31). Surprisingly, we saw a marginal effect of rAAV-IL-10 treatment on in vitro cytokine production profiles with the doses used in these studies. Indeed, we previously observed that very high doses (1 × 10^10 IU) of rAAV-IL-10 treatment can greatly reduce the in vitro stimulated production of IL-2, IL-4, IL-10, and TNF-α (26). In this study much more modest reductions in mitogen stimulated cytokine production to the point of being statistically insignificant. Because the differences in cytokine production were so small and, in addition, since IL-2 production was, in reality, elevated by rAAV-IL-10 therapy in these studies, we cannot arrive at consensus conclusions about the affect of cytokine production on diabetes development in this model.

As in our previous study, we saw reduced insulitis using IL-10 treatment and an alteration in IAA index values. Although these values do not give a direct link to the mechanism of protection, they are accurate tools for evaluating disease development and immune responses against islet cell Ags. Clearly, reduced insulitis scores of the high dose IL-10-treated mice can explain the preservation of β-cell function and diabetes prevention, and why the IAA level in the same animals is lower. A still unresolved issue is what effect late treatment (i.e., 12 wk of age) with rAAV-IL-10 has on the natural history and composition of the insulitis lesion. In other words, does the lesion resolve by this treatment and, if so, in what duration of time? Unfortunately, this issue was not directly addressed by this study, but will be subject to future evaluation. Indeed, the ability for rAAV-IL-10 to prevent insulitis at 12 wk of age was surprising. In studies evaluating the time required for detection of serum IL-10 following i.m. delivery of NOD mice with rAAV-IL-10, levels averaging 230 pg/ml were observed 2 wk.
postinjection, while no IL-10 was detectable in saline-treated animals under the same time frame (Y. Clare Zhang et al., unpublished observations). Hence, it would appear that despite the kinetics of rAAV expression, the production levels were sufficient to prevent diabetes in these animals.

In summary, this study elucidated the time and dose dependencies as well as the mechanism by which rAAV-IL-10 gene therapy prevents type 1 diabetes in the NOD model. Despite the remarkable success of this therapy, further consideration needs to be exercised in terms of applying this model to humans. Recombinant AAV gene therapy itself, however, has proven to be a very effective method for introducing the protein of interest for long term expression. It is a promising approach that will be applied more frequently in studies aimed at preventing diabetes and as an effective tool for understanding the protein’s effects in vivo.

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References