Published in final edited form as:

Exp Hematol. 2011 October ; 39(10): 977–985. doi:10.1016/j.exphem.2011.06.008.

Differential Outcomes in Pre-diabetic vs. Overtly Diabetic NOD Mice Nonmyeloablatively Conditioned with Co-stimulatory Blockade

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Abstract

Objective—Autoimmune diabetes can be reversed with mixed chimerism. However, the myelotoxic agents currently required to establish chimerism have prevented the translation of this approach to the clinic. Here, we investigated whether multimodal co-stimulatory blockade would enhance chimerism and promote islet allograft tolerance in spontaneously diabetic NOD mice.

Methods—Pre-diabetic and spontaneously diabetic NOD mice were preconditioned with anti-CD8 monoclonal antibody (mAb) prior to conditioning with 500 cGy total body irradiation and transplantation with 30×10^6 B10.BR bone marrow cells. Overtly diabetic animals were conditioned similarly and transplanted with 300–400 B10.BR islets. After irradiation, both groups of recipients were treated with anti-CD154, anti-OX40L, and anti-ICOS mAbs. Urine, blood glucose levels, and chimerism were monitored.

Results—Conditioning of non-obese diabetic (NOD) mice with co-stimulatory blockade significantly enhanced engraftment, with 61% of mice engrafting at 1 month. Eleven of twelve chimeric animals with engraftment at 1 month remained diabetes-free over a 12 month follow-up, whereas non-chimeric animals progressed to diabetes. In contrast, similar conditioning prolonged islet allograft survival in only 2 of 11 overtly diabetic NOD recipients. Chimerism levels in the 9 islet rejector animals were 0%.

Conclusion—Although nonmyeloablative conditioning reversed the autoimmune process in pre-diabetic NOD mice, the same regimen was significantly less effective in establishing chimerism and reversing autoimmune diabetes in spontaneously diabetic NOD mice.

INTRODUCTION

Type 1 diabetes is an immune-mediated disease characterized by the destruction of insulin-secreting beta cells in the pancreas. Mixed allogeneic chimerism has been shown to prevent

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type 1 diabetes in humans and mice [1,2]. Chimerism has also been shown to halt the progression of numerous other autoimmune diseases, including rheumatoid arthritis [3], psoriasis, lupus, hyperthyroidism, dermatitis, and Crohn's disease [1] in humans, and encephalomyelitis [4] and insulinitis in mice [5]. The morbidity and mortality associated with current conditioning protocols have prevented the widespread use of chimerism as an accepted clinical therapy [6]. A major goal of diabetes research is the induction of tolerance in diabetic recipients to prevent disease recurrence. The development of safe, nontoxic approaches to induce tolerance and reverse the autoimmunity would be a transformational advance in the field.

The underlying autoimmunity in nonobese diabetic (NOD) mice has many distinct features [7]. Impaired thymic selection, impaired co-stimulation from accessory cells, and defective regulatory T cells (T_{reg}) are associated with the increased number of autoreactive NOD T cells [8–10]. Defects in NOD T cell activation and function are responsible for elevated IFN- γ and decreased interleukin-4 (IL-4) production in $CD4^+$ T cells [11]. In addition, pre-diabetic NOD mice require increased cell numbers and higher total body irradiation (TBI) doses to establish chimerism compared to disease-resistant mouse strains [5]. Treatment of NOD mice with anti-CD8 and anti-CD154 mAbs synergistically enhanced engraftment. We therefore hypothesized that multimodal co-stimulation targeting specific cell populations could enhance allogeneic engraftment.

T cell co-stimulatory blockade has been demonstrated to promote tolerance in rodent models of cardiac, hepatic, islet, renal, lung, and bone marrow transplantation (BMT) [11,12]. Demirci et al. showed that C57BL/6 mice treated with anti-OX40L, mCTLA-4Ig and anti-CD154 exhibited long-term skin allograft survival, while untreated mice showed prompt rejection [13]. Similarly, Nanji et al. demonstrated that islet allograft survival was prolonged when C57BL/6 mice were given monotherapies consisting of anti-CTLA4-Ig, anti-CD40L, or rapamycin alone. When each of the monotherapies was combined with anti-ICOS, significant islet allograft prolongation occurred, showing the importance of ICOS signaling [14].

In the present studies, we found that preconditioning of pre-diabetic NOD mice with anti-CD8 combined with co-stimulatory blockade with anti-CD154, anti-OX40L, and anti-ICOS mAb significantly promotes allogeneic engraftment and prevents diabetes-onset. In distinct contrast, the majority of overtly diabetic NOD recipients that were conditioned and transplanted similarly rejected their marrow and islet grafts. Moreover, the absolute numbers of forkhead box P3 (FoxP3 $^+$) T_{reg} were elevated in the BM and spleen of islet acceptor animals. Taken together, these data might implicate a role for $CD4^+/CD25^+$ T_{reg} in establishing chimerism and tolerance *in vivo*. Understanding the mechanism underlying the autoimmune process will aid in the development of immune-based nonmyeloablative conditioning approaches that may impact the clinical application of chimerism for treatment of type 1 diabetes.

METHODS

Animals

Eight to ten-week old female NOD Ltj (H-2 g) and B10.BR SgSnJ (H-2 k) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained under the National Institutes of Health animal care guidelines in a pathogen-free barrier facility at the Institute for Cellular Therapeutics (ICT) in Louisville, KY,

Reagents and antibodies

Anti-CD8 mAb (hybridoma 53-6.7) was produced at ICT. All other mAb were purchased from BD Biosciences (San Diego, CA).

In vivo mAb conditioning and BMT

Pre-diabetic NOD female recipients were pretreated intraperitoneally with previously titrated anti-CD8 mAb on day -3 (Fig. 1A) [15]. Recipients were irradiated with 500 cGy TBI on day 0. NOD recipients were infused with 30×10^6 donor B10.BR bone marrow cells (BMC) via lateral tail vein at least six hours after irradiation. Recipients were conditioned intraperitoneally with and without anti-CD154 mAb (0.5 mg/mouse) (MR-1: Bioexpress; Kaysville, UT), anti-OX-40L mAb (0.5 mg/mouse) (RM134L: Bioexpress) and anti-ICOS mAb (0.5 mg/mouse) (17G9: Bioexpress) on day 0. Subsequent intraperitoneal injections consisted with and without anti-CD154 mAb (0.25 mg/mouse) anti-OX-40L mAb (0.25 mg/mouse) and anti-ICOS mAb (0.25 mg/mouse) on day +1, +2, and +3.

Chimerism typing and multilineage analysis

NOD recipients were characterized for donor B10.BR (H-2^k) (AF3-12.1) and recipient NOD (H-2^{g7}) (SF1-1.1) engraftment by flow cytometry monthly [2]. Peripheral blood (PB) cells were stained with appropriate directly conjugated mAb and then analyzed using a flow cytometer (FACScan or LSR; Becton Dickinson, Mountain View, CA).

Islet harvest and transplantation

Spontaneously diabetic NOD mice were treated with antibody and conditioned with 500 cGy TBI according to Fig. 3A. Six hours after irradiation, NOD recipients were infused with 30×10^6 donor B10.BR BMC. One day after donor B10.BR BMC infusion, diabetic NOD mice were transplanted with B10.BR donor islets. To isolate islets, B10.BR (H-2^k) donor pancreata were digested with 0.2 mg/mL Liberase and then subjected to a layered Ficoll gradient. Suspended donor islets were collected from each layer, aspirated into sterile tubing, and transplanted under the recipient's right renal capsule.

Intraperitoneal glucose tolerance assay

Glucose tolerance tests were performed 4 months after islet transplantation. Animals were fasted overnight. Blood was isolated and measured using the Prestige glucose meter (PGM) (Home Diagnostics; Fort Lauderdale, FL). Following baseline measurements, animals were injected intraperitoneally with 5% glucose (1 mg/g body weight) in saline. Blood samples were collected at selected time points and analyzed.

Diabetes monitoring and islet allograft rejection criteria

Urine from NOD mice was tested weekly for glycosuria using Chemstrip uGK test strips (Roche; Indianapolis, IN). In some experiments, blood glucose levels were monitored in islet allograft recipients using the PGM. Animals with sustained diabetes were euthanized and their native pancreata examined histologically.

Histological analysis

Histological analysis was carried out on native pancreata. Tissues were fixed in formaldehyde and stained with aldehyde fuchsin for islet identification and hematoxylin and eosin to observe inflammation prior to light microscopy.

RESULTS

Anti-ICOS conditioning enhances allogeneic engraftment in pre-diabetic mice

We previously reported that preconditioning of NOD mice with anti-CD8 followed by anti-CD154 mAb treatment synergistically enhanced allogeneic stem cell engraftment [2]. Previous studies have demonstrated that blockade of CD154 or ICOS can also promote allogeneic bone marrow (BM) engraftment [16,17]. We therefore examined whether engraftment could be enhanced through combined blockade of CD154, OX-40L and ICOS. Pre-diabetic NOD recipients were preconditioned with anti-CD8 prior to total body irradiation, infused with B10.BR donor BMC and treated with anti-CD154, anti-OX40L with and without anti-ICOS (Fig. 1A). Preconditioning of NOD mice with anti-CD8 mAb followed by anti-CD154, anti-OX40L, and anti-ICOS treatment enhanced engraftment considerably, with 63% (12 of 19) of mice engrafting with 500 cGy of TBI at one month. Only 23% of mice (3 of 13) preconditioned with anti-CD8 mAb and treated with anti-CD154 and anti-OX40L mAb engrafted with 500 cGy of TBI. NOD mice treated with anti-CD8, 500 cGy of TBI, and saline alone ($n = 10$) did not engraft (Fig. 1B). No engraftment was observed with TBI doses lower than 500 cGy (data not shown). Of the 12 animals that engrafted with anti-CD8 mAb, anti-CD154, anti-OX40L, and anti-ICOS conditioning and 500 cGy TBI, 6 animals displayed long-term donor chimerism with an average chimerism of 12.5% at 8 months post BMT (Fig. 1C). The remaining 6 animals displayed only transient donor chimerism for up to 4 months post-BMT, with chimerism $\leq 1\%$ by 5 months post-BMT (Fig. 1C). The 7 of 19 animals that were conditioned in the same manner but did not engraft had chimerism levels $<1\%$ (Fig. 1C). To confirm that chimeric recipients had engraftment of the pluripotent hematopoietic stem cell, flow cytometric analysis was carried out 2 months after BMT. Multilineage typing in chimeric recipients was similar to that for control naïve B10.BR mice (Fig. 1D), with CD11c⁺ dendritic cells, Mac-1⁺ macrophages, Gr-1⁺ granulocytes, Dx5⁺ NK cells, B220⁺ B cells, as well as CD4⁺, CD8⁺ T cells present (Fig. 1E,F).

Co-stimulatory blockade prevents the onset of diabetes in prediabetic animals

We next determined whether our conditioning approach would protect pre-diabetic NOD mice from developing diabetes. We previously demonstrated that chimeric pre-diabetic NOD mice failed to progress to develop diabetes [2]. In the present study, all animals (12 of 12) conditioned with anti-CD8 plus anti-CD154, anti-OX40L, anti-ICOS, 500 cGy TBI and transplanted with 30×10^6 donor B10.BR BMC remained diabetes-free over a 7 month period irrespective of whether the chimerism was durable (Fig. 2). All 6 long-term chimeric animals remained diabetes-free over a seven month period. Interestingly, only 1 of the 6 transiently chimeric animals developed diabetes at 8 months though these animals had lost donor chimerism by 5 months post-BMT. Sixty percent (3 of 5) of non-chimeric animals treated similarly developed diabetes at 7 months and 100% developed diabetes at 12 months (5 of 5). All animals conditioned with 500 cGy TBI and transplanted with 30×10^6 B10.BR BMC ($n = 5$) or 500 TBI alone ($n = 7$) developed diabetes by 6 months (Fig. 2).

Chimerism reverses active autoimmune diabetes in surviving islet allografts (B10.BR → NOD)

In striking contrast with pre-diabetic NOD recipients, long-term (> 6 months) donor islet allograft survival was observed in only 2 of the 11 recipients (mean survival = 190 days) conditioned with anti-CD8 mAb, anti-CD154, anti-OX40L, and anti-ICOS treatment plus cGy TBI and transplanted with 30×10^6 donor B10.BR BMC (Fig. 3A). The other 9 transplanted recipients rejected their islet allografts with a mean survival of 27.5 days (Fig. 3B). Spontaneously diabetic control recipients that were not transplanted with islet allografts but received antibody treatment and 500 cGy TBI failed to return to normoglycemic levels

(Fig. 3B). Hematoxylin and eosin (H&E) staining of islet acceptor pancreatic tissue revealed preservation of islet architecture with few mononuclear infiltrates (Fig. 4A,B), while H&E staining of rejector pancreatic tissue showed dense mononuclear cell infiltration and islet destruction (Fig. 4C,D). To determine whether chimerism was critical in acceptance of transplanted islet allografts, transplanted recipients were typed for the percentage of donor PB chimerism by flow cytometric analysis at 30 days and monthly thereafter. Chimerism directly correlated with diabetes-prevention. NOD mice that rejected their islet grafts showed no detectable donor chimerism. Islet acceptor mice demonstrated mean donor chimerism of 81.6% at 1 month (Fig. 4E). The chimerism was durable and was associated with islet allograft acceptance for the remainder of the 6 month observation period (Fig. 4E).

To provide an accurate assessment of glucose homeostatic regulation and to confirm that the transplanted islet allografts were functional, intraperitoneal glucose tolerance tests (IPGTT) were performed 16 weeks after transplantation. Fasting blood glucose baseline measurements from islet acceptor animals were not significantly different from that of pre-diabetic NOD or control naïve C57BL/6 animals (Fig. 4F). Islet transplanted recipients and untransplanted pre-diabetic control NOD mice displayed normal glucose homeostasis when subjected to a glucose challenge, whereas diabetic NOD mice that rejected their graft maintained hyperglycemic levels throughout the assay (Fig. 4F).

Increased numbers of CD8⁺/CD4⁺/CD25⁺/FoxP3⁺ T_{reg} were detected in animals with long-term surviving grafts

The expression of FoxP3 in T cells is associated with regulatory functions [18]. Whereas CD4⁺/CD25⁺/FoxP3⁺ T cells are activated effector T cells (T_{eff}), CD4⁺/CD25⁺/FoxP3⁺ T_{reg} display regulatory properties. To determine the influence of FoxP3⁺ T_{reg} on islet allograft acceptance, we performed flow cytometric analysis for CD8⁺/CD4⁺/CD25⁺/FoxP3⁺ T_{reg} and CD8⁺/CD4⁺/CD25⁺/FoxP3⁺ T_{eff}. No significant differences were observed in the percentages of CD8⁺/CD4⁺/CD25⁺/FoxP3⁺ T_{reg} in PB, BM, or spleen of chimeric islet acceptor animals compared to diabetic and islet rejector animals, (Fig. 5A). A small increase in the percentage of CD8⁺/CD4⁺/CD25⁺/FoxP3⁺ T_{reg} was observed in PB of naïve/pre-diabetic NOD mice. A moderate increase in newly activated CD8⁺/CD4⁺/CD25⁺/FoxP3⁺ T_{eff} was also observed in the spleens of islet acceptor animals.

The absolute numbers of CD8⁺/CD4⁺/CD25⁺/FoxP3⁺ T_{reg} in the BM and spleens of islet acceptor animals was 7.8 fold and 5.9 fold higher than that found in islet rejector animals, respectively (Fig. 5B). Absolute numbers of CD8⁺/CD4⁺/CD25⁺/FoxP3⁺ T_{reg} in the BM and spleens of islet acceptor animals was only 2.8 fold and 2.6 fold higher than that found in diabetic animals, respectively (Fig. 5B). Moreover, absolute numbers of CD8⁺/CD4⁺/CD25⁺/FoxP3⁺ T_{reg} in the PB of naïve animals was 4.3 fold higher than observed in diabetic animals, nearly 9.8 fold higher than in rejector animals, and only 1.7 fold higher than islet acceptor animals. We also observed an increase in the number of newly activated CD8⁺/CD4⁺/CD25⁺/FoxP3⁺ T_{eff} in both BM and spleen of islet acceptor animals. As the T_{reg}/T_{eff} balance is important in influencing local inflammation, we determined that T_{reg}/T_{eff} ratio in PB, BM, and spleen from islet acceptor animals was 3.61, 4.37, and 1.54, respectively. The T_{reg}/T_{eff} ratio in PB, BM, and spleen from islet rejector animals was 2.70, 3.07, and 2.38, respectively. Taken together, these data might suggest an association between T_{reg} expression and islet-allograft acceptance.

DISCUSSION

NOD mice spontaneously develop insulin-dependent diabetes and have been a beneficial model in studying the *in vivo* control of autoimmunity on restorative interventions in type 1 diabetes. Establishment of mixed chimerism reverses insulinitis in pre-diabetic mice and is

associated with diabetes-prevention [5]. However, the capacity to tolerize host T cells of diabetic NOD mice remains unclear [19]. As such, NOD mice require more intense conditioning therapies and increased BMC doses compared to non-autoimmune controls [5].

Co-stimulatory blockade during T cell activation induces anergy or antigen-driven apoptosis of alloreactive cells [20]. Previous studies demonstrated that preconditioning of NOD mice with anti-CD8 mAb alone can significantly reduce the minimum TBI dose required for engraftment. Treatment of NOD mice with anti-CD8 mAb plus anti-CD154 synergistically enhanced engraftment [2]. Blockade of ICOS signaling is synergistic with other co-stimulatory blocking agents or conventional immunosuppression in achieving long-term graft acceptance and impairing chronic allograft rejection [21]. Nanji et al. demonstrated that monotherapy with either anti-ICOS or anti-CD154 alone does not reduce the onset of autoimmune diabetes, but when combined, the agents work synergistically to promote islet allograft acceptance across fully major histocompatibility complex (MHC) -mismatched barriers and prevents diabetes onset in pre-diabetic NOD mice [14,22]. Chitnis *et al.* demonstrated that anti-OX40L monoclonal antibody therapy in CD28-deficient mice could protect against Experimental Autoimmune Encephalomyelitis [23]. In the present studies, we found that preconditioning of NOD mice with anti-CD8 mAb in the context of CD154, ICOS, and OX40L co-stimulatory blockade enhanced engraftment. These data suggest that the blockade of CD154, OX40, and ICOS targets nonoverlapping recipient alloreactive populations, perhaps resulting in the elimination or anergy of these cells in prediabetic mice.

It is of note that animals with transient chimerism and those that retained elevated chimerism levels remained diabetes-free 7 months post-BMT. Li et al. demonstrated that when NOD mice were conditioned nonmyeloablatively with a single dose of cyclophosphamide, overt diabetes could be prevented in all chimeric animals [5]. Here we demonstrate that although diabetes can be prevented in chimeric NOD mice, NOD mice that showed macrochimerism for longer than 2 months but later lost donor chimerism still remained diabetes-free. In addition, animals that received TBI alone or TBI with BM but never engrafted, demonstrated enhanced susceptibility to diabetes onset. Though both chimeric and non-chimeric animals were treated similarly, only the chimeric animals remained diabetes-free, suggesting that the tolerant effect was a result of antigen specificity rather than a non-specific effect from antibody treatment. It is possible that microchimerism leading to clonal exhaustion may be operational in contributing to diabetes-prevention. Microchimerism has been shown by some to promote tolerance to organ allografts through central or peripheral mechanisms [24].

Although a number of immune-based approaches have delayed the development of diabetes in NOD mice, few of these have been effective in preventing the long-term destruction of islet allografts in overtly diabetic NOD mice [25,26]. Molano et al. demonstrated that islet graft survival could be prolonged with CD40-CD154 blockade in spontaneously diabetic NOD mice [27]. However, the islets were eventually rejected in contrast with normal mice. Similarly, in our present studies we found that co-stimulatory blockade and BM transplantation prevented recurrent diabetes in overtly diabetic NOD recipients of BMC plus islet allografts. However, only 2 of 11 animals demonstrated long-term tolerance, suggesting increased resistance to BM and islet allograft acceptance. Pearson et al. hypothesized that there are two distinct non-overlapping mechanisms that control both autoimmunity and transplantation tolerance and that the genetic disposition for normalizing transplantation tolerance is greater than the genetic disposition for preventing autoimmune diabetes [28]. Collectively, these data suggest that NOD mice appear to have a defect in costimulation blockade-based tolerance induction [29].

In addition to defects in co-stimulatory blockade induced-tolerance, defects in NK/T and CD4⁺/CD25⁺/FoxP3⁺ regulatory cells have been shown to contribute to the autoimmune process in NOD mice [30,31]. CD4⁺ T cells depleted of the CD25⁺ subpopulation from pre-diabetic animals are diabetogenic when transferred into immunodeficient NOD mice [32]. Alyanakian et al. demonstrated that the adoptive transfer of certain depleted regulatory T cell subsets results in differential control of autoimmunity [10]. When *in vitro* assays were carried out, they found that both CD3 antibody-induced proliferation of CD4⁺/CD25⁻ T cells and cytokine production could be suppressed, further supporting the hypothesis of variation in T_{reg} subset activities [10].

In summary, blockade of CD154, ICOS, and OX40L co-stimulatory molecule interactions enhances engraftment. This treatment protocol promoted durable chimerism, and prevented the onset of diabetes in pre-diabetic animals. Diabetes onset was prevented in pre-diabetic NOD mice that showed chimerism at 1 month post-BMT, while animals with no observed chimerism at 1 month post BMT progressed to develop diabetes. In striking contrast, the antibody treatment protocol combined with simultaneous islet transplantation only reversed the active autoimmune process in 2 of 11 animals with overt diabetes. Though these animals demonstrated elevated levels of FoxP3⁺ T_{reg} in BM and spleen, additional conditioning approaches are clearly required to reverse the observed autoimmunity. Huang et al. demonstrated that chimeric T_{reg} can enhance donor-specific stem cell engraftment *in vivo* and can significantly suppress donor antigen-induced T cell proliferation *in vitro* in prediabetic NOD mice [33]. In these and other experimental models, elevated T_{reg} in BM and spleen may prolong islet allograft survival in islet transplanted chimeric animals [34]. Understanding the mechanism of underlying autoimmune process will aid in the development of nonmyeloablative conditioning approaches that may impact on the clinical application of chimerism for treatment of type 1 diabetes and induction of tolerance to islet allografts.

Acknowledgments

The authors thank Dr. Haval Shirwan for review of the manuscript and helpful comments; Carolyn DeLautre for manuscript preparation; and the staff of the University of Louisville animal facility for outstanding animal care. This work was supported in part by NIH T32HL076138; NIH RO1 DK069766; JDRF 1-2006-146; and the American Society of Hematology scholar award. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the Commonwealth of Kentucky Research Challenge Trust Fund; the W. M. Keck Foundation; and The Jewish Hospital Foundation. Research was conducted in compliance with the Animal Welfare Act Regulations and other Federal statutes relating to animals and experiments involving animals and adheres to the principles set forth in the Guide for Care and Use of Laboratory Animals, National Research Council, 1996.

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C

D

Figure 1. Co-stimulatory blockade with anti-OX40L and anti-ICOS mAbs enhances B10.BR allogeneic engraftment in NOD mice

A: Mice were preconditioned with anti-CD8 mAb on day -3. On day 0, recipients were transplanted with 30×10^6 B10.BR BMC 4 to 6 h after TBI as well as single daily dose of anti-CD154, anti-OX40L, and anti-ICOS mAbs on days 0, 1, 2, and 3. **B:** NOD mice received BMC with vehicle ($n = 10$), BMC with anti-CD8, anti-CD154, anti-OX40L ($n = 13$), or BMC with anti-CD8, anti-CD154, anti-OX40L, and anti-ICOS mAbs in addition to 500 cGy TBI ($n = 19$). Recipient mice were monitored for engraftment 30 days after BMC transplantation. **C:** NOD mice received BMC with anti-CD8, anti-CD154, anti-OX40L, and anti-ICOS in addition to a 500 cGy TBI dose ($n = 12$). Animals with transient donor chimerism were monitored monthly for 5 months ($n = 6$) ($\circ$). Animals with durable long-term chimerism ($n = 6$) ($\blacksquare$) were monitored monthly for 10 months. Animals with no engraftment were monitored for 2 months ($n=7$) ($\blacktriangle$). Data represents the means $\pm$ SE of combined data obtained from independent determinations. **D–F:** Multilineage typing of

representative pre-diabetic chimeric NOD recipient treated with anti-CD8, anti-CD154, anti-OX40L, and anti-ICOS, donor BM and 500 cGy TBI and control B10.BR recipient in both bar graph (**D**) and dotplot format (**E–F**). Multilineage data were derived from PB 2 months after BM transplantation and analyzed using both lymphoid and myeloid gates.

Figure 2. Incidence of Diabetes in age-matched naïve and conditioned NOD mice

The incidence of diabetes was compared between the various treatment groups listed in the figure legend. The most robust diabetes-prevention was in the group that was durably chimeric. The progression of diabetes was monitored weekly in control mice and all treatment groups. Mice with blood glucose levels greater than 250 mg/dL after 3 consecutive measurements were considered diabetic.

Figure 3. Diabetes is reversed in some conditioned diabetic mice transplanted with islet allografts
A: Diabetic NOD recipients were preconditioned with anti-CD8 mAb on day -3, transplanted with 30×10⁶ B10.BR donor BMC on day 0 and received a 500 cGy TBI dose 6 h later. Twenty-four hours after BM transplant, NOD mice were transplanted with 300–400 B10.BR donor islet allografts, and on days 0–3 received anti-CD154, anti-OX40L and anti-ICOS. **B:** Blood glucose measurements were carried out in islet allograft acceptor mice (○) (n = 2), islet allograft rejector mice (●) (n = 9), and control mice not transplanted with donor islets (△) (n = 2) to assess islet allograft survival in transplanted recipients. Rejection was confirmed with 3 consecutive non-fasting blood glucose readings over 250 mg/dL. Values are expressed as mean ± SE.

Figure 4. Histological analysis, donor chimerism, and glucose challenge in islet acceptor NOD mice

Hematoxylin and eosin staining was carried out on pancreatic tissue obtained either 6 months after islet transplantation in acceptor animals (**A–B**) or 2–3 months in rejector animals (**C–D**). H&E staining revealed mononuclear infiltration with preservation of islet architecture (200 $\times$) in pancreatic tissue from acceptor animals, while the majority of beta cells from rejected pancreatic tissue showed strong mononuclear infiltration (**C**) and large to small vacuoles indicating destruction of islet architecture (**D**) (200 $\times$). **E**: Islet allograft

transplanted animals were monitored for donor chimerism 1 to 6 months post-BMT in peripheral blood of islet acceptor animals (○) (n = 2) and islet rejector animals (●) (n = 9). **F**: Glucose tolerance tests were carried out in islet allograft acceptor mice (○) (n = 2) 16 weeks after B10.BR BM and islet allograft transplantation. Normal naïve C57BL/6 mice (Δ) (n = 5), pre-diabetic naïve NOD mice (■) (n = 9), and diabetic NOD mice (●) (n = 4) were evaluated as controls for comparison. Values are expressed as mean ± SE (blood glucose (mg/dL)).

Figure 5. Expression of $CD8^-/CD4^+/CD25^+/FoxP3^+$ in PB, BM and spleens of islet allograft rejector animals and recipients carrying long-term surviving grafts

Peripheral blood, BM, and splenocytes were isolated from naïve NOD, diabetic NOD, NOD recipients with long-term surviving islet allografts, or from NOD recipients that rejected their islet allografts and stained for surface markers: CD8, CD4, and CD25. Cells were fixed and permeabilized overnight and stained for FoxP3 the following day. Stained cells were then analyzed by flow cytometry. Percentages (A) and absolute number (B) of $CD8^-/CD4^+/CD25^+/FoxP3^+ T_{reg}$ and newly activated $CD8^-/CD4^+/CD25^+/FoxP3^- T_{eff}$. The values are expressed as mean $\pm$ SE of independent experiments from 3 naïve animals, 3 diabetic animals, 2 islet acceptor animals, and 4 islet rejector animals.