

AAV8-mediated Gene Transfer of Interleukin-4 to Endogenous β -Cells Prevents the Onset of Diabetes in NOD Mice

Khaja K Rehman¹, Massimo Trucco², Zhong Wang¹, Xiao Xiao³ and Paul D Robbins¹

¹Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA; ²Department of Pediatrics, Children's Hospital of Pittsburgh, University of Pittsburgh School of Medicine Pittsburgh, Pennsylvania, USA; ³Division of Molecular Pharmaceutics, University of North Carolina School of Pharmacy, Chapel Hill, North Carolina, USA

We have demonstrated the ability to deliver and express genes specifically in β -cells for at least 6 months, using a murine insulin promoter (mIP) in a double-stranded, self-complementary AAV vector (dsAAV8-mIP). In this study, we evaluated the effects of dsAAV8-mIP-mediated delivery of interleukin 4 (mIL-4) to endogenous β -cells in nonobese diabetic (NOD) mice. In 4-week-old NOD mice, the extent of gene transfer and expression in endogenous β -cells after ip delivery of dsAAV8-mIP-enhanced green fluorescent protein (eGFP) was comparable to normal BALB/c mice. Further, after IP delivery of dsAAV8-mIP-IL4, expression of mIL-4 was detected in islets isolated from the treated mice and cultured. AAV8-mIP-mediated gene expression of mIL-4 in endogenous β -cells of 4- and 8-week-old NOD mice prevented the onset of hyperglycemia in NOD mice and reduced the severity of insulinitis. Moreover, expression of mIL-4 also maintained the level of CD4⁺CD25⁺FoxP3⁺ cells, and adoptive transfer of splenocytes from nondiabetic dsAAV8-mIP-IL-4 mice to NOD $scid$ mice was able to block the diabetes induced by splenocytes co-adoptively transferred from nondiabetic dsAAV-mIP-eGFP mice. Taken together, these results demonstrate that local expression of mIL-4 in islets prevents islet destruction and blocks autoimmunity, partly through regulation of T-cell function.

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INTRODUCTION

Autoimmune type 1 diabetes (T1D) is an organ-specific disease, characterized by infiltration of autoreactive T cells into the pancreas and leading to destruction of pancreatic islet β -cells. Approximately 5–10% of those with diabetes in North America have the type 1 disease. It is prominently found among young children (~40 in every 10,000 children), the peak age of onset being 11–12 years. Insulin replacement can ameliorate the symptoms of the disease but has no effect on the autoimmune process;

eventually complications set in, such as nephropathy, micro-vascular disease, blindness, and atherosclerosis. In order to prevent the progression of T1D in its early stages, therefore, the autoimmune component of the disease has to be controlled. A murine model for human T1D is the nonobese diabetic (NOD) mouse which spontaneously develops insulinitis at ~4 weeks of age (depending on housing conditions and type of food), followed by the onset of hyperglycemia between 12 and 18 weeks of age.¹

T1D is mediated by T cells; this is shown by the fact that it can be prevented by treatment with anti-T-cell antibodies^{2,3} and can be transferred to prediabetic animals by transfer of T cells from diabetic mice.^{4,5} T1D develops, in part, from a deficiency in the function of regulatory T (T-reg) cells, resulting in their failure to control pathogenesis.⁶ TH1 cells appear to be responsible for mediating autoimmunity in NOD mice, whereas endogenous TH2 and T-reg cells have the ability to prevent the development of diabetes in the mice.^{4,7} The inflammatory process in early diabetes is initiated and propagated by TH1-secreted cytokines such as interferon and suppressed by TH2-secreted anti-inflammatory cytokines such as interleukin-4 (IL-4) and IL-10. Numerous studies suggest that the intrinsic TH1/TH2 balance in NOD mice is tilted toward TH1, and manipulations that correct this balance result in protection from destructive insulinitis and diabetes.^{8,9}

Delivery of TH2 cytokines prevents the onset of diabetes in NOD mice,^{4,7} low-dose streptozotocin-treated mice,¹⁰ and BioBreeding rats.¹¹ IL-4 and IL-10, in particular, have been delivered therapeutically as recombinant proteins,⁴ by gene transfer of expression plasmids,^{7,12–14} by transplantation of genetically modified dendritic cells,^{8,15} or by gene transfer using recombinant adeno-associated virus (AAV) vectors.^{16,17} Additionally, transgenic NOD mice expressing IL-4 specifically in β -cells have been used to demonstrate that pancreas-specific expression of these TH2 cytokines was sufficient to block the development of hyperglycemia.^{18,19} These results suggest that the TH2 cytokines IL-10 and, even more so IL-4, may play therapeutic roles by preventing or reversing T1D.

Given the short half-life of anti-inflammatory mediators such as recombinant cytokines, systemic administration of these

Correspondence: Paul D. Robbins, Department of Microbiology and Molecular Genetics, University of Pittsburgh, School of Medicine, W1246, Biomedical Science tower, Pittsburgh, Pennsylvania 15261, USA. E-mail: probb@pitt.edu

molecules requires repeated administration, which can result in nonspecific side effects. Sustained, systemic expression of cytokines has been achieved by gene transfer to different tissues such as muscle, but there is a concern that the systemic cytokine levels may have adverse effects on normal immune function. Against this background, approaches involving delivery of anti-inflammatory or anti-apoptotic agents to endogenous islets have been investigated. Adenoviral vectors are effective for the transduction of both human and rodent islets in culture, resulting in gene transfer of the β -cells within the intact islet without interfering with β -cell function.^{20–22} Adenoviral vectors are also able to transduce endogenous islets *in vivo*, albeit at a low efficiency. The disadvantage of adenoviral vectors is that gene expression is transient, partly because of the inherent immunogenicity of first-generation adenoviral vectors. AAV-based vectors can infect both dividing and nondividing cells resulting in long-term efficient transgene expression in animal models with limited immunogenicity.^{22,23} The use of self-complementary (double stranded) AAV vectors partially overcomes the need for high multiplicity of infection of single-stranded AAVs, thereby conferring rapid and efficient transduction. Also, the efficiency of AAV infection has been improved through the use of certain serotypes of AAV that have different tropisms *in vivo*. We have earlier demonstrated the efficient transduction of both rodent and human islets with dsAAV vectors packaged in AAV2, 6, and 8 capsids in culture.²³ We have also demonstrated that intravenous, intraperitoneal (IP), or intraductal injection of AAV6 and, to an even greater degree AAV8, results in efficient transduction of endogenous murine islets.²⁴ Further, we have demonstrated that the use of a murine insulin promoter (mIP) with the AAV vector results in β -cell-specific transgene expression for >6 months without producing any apparent side effects.²⁴

In this study, we have investigated the therapeutic effects of dsAAV8 gene transfer of murine IL-4 into endogenous β -cells of 4- and 8-week-old NOD mice. Expression of IL-4, but not IL-10 or I κ B, in β -cells reduced insulinitis, maintained the number of CD4⁺CD25⁺FoxP3⁺ cells, and prevented the onset of hyperglycemia in NOD mice. Adoptive transfer of splenocytes from the nondiabetic dsAAV8-mIP-IL-4 mice to NOD*scid* mice was able to block the diabetes induced by splenocytes co-adoptively transferred from nondiabetic dsAAV-mIP-eGFP mice. Taken together, these results demonstrate that local expression of IL-4 in islets prevents islet destruction by blocking autoimmunity. The mechanism for blocking of autoimmunity is through regulation of diabetogenic T-cells, partly by the maintenance of CD4⁺CD25⁺FoxP3⁺ cells. These results also demonstrate that dsAAV8-mIP-mediated gene transfer to endogenous NOD β -cells can be used for examining the role of specific agents that could enhance or prevent T1D.

RESULTS

IP delivery of dsAAV8 transduces pancreatic islets, and specifically β -cells

We have demonstrated earlier that IP, systemic (intravenous), or intraductal administration of dsAAV8-CMV-GFP results in significant transduction of both exocrine and endocrine cells of the pancreas along with other organs in both C57BL/6 and BALB/c mice.²⁴ The

murine insulin promoter (mIP), shown to be β -cell-specific in the dsAAV vector, was effective in limiting enhanced green fluorescent protein (eGFP) expression exclusively to the pancreatic β -cells.^{24,25} The ability to produce gene expression specifically in endogenous β -cells at certain time points makes it possible to examine the efficacy of specific gene products in regulating the progression of diabetes in NOD mice. Initially, we investigated the ability of dsAAV8 carrying the mIP-eGFP cassette to transduce pancreatic β -cells in young (4 week old) female NOD mice as compared to normal BALB/C mice. As shown in **Figure 1**, dsAAV8-mIP-eGFP transduction and expression of eGFP in pancreatic isolated islets in young female NOD mice (**Figure 1c**) was as efficient as in normal BALB/C mice (**Figure 1b**), and was specifically expressed in β -cells (**Figure 1d–f**). Moreover, the GFP expression was absent in exocrine pancreatic tissue, liver, and spleen (**Figure 1g–i**), and no eGFP expression was seen in vehicle control (**Figure 1a**), which is in agreement with our earlier results.²⁴

Expression of murine IL-4 (mIL-4) in islets after transduction with dsAAV8-mIP-IL-4 in culture

In order to determine whether local expression of IL-4 in the endogenous β -cells in NOD mice could block the progression of diabetes, we generated a dsAAV vector carrying the mIL-4 cDNA under the regulation of the mIP. The ability of the dsAAV8-mIP-IL-4 to express IL-4 after infection of β -cells was tested initially by infecting a group of 100 islet equivalents, isolated from BALB/C mice, with 10,000 v.g. of AAV-mIP-IL-4 per islet cell

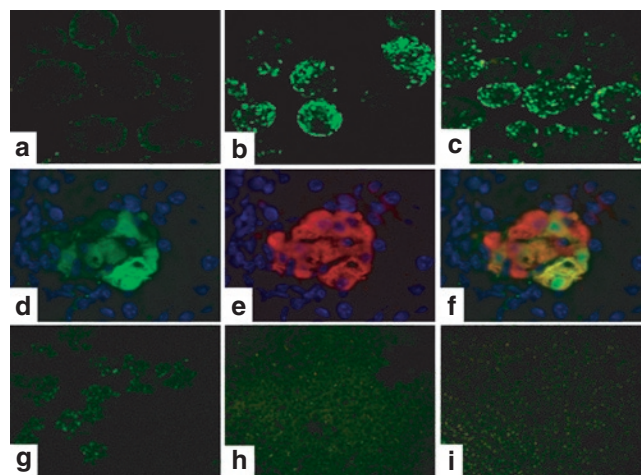


Figure 1 Expression of enhanced green fluorescent protein (eGFP) in pancreatic islets transduced with dsAAV8 *in vivo*. 4×10^{11} vector genome of dsAAV8 expressing eGFP under mIP was injected intraperitoneally (IP), into BALB/c and nonobese diabetic (NOD) mice. Two weeks later the islets were isolated as described in Materials and Methods. Expression of eGFP in isolated islets was visualized using two-photon laser scanning confocal microscopy. **(a)** eGFP expression was similar in **(b–c)** dsAAV8-treated islets from both BALB/c and NOD mice, whereas the islets from the mice that had received vehicle control showed no eGFP. Further, the eGFP expression was specifically present in β -cells as shown by **(d)** double immunofluorescence staining of GFP, **(e)** similar staining of insulin, or **(f)** a merged image of both. Moreover, the GFP expression was completely absent in **(g–i)** exocrine pancreatic tissue, liver, and spleen. The photographs are representative of two independent experiments, each performed in duplicate. Magnification **a–c** and **g–i**, $\times 100$; **d–f**, $\times 1,000$. AAV, adeno-associated virus.

in vitro in triplicate in a 12-well plate. At 4 and 6 days after infection, the level of IL-4 secreted into the medium was determined using enzyme-linked immunosorbent assay. As shown in **Figure 2a**, the levels of IL-4 secreted by the β -cells increased with time, whereas only negligible amounts of IL-4 were detected in islets transduced with AAV expressing GFP, and in mock-infected (control) islets. Similarly, significant amounts of IL-4 were detected in islet homogenates infected with dsAAV expressing IL-4 as compared to mock (control) and GFP-transduced islets (**Figure 2b**).

Subsequently, in order to demonstrate expression of IL-4 after transduction of endogenous islets, 4-week-old female NOD mice were injected IP with dsAAV8-mIP-IL-4. The islets (100) from five mice were isolated 2 weeks after injection and cultured in a 12-well plate. As shown in **Figure 2c**, significant expression of IL-4 (**Figure 2c**) was detected in NOD islets infected with dsAAV8-mIP-IL-4 as compared to eGFP (control). Although it is possible that some of the IL-4 expression could have been from endogenous cells, the major part of the IL-4 expression is exogenous IL-4 from the AAV-transduced β -cells. Also, it is important to note that no expression of IL-4 was detected in the sera of mice in any of the three groups (data not shown).

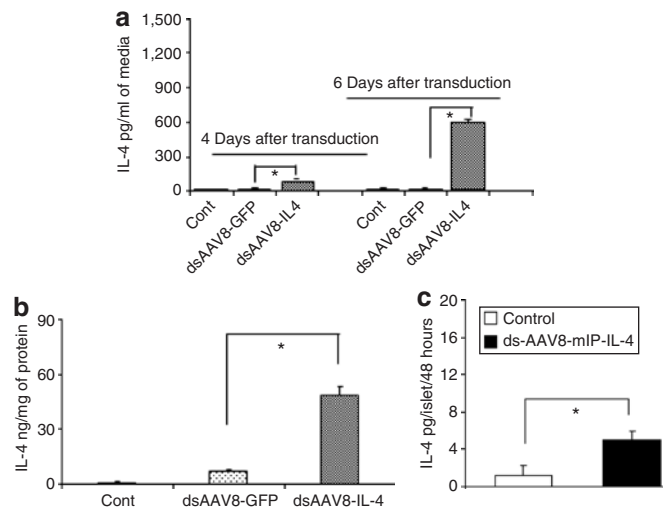


Figure 2 Murine interleukin-4 (mIL-4) secretion in media from dsAAV8mIP-eGFP- and mIL-4-transduced islets. Groups of 100 isolated islets each were transduced with double-stranded adeno-associated virus type 8 (dsAAV8) expressing either GFP or mIL-4 under mIP, and subsequently cultured in Roswell Park Memorial Institute (RPMI) medium for 6 days. The mIL-4 secreted into the medium was assayed using enzyme-linked immunosorbent assay. The mIL-4 released into the medium by (a) enhanced green fluorescent protein (eGFP)- and AAV8-mIL-4-transduced islets on days 4 and 6 of culture, and (b) in the islet lysate on day 6 respectively post-transduction and culture. The secretion of mIL-4 from dsAAV8mIP-eGFP and mIL-4 *in vivo*-transduced nonobese diabetic (NOD) islets. 4×10^{11} vector genome of dsAAV8-mIP expressing either eGFP or mIL-4 was injected intraperitoneally into the mice. The animals were killed, and the islets were subsequently isolated as described in Materials and Methods. A group of 100 isolated islets was cultured in RPMI medium for 48 hours and the levels of mIL-4 in the medium were determined as above. (c) mIL-4 released by eGFP (control)- and mIL-4-transduced islets into 48-hour culture media. The data represent the mean \pm SEM of two independent experiments, each carried out in triplicate (* $P < 0.001$).

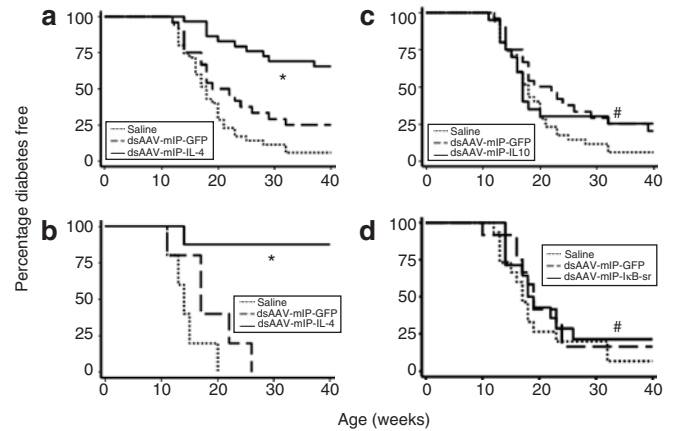


Figure 3 Murine interleukin-4 (mIL-4) expression in pancreatic islet β -cells of female nonobese diabetic (NOD) mice reduced the onset of hyperglycemia. Four-week-old female NOD mice received dsAAV8 expressing vehicle ($n = 35$), green fluorescent protein (GFP) ($n = 24$), mIL-4 ($n = 29$), mIL-10 ($n = 20$), or mIL-4 super-repressor ($n = 13$), while 8-week-old NOD mice received dsAAV-mIL-4 ($n = 10$). The onset of diabetes was monitored as described in Materials and Methods. A significant higher number of (a) 4-week-old and (b) 8-week-old mice treated with dsAAV8-mIP-IL-4 were normal as compared to those that had received enhanced GFP and vehicle control. There was no difference in the incidence of diabetes in (c) mIL-10- and (d) mIL-4-sr-treated mice as compared to controls. A P value of < 0.05 by log rank test analysis was used to indicate a statistically significant percentage of normo-glycemic animals. * $P < 0.001$, #nonsignificant. dsAAV, double-stranded adeno-associated virus.

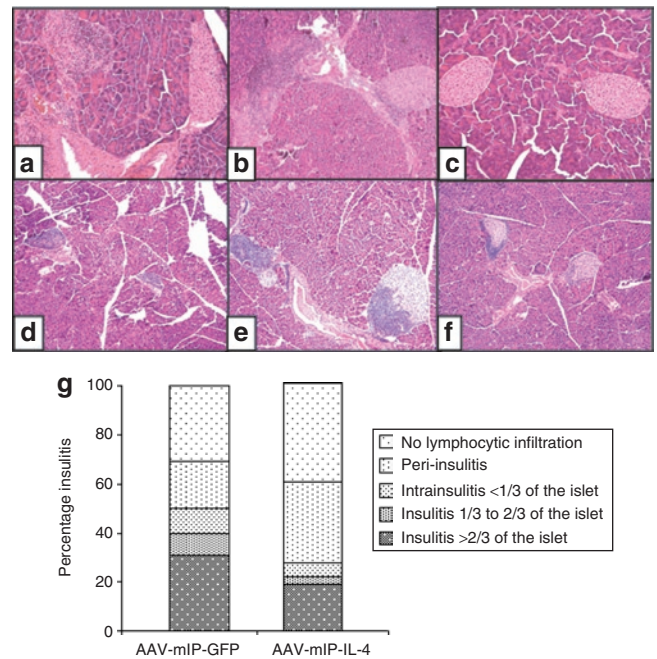


Figure 4 Histochemical analysis of infiltrating leukocytes in 20-week-old normoglycemic mice. Photomicrographs of hematoxylin and eosin for infiltrating leukocytes in 8-week normoglycemic female nonobese diabetic mice showed mild mononuclear cell infiltration in and around the islets in saline- and green fluorescent protein (GFP)-treated mice (a,b), whereas there was no mononuclear cell infiltration in the dsAAV8-mIP-IL-4-treated mice (c). However, in 21-week-old female nonobese diabetic mice showed mild to severe mononuclear cell infiltration in and around the islets in saline- and green fluorescent protein (GFP)-treated mice (d,e), whereas (f) the dsAAV8-mIP-IL-4-treated mice showed limited peri-islet mononuclear cell infiltration in significantly fewer number of islets. (g) The insulinitis score was determined as described in Materials and Methods. Original magnification $\times 100$. AAV, adeno-associated virus; IL, interleukin.

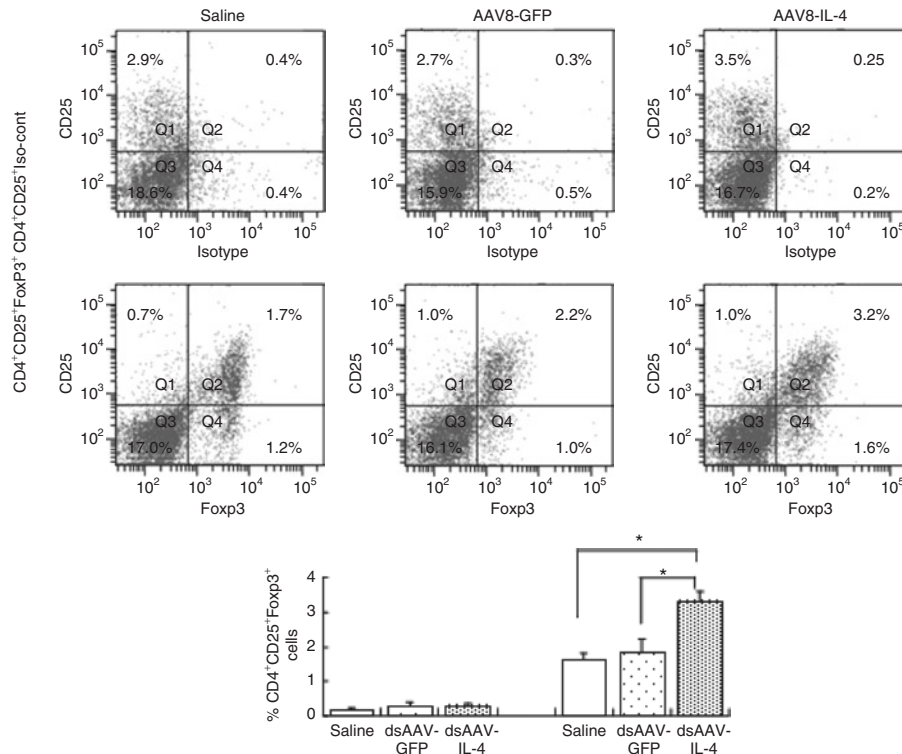


Figure 5 CD4⁺CD25⁺FoxP3⁺ T cells from spleens of 8-week-old female nonobese diabetic (NOD) mice treated with dsAAV8-mIP-IL-4. Four-week-old NOD mice received dsAAV8-mIP expressing either mIL-4 or enhanced green fluorescent protein (eGFP). Four weeks after the injection, the animals were killed, the spleens were collected, and the CD4⁺CD25⁺FoxP3⁺ T cells were analyzed by fluorescence-activated cell sorting. Splenocytes were stained for CD4⁺CD25⁺ and isotype control for FoxP3 (top panel); and splenocytes were stained for CD4⁺CD25⁺FoxP3⁺ (bottom panel). The bar diagram shows the percentage of CD4⁺CD25⁺FoxP3⁺ cells in splenocytes of control, AAV-GFP-, and AAV-IL4-injected mice. The data are mean \pm SD of two independent experiments, each carried out in triplicate. (* $P < 0.04$). AAV, adeno-associated virus; IL, interleukin; mIL, murine IL.

Prevention of onset of diabetes in NOD mice treated with dsAAV-mIP-IL-4

NOD mice spontaneously develop insulinitis ~4 weeks of age, followed by the onset of hyperglycemia between 12 and 18 weeks of age in 80–90% of the female animals.¹ It has been reported that the inflammatory process in early diabetes is initiated and propagated by the effect of TH1-secreted cytokines such as interferon- γ , and suppressed by TH2-secreted anti-inflammatory cytokines such as IL-4 and IL-10.^{4,7,12–14,26} We wanted to determine the possible therapeutic effects of IL-4 expression from endogenous β -cells on the course of T1D in NOD mice. For this purpose, 4-week-old female NOD mice (at the time point of onset of insulinitis) were injected IP with 4×10^{11} v.g. of the recombinant AAV vector expressing mIL-4. Blood glucose was monitored weekly until the experiment was terminated when the animals were 40 weeks of age. As shown in **Figure 3a**, dsAAV-mediated intrapancreatic β -cell expression of IL-4 significantly reduced the frequency of onset of hyperglycemia as compared to GFP and vehicle control mice. However, in the GFP control mice, the onset of disease was only slightly delayed as compared to the saline controls (**Figure 3a**). In contrast, treatment with dsAAV vectors expressing IL-10 and I κ B super-repressor appeared to have no therapeutic effect of preventing or delaying the onset of the disease (**Figure 3c** and **d**).

In order to determine the possible therapeutic effects of IL-4 expression in NOD mice with progressive expansion of insulinitis,

8-week-old female NOD mice were injected IP with 4×10^{11} v.g. of dsAAV-mIP-IL-4 and monitored for blood glucose. As shown in **Figure 3b**, the β -cell-specific expression of mIL-4 even in 8-week-old mice significantly reduced the onset of hyperglycemia as compared to saline and eGFP controls.

Evaluation of insulinitis by histological analysis

The extent of insulinitis in the 8- and 21-week-old euglycemic mice was examined by histological analysis of pancreatic sections. In 8-week-old mice, there was mild to severe mononuclear cell infiltration in and around the islets in the saline- and GFP-treated mice (**Figure 4a** and **b**) whereas the dsAAV8-IL-4-treated mice showed limited mononuclear cell infiltration in significantly fewer numbers of islets (**Figure 4c**). In the 21-week-old NOD mice there was 50–100% of mononuclear cell infiltration in the islets in saline and eGFP control mice (**Figure 4d** and **e**). In contrast, expression of mIL-4 in the β -cells limited the insulinitis to the periphery of the islets (peri-insulinitis; **Figure 4f**).

Overall, the insulinitis score at 21 weeks of age in dsAAV-mIP-IL-4 recipient mice (1.365 ± 1.205) was lower than that in mice that had received dsAAV-mIP-eGFP (2.009 ± 1.438 ; **Figure 4g**). The percentage of total intact islets and those with mild peri-insulinitis in dsAAV-mIP-IL-4 recipient mice was 73%, which was ~37% higher than in eGFP recipient mice (36%; **Figure 4g**). Most of the insulinitis (33%) in AAV-mIP-IL-4 recipient mice occurred in the range of moderate insulinitis (insulinitis score 1); whereas 53% of

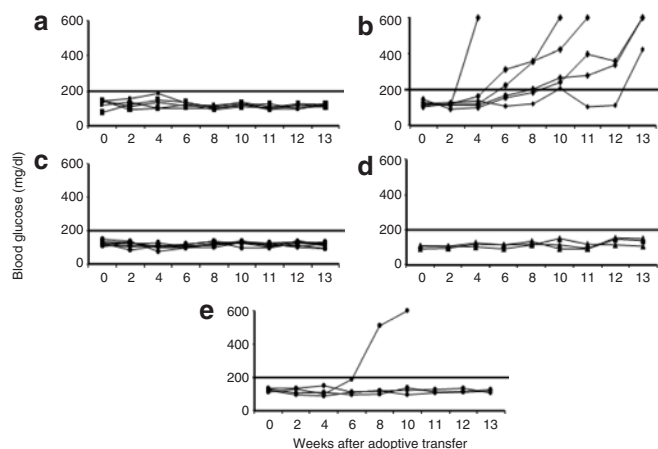


Figure 6 Adoptive transfer of diabetes in *NOD^{scid}*. Splenocytes isolated from normal 21-week dsAAV-mIP-GFP mice or normal 21- and 40-week mIL-4-transduced mice were injected intraperitoneally into *NOD^{scid}* mice, and the blood glucose levels of these mice were monitored weekly. The *NOD^{scid}* mice that received saline only did not develop disease (**a**). The *NOD^{scid}* mice that received splenocytes from green fluorescent protein (GFP)-treated mice became diabetic (**b**), whereas the splenocytes from 22- and 40-week mIL-4-treated mice did not transfer the disease to *NOD^{scid}* mice (**c-d**). In order to determine the suppressive effects of mIL-4-treated nonobese diabetic (NOD) mice splenocytes, co-adoptive transfer was performed as described in Materials and Methods. Three of the four mice that received splenocytes both from mIL-4-treated and from enhanced eGFP-treated mice did not develop diabetes (**e**). AAV, adeno-associated virus; IL, interleukin; mIL, murine IL.

the islets measured in AAV-eGFP recipient mice displayed severe insulinitis (**Figure 4g**).

Expression of IL-4 in pancreatic β -cells modulates CD4⁺CD25⁺FoxP3⁺ T-reg cells

In order to examine the mechanism through which the suppression of disease onset is mediated by IL-4 expression in the β -cells, we initially analyzed whether there was a change in the T-reg cell population in the spleen. As shown in **Figure 5**, mIL-4 expression in pancreatic β -cells for 4 weeks resulted in normal levels of CD4⁺CD25⁺FoxP3⁺ T cells ($3.3\% \pm 0.29$) in the splenocytes. In contrast, there was significant ($P < 0.04$) decrease in the CD4⁺CD25⁺FoxP3⁺ T-cell population in the AAV-GFP ($1.83\% \pm 0.38$) and saline control ($1.6\% \pm 0.21$) mice (**Figure 5**).

Splenocytes from mice treated with dsAAV8-mIP-IL-4 are unable to adoptively transfer diabetes

We addressed the possibility that T-reg cells are able to confer protection from diabetes because of modulation by IL-4. In order to investigate this possibility, adoptive transfer experiments were performed. Splenocytes were taken from normoglycemic 21-week-old NOD mice that had received dsAAV expressing mIL-4, and these splenocytes were injected into *NOD^{scid}* mice. As shown in **Figure 6c** and **d**, the total splenocytes from dsAAV8-mIP-IL-4-treated NOD mice were unable to transfer the disease, whereas the splenocytes from dsAAV-mIP-GFP-treated (but euglycemic) mice rapidly transferred the disease (**Figure 6b**). The control, saline-injected *NOD^{scid}* were also euglycemic (**Figure 6a**).

In order to determine whether T cells from splenocytes of 21-week-old normal AAV-mIL-4-treated NOD mice would inhibit transfer of disease by diabetogenic T cells from eGFP-treated mice, an adoptive co-transfer experiment was performed. An equal number of splenocytes from the AAV-mIL-4-treated mice were co-administered with splenocytes from the AAV-eGFP-treated mice. As shown in **Figure 6e**, three of the four *NOD^{scid}* mice were protected from development of diabetes. These results suggest that local expression of mIL-4 from the islets modulates T-reg cell subsets. However, there may be additional mechanisms through which localized expression of IL-4 in β -cells suppresses onset of hyperglycemia.

DISCUSSION

We have demonstrated earlier that dsAAV8-based vectors can confer long-term, stable gene transfer and expression in pancreatic β -cells when delivered IP, intravenously, or intraductally into wild-type C57BL/6 and BALB/c mice.²⁴ In this study, we demonstrate the efficient transfer of genes to endogenous β -cells in NOD mice using a dsAAV8-mIP-eGFP vector injected IP, the results being similar to those in our earlier study in BALB/c mice.²⁴ The ability to genetically modify endogenous β -cells in a stable manner facilitates the analysis of the roles played by specific gene products in regulating the progression of T1D in animal models. Gene transfer using an mIP and ensuring expression of transgene exclusively in endogenous β -cells is more advantageous than using transgenic mice, because gene delivery can be undertaken at different stages of insulinitis to examine therapeutic efficacy. Also, it is significantly easier to use AAV gene transfer to deliver transgenes to islets than to generate and breed transgenic mice. Therefore, we initiated experiments to examine the therapeutic efficacy of specific immunosuppressive gene products, delivered exclusively to endogenous β -cells, in preventing diabetes in NOD mice. We have demonstrated that, in comparison with controls, dsAAV-mediated delivery of the mIL-4 gene to the endogenous β -cells of 4- and 8-week old NOD mice reduces insulinitis and prevents onset of hyperglycemia.

It has been shown that TH1 cells are responsible for autoimmunity in NOD mice, and that endogenous TH2 cells can protect mice against the development of diabetes.^{8,9,18,27} The inflammatory process in early diabetes is reported to be initiated and propagated, in part, by TH1 cytokines, and suppressed by TH2 anti-inflammatory cytokines. For example, systemic administration of IL-4 limits insulinitis and T1D by reversing CD4 T-cell hyporesponsiveness and potentiating TH2 cell function.^{9,28,29} Systemic administration of recombinant IL-4 to young NOD mice or expression of IL-4 within the islets of transgenic NOD mice reduced the incidence of diabetes.²⁷⁻²⁹ The protection was attributed to a reversal in CD4 T-cell hyporesponsiveness and the capacity to produce IL-4.^{27,29} It has also been reported that IL-4 expressed in the islets in transgenic mice does not prevent the generation of pathogenic islet responses, but instead induces islet Ag-specific TH2 T-cells that block the action of diabetogenic T cells in the pancreas.¹⁹

In examining the mechanism of regulation in NOD mice, we observed that no disease developed in *NOD^{scid}* mice that received splenocytes from 21- and 40-week-old dsAAV-IL-4-treated NOD mice. Also, no disease developed after co-adoptive transfer of

splenocytes from 21-week-old dsAAV-IL-4-treated NOD mice mixed with diabetogenic splenocytes. This is in contrast to the finding that NOD scid mice developed diabetes after adoptive transfer of splenocytes from disease-free AAV-mIP-eGFP mice. These results demonstrate the presence of regulatory splenocytes, presumably T-cells, in the AAV-IL4-treated mice. Consistent with this result is the observation that mIL-4 expression in pancreatic β -cells resulted in normal levels of CD4⁺CD25⁺FoxP3⁺ T cells in the spleen. It would appear, therefore, that β -cell-specific expression of IL-4 inhibits disease-causing lymphocytes, partly by maintaining the number of T-reg cells.

T-reg cells arise in the thymus as a consequence of positive selection, and they play an active role in the maintenance of immune homeostasis. Recent studies have shown that there is a reduction in the number of CD4⁺CD25⁺ T-reg cells in NOD mice as disease progresses.^{30,31} The regulatory activity is confined to CD62L- and/or CD25-expressing CD4⁺ T-cell subsets.^{32,33} In addition, CD4⁺CD25⁺ T cells effectively protected NOD scid mice in co-transfer experiments with diabetogenic CD28^{-/-} cells.³¹ Our results demonstrate that CD4⁺CD25⁺ T-reg cells in mice are affected after 4 weeks of IL-4 expression in β -cells, whereas a significantly smaller T-reg cell population was found in saline and GFP control mice. Moreover, the T-reg cells decreased more prominently with age in control mice as compared to IL-4-recipient mice (data not shown).

Immunization with self-antigens or antigenic epitopes derived from these β -cell products can prevent the onset of diabetes in NOD mice and in various models of induced diabetes, by generation of autoreactive T-reg cells.³⁴⁻³⁹ The precise role of individual effector molecules produced by T-reg cells appears to be a function of the experimental model system employed, the nature of the immunizing antigen, and the presence of immunosuppressive agents such as tumor growth factor- β , IL-10, IL-4, and cytotoxic T-lymphocyte antigen 4.³⁴⁻³⁹ We speculate that the dose and route of administration of IL-4 also mediates the regulation of the immune response, leading to the prevention of diabetes.

It is also possible that there is low-level exogenous IL-4 expression in the thymus of AAV-transduced mice which could be modulating the immune response to islet antigens. We have examined eGFP and insulin expression in the thymus from the INS2 promoter in a knock-in mouse model carrying an INS2-C-emeraldGFP for analysis of eGFP expression.⁴⁰ Analysis of insulin expression in the thymus of these mice by immunohistochemical analysis clearly showed expression as expected, but no eGFP-positive cells could be detected in the thymus. It is nevertheless possible that a low level of IL-4 expression in the thymus of the AAV-transduced mice could be mediating the progression of diabetes.

We have demonstrated that gene transfer of I κ B super-repressor and mIL-10 by AAV to endogenous NOD β -cells was unable to delay the onset of hyperglycemia. These results with AAV gene transfer of IL-10 and I κ B demonstrate the specificity of localized mIL-4 expression in blocking the onset of hyperglycemia. It is important to note that dsAAV-mIP-eGFP gene transfer to β -cells slightly delayed the onset of the disease as compared to the saline control. It is possible that the marginal, nonsignificant delay in onset of diabetes in AAV8-mIP-eGFP control-treated NOD could be attributable either to the effect of eGFP expression or to AAV infection.

In conclusion, in this study we have demonstrated the ability of dsAAV8 to transduce endogenous β -cells when delivered IP into NOD mice. We have also demonstrated its ability to prevent the onset of diabetes by local expression of mIL-4 from the islets. That is, the delivery of anti-inflammatory cytokines, cytoprotective antioxidants, and anti-inflammatory enzymes to the islets, along with antiapoptotic molecules or growth-promoting factors by dsAAV, may prevent the onset of T1D and offer a promising form of immunotherapy.

MATERIALS AND METHODS

Experimental animals. Four-week-old female NOD/LTJ mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in the specific pathogen-free animal facility at the University of Pittsburgh. All the studies were performed in accordance with the US Department of Agriculture and National Institutes of Health regulations. All animal experiments were conducted and monitored under protocols reviewed and approved by the Institutional Animal Care and Use Committee.

Construction of AAV vector. The AAV vectors, dsAAV-CMV-GFP were generated by triple plasmid transfection of 293 cells as described earlier.⁴¹⁻⁴³ The AAV vector plasmid dsAAV-mIP-GFP was made by replacing the cytomegalovirus promoter of dsAAV-CMV-GFP with a 1.13-kilobase mouse preproinsulin gene II promoter. mIP was obtained by PCR from plasmid Ad.Ins-C-GFP, as published earlier,²⁴ including full-length promoter, intron 1, and noncoding sequence of exon 1 and exon 2 of mouse preproinsulin gene II.^{24,27,44,45} The pseudotyped AAV packaging plasmid contained the AAV8 serotype capsid gene coupled with the AAV2 rep gene.^{41,46} The AAVs were purified two times by CsCl gradient ultracentrifugation,⁴³ and the titers of vector genome (v.g.) particles were determined using a standard dot-blot assay.⁴³ The double-stranded AAV vector expressing murine IL-4 (mIL-4) and IL-10 (mIL-10) was generated by replacing the GFP expression cassette with mIL-4 and mIL-10 (obtained by PCR from plasmid pAdlox-mIL-4 and IL-10).^{47,48} Murine I κ B super-repressor was a kind gift from Denis Guttridge (Ohio State University). To test the functional efficacy of these AAVs, a group of 100 isolated islets were transduced with 10,000 v.g. per islet cell and incubated at 37°C for 48 hours. The medium was replaced with fresh medium, and the mIL-4 levels were determined after 4 and 6 days of transduction and culture in media and islet lysates at the end of 6 days of culture (IL-10 and I κ B super-repressor data not shown).

In vivo AAV transduction of pancreatic islets, islet isolation, and culture. AAV8-mIP-GFP or AAV8-mIP-IL-4 at a dose of 4×10^{11} viral genomes (v.g.) per mouse, or saline was injected IP into 4-week-old female NOD/LTJ mice. Two weeks later, islets were isolated from the mouse pancreata by intraductal collagenase digestion (Type IX, 1.75 mg/ml; Sigma Chemical, St. Louis, MO) as described.^{23,49} The isolated islets were further purified by Ficoll density gradient centrifugation and were handpicked under a stereomicroscope. The purity of the islets was determined by dithizone staining, and was found to be >95% in all the isolates. Islets of $\sim 150 \mu\text{m}$ in diameter, as determined by a standard algorithm, were expressed as islet equivalents, and a group of 100 islet equivalents was handpicked, incubated for 48 hours in Roswell Park Memorial Institute medium-1640 supplemented with 20 mmol/l L-glutamine, 100 μg /ml streptomycin, 100 U/ml penicillin, and 10% heat-inactivated fetal bovine serum in a humidified 5% CO₂ incubator at 37°C. The level of secreted mIL-4 was determined using standard enzyme-linked immunosorbent assay. The GFP expression was analyzed using two-photon confocal microscopy.^{23,49}

IP injection of AAV-mIP-IL-4, IL-10, I κ B super-repressor, or eGFP in female NOD mice. 4×10^{11} v.g. of AAV8-mIP-GFP or mIL-4 vector genomes were injected in 1 ml of saline IP into 4- and 8-week-old female NOD/LTJ

mice. Similarly, 4-week-old female NOD received either AAV8-mIL-10 or AAV8-I κ B super-repressor IP. The controls received 1 ml of vehicle buffer (saline). The blood glucose levels of the animals were monitored in blood drawn from the tail vein, and only mice with blood glucose >300 mg/dl (Ascensia BREEZE; Bayer) on at least two different days were considered as being hyperglycemic.

Flow cytometric analysis of T-reg cells in mIL-4-treated NOD mice. Groups of 4-week-old female NOD/LTJ mice were injected IP with buffer or 4×10^{11} v.g. of AAV expressing either GFP or mIL-4. Eight weeks after IP delivery of dsAAV expressing eGFP or mIL-4, the mice were killed and their spleens were removed. Splenocytes were extracted and were immunostained for surface expression of CD4 and CD25, using appropriate concentrations of fluorescein isothiocyanate-conjugated anti-mouse CD4 and allophycocyanin-conjugated anti-mouse CD25 antibodies, as described in the manufacturer's protocol (eBioscience, San Diego, CA). The staining was performed at 4°C for 30 minutes, with antibodies suspended in staining buffer. After washing to remove unbound antibodies, intracellular detection of FoxP3 was performed by incubating a cross-reactive, directly conjugated murine FoxP3 antibody at 4°C for 30 minutes [phycoerythrin-conjugated, anti-mouse/rat FoxP3, clone FJK-16s and the permeabilization and fix/perm buffers that accompany the antibody (FoxP3 Staining Set; eBioscience)]. A phycoerythrin-conjugated rat IgG2a antibody was used as the isotype control. Following two additional washes in permeabilization buffer, the cells were resuspended in fluorescence-activated cell sorting buffer and maintained at 4°C before analysis. The percentage of T-reg cells was calculated in terms of the percentage of CD4⁺CD25⁺FoxP3⁺ cells within the overall cell population.

Adoptive transfer of splenocytes. Groups of six normo-glycemic female NOD/LTJ mice each, 21 and 40 (mIL-4 recipients) weeks old, pretreated with an IP injection of AAV expressing either GFP or mIL-4, were used as donor mice. The mice were killed, their spleens were removed, and equal numbers of splenocytes (20×10^6 /mouse, six donors per six recipients) were infused IP into recipient NODscid mice. In order to study the suppressive effects of T-reg cells on disease-transferring T cells, co-adoptive transfer experiments were performed. Ten million splenocytes from dsAAV-IL-4- and eGFP-treated mice were injected IP into NODscid mice. It has been reported in the literature that 10 million splenocytes injected systemically are sufficient to transfer disease into NODscid mice.⁵⁰ The blood glucose levels of all the animals were monitored in blood drawn from the tail vein, and only mice with blood glucose levels >300 mg/dl (Ascensia BREEZE; Bayer) on at least two different days were considered to be hyperglycemic.

Histochemistry. Eight- and twenty-one-week euglycemic NOD mice were killed, and the pancreata were collected for immunohistochemistry, and 5- μ m sections were prepared. The 5- μ m sections were deparaffinized, and histological analysis of infiltrating mononuclear cells was performed by routine hematoxylin and eosin staining followed by examination under a light microscope for determining insulinitis scores. The mice were assigned insulinitis scores by investigators who were blinded as to the treatment groups, and 20–40 islets were examined for each group. Instead of scoring intra-islet insulinitis merely as present or not present, we adopted a classification scheme for a more accurate statistical analysis: 0 = no lymphocytic infiltration; 1 = peri-insulinitis; 2 = intrainsulinitis affecting less than one-third of the islet area; 3 = insulinitis comprising one-third to two-third of the islet; 4 = insulinitis comprising more than two-third of the islet. The histology score index was calculated by dividing the sum of all individual islet scores by the total number of islets evaluated.⁵¹

Statistical analysis. Statistical analysis was performed using the Stata 8.2 (STATA, College Station, TX) software package, and the data collected

were expressed as mean \pm SEM. The percentage of nondiabetic mice was calculated by Kaplan–Meier survival analysis. A *P* value of <0.05, arrived at using analysis of variance and log rank test, was considered to indicate statistically significant difference.

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REFERENCES

- Shoda, LK, Young, DL, Ramanujan, S, Whiting, CC, Atkinson, MA, Bluestone, JA *et al.* (2005). A comprehensive review of interventions in the NOD mouse and implications for translation. *Immunity* **23**: 115–126.
- Chatenoud, L, Thervet, E, Primo, J and Bach, JF (1994). Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. *Proc Natl Acad Sci USA* **91**: 123–127.
- Ogawa, N, Minamimura, K, Kodaka, T and Maki, T (2006). Short administration of polyclonal anti-T cell antibody (ALS) in NOD mice with extensive insulinitis prevents subsequent development of autoimmune diabetes. *J Autoimmun* **26**: 225–231.
- Cameron, MJ, Arreaza, GA, Zucker, P, Chensue, SW, Strieter, RM, Chakrabarti, S *et al.* (1997). IL-4 prevents insulinitis and insulin-dependent diabetes mellitus in nonobese diabetic mice by potentiation of regulatory T helper-2 cell function. *J Immunol* **159**: 4686–4692.
- Maki, T, Ichikawa, T, Blanco, R and Porter, J (1992). Long-term abrogation of autoimmune diabetes in nonobese diabetic mice by immunotherapy with anti-lymphocyte serum. *Proc Natl Acad Sci USA* **89**: 3434–3438.
- Thomas, D, Zaccane, P and Cooke, A (2005). The role of regulatory T cell defects in type 1 diabetes and the potential of these cells for therapy. *Rev Diabet Stud* **2**: 9–18.
- Lee, M, Koh, JJ, Han, SO, Ko, KS and Ki, SW (2002). Prevention of autoimmune insulinitis by delivery of interleukin-4 plasmid using a soluble and biodegradable polymeric carrier. *Pharm Res* **19**: 246–249.
- Pennline, KJ, Roque-Gaffney, E and Monahan, M (1994). Recombinant human IL-10 prevents the onset of diabetes in the nonobese diabetic mouse. *Clin Immunol Immunopathol* **71**: 169–175.
- Rapoport, MJ, Jaramillo, A, Zipris, D, Lazarus, AH, Serreze, DV, Leiter, EH *et al.* (1993). Interleukin 4 reverses T cell proliferative unresponsiveness and prevents the onset of diabetes in nonobese diabetic mice. *J Exp Med* **178**: 87–99.
- Wood, SC, Rao, TD and Frey, AB (1999). Multidose streptozotocin induction of diabetes in BALB/cBy mice induces a T cell proliferation defect in thymocytes which is reversible by interleukin-4. *Cell Immunol* **192**: 1–12.
- Zipris, D and Karnieli, E (2002). A single treatment with IL-4 via retrovirally transduced lymphocytes partially protects against diabetes in BioBreeding (BB) rats. *JOP* **3**: 76–82.
- Hayashi, T, Yasutomi, Y, Hasegawa, K, Sasaki, Y and Onodera, T (2003). Interleukin-4-expressing plasmid DNA inhibits reovirus type-2-triggered autoimmune insulinitis in DBA/1J suckling mice. *Int J Exp Pathol* **84**: 101–106.
- Ko, KS, Lee, M, Koh, JJ and Kim, SW (2001). Combined administration of plasmids encoding IL-4 and IL-10 prevents the development of autoimmune diabetes in nonobese diabetic mice. *Mol Ther* **4**: 313–316.
- Wolfe, T, Bot, A, Hughes, A, Möhrle, U, Rodrigo, E, Jaume, JC *et al.* (2002). Endogenous expression levels of autoantigens influence success or failure of DNA immunizations to prevent type 1 diabetes: addition of IL-4 increases safety. *Eur J Immunol* **32**: 113–121.
- Feili-Hariri, M, Falkner, DH, Gambotto, A, Papworth, GD, Watkins, SC, Robbins, PD *et al.* (2003). Dendritic cells transduced to express interleukin-4 prevent diabetes in nonobese diabetic mice with advanced insulinitis. *Hum Gene Ther* **14**: 13–23.
- Goudy, K, Song, S, Wasserfall, C, Zhang, YC, Kapturczak, M, Muir, A *et al.* (2001). Adeno-associated virus vector-mediated IL-10 gene delivery prevents type 1 diabetes in NOD mice. *Proc Natl Acad Sci USA* **98**: 13913–13918.
- Kapturczak, MH, Flotte, T and Atkinson, MA (2001). Adeno-associated virus (AAV) as a vehicle for therapeutic gene delivery: improvements in vector design and viral production enhance potential to prolong graft survival in pancreatic islet cell transplantation for the reversal of type 1 diabetes. *Curr Mol Med* **1**: 245–258.
- Gallichan, WS, Balasa, B, Davies, JD and Sarvetnick, N (1999). Pancreatic IL-4 expression results in islet-reactive Th2 cells that inhibit diabetogenic lymphocytes in the nonobese diabetic mouse. *J Immunol* **163**: 1696–1703.
- Mueller, R, Krahl, T and Sarvetnick, N (1996). Pancreatic expression of interleukin-4 abrogates insulinitis and autoimmune diabetes in nonobese diabetic (NOD) mice. *J Exp Med* **184**: 1093–1099.
- Csete, ME, Afra, R, Mullen, Y, Drazan, KE, Benhamou, PY and Shaked, A (1994). Adenoviral-mediated gene transfer to pancreatic islets does not alter islet function. *Transplant Proc* **26**: 756–757.
- Csete, ME, Benhamou, PY, Drazan, KE, Wu, L, McIntee, DF, Afra, R *et al.* (1995). Efficient gene transfer to pancreatic islets mediated by adenoviral vectors. *Transplantation* **59**: 263–268.
- Flotte, T, Agarwal, A, Wang, J, Song, S, Fenjves, ES, Inverardi, L *et al.* (2001). Efficient *ex vivo* transduction of pancreatic islet cells with recombinant adeno-associated virus vectors. *Diabetes* **50**: 515–520.

23. Rehman, KK, Wang, Z, Bottino, R, Balamurugan, AN, Trucco, M, Li, J *et al.* (2005). Efficient gene delivery to human and rodent islets with double-stranded (ds) AAV-based vectors. *Gene Ther* **12**: 1313–1323.
24. Wang, Z, Zhu, T, Rehman, KK, Bertera, S, Zhang, J, Chen, C *et al.* (2006). Widespread and stable pancreatic gene transfer by adeno-associated virus vectors via different routes. *Diabetes* **55**: 875–884.
25. Watkins, S, Geng, X, Li, L, Papworth, G, Robbins, PD and Drain, P (2002). Imaging secretory vesicles by fluorescent protein insertion in propeptide rather than mature secreted peptide. *Traffic* **3**: 461–471.
26. Raz, I, Eldor, R and Naparstek, Y (2005). Immune modulation for prevention of type 1 diabetes mellitus. *Trends Biotechnol* **23**: 128–134.
27. Debray-Sachs, M, Carnaud, C, Boitard, C, Cohen, H, Gresser, I, Bedossa, P *et al.* (1991). Prevention of diabetes in NOD mice treated with antibody to murine IFN- γ . *J Autoimmun* **4**: 237–248.
28. Inobe, JI, Chen, Y and Weiner, HL (1996). *In vivo* administration of IL-4 induces TGF- β -producing cells and protects animals from experimental autoimmune encephalomyelitis. *Ann NY Acad Sci* **778**: 390–392.
29. Tominaga, Y, Nagata, M, Yasuda, H, Okamoto, N, Arisawa, K, Moriyama, H *et al.* (1998). Administration of IL-4 prevents autoimmune diabetes but enhances pancreatic insulinitis in NOD mice. *Clin Immunol Immunopathol* **86**: 209–218.
30. Green, EA, Choi, Y and Flavell, RA (2002). Pancreatic lymph node-derived CD4⁺CD25⁺ Treg cells: highly potent regulators of diabetes that require TRANCE-RANK signals. *Immunity* **16**: 183–191.
31. Salomon, B, Lenschow, DJ, Rhee, L, Ashourian, N, Singh, B, Sharpe, A *et al.* (2000). B7/CD28 costimulation is essential for the homeostasis of the CD4⁺CD25⁺ immunoregulatory T cells that control autoimmune diabetes. *Immunity* **12**: 431–440.
32. Herbelin, A, Gombert, JM, Lepault, F, Bach, JF and Chatenoud, L (1998). Mature mainstream TCR alpha beta⁺CD4⁺ thymocytes expressing L-selectin mediate “active tolerance” in the nonobese diabetic mouse. *J Immunol* **161**: 2620–2628.
33. Lepault, F and Gagnerault, MC (2000). Characterization of peripheral regulatory CD4⁺ T cells that prevent diabetes onset in nonobese diabetic mice. *J Immunol* **164**: 240–247.
34. Belghith, M, Bluestone, JA, Barriot, S, Mègret, J, Bach, JF and Chatenoud, L (2003). TGF- β -dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. *Nat Med* **9**: 1202–1208.
35. Bergerot, I, Arreaza, GA, Cameron, MJ, Burdick, MD, Strieter, RM, Chensue, SW *et al.* (1999). Insulin B-chain reactive CD4⁺ regulatory T-cells induced by oral insulin treatment protect from type 1 diabetes by blocking the cytokine secretion and pancreatic infiltration of diabetogenic effector T-cells. *Diabetes* **48**: 1720–1729.
36. Maron, R, Melican, NS and Weiner, HL (1999). Regulatory Th2-type T cell lines against insulin and GAD peptides derived from orally- and nasally-treated NOD mice suppress diabetes. *J Autoimmun* **12**: 251–258.
37. Maron, R, Palanivel, V, Weiner, HL and Harn, DA (1998). Oral administration of schistosoma egg antigens and insulin B-chain generates and enhances Th2-type responses in NOD mice. *Clin Immunol Immunopathol* **87**: 85–92.
38. Tian, J, Clare-Salzler, M, Herschenfeld, A, Middleton, B, Newman, D, Mueller, R *et al.* (1996). Modulating autoimmune responses to GAD inhibits disease progression and prolongs islet graft survival in diabetes-prone mice. *Nat Med* **2**: 1348–1353.
39. von Herrath, MG, Dyrberg, T and Oldstone, MB (1996). Oral insulin treatment suppresses virus-induced antigen-specific destruction of beta cells and prevents autoimmune diabetes in transgenic mice. *J Clin Invest* **98**: 1324–1331.
40. Ben-Yehudah, A, Reinhart, B, Navara, C, Kotzok, J, Witelch, S, Schatten, G *et al.* (2005). Specific dynamic and noninvasive labeling of pancreatic beta cells in reporter mice. *Genesis* **43**: 166–174.
41. Rabinowitz, JE, Rolling, F, Li, C, Conrath, H, Xiao, W, Xiao, X *et al.* (2002). Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. *J Virol* **76**: 791–801.
42. Wang, Z, Ma, H, Li, J, Sun, L, Zhang, J and Xiao, X (2003). Rapid and highly efficient transduction by double-stranded adeno-associated virus vectors *in vitro* and *in vivo*. *Gene Ther* **10**: 2105–2111.
43. Xiao, X, Li, J and Samulski, RJ (1998). Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J Virol* **72**: 2224–2232.
44. Geng, X, Li, L, Watkins, S, Robbins, PD and Drain, P (2003). The insulin secretory granule is the major site of K(ATP) channels of the endocrine pancreas. *Diabetes* **52**: 767–776.
45. Wentworth, BM, Schaefer, IM, Villa-Komaroff, L and Chirgwin, JM (1986). Characterization of the two nonallelic genes encoding mouse preproinsulin. *J Mol Evol* **23**: 305–312.
46. Rutledge, EA, Halbert, CL and Russell, DW (1998). Infectious clones and vectors derived from adeno-associated virus (AAV) serotypes other than AAV type 2. *J Virol* **72**: 309–319.
47. Kim, SH, Kim, S, Evans, CH, Ghivizzani, SC, Oligino, T and Robbins, PD (2001). Effective treatment of established murine collagen-induced arthritis by systemic administration of dendritic cells genetically modified to express IL-4. *J Immunol* **166**: 3499–3505.
48. Kim, SH, Lechman, ER, Bianco, N, Menon, R, Keravala, A, Nash, J *et al.* (2005). Exosomes derived from IL-10-treated dendritic cells can suppress inflammation and collagen-induced arthritis. *J Immunol* **174**: 6440–6448.
49. Rehman, KK, Bertera, S, Bottino, R, Balamurugan, AN, Mai, JC, Mi, Z *et al.* (2003). Protection of islets by *in situ* peptide-mediated transduction of the I κ B kinase inhibitor Nemo-binding domain peptide. *J Biol Chem* **278**: 9862–9868.
50. Fuchtenbusch, M, Larger, E, Thebault, K and Boitard, C (2005). Transfer of diabetes from prediabetic NOD mice to NOD-SCID/SCID mice: association with pancreatic insulin content. *Horm Metab Res* **37**: 63–67.
51. Rietz, C, Screpanti, V, Brenden, N, Böhme, J and Fernández, C (2003). Overexpression of bcl-2 in T cells affects insulinitis in the nonobese diabetic mouse. *Scand J Immunol* **57**: 342–349.