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Induction of Robust Diabetes Resistance and Prevention of Recurrent Type 1 Diabetes Following Islet Transplantation by Gene Therapy

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We have previously shown that the development of type 1 diabetes (T1D) can be prevented in nonobese diabetic (NOD) mice by reconstitution with autologous hemopoietic stem cells retrovirally transduced with viruses encoding MHC class II I-A β-chain molecules associated with protection from the disease. In this study we examined whether a blockade of the programmed death-1 (PD-1)-programmed death ligand-1 (PD-L1) pathway, a major pathway known to control diabetes occurrence, could precipitate T1D in young NOD mice following reconstitution with autologous bone marrow retrovirally transduced with viruses encoding protective MHC class II I-A β-chain molecules. In addition, we examined whether the expression of protective MHC class II alleles in bone marrow-derived cells was sufficient to induce resistance to diabetes in mice with pre-existing disease following islet transplantation. Protection from the occurrence of T1D diabetes in young NOD mice by the expression of protective MHC class II I-A β-chain molecules in bone marrow-derived hemopoietic cells was resistant to induction by PD-1-PD-L1 blockade. Moreover, reconstitution of NOD mice with pre-existing T1D autologous hemopoietic stem cells transduced with viruses encoding protective MHC class II I-A β-chain alleles allowed for the successful transplantation of syngeneic islets, resulting in the long-term reversal of T1D. Reversal of diabetes was resistant to induction by PD-1-PDL-1 blockade and depletion of CD25+ T cells. These data suggest that expression of protective MHC class II alleles in bone marrow-derived cells establishes robust self-tolerance to islet autoantigens and is sufficient to prevent the recurrence of autoimmune diabetes following islet transplantation. The Journal of Immunology, 2007, 179: 6762–6769.

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3 Abbreviations used in this paper: T1D, type 1 diabetes; GAD 65, glutamic acid decarboxylase 65; GVHD, graft-vs-host disease; MBP, myelin basic protein; PD-1, programmed death-1; PD-L, programmed death ligand.

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tolerance as seen with mixed cellular chimerism (30, 31). In addition, we have shown that the induction of molecular chimerism can be used to prevent the development of autoimmunity in T1D (31, 33). The expression of diabetes-resistant protective MHC class II I-A β-chain molecules in NOD mice following retroviral transduction of autologous bone marrow hemopoietic stem cells prevented the development of autoimmune T cells by intrathymic deletion and protected the mice from the development of insulin and diabetes (31). These data suggest that T1D could be prevented in individuals expressing MHC alleles associated with susceptibility to disease by restoring protective MHC class II expression through the genetic engineering of autologous hemopoietic stem cells.

To further examine the potential clinical utility of using the induction of molecular chimerism to treat T1D, we set out to determine whether protection from disease is robust. Programmed death-1 (PD-1) binds two ligands, programmed death ligand-1 (PD-L1) and programmed death ligand-2 (PD-L2). PD-1-PD-L interactions control the induction and maintenance of peripheral T cell tolerance (reviewed in Ref. 34). Studies have shown that PD-1 or PD-L1 but not PD-L2 blockade rapidly precipitated diabetes in prediabetic NOD mice regardless of age and is thus a major pathway that controls diabetes development (35). We therefore used PD-1-PD-L1 blockade as a way to measure whether the protection from disease is robust. We also wished to examine whether this approach, in addition to preventing the development of T1D in genetically prone individuals without pre-existing disease, can be used to treat individuals with pre-existing disease and prevent recurrence after islet transplantation. Accordingly, we examined whether the induction of molecular chimerism and the expression of protective MHC class II alleles on bone marrow-derived cells from NOD mice with pre-existing disease is sufficient to prevent the recurrence of T1D in these mice after treatment and allow for the long-term restoration of normoglycemia through islet transplantation. Our results indicate that protection from diabetes using this approach is resistant to induction through PD-1-PD-L1 blockade. Moreover, expression of the protective MHC class II I-Aβ1 chain on hemopoietic cells following retroviral transduction of bone marrow is sufficient to prevent the destruction of transplanted syngeneic islets and therefore the recurrence of diabetes in NOD mice with pre-existing T1D despite the presence of peri-insulins within the islet transplant.

Materials and Methods

**Mice**

NOD/LtJ (NOD) and NOD.CB17-PrkdscID/J (NOD.ScID) mice were purchased from The Jackson Laboratory. All experiments were performed in accordance with institutional guidelines.

**Vector construction and production of recombinant retroviruses**

The construction of the vector encoding MMP-IAβ/H9252-GFP and the production of a recombinant retrovirus was described previously (31). Viruses preparations tested negative for helper virus contamination. The titer of retroviral supernatants used in this study were between 3 and 5 × 10⁶ infectious particles per ml. The control MMP-GFP retrovirus was kindly provided by Dr. R. Mulligan through the Harvard Gene Therapy Initiative, Harvard Institute of Human Genetics (Boston, MA).

**Bone marrow transduction and transplantation**

Bone marrow cells were harvested from NOD mice as described previously (29). Briefly, 3- to 4-wk-old female NOD mice were injected via the tail vein with 150 mg/kg 5-fluorouracil. Seven days later the mice were sacrificed and bone marrow was harvested from the hind limbs by flushing with sterile medium. The bone marrow was filtered through sterile nylon monofilament mesh, and RBC lysed. The cells were then transduced as described (31). At the end of the transduction period the frequency of mature lymphocytes in the bone marrow preparation was usually <1%. A multiplicity of infection of 2 for MMP-IAβ-d-GFP- and 3 for MMP-GFP-transduced cells was used. After 96 h in culture, cells were harvested, washed, and 4 × 10⁶ cells were injected i.v. into preconditioned recipients. Recipients were preconditioned with 11 gray of whole body irradiation 1 day before bone marrow transplantation.

**Antibodies and PD-1-PDL-1 blockade**

A mAb specific for I-Aβ (39-10-8) was obtained from BD Pharmingen. mAbs specific for FoxP3 (FJK-16s) were obtained from eBioscience. A rabbit anti-mouse CD3 Ab was obtained from Cell Marque. Guinea pig anti-porcine insulin Abs were obtained from DakoCytomation. The anti-mouse PD-L1 mAb (MH6: rat IgG2a) was generously provided by Dr. Miyuki Azuma (36) and manufactured and purified by BioExpress. Rat IgG (Sigma-Aldrich) was used as a control. Anti-mouse PD-L1 mAb or rat IgG were given i.p. as follows: 500 μg on day 0 followed by 250 μg on days 2, 4, 6, 8, and 10 (35).

**In vivo depletion of CD25+ T cells**

To deplete CD25+ T cells, mice were injected i.p. with 0.5 mg of the anti-CD25 Ab PC61 (manufactured and purified by BioExpress) every 3 days. A total of four doses was given. Depletion of CD25+ T cells was confirmed by flow cytometry after staining blood cells with the anti-CD25 Ab 7D4 (BD Pharmingen) 5 days after the completion of Ab treatment. Staining with 7D4 is not blocked by PC61.

**Blood glucose levels and insulin treatment**

Blood glucose levels were monitored weekly before and daily after the onset of diabetes using a BD Logic blood glucose monitor (BD Biosciences). Two consecutive readings of ≥250 mg/dl were considered to be indicative of the development of hyperglycemia. Diabetic NOD mice were maintained with a daily injection of 2–6 U of insulin, a 1:1 mixture of Humulin U and Humulin L (Eli Lilly).

**Islet transplantation**

After naive female NOD mice developed T1D spontaneously, mice were reconstituted with retrovirally transduced bone marrow cells. Five to six weeks after reconstitution, the mice were confirmed to be diabetic by suspending insulin injections and demonstrating that the blood glucose level was ≥250 mg/dl. Mice were then transplanted with ~1000 islets from female NOD.SCID donors under the renal capsule as described previously (37). This islet dose was used to allow us to observe a transient reversal of hyperglycemia in control mice and to rule out technical failures of the islet transplant procedure. Islet graft function was assessed by blood glucose measurements daily for 1 wk and then three times a week until the mice were sacrificed. Reversal of T1D was defined as blood glucose levels of <200 mg/dl after islet transplantation. Graft rejection was defined as blood glucose levels of ≥250 mg/dl on two consecutive days. Rejection was also confirmed by histology.

**ELISPOT**

Cytokine ELISPOT assays were performed as described previously (31). Briefly, BVD-1D11 (anti-IL-4) and R4-6A2 (anti-IFN-γ) (Invitrogen Life Technologies) were used at 10 μg/ml to coat plates. Splenocytes (10⁶) were added to each well. Cells were cultured overnight with 0.1 mg/ml insulin (Sigma-Aldrich) and the myelin basic protein (MBP) peptide (peptide 85–99; ENPVHVFKNIVTPR) or the glutamic acid decarboxylase 65 (GAD 65) peptide (peptide 206–220; TYELAPVFILLEYVT). Peptides were obtained from Invitrogen Life Technologies. Cytokine production was detected using 10 μg/ml biotinylated BVD-2G4 (anti-IL-4) and XMG1.2 (anti-IFN-γ) (Invitrogen Life Technologies). Assays were performed in triplicate and averaged. To calculate the specific response to GAD 65 and insulin, the average background response to MBP protein was subtracted. There was no significant difference in the response to MBP peptide between the groups (not shown).

**Histology**

Ninety days after islet transplantation or when the mice became diabetic, islet grafts along with the kidney were collected from recipients reconstituted with either MMP-IAβ-d-GFP- or MMP-GFP-transduced bone marrow and fixed in buffered formalin. Immunohistochemistry staining was performed using 5-μm thick paraffin-embedded tissue sections. Briefly, slides were deparaffinized through xylene, passed through graded alcohol, and put in distilled water. Slides were then pretreated with 1.0 mM EDTA (pH 8.0) (Zymed Laboratories) in a steam pressure cooker (Biocare Medical Decloaking Chamber) followed by washing in distilled water. All further steps were performed at room temperature in a hydrated chamber. Slides were
pretreated with Peroxidase Block (DakoCytomation) for 5 min to quench endogenous peroxidase activity. Primary rabbit anti-CD3 Ab (Cell Marque) or primary rat anti-FoxP3 Ab (eBioscience) were applied in DakoCytomation diluent for 1 h. Slides were washed in 50 mM Tris-Cl (pH 7.4) and detected with an anti-rabbit EnVision + kit (DakoCytomation) or polyclonal biotinylated rabbit anti-rat Ig (DakoCytomation) as per the manufacturer’s instructions. After further washing, immunoperoxidase staining was developed using a DAB chromogen (3,3′-diaminobenzidine in chromogen solution; DakoCytomation) and counterstained with hematoxylin. For insulin staining, tissue sections were stained with guinea pig anti-porcine insulin Ab for 15 min and stained with a biotinylated goat anti-guinea pig secondary Ab (Vector Laboratories) for 30 min. Sections were developed using an avidin-biotin complex (ABC) solution and 3,3′-diaminobenzidine (Vector Laboratories) and counterstained with hematoxylin. Samples were then analyzed by bright field microscopy.

Results
Expression of retrovirally encoded MHC class II-GFP fusion genes following reconstitution of NOD mice with transduced syngeneic bone marrow

To examine the role of MHC class II in providing protection from a recurrence of diabetes following islet transplantation, genetic engineering of bone marrow hemopoietic stem cells was used to achieve the expression of retrovirally encoded MHC class II β-chains associated with protection from disease in autologous hemopoietic cells of NOD mice. Bone marrow was harvested from 3- to 4-wk-old female NOD/LtJ mice (hereafter referred to as NOD mice) treated (median onset time 4.2%) with control MMP-GFP-transduced bone marrow developed hyperglycemia in 134 days following anti-I-Ad treatment (median onset time 130 days, n = 6; Fig. 2). This ratio was similar to that observed following infection with MMP-GFP (~17%), a control virus that carries only the gene encoding GFP. Mock-transduced bone marrow cells remained negative (Fig. 1A).

NOD mice expressing protective MHC class II alleles in bone marrow-derived cells are resistant to diabetes induction by PD-1-PD-L1 blockade

To determine whether the prevention of disease occurrence conferred by the expression of protective MHC class II β-chains is robust, we examined whether blockade of the PD-1-PD-L1 pathway could precipitate the occurrence of T1D. To this end, 4-wk-old NOD mice were lethally irradiated and reconstituted the following day with either MMP-IAβ-d-GFP- or MMP-GFP-transduced bone marrow cells as described above. Four weeks after reconstitution, recipient mice were treated with anti-PD-L1 mAb or rat IgG as a control. The frequency of cells expressing the retrovirally transduced MHC-GFP fusion protein was similar to that reported previously (31). Approximately 67% of mice reconstituted with control MMP-GFP-transduced bone marrow developed hyperglycemia in 134 days following control rat IgG treatment (median onset time = 130 days, n = 6; Fig. 2). This ratio was similar to that of untreated naive NOD mice in our colony (not shown). Consistent with our previous data showing that a PD-1-PD-L1 blockade precipitates diabetes in an accelerated fashion (35), 75% of mice reconstituted with control MMP-GFP-transduced bone marrow developed hyperglycemia within 85 days following anti-PD-L1 treatment (median onset time = 32 days, n = 6, p < 0.001 between groups; Fig. 2). In contrast, mice reconstituted with MMP-IAβ-d-GFP-transduced bone marrow remained normoglycemic for at least 180 days following control rat IgG treatment (median onset time >180 days, n = 9), at which time point the experiment was terminated (Fig. 2). Moreover, 10 of 11 mice reconstituted with MMP-IAβ-d-GFP-transduced bone marrow remained normoglycemic for 180 days following anti-PD-L1 treatment (median onset time >180 days, n = 11; Fig. 2). These data suggest that the prevention of T1D in young NOD mice induced by molecular chimerism is resistant to precipitation by a PD-1-PD-L1 blockade.

Expression of protective MHC class II genes in NOD mouse bone marrow-derived cells prevents recurrence of T1D following islet transplantation

We next set out to examine the effectiveness of inducing molecular chimerism by examining whether the expression of retrovirally transduced diabetes-protective MHC class II genes in bone marrow-derived cells can prevent diabetes recurrence following islet transplantation. Fourteen- to 22-wk-old spontaneously diabetic female NOD mice with pre-existing disease (blood glucose level of >250 mg/dl) were maintained with daily injections of 2–6 U of a 1:1 mixture of Humulin U and Humulin L. The mice were lethally irradiated (11 gray) and reconstituted with 4 × 10^6 MMP-IAβ-d-GFP or MMP-GFP virus-transduced syngeneic bone marrow cells from 3 to 4 wk old nonidiabetic female donor NOD mice. Four weeks after bone marrow transplantation, 20.3 ± 3.7% (n = 16) of PBMC from mice reconstituted with MMP-GFP-transduced bone marrow expressed GFP. In these mice, both MHC class II- and as well as class II- cells derived from transduced progenitors expressed GFP (Fig. 1B). In mice reconstituted with MMP-IAβ-d-GFP-transduced bone marrow, 19.5 ± 4.2% (n = 16) of PBMC...
from mice expressed GFP (Fig. 1B), indicating similar levels of transduction compared with control mice. Although both MHC class II and well as class II cells derived from transduced progenitors expressed GFP in these mice, we wish to point out that the transduced MHC class II β-chain-GFP fusion protein is expressed on the surface of cells committed to MHC class II expression as previously reported (31). At this time point, the experiments were terminated (p < 0.01 between groups, median time of onset >90 days after islet transplantation, n = 6; Fig. 3). One mouse from the group of six mice reconstituted with MMP-IAβ-d-GFP-transduced bone marrow developed recurrent diabetes 42 days after islet transplantation. Interestingly, the mouse reconstituted with MMP-IAβ-d-GFP that developed recurrent T1D 42 days after islet transplantation showed a dramatically lower frequency of MMP-IAβ-d-GFP-expressing cells in its blood based on GFP fluorescence as assessed by flow cytometry when compared with that of other mice in the same group (<5% of nucleated cells in blood).

Expression of retrovirally encoded MHC class II-GFP fusion genes in NOD mouse bone marrow-derived cells prevents the destruction of islet grafts by autoreactive T cells

We next examined transplanted islets histologically to further assess the degree of protection from recurrent disease. Mice reconstituted with MMP-IAβ-d-GFP or control MMP-GFP-transduced bone marrow that received an islet transplant were sacrificed 90 days after transplantation or when the mice developed recurrent diabetes (n = 6 per group). Tissue sections of islet grafts were then prepared and stained with H&E or anti-insulin, anti-CD3, and anti-FoxP3 Abs (Fig. 4). Islet grafts under the kidney capsule of mice reconstituted with MMP-IAβ-d-GFP-transduced bone marrow showed normal levels of insulin storage in the presence of peri-insulitis. In contrast, islets from mice reconstituted with MMP-IAβ-d-GFP-transduced bone marrow showed increased or absent insulin storage (Fig. 4). Cellular infiltrates in the insulitic lesions consisted primarily of CD3 T cells (Fig. 4).

FIGURE 2. Induction of molecular chimerism in young NOD mice is resistant to the T1D induced by anti-PD-L1 treatment. Bone marrow cells were harvested from 3- to 4-wk-old NOD mice treated with 5-fluorouracil (150 mg/kg) 7 days earlier and then transduced with a retrovirus carrying either MMP-IAβ-d-GFP (open squares (n = 9) and open diamonds (n = 11)) or MMP-GFP (filled squares (n = 6) and filled diamonds (n = 8)) alone as a control. Four-week-old recipient NOD mice were then lethally irradiated and reconstituted the following day with either MMP-IAβ-d-GFP or MMP-GFP-transduced bone marrow. Four weeks after reconstitution, recipient mice were treated with anti-PD-L1 mAb (diamonds) or rat IgG (squares) as a control. Shown is the proportion of normoglycemic mice. The data are representative of two independent experiments.

FIGURE 3. NOD mice with pre-existing diabetes reconstituted with MMP-IAβ-d-GFP-transduced bone marrow are protected from recurrent diabetes following islet transplantation. Diabetic NOD mice were reconstituted with either MMP-IAβ-d-GFP- (squares, n = 6) or control MMP-GFP-transduced bone marrow (triangles, n = 6). Five to 6 wk after reconstitution, islet transplantations were performed and blood glucose levels were measured as described in the text. Shown is the proportion (combined results of three independent experiments) of normoglycemic mice after islet transplantation.

FIGURE 4. Prevention of islet graft rejection in diabetic NOD mice reconstituted with MMP-IAβ-d-GFP-transduced bone marrow following islet transplantation. NOD mice reconstituted with either MMP-IAβ-d-GFP or MMP-GFP-transduced bone marrow were sacrificed 90 days after islet transplantation or when the mice developed recurrent diabetes. Kidneys with islet grafts were then harvested, fixed, sectioned, and stained with hematoxylin and eosin (HE) or anti-insulin, anti-CD3, and anti-FoxP3 Abs. Islet grafts from mice reconstituted with MMP-GFP-transduced bone marrow with recurrent diabetes and a blood glucose level of 250–300 mg/dl on day 1 after islet transplantation (far left column) or with a blood glucose level >300 mg/dl on day 3 after islet transplantation (second column from left) showed significant mononuclear cell infiltration, CD3 T cell infiltration, and increased (far left column) or absent (second column from left) storage of insulin. In contrast, islets from mice reconstituted with MMP-IAβ-d-GFP-transduced bone marrow showed normal levels of insulin storage in the presence of peri-insulitis. Shown are representative sections from three independent experiments.
FoxP3⁺ cells were only found in one mouse in the early stage of rejection (Fig. 4, far left column). In contrast, islet grafts from mice reconstituted with MMP-IAβ-d-GFP-transduced bone marrow showed either normal islets without insulitis or nondestructive peri-insulitis in which CD3⁺ T cell infiltration was observed around islets (Fig. 4). Insulin storage appeared to be intact in all transplanted islets, including those with peri-insulitis. FoxP3⁺ cells were found in those islet grafts with peri-insulitis, suggesting that FoxP3⁺ regulatory cells might play a role in preventing the rejection of islet grafts in these mice. Overall, these data suggest that expression of the diabetes-protective MMP-IAβ-d-GFP gene prevents the destruction of islet grafts by autoreactive T cells.

_T cells in diabetic NOD mice reconstituted with MMP-IAβ-d-GFP-transduced bone marrow following islet transplantation are not responsive to islet self-Ags_

We also examined the frequency of self-Ag reactive T cells in the spleens of islet transplant recipients sacrificed for histological analysis of their islet grafts. Splenocytes were stimulated overnight with either insulin (whole protein), peptide 206–220 from GAD 65, or peptide 85–99 from MBP as a control in ELISPOT assays. Both the GAD 65 and MBP peptides bind I-A²⁷ (38). The GAD 65 peptide has been proposed to be a potential self-Ag peptide in NOD mice (39). NOD mice with pre-existing diabetes that were reconstituted with MMP-GFP-transduced bone marrow contained a high frequency of splenocytes that were able to secrete IFN-γ (298 ± 82/10⁶ cells) and IL-4 (100 ± 67/10⁶ cells) in response to the GAD 65 peptide (Fig. 5, n = 4). In contrast, we were unable to detect significant production of either IFN-γ (9 ± 8/10⁶ cells, p < 0.01) or IL-4 (7 ± 7/10⁶ cells, p < 0.01) in response to GAD 65 peptide by splenocytes from mice reconstituted with MMP-IAβ-d-GFP-transduced bone marrow (Fig. 5). Similarly, a significantly higher number of splenocytes from NOD mice with pre-existing diabetes that were reconstituted with MMP-GFP-transduced bone marrow produced IFN-γ (815 ± 84/10⁶ cells) and IL-4 (305 ± 83/10⁶ cells) in response to insulin, while cells from NOD mice reconstituted with MMP-IAβ-d-GFP-transduced bone marrow had significantly lower number of splenocytes producing IFN-γ (21 ± 7/10⁶ cells, p < 0.01) and IL-4 (11 ± 9/10⁶ cells, p < 0.01) in response to insulin (Fig. 5). These data suggest that expression of a protective MHC class II β-chain in bone marrow-derived cells is able to prevent the recurrence of autoimmunity in diabetic NOD mice.

_Prevention of diabetes recurrence following islet transplantation in mice expressing protective MHC class II alleles is resistant to diabetes induction by PD-1-PD-L1 blockade and the depletion of CD25⁺ cells_

To determine whether the prevention of disease recurrence following islet transplantation conferred by the expression of protective MHC class II β-chains is robust, we examined whether blockade of the PD-1-PD-L1 pathway could precipitate the recurrence of T1D. Fourteen- to 22-wk-old spontaneously diabetic female NOD mice with pre-existing disease (blood glucose >250 mg/dl) were maintained with daily injections of 2–6 U of insulin, lethally irradiated (11 gray), and reconstituted with 4 × 10⁵ MMP-IAβ-d-GFP virus transduced syngeneic bone marrow cells from 3- to 4-wk-old nondiabetic female donor NOD mice as described above. Four weeks after bone marrow transplantation, the mice were then transplanted with ~1000 NOD-SCID islets under the left kidney capsule and blood glucose levels were then monitored as described above. At the time of islet transplantation, day 0, the mice were treated with 500 µg of anti-PD-L1 mAb or rat IgG followed by 250 µg on days 2, 4, 6, 8, and 10. As observed for young mice in which expression of protective MHC class II chains prevented T1D induction by PD-1-PD-L1 blockade (Fig. 2), mice reconstituted with MMP-IAβ-d-GFP-transduced bone marrow in which normoglycemia was restored following islet transplantation were protected from PD-1-PD-L1 blockade-induced diabetes (Fig. 6A).

Based on the observation that FoxP3⁺ cells are observed in islet grafts from mice reconstituted with MMP-IAβ-d-GFP-transduced bone marrow that exhibit nondestructive insulitis (Fig. 4), we next set out to determine whether the maintenance of normoglycemia following islet transplantation in mice reconstituted with MMP-IAβ-d-GFP-transduced bone marrow is controlled by regulatory T cells. To this end, spontaneously diabetic NOD mice were reconstituted with MMP-IAβ-d-GFP-transduced bone marrow and then transplanted with NOD-SCID islets as described above. On the day of islet transplantation, mice were treated with the anti-CD25-depleting Ab PC61 or rat Ig as a control and the depletion of CD25⁺ T cells was analyzed as described in Materials and Methods. Analysis of CD25 depletion by cell surface staining and flow cytometry revealed that PC61 treatment resulted in the depletion of >95% of CD25⁺ T cells in blood (not shown). Although FoxP3⁺ cells are present in islet grafts from mice reconstituted
merism in prediabetic individuals is robust and able to resist re-establishment of self-tolerance induced through molecular chimerism. This pathway it would be directly possible to test whether the disease occurrence (35). We therefore hypothesized that by blocking in young NOD mice and thus plays an important role in controlling that is involved in the induction and maintenance of peripheral T cells. The PD-1-PD-L1 pathway provides an important negative signal during immune responses precipitated after the induction of a molecular chimerism by blocking the interaction of PD-1 and PD-L1 suggesting that protection against recurrent diabetes is also robust.

Expression of a diabetes-protective MHC class II β-chain prevents the function of self-reactive T cells that respond to islet autoantigens. Islet grafts from these mice appeared either completely devoid of infiltration or contained a nondestructive peri-insulitis. In either case, insulin storage and islet architecture were normal. Our inability to detect functional autoreactive T cells in these mice by ELISPOT assays may be due to either negative selection of autoreactive clones, regulation of these cells by peripheral mechanisms, or both. However, depletion of CD25+ cells did not precipitate diabetes in these mice. Although this would suggest that CD25+ regulatory T cells do not play a role, it is important to keep in mind that activated T cells also express CD25; therefore, the lack of disease induction could be related to the depletion of recently activated effectors. Alternatively, it is possible that the expression of protective MHC class II genes in bone marrow-derived cells leads to the negative selection of autoreactive T cells. Indeed, we have previously demonstrated that the induction of molecular chimerism by the reconstitution of NOD mice with autologous bone marrow transplanted with retroviruses carrying the MHC class II β-chain genes associated with protection from diabetes prevents the recurrence of autoimmunity following islet transplantation. As observed for young mice in which the expression of protective MHC class II chains prevented disease, prevention of the recurrence of T1D following islet transplantation was resistant to disease induction by blocking the interaction of PD-1 and PD-L1 suggesting that protection against recurrent diabetes is also robust.

Discussion

Previously, we have shown that the development of T1D can be prevented in NOD mice by inducing a state of mixed chimerism through the reconstitution of conditioned recipients with autologous hemopoietic stem cells retrovirally transduced with viruses encoding MHC class II I-A β-chain molecules associated with protection from disease (31). In this study we set out to examine the potential clinical utility of using this approach to treat T1D. We reasoned that in terms of advancing this approach toward clinical applicability it would be important to determine whether the prevention of disease is robust and whether molecular chimerism can be used to prevent recurrent disease in individuals with preexisting T1D. We examined whether the protection from disease occurrence is robust by determining whether the disease could be precipitated after the induction of a molecular chimerism by blocking the interaction of PD-1 and PD-L1. The PD-1-PD-L1 pathway provides an important negative signal during immune responses that is involved in the induction and maintenance of peripheral T cell tolerance (34). It has been shown that a blockade of PD-1 or PD-L1 by mAb treatment results in the rapid precipitation of T1D in young NOD mice and thus plays an important role in controlling disease occurrence (35). We therefore hypothesized that by blocking this pathway it would be directly possible to test whether the re-establishment of self-tolerance induced through molecular chimerism in prediabetic individuals is robust and able to resist interventions that normally affect the ability to maintain peripheral tolerance. Anti-PD-L1 mAb treatment failed to precipitate the occurrence of T1D in young NOD mice following reconstitution with autologous bone marrow retrovirally transduced with viruses encoding protective MHC class II I-A β-chain molecules. These data suggest that the induction of molecular chimerism by gene therapy is a robust way to re-establish self-tolerance in prediabetic NOD mice. Moreover, these data suggest that the protection provided is able to resist alterations in peripheral tolerance induced by the blockade of the PD-1-PD-L1 pathway and that the tolerance induced through this approach involves the deletion of self-reactive T cells as we have suggested previously (31).

A major issue in the treatment of T1D is the ability to prevent the recurrence of autoimmunity. Indeed, the inability to prevent the recurrence of autoimmunity has significantly impacted the ability to treat T1D through islet transplantation. Our data suggest that the reconstitution of NOD mice with pre-existing disease using autologous bone marrow transplanted with retroviruses carrying the MHC class II β-chain genes associated with protection from diabetes prevents the recurrence of autoimmunity following islet transplantation. As observed for young mice in which the expression of protective MHC class II chains prevented disease, prevention of the recurrence of T1D following islet transplantation was resistant to disease induction by blocking the interaction of PD-1 and PD-L1, and PD-L1 suggesting that protection against recurrent diabetes is also robust.

Fourteen- to 22-wk-old spontaneously diabetic female NOD mice with pre-existing disease (blood glucose of >250 mg/dl) were maintained with daily injections of 2–6 U of insulin, lethally irradiated (11 gray), and reconstituted with $4 \times 10^6$ MMP-IAb-d-GFP virus-transduced syngeneic bone marrow cells as described above. Four weeks after bone marrow transplantation, the mice were transplanted with NOD-SCID islets under the left kidney capsule and blood glucose levels were then monitored as described above. A. At the time of islet transplantation, day 0, the mice were treated with anti-PD-L1 mAb (inverted triangles, $n = 5$) and rat IgG (triangles, $n = 5$) as described in Results. Shown is the proportion of normoglycemic mice. B. Spontaneously diabetic NOD mice were reconstituted with MMP-IAb-d-GFP-transduced bone marrow and then transplanted with NOD-SCID islets as described in Results. On the day of islet transplantation, mice were treated with the anti-CD25-depleting Ab PC61 (open circles, $n = 5$) or rat Ig (filled squares, $n = 5$). Shown is the proportion of normoglycemic mice.

with MMP-IAb-d-GFP-transduced bone marrow, treatment with PC61 failed to precipitate diabetes (Fig. 6B).

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bone marrow, although these mice remained normoglycemic. This result may suggest that although the thymus is not able to produce newly developing autoreactive T cells in molecular chimeras due to central deletion, the pre-existing self-reactive memory cell pool may be large enough to survive irradiation and persist but does not destroy the islet grafts in the recipients. Indeed, we have shown that existing T cells are resistant to irradiation (45). It is possible that, in the mice with advanced TID, peripheral autoreactive T cells are not susceptible to irradiation or deletion, accounting for peri-insulitis in the islet grafts. However, the peri-insulitis observed appears to be nondestructive, which may be due to regulation by Foxp3+ cells observed infiltrating into the islet grafts. We are presently attempting to define the role of islet graft-infiltrating Foxp3+ cells.

We did observe an instance in which a mouse reconstituted with MMP-IAβ-d-GFP-transduced bone marrow showed no significant cell infiltration within the transplanted islet graft and intact insulin storage. In this animal, we did not initially observe severe hyperglycemia, and the blood glucose level was just above the cutoff point of 250 mg/dL when reconstituted with MMP-IAβ-d-GFP-transduced bone marrow. This supports the idea that the size of the pre-existing autoreactive T cell pool might be a key factor in the development of peri-insulitis in the islet grafts of mice reconstituted with MMP-IAβ-d-GFP-transduced bone marrow. Indeed, as suggested by other investigators, there may be a qualitative change in autoreactive T cells after the development of overt diabetes in the NOD (46). The absence of Foxp3+ cells in the islet graft of this mouse further indicates that the mechanism of protection from recurrent diabetes involved the deletion of autoreactive T cells. If the pre-existing autoreactive T cell pool is relatively small, as in this instance, lethal irradiation may reduce the existing mature autoreactive T cell pool and prevent the development of peri-insulitis. In contrast, if the pre-existing autoreactive T cell pool is large as those in the mice in which glucose levels were much higher at the time of reconstitution, irradiation-resistant autoreactive T cells may persist in a significant number and will contribute to the development of peri-insulitis. We also observed that one of the six mice reconstituted with MMP-IAβ-d-GFP developed recurrence of TID 42 days after islet transplantation. This particular mouse showed dramatically lower levels of MMP-IAβ-d-GFP expression in its blood when compared with that of other mice in the same group, which may have led to the failure to re-establish tolerance to self. This result suggests that a threshold level of protective MMP-IAβ-d-GFP expression must be achieved to overcome autoimmune diabetes. Indeed, we have recently shown in molecular chimeras that persistence of Ag is important in maintaining tolerance (47).

Many gene therapy strategies aimed at preventing autoimmune diabetes by the introduction of cytokine genes or by the blockade of costimulatory molecules have achieved promising results in animal models (48–61). However, to our knowledge gene therapy strategies have in general been unsuccessful in establishing a long-term reversal of diabetes in subjects with well-established pre-existing TID (61). Most importantly, many previously described strategies attempt to prevent autoimmunity by modifying cytokine or costimulatory profiles but do not address the issue of preventing the generation and development of autoreactive T cells themselves. Such strategies will likely cause nonspecific immunosuppression in recipients. By using gene therapy to introduce a diabetes-protective MHC class II β-chain allele, we suggest it may be possible to specifically target autoreactive T cells while sparing general immunocompetence (30, 31).

Islet transplantation is a promising way to cure TID. However, for islet transplantation to be successful as a long-term cure for TID (2, 3, 62) it is necessary to overcome the underlying autoimmunity that leads to rejection of the transplanted islets (4). Our results indicate that the introduction of a diabetes-protective MHC class II β-chain on bone marrow cells is sufficient to reverse disease following islet transplantation and prevent its recurrence. Because this approach involves genetic modification of autologous hematopoietic progenitors to establish molecular rather than cellular chimerism, problems associated with the transplantation of allogeneic bone marrow, such as GVHD, are eliminated. We suggest that these results may therefore be of clinical significance in the context of islet transplantation for patients with TID. Our results are a proof of principle that the induction of molecular chimerism can be used to treat TID. A major component of moving this approach forward is to develop nonmyeloablative conditioning regimens that would permit the engraftment of genetically modified bone marrow. For individuals with preexisting disease, an interesting issue will be whether the ability of nonmyeloablative conditioning regimens to control or reduce preexisting autoreactive T cells has an impact in terms of being able to prevent disease recurrence. As suggested by others in the context of alloreactivity, conditioning regimens that permit T cell apoptosis may play an important role in reducing the size of autoreactive T cell clones and the development regulatory T cells that permit long-term graft survival (63, 64). In the context of TID, using conditioning regimens that result in a debulking of pre-existing autoreactive T cells and the development or expansion of regulatory T cells may also be important for the success of the approach we have developed.

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Disclosures
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References


