Recovery from Overt Type 1 Diabetes Ensues When Immune Tolerance and β Cell Formation Are Coupled with Regeneration of Endothelial Cells in the Pancreatic Islets

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ABSTRACT

Immune modulation of pancreatic inflammation induces recovery from type 1 diabetes (T1D) but remission was not durable perhaps due to an inability to sustain the formation and function of new pancreatic β-cells. We have previously shown that Ig-GAD2, carrying glutamic acid decarboxylase (GAD) 206-220 peptide, induced in hyperglycemic mice immune modulation that was able to control pancreatic inflammation, stimulate β-cell regeneration and prevent T1D progression. Herein, we show that the same Ig-GAD2 regimen given to mice with overt T1D is unable to reverse the course of disease despite eradication of Th1 and Th17 cells from the pancreas. However, the regimen is able to sustain recovery from T1D when Ig-GAD2 was accompanied with transfer of bone marrow (BM) cells from healthy donors. Interestingly, alongside immune modulation there was concomitant formation of new β and endothelial cells in the pancreas. The new β-cells were of host origin while the donor BM cells gave rise to the endothelial cells. Moreover, transfer of purified BM endothelial progenitors instead of whole BM cells sustained both β and endothelial cell formation and reversal of diabetes. Thus, overcoming T1D requires both immune modulation and repair of the islet vascular niche to preserve newly formed β-cells.
INTRODUCTION

T1D is a chronic disease in which the insulin-producing β-cells of the pancreatic islets are destroyed by inflammatory T lymphocytes of the immune system (1; 2). Broad-based T-cell-targeted therapies, such as anti-CD3 monoclonal antibodies, were able to reverse established overt T1D in the NOD mouse (3). In humans however, while the regimen preserved C-peptide responses, disease rebounds even when the antibody was used in a non-Fc receptor binding form (4; 5). Non-specific activation of T cells was perhaps responsible for the return of inflammation and β-cell dysfunction. Thus, we reasoned that antigen-specific therapy that targets mostly self-reactive T cells with minimal interference with other specificities would be effective against the disease. In a previous study, we expressed the suppressive GAD206-220 peptide (6) on an Ig molecule and the resulting Ig-GAD2 was able to prevent disease progression in NOD mice that were diagnosed with insulitis (7). Moreover, Ig-GAD2 was effective against the disease even when the treatment was applied at the hyperglycemic stage where blood glucose level (BGL) began to rise between 160-250 mg/dl (7). Interestingly, the animals restored normoglycemia (≤140 mg/dl) which was long-lasting due to effective immune modulation of pancreatic inflammation and, most importantly, stimulation of β-cell division and generation of healthy islets (7). These observations prompted us to test Ig-GAD2 for treatment of overt T1D (BGL ≥ 300 mg/dl) which would be more relevant to human circumstances. However, the regimen failed to sustain β-cell regeneration and overcome overt T1D despite induction of immune modulation and eradication of pancreatic infiltration. Given that β-cell mass is diminished at the diabetic stage and that BM transplantation sustained regeneration of endogenous β-cells in Streptozotocin (STZ)-models of diabetes (8; 9), we sought to determine whether enrichment with donor BM cells during treatment with Ig-GAD2 would restore β-cell regeneration and counter
overt diabetes. This was indeed feasible as β cell formation ensued and the mice recovered from overt T1D. Surprisingly, however, there was engraftment of endothelial cells (ECs) and these were of donor BM origin while the newly formed β cells were derived from host cells. Moreover, substitution of whole BM with endothelial cell progenitors (EPCs) during treatment with Ig-GAD2 allowed for restoration of both endothelial and β-cells and recovery from overt T1D. These findings indicate that recovery from overt T1D necessitates repair of both β cell mass and the islet endothelial niche.
RESEARCH DESIGN AND METHODS

Mice. NOD and NOD.GFP mice expressing the green fluorescence protein under the β-actin promoter were previously described (10) were maintained in the Animal Facility at the Medical Sciences Building under barrier conditions. All animals were used according to the guidelines of the University of Missouri Animal Care and Use Committee.

Treatment with Ig-GAD2 and donor BM. Mice began BGL monitoring at 10 weeks of age, and those displaying ≥300 mg/dl for 2 consecutive weeks (considered overtly diabetic) were enrolled in the treatment regimen. The mice were first given 2 sustained release insulin implants (LinShin, Toronto, Ontario, Canada) inserted subcutaneously in the abdomen to temporarily maintain normoglycemia for 2-3 weeks. The mice were then given 300 µg Ig-GAD2 intraperitoneal (i.p.) 3 times weekly for 5 weeks and then once a week for another 5 weeks. Donor BM cells were isolated from the femur and tibia of healthy (non diabetic) NOD mice, and 10 x 10^6 cells were transferred intravenously (i.v) weekly on week 2, 3, and 4 post diagnosis. The mice were monitored for BGL until day 120. In some experiments (Fig. 1A), treatment with Ig-GAD2 alone was applied to hyperglycemic mice where BGL were 160-250 mg/dl for 2 consecutive measurements 3 days apart.

Treatment with Ig-GAD2 and donor EPCs. This regimen was similar to treatment with Ig-GAD2 and donor BM except that EPCs substituted for whole BM cells. FLK-1^+ EPCs were given at 5 x 10^4 per injection while FLK-1^- EPCs were given at 3 x 10^6 cells per injection.
**Purification of EPCs.** BM cells were harvested from healthy or diabetic (sick) mice and depleted of lineage+ (Lin⁺) cells using the lineage cell depletion kit according to manufacturer’s instruction (Miltenyi Biotec). The Lin⁻ cells were stained with anti-c-Kit, and anti-FLK-1 antibodies as well as with 7-amino-actinomycin D (7-AAD) and sorted into c-Kit⁺7-AAD⁻FLK-1⁺ or c-Kit⁺7-AAD⁻FLK-1⁻ cells.

**Cell surface staining.** For detection of PECAM1, FLK-1, c-Kit and CD45 the cells were stained with marker-specific antibodies, including phycoerythrin (PE)-cy7-conjugated anti-PECAM1 (390), allophycocyanin (APC)-conjugated anti-FLK1 (Avas12a1) (both from eBiosciences), PE-cy7-conjugated anti-c-Kit (2B8) and APC-cy7-conjugated anti-CD45 (2D1) (both from BD Pharmingen) antibodies. For detection of apoptotic cells, cells were stained with 7-AAD (EMD Biosciences).

**Intracellular staining.** For detection of intracellular IFNγ, IL-10 and IL-17 in CD4⁺ T cells, the cultures were stimulated with PMA (50ng/ml) and ionomycin (500ng/ml) for 4h in presence of Brefeldin A (10µg/ml), and then stained with Peridinin-chlorophyll-protein (PerCP)-cy5.5-conjugated anti-CD4 (RM4-5), PE-conjugated anti-Vβ8.1/8.2 (MR5-2) and FITC-conjugated anti-CD8 (RPA-T8) antibodies (all from BD Pharmingen). Subsequently, the cells were fixed with 2% formaldehyde, permeabilized with 0.2% saponin and stained with PE-cy7-conjugated anti-IFNγ (XMG1.2), APC-conjugated anti-IL-10 (JES5-16E3) or APC-conjugated anti-IL-17 (eBio17B7) antibody (all from eBiosciences).
**Flow cytometry analyses.** The samples were read using a Beckman Coulter CyAn ADP and data were analyzed using Summit V4.3 (Dako). Cell sorting (> 98% purity) was performed using a Beckman Coulter MoFlo XDP sorter.

**Sample preparation for histologic analyses.** Pancreata were frozen in tissue freezing medium (Triangle Biomedical Sciences), and non-serial 8-µm thick sections were cut 150-µm apart to avoid over counting of the labeled cells. The sections were fixed in 4% formaldehyde for 10 min before histological procedures. For detection of enhanced GFP expression in tissues, pancreata were fixed in 4% formaldehyde for 4h at 4°C and immersed in 30% sucrose overnight before freezing. Analysis of insulitis used H&E staining as previously described (7). Each experiment used 3-6 sections per pancreas.

**Immunohistochemistry.** For detection of β-cells, pancreatic sections were incubated with HRP-conjugated anti-insulin affibody molecule (Abcam, 1:200) at room temperature (RT) for 45min, and the insulin⁺ cells were identified by incubating the slides with DAB chromogen and substrate (ScyTek) for 5 min. The cell nuclei were counterstained with hematoxylin.

**Immunofluorescence.** Pancreatic sections were treated with a PBS solution containing 1% BSA, 10% goat or donkey serum, and 0.2% Triton-X100 at RT for 1h. The sections were then incubated overnight at 4°C with primary antibodies, including rabbit anti-insulin (Santa Cruz, 1:200), guinea pig anti-insulin (Abcam, 1:300), rabbit anti-PECAM1 (Abcam, 1:100), goat anti-PECAM1 (Santacruz, 1:100), rabbit anti-ki-67 (Abcam, 1:300) and goat anti-VEGF (Santa Cruz, 1:200). The slides were washed with 3 changes of 0.02% Triton-X100 in PBS and then stained.
for 1 hour at RT with the corresponding secondary antibodies, including Texas Red-conjugated goat anti-rabbit IgG (1:200), fluorescein isothiocyanate (FITC)-conjugated goat anti-guinea pig IgG (1:200), FITC-conjugated donkey anti-goat IgG (1:200) (all from Santa Cruz), DyLight 405-conjugated donkey anti-rabbit IgG (1:200) and DyLight 549-conjugated donkey anti-goat IgG (1:300) (all from Jackson ImmunoResearch). In some experiments, the cell nuclei were counterstained with DAPI (Santa Cruz). The images were visualized and acquired with a Zeiss fluorescence microscope or an Olympus DSU confocal microscope. The number of β-cells, islets, and endothelial cells was scored with a computer-assisted Image Pro Plus program (Media Cybernetics, Silver Spring, MD).

**Measurement of β-cell mass.** Pancreata were weighed and used to prepare frozen sections. The slides were stained for insulin by immunohistochemistry and β-cell mass was determined by point-counting morphometry as well as pixel based technology as described (11). Despite that β-cell mass quantification was validated by both technologies figure 3 incorporates only data calculated by pixel based technology. Briefly, cross-sectional areas of insulin+ cells were measured at 150 µm intervals and normalized to total pancreatic area using the Image-Pro Plus program. β-cell mass is expressed in mg after normalization to total pancreatic mass. For each pancreas, 30 sections were cut and used for different experiments. For determination of β-cell mass, 6 non-serial sections (5th, 10th, 15th, 20th, 25th and 30th) spanning both proximal and distal areas of the pancreas were used.

**Laser capture microdissection.** Pancreatic sections were stained with insulin or PECAM1 and thoroughly dehydrated with the Arcturus dehydration component. The insulin+ or PECAM1+
cells were dissected with the CapSure HS LCM caps and the Autopix 100 laser capture microdissection system by following the manufacturer’s instructions. For each individual mouse, cells were dissected from 3-10 non-serial sections. Genomic DNA was extracted from the dissected cells using the PicoPure DNA extraction kit. All the reagents are from Applied Biosystems.

**Detection of Y chromosome by PCR.** Detection of Y chromosome and beta-actin was performed using 20 ng DNA template and Maxima qPCR master mix (Fermentas). The sequences of the primers are listed in supplementary Table 1.

**Quantitative PCR analysis.** Total RNA was extracted from pancreatic islets using the TRI RNA isolation reagent (Sigma). Quantitative PCR was performed using the Power SYBR Green kit and the StepOnePlus instrument (all from Applied Biosystems). The relative quantity (RQ) was calculated based on the ∆∆CT after normalization with the internal control 18S ribosome RNA expression. The sequences of the primers for T cell transcription factors and genes involved in endothelial cell function are listed in supplementary Table 1.

**Statistical analyses.** P values associated with all pair wise comparisons were calculated based on Student’s t-test for independent groups. Error bars were defined using standard error of mean.
RESULTS

Ig-GAD2 driven immune modulation is not sufficient to overcome overt type 1 diabetes. In a prior study, we have shown that Ig-GAD2 can restore normoglycemia in NOD mice that began to display a rise in BGL (160-250 mg/dl) and are referred to as hyperglycemic (7). The immune mechanisms underlying protection against the disease manifest in the form of reduced cell infiltration in the pancreas (PN) associated with eradication of both Th1 and Th17 cells (7). However, while no Th17 cells were observed in the pancreatic lymph nodes (PLN) or the spleen (SP), there were residual Th1 cells retained in the latter organ, but these were non-pathogenic (7). We therefore tested the regimen against overt T1D (BGL ≥ 300 mg/dl), which would be more relevant to human circumstances. To our surprise however, there was no restoration of normoglycemia in overtly diabetic mice, despite that Ig-GAD2-driven immune modulation was similar to the hyperglycemic mice that recovered from disease (Fig. 1). Indeed, while all hyperglycemic mice displayed a reduction in BGL, none of the diabetic animals recovered from diabetes (Fig. 1A). More intriguing, the sick animals displayed eradication of Th17 cells and retention of Th1 cells in the SP (Fig. 1B-D). In fact, the Ig-GAD2-treated diabetic mice had increased frequency of CD4+CD8−Vβ8.1/8.2+ T cells producing IFNγ and/or IL-10 (Fig. 1B), but diminished Th17 cells in the SP and PLN relative to untreated sick animals (Fig. 1C). Moreover, there were reduced Th1 or Th17 cells in the pancreas because the mRNA for their signature transcription factors, T-bet and RORγt respectively, were significantly diminished (Fig. 1D). Overall, Ig-GAD2-driven immune modulation is not sufficient to restore normoglycemia in overtly diabetic mice.
Transfer of BM cells alongside Ig-GAD2 treatment overcomes overt T1D. It has been previously shown that adult β-cells are formed mainly by self-duplication (12). Given the fact that in diabetic mice most of the β-cells are destroyed and self-duplication would be minimal we reasoned that Ig-GAD2 treatment would overcome overt T1D if β-cell regeneration is restored. Since BM transplantation has been shown to support regeneration of endogenous β-cells and restore normoglycemia in STZ-induced diabetes (8; 9), it is logical to envision that enrichment with BM cells during suppression of inflammation with Ig-GAD2 would counter overt diabetes. To test this premise, we combined BM cell transfer from healthy donors with a 70-day Ig-GAD2 treatment (Fig. 2A) and assessed for reduction in BGL. Fig. 2B shows that the majority of the mice given both Ig-GAD2 and BM transfer (Ig-GAD2+BM) returned to BGL below 300 mg/dl whether the BM was from male or female donors (Fig. 2B). Despite the fact that some of the mice restored normoglycemia (BGL < 140 mg/dl) while others reduced their BGL only to hyperglycemic levels (160-250 mg/dl), they were all used for mechanistic analyses without distinction. Furthermore, the treatment ablated insulin-resistance associated with the onset of diabetes (Supplementary Fig.1) as was observed in models of non-antigen-specific regimens (13; 14). No protection was observed in mice given Ig-GAD2 or BM alone (Fig. 2B). The enrichment with BM cells sustained protection against disease without impacting Ig-GAD2-mediated immune modulation. Indeed, the diabetic mice treated with the combination (Ig-GAD2+BM) regimen, like those recipient of Ig-GAD2 alone, had increased frequency of CD4^+CD8^−Vβ8.1/8.2^+ T cells producing IFNγ and/or IL-10 (Fig. 2C), but diminished Th17 cells in the SP and PLN (Fig. 2D). In contrast, mice recipient of BM alone which remained sick had no increase in IFNγ^- and IL-10-producing cells or decrease in Th17 cells (Fig. 2, C and D). Moreover, in the pancreas of Ig-GAD2+BM groups the mRNA for T-bet and RORγt was
significantly diminished relative to animals recipient of BM alone (Fig. 2E) indicating that both Th1 and Th17 cells were minimal in this site. Overall, addition of BM transfer to the Ig-GAD2 regimen sustained protection against diabetes without impacting immune modulation.

**BM transfer synergizes with Ig-GAD2 to drive formation of healthy islets.** Since Ig-GAD2+BM but not Ig-GAD2 alone reduced BGL, it is possible that addition of BM transfer sustained regeneration of β-cells that were able to thrive under minimal inflammation curtailed by Ig-GAD2. To test these premises, the mice treated with Ig-GAD2+BM which displayed consistent return to BGL below 300 mg/dl compared to those recipient of Ig-GAD2 or BM alone (Fig. 3A) were examined for reduction in pancreatic infiltration and formation of healthy islets. The results show that the mice recipient of Ig-GAD2+BM had islets that were mostly free of insulitis or islets that had minimal infiltration in the form of peri-insulitis (Fig. 3, B and C). Again, the mice recipient of Ig-GAD2 alone which were unable to recover from diabetes had islets with no or peri-insulitis indicative of effective immune modulation. In contrast, the animals recipient of BM alone had mostly severe insulitis indicating that the BM transfer without Ig-GAD2 does not directly modulate pancreatic inflammation (Fig. 3, B and C). Moreover, while the mice treated with Ig-GAD2+BM had structured islets with abundant insulin-positive cells, those given Ig-GAD2 or BM alone had less islets with fewer β-cells like untreated recently diagnosed diabetic mice (Fig. 3D). Compiled results indicate a significant increase in the number of insulin-producing β-cells, the number of islets with more than 10 insulin-positive cells, and the mass of β-cells in Ig-GAD2+BM-treated mice that were not evident in animals recipient of Ig-GAD2 or BM alone (Fig. 3, E-G). Since many islets in new onset diabetic NOD mice have no insulin\(^+\) (degranulated) cells (15) and our detection quantified only those with insulin\(^+\) cells, it is
possible that previously degranulated β-cells regained insulin-producing function in Ig-
GAD2+BM treated mice. Moreover, previous findings (7) and results below showing evidence
of significant β-cell division suggest a role for β-cell regeneration in the increase of insulin-
producing β-cells and healthy islets. Thus, the enrichment with BM cells synergized with Ig-
GAD2-driven immune modulation to sustain an increase in the number of β-cells that were able
to thrive and maintain the reduction in BGL.

Mice recipient of BM transfer and Ig-GAD2 treatment display increased endothelial cell
numbers in the pancreatic islets. The observation that BM transfer alongside Ig-GAD2
treatment sustains formation of healthy islets raises the question as to whether the newly formed
β-cells originate from the donor BM cells. To test this premise, we used NOD.GFP mice (10) as
a source of BM during treatment with Ig-GAD2 and assessed the insulin-producing β-cells for
GFP expression. The results show that there was no GFP/insulin colocalization at any time point
during Ig-GAD2+BM treatment (Supplementary Fig. 2, left panels). Furthermore, the GFP+
cells, which were abundant in the diabetes-free mice, were minimal in those recipient of the
same regimen but remained diabetic (Supplementary Fig. 2, right panels). Thus, the BM transfer
did not appear to serve as a source of insulin-producing β-cells but yielded engraftment of GFP+
cells in the islets of the recovering mice. Therefore, the β-cells did not originate from the donor
cells as was observed in other models (8; 9; 16; 17). The question then is whether the GFP+
engraftment represents cells that could not be provided by the host’s BM but are required for
maintenance of endogenous β-cells. Given that the islets are highly vascularized with endothelial
networks essential for optimal β-cell function (18-23), and that the number of functional ECs are
compromised in diabetic mice (24-28), it is logical to envision that the donor GFP+ cells
represent ECs. The results showing a significant decrease in the frequency of both circulating and intra-islet PECAM1+ ECs in diabetic versus healthy mice (Fig. 4) support the postulate. Indeed, there was a dramatic decrease in the frequency of peripheral blood ECs as the mice progressed towards overt diabetes (Fig. 4, A and B). Similarly, the frequency of ECs in the pancreatic islets diminished as the mice became diabetic, a phenomenon that correlates with the loss of β-cells (Fig. 4, C and D). This indicates that the frequency of ECs is diminished both in the peripheral blood and the pancreas in diabetic mice. Interestingly, the mice recipient of Ig-GAD2+BM but not those given Ig-GAD2 or BM alone restored the PECAM1+ ECs in the islets (Fig. 5A). Moreover, when the expression of genes encoding VE-cadherin (Cdh5), angiopoietin receptor (Tie1) and VEGF receptor 1 (Flt1) which represent functional markers for ECs were analyzed, there was a significant mRNA increase for these genes in the mice recipient of Ig-GAD2+BM relative to untreated diabetic animals (Fig. 5B). Those given Ig-GAD2 or BM alone did not display a similar increase in the expression of the genes (Fig. 5B). The increase in ECs likely fosters angiogenesis for better islet vascularization and thriving of β-cells. In fact, there was a strong up-regulation of genes encoding angiogenic factors, including VEGFa (vegfa), angiopoietin 1 (angpt1), and angiopoietin 2 (angpt2) in the pancreas of diabetes-free mice treated with Ig-GAD2+BM (Supplementary Fig. 3A). Furthermore, the newly-formed β-cells produced VEGFa (Supplementary Fig. 3B), which is critical for development of endothelial cells and islet vascularization (29; 30). The symbiotic relationship among endothelial and β cells is further evidenced by the parallel restoration of β-cell division in the Ig-GAD2+BM cell transfer mice (Fig. 5C). Indeed, the β-cells displayed significant staining for the proliferation marker ki-67 when compared to resting β-cells in normal mice or to residual β-cells in untreated diabetic mice (Fig. 5C). Indeed, the absolute cell number of ki-67+ insulin+ cells was significantly higher in the
Ig-GAD2+BM treated versus control new onset diabetic mice (Fig. 5C). This is further supported by a similar increase in the ratio of ki-67+insulin+ over total insulin+ cells (Fig.5C). These results suggest that BM transfer during treatment with Ig-GAD2 sustained repair of the endothelial network leading to efficient regeneration of β-cells. The latter were able to produce the vital angiogenic factor VEGFa to maintain symbiosis and the health of the islets.

**Donor BM transfer gives rise to islet endothelial cells.** To test whether the engrafted donor BM derived GFP+ cells represent ECs we examined the GFP+ cells for expression of the endothelial marker PECAM1 and for localization relative to insulin-producing β cells. The results show that in the diabetes-free mice there were GFP+ cells in the islets that expressed PECAM1 as indicated by the colocalization of the two markers at both day 30 and 60 of treatment (Fig. 6A). Such colocalization was not observed in mice recipient of the same regimen that remained diabetic. Also, the GFP+PECAM1+ cells did not colocalize with insulin staining, indicating that the BM transfer gives rise to ECs during protection against T1D. These observations are supported by the detection of Y chromosome in the endothelial but not in β-cells when the BM transfer was from male donors. Indeed, Y chromosome was detectable when the DNA was extracted from bulk pancreatic cells in mice recipient of Ig-GAD2+BM (Fig. 6B). More specifically, when PECAM1+ and insulin+ cells were micro-dissected using a laser-capture system (Supplementary Fig. 4) and their genomic DNA was analyzed by PCR, Y chromosome was detected in PECAM1+ but not insulin+ cells and this was restricted to diabetes-free mice given Ig-GAD2+BM transfer (Fig. 6, C and D). These results indicate that donor BM gives rise to ECs that are required for recovery from diabetes.

**Endothelial progenitor cells substitute for BM transfer and assist Ig-GAD2 for reversal of T1D.** EPCs are diminished in diabetic mice and human subjects (24-28). The transfer of BM
alongside Ig-GAD2 treatment gave rise to pancreatic ECs (Fig. 6). These observations suggest that BM transfer during treatment with Ig-GAD2 likely provides EPCs that give rise to the increased frequency of mature ECs. If this were to be the case, transfer of donor EPCs instead of whole BM alongside Ig-GAD2 should yield mature ECs able to assist β cells to thrive, and restore normoglycemia. To test this premise, we first determined the frequency of EPCs in our NOD colony and found that the BM lineage-negative (Lin−) population expressing the EPC markers c-Kit (31) and FLK-1(32; 33) was significantly reduced in the diabetic versus age-matched healthy mice (Fig. 7, A and B). We then sorted these EPCs from BM of healthy NOD.GFP mice and determined whether these cells can substitute for whole BM to reverse T1D. Indeed, when sorted GFP+Lin−c-Kit+FLK-1+ (hFLKI1+) cells from healthy donors replaced whole BM transfer during treatment with Ig-GAD2, most of the mice recovered from the disease while the control group given Lin−c-Kit+FLK-1− (hFLKI1−) cells had a much lower recovery rate despite receiving a 60-times higher cell number (Fig. 7C). In addition, no significant recovery from disease was observed when the hFLKI1+ cells were transferred without Ig-GAD2 (Fig. 7C).

It has previously been shown that, EPCs from diabetic subjects display impaired ability to differentiate into functional ECs (24). Thus, it is understandable that the diabetic mice were unable to utilize endogenous EPCs and required transfer of cells from healthy donor to recover from T1D upon treatment with Ig-GAD2. In fact, when the FLK-1+ cells were derived from sick NOD.GFP mice (sFLKI1+), there was minimal recovery from the disease as compared to the group recipient of Ig-GAD2+hFLKI1+ (p = 0.003840, area under the ROC curve) (Fig. 7C). Furthermore, there was no evident GFP+ cells in the islets of these mice which explains the lack of increase in PECAM1+ cells (compare right to left panel in Fig. 7D). In fact, similar results were observed in the mice that did not recover from diabetes under the Ig-GAD2+FLK-1− or
FLK-1⁺ cells without Ig-GAD2 (Fig. 7D). These results indicate that EPCs can substitute for BM transfer and give rise to mature ECs that help β-cells thrive and restore normoglycemia. Furthermore, maturation of the EPCs and increase in ECs occurs only when EPCs originate from healthy donors, which explains the inability of diabetic mice to utilize their own EPCs for repair of the pancreatic endothelial network.
DISCUSSION

Recent advances have shown that treatment with anti-CD3 antibody can reverse overt diabetes (34; 35). However, because of potential interference with the function of the immune system, open-ended treatment regimens are undesirable (36). In this context, antigen-specific approaches (37-42) which target autoreactive T cells and circumvent interference with immunity provide an alternative that could be effective against the disease. We and others have tested cell or protein-based antigen-specific regimens at advanced stages of the disease and promising outcomes were observed (7; 43; 44). In fact, Ig-GAD2 was able to trigger β-cell regeneration which prevented progress to overt diabetes (7). The present study was designed to test Ig-GAD2 for efficacy against overt T1D. However, despite the induction of an immune modulation similar to the hyperglycemic stage, the regimen was not able to trigger β-cell regeneration or overcome overt T1D. Given that β-cells are able to self-renew (12) we thought that either the differentiation of stem cell progenitors into insulin-producing β-cells is defective at this stage of the disease or there was minimal residual β-cells to support division and formation of sufficient β-cell mass. Although the use of BM as a source of β cell progenitors is debatable (8; 9; 16; 17; 45), repair of injured islet was feasible by BM transplantation (8; 9). Thus, we supplemented Ig-GAD2 treatment with BM transfer and found that recovery from disease is attainable. This was intriguing and prompted us to determine the mechanism underlying protection against disease. Interestingly, the islets had increased β-cell mass but the proliferating insulin-producing cells were of endogenous origin rather than donor BM-derived cells. Surprisingly, alongside the newly formed β-cells there was engraftment of donor BM-derived cells that did not produce insulin or colocalize with the β cells.
It has previously been shown in human patients with recent onset of T1D that the number of EPCs is significantly reduced in the peripheral blood compared with sex-matched healthy control subjects (24; 25). More importantly, EPCs in these patients displayed impaired migratory capacity (27) and damage repair potential (26), and failed to differentiate into functional vasculatures (24; 25). Given that our NOD mice displayed diminished frequency of EPCs at the onset of diabetes as was observed by others (25; 28), we suspected that the engrafted donor BM-derived cells represent mature ECs. This was indeed the case as the BM-derived GFP cells present in the islets of treated diabetes-free mice expressed the EC marker PECAM1. Moreover, PECAM1 expression tightly colocalized with donor GFP but not with newly formed endogenous β-cells. Since recovery from diabetes occurred only when the treatment is accompanied with BM transfer it is likely that repair of the islet vascular system was required to maintain symbiosis among β and ECs (18-23; 29). This could explain the unexpected engraftment of donor BM-derived endothelial cells in the pancreas of STZ mouse models of experimental diabetes (8; 9) especially since the animals have undergone irradiation prior to BM transplantation which destroys endogenous stem cell precursors. The fact that β-cells produced VEGFa and the angiogenic activity in the pancreas became evident, it is likely that repair of the islet vascular niche is required for β-cells to thrive and for insulin delivery into the circulation. Along this line of reasoning we substituted whole BM with EPCs (Lin<sup>-c-Kit</sup><sup>+</sup>FLK-1<sup>+</sup>) during treatment with Ig-GAD2 and observed formation of donor-derived ECs alongside the generation of endogenous β-cells in the diabetes-free mice. Again, this suggests that under tight control of the immune inflammatory process, the symbiotic relationship between β and ECs ensues and both types of cells contribute to the makeup of an environment suitable for formation of β-cells, maturation of ECs, and maintenance of healthy islets. Studies with anti-CD3 antibody indicated that despite
control of the inflammatory process β-cell proliferation/regeneration was minimal or at a very slow rate (15; 46; 47) with evident re-granulation of residual insulin-negative β-cells (15). However, when eradication of inflammation was accompanied with exogenous epidermal growth factor (EGF), β-cell neogenesis and proliferation ensued and sustained reversal of overt T1D (48). The findings in our study suggest that the low frequency of EPC and the defective function of residual pancreatic endothelial network in diabetics compromise not only the generation of new β-cells but also the restoration of optimal function, hence the return of disease observed in human trials upon termination of anti-CD3 antibody treatment (4; 5). In summary, antigen-specific therapy and supplementation with EPCs may overcome the limitation associated with long-term antibody administration and the repair of the islet endothelial niche. Whether effective control of immune inflammation by multiple Ig chimeras and diverse mechanisms (7; 39; 40; 49; 50) upon early diagnosis of disease would overcome the need for stem cell enrichment remains to be determined.
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X.W. performed the experiments and data analysis, F.B.G. designed cell sorting and assisted with flow cytometry analysis, A.M.V. assisted with analysis of diabetes, L.M.R. purified the Ig chimeras and assisted with analysis of diabetes, S.Z. performed laser capture and microdissection J.A.C, M.D., and C.M.H. assisted in the design of experiments and reviewed data. H.Z. designed and supervised the study and wrote the manuscript. H.Z. is the guarantor of this work, had full access to all the data, and takes full responsibility for the integrity of data and the accuracy of data analysis.
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FIGURE LEGENDS

FIG. 1. Ig-GAD2 treatment could not overcome overt T1D despite induction of immune tolerance. A: Hyperglycemic (BGL = 160-250 mg/dl) and diabetic (BGL ≥ 300 mg/dl) NOD mice (6 per group) were given Ig-GAD2 and their blood glucose levels (BGL) were monitored for a period of 70 days. B and C: The mice of the diabetic group were sacrificed on day 70 and the spleen (SP) and pancreatic lymph node (PLN) cells were harvested, stimulated with PMA and ionomycin and stained for surface CD4, CD8 and Vβ8.1/8.2 as well as intracellular IFNγ, IL-10 and IL-17. A group of mice sacrificed upon diagnosis of diabetes (untreated) is used as control. B: shows representative FACS plots (top) and individual mice (bottom) depicting CD4<sup>+</sup>CD8<sup>−</sup>V<sub>β</sub>8.1/8.2<sup>+</sup> cells producing IFNγ and IL-10. C: Shows the frequency of CD4<sup>+</sup>CD8<sup>−</sup>IL-17<sup>+</sup> cells. D: Shows the relative quantity (RQ) of mRNA expression for RORγt (rorc) and T-bet (tbx21) in the pancreatic islets of untreated and Ig-GAD2 treated mice. Each bar represents mean ± SEM of 3-6 mice. ** P < 0.01, * P < 0.05.

FIG. 2. Healthy donor BM cells transferred during immune modulation restore normoglycemia in diabetic mice. A: Shows a schematic representation of the treatment regimen in which diabetic mice received 2 insulin pellet implants on day 1 of the regimen and Ig-GAD2 and BM transfer as indicated. B: shows percent of mice with BGL < 300 mg/dl from groups recipient of BM transfer alone (BM), Ig-GAD2 alone (Ig-GAD2), or Ig-GAD2 plus male or female BM. n indicates the number of mice. C and D: The SP and PLN cells of the mice recipient of BM or Ig-GAD2 + BM were harvested on day 70, stimulated with PMA and Ionomycin, and analyzed for cytokine production. C: Shows representative FACS plots (left) and individual mice (right) depicting CD4<sup>+</sup>CD8<sup>−</sup>V<sub>β</sub>8.1/8.2<sup>+</sup> cells producing IFNγ and IL-10. D: Frequency of IL-17-producing
CD4⁺CD8⁻ cells. E: shows the RQ of mRNA expression for RORγt (rorc) and T-bet (tbx21) in the pancreatic islets of mice recipient of BM or Ig-GAD2+BM. Each bar represents mean ± SEM of 4-8 mice. ** P < 0.01, * P < 0.05.

**FIG. 3.** Mice recipient of BM transfer during treatment with Ig-GAD2 display increased insulin-producing pancreatic β cells. A: Blood glucose levels (BGL) in mice grafted with insulin pellets and given BM (n=8), Ig-GAD2 (n=7), or Ig-GAD2 + BM (n=17). B: Representative H&E staining (100X) of pancreatic sections from mice recipient of BM, Ig-GAD2 or Ig-GAD2+BM (sacrificed on day 70) (n=6 per group). Unmanipulated 4-6 week-old healthy (BGL ≤140 mg/dl), hyperglycemic (BGL = 160-250 mg/dl), and recent onset diabetic (BGL ≥ 300mg/dl) mice are included as control. C: Shows islet infiltration severity scores of the indicated groups. D: Shows representative immunohistochemistry staining (100X) for insulin (brown) with nuclei counterstained with hematoxylin (blue). E: Quantification of insulin⁺ cells per islet. F: Number of islets that contain more than 10 insulin⁺ cells. G: Mass of β-cells. Results in (E-G) are based on analysis of 3-6 non-serial sections per pancreas for 6 mice in each group. Specifically, at least 60 islets were counted for each of the new onset diabetic, BM, or Ig-GAD2 treated groups of mice. For the hyperglycemic and Ig-GAD2+ BM treated groups, at least 300 islets were counted per group. Error bars, mean ± SEM.. * P < 0.05, ** P < 0.01.

**FIG. 4.** Decline of blood and pancreatic endothelial cells during progression to diabetes. A and B: Peripheral blood cells from un-manipulated 4-6 week-old healthy (n=22), 8-14 week-old hyperglycemic (n=12) and 15-30 week-old diabetic (n=9) mice were stained with anti-CD45, anti-PECAM1 and 7-AAD, and PECAM1 expression was analyzed on 7-AAD⁻CD45⁻ gated
cells. A: Shows representative FACS plots and B: Shows results of individually tested mice in each group. C and D: Non-serial pancreatic sections from the same groups of mice were stained for insulin (green) and PECAM1 (red) and analyzed by immunofluorescence microscopy. C: shows a representative staining within the boundary of an islet area. Scale bars are 50µm. D: shows the number of PECAM1<sup>+</sup> cells per islet. This was determined by nuclear counterstaining with DAPI. At least 40 islets from 3-6 non-serial sections per mouse were analyzed. Each bar represents mean ± SEM of 5 mice per group. * P < 0.05, ** P < 0.01.

**FIG. 5.** Restoration of endothelial cells parallels with β-cell regeneration. A: Shows fluorescence microscopy images of PECAM1 (red) and insulin (green) immunostaining of pancreatic sections from mice given Ig-GAD2, BM or Ig-GAD2+BM. Islet boundary is depicted by dashed lines. Scale bars are 50µm. The bar graph represents quantification of PECAM1<sup>+</sup> cells per islet (5 mice per group). 10-30 islets from 3-6 non-serial sections per mouse were examined. B: Quantitative PCR analysis for cdh5, tie1 and flt1 expression in pancreatic islets of the mice described in (A) as well as untreated healthy and diabetic controls (4-8 mice per group). C: Shows confocal microscopy images of insulin (green) and ki-67 (red) staining of pancreatic sections from Ig-GAD2+BM treated mice (day 60 of treatment), healthy (untreated 4-6 week-old) and diabetic (day 1 of diagnosis) mice. The arrows depict insulin<sup>+</sup>ki-67<sup>+</sup> cells. Scale bars are 10µm. The bar graphs represent the number of insulin<sup>+</sup>ki-67<sup>+</sup> cells per section (left panel) and the ratio of insulin<sup>+</sup>ki-67<sup>+</sup> over total insulin<sup>+</sup> cells per pancreas (right panel). A total of 3,000 and 7,000 insulin<sup>+</sup> cells were counted from 30-40 non-serial sections for the diabetic and Ig-GAD2+BM mice (6 per group), respectively. The bars in the panels represent the mean ± SEM. * P < 0.05, ** P < 0.01.
FIG. 6. Donor bone marrow gives rise to pancreatic endothelial cells during suppression of diabetes. A: Shows confocal microscopy images for insulin (blue), PECAM1 (red) and GFP (green) from mice given Ig-GAD2 plus NOD.GFP BM (5 mice per time point). The arrows indicate co-localization of PECAM1 and GFP. Scale bars are 10µm. B-D: PCR analysis of Y chromosome (Y) using genomic DNA extracted from raw pancreatic sections (B), micro-dissected PECAM1+ (C) or insulin+ cells (D) of mice given Ig-GAD2+male BM (sacrificed on day 60 post treatment). DNA extracted from unmanipulated male (♂) and female (♀) mice was included for control purposes. In (C) lane 6 represents DNA from a Ig-GAD2 + male BM-treated mouse that did not recover from diabetes. In (D) lanes 1-9 represent the percentage of male DNA diluted with female DNA. Lanes 10-12 represent DNA from three individual diabetes-free mice given Ig-GAD2 + male BM.

FIG. 7. Transfer of endothelial cell progenitors during treatment with Ig-GAD2 sustains β cell regeneration and restores normoglycemia. A: Shows representative FACS plots depicting FLK-1 expression on Lin−c-Kit7-AAD− BM cells from age-matched healthy and diabetic mice. B: shows FLK-1 expression on Lin−c-Kit7-AAD− BM cells of individually tested mice described in (A). C and D: FACS-sorted Lin−c-Kit7FLK-1+ (FLK-1+) or Lin−c-Kit7FLK-1− (FLK-1−) BM cells from healthy (hFLK-1+) or diabetic (sFLK-1+) NOD.GFP donors were used with or without Ig-GAD2 for treatment of diabetic NOD mice. Some of the mice were monitored for diabetes for 100 days (C), and others (D) were sacrificed on day 60 post treatment and used for visualizing insulin (blue), PECAM1 (red) and GFP by confocal microscopy (at least 20 sections per group). The arrows indicate co-localization of PECAM1 and GFP. Scale bars are 10µm. ** P < 0.01.
Diabetes
Diabetes
SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Treatment with Ig-GAD2+BM ablates insulin resistance in diabetic NOD mice. Six-hour-fasting mice were injected i.p. with 0.75 units per kg of regular human insulin (Novolin; Novo Nordisk, Clayton, NC). The blood glucose level was then measured at the indicated time points. The results are presented as the percentage of initial blood glucose level.

Supplemental Figure 2. Insulin producing-β cells in Ig-GAD2+BM-treated mice are not derived from donor cells. NOD mice given Ig-GAD2 and BM from NOD.GFP donors (Ig-GAD2+GFP BM) according to the regimen described in Figure 2 were sacrificed on day 30, 60 and 120 post treatment and pancreatic sections from 3 diabetic or 3 diabetes-free mice were stained for insulin at each time point. Data shows representative confocal microscopy visualizing insulin (red) and GFP (green). The dashed line depicts the boundary of the islet area. Scale bars are 10µm.

Supplemental Figure 3. Enhanced production of angiogenic factors during restoration of normoglycemia. A: Diabetic mice were given Ig-GAD2+BM treatment regimen and RNA was extracted from their pancreata on day 60 and used to analyze expression of vegfa, angpt1 and angpt 2. RNA from the pancreata of healthy and diabetic mice was included for control purposes. Each bar represents relative fold change of gene expression (mean ± SEM) analyzed by quantitative PCR in 5 mice per experimental group. B: shows representative confocal microscopy visualizing VEGFa (green) and insulin (red) in pancreatic sections from Ig-GAD2+BM-treated mice (sacrificed on day 60, n=3). At least 30 islets from 3-4 non-serial sections per mouse were analyzed. The scale bars are 10µm. * P < 0.05, ** P < 0.01.
Supplemental Figure 4. Microdissection of insulin^+ or PECAM1^+ cells. Pancreatic sections were stained for either insulin or PECAM1 (brown) and the cell nuclei were counterstained with hematoxylin (blue). Data shows representative light microscopy depicting the cell capture area before and after the procedure. The microdissected cells were then used for extraction of genomic DNA and PCR analysis.
Diabetes
Diabetes

Before microdissection | After microdissection | Microdissected cells

Insulin

130x94mm (300 x 300 DPI)

PECAM1
Wan et al., Supplementary Table 1

| Primers for detection of Y chromosome | Forward | Reverse |
|--------------------------------------|---------|---------|
| Y chromosome                         | 5’-GGTGAGAGGCACAAGTTGG-3’ | 5’-ATCTCTGTGCCTCCTGGAAA-3’ |
| Beta-actin                           | 5’-GCTTCTTTTCAGCTCTTGGCGGTTGC-3’ | 5’-GTGTCCGTCTGAGTGATCCTCAG-3’ |

| Primers for detection of genes associated with endothelial cell function and T cell transcription factors | Forward | Reverse |
|-------------------------------------------------------------------------------------------------|---------|---------|
| angpt1                                             | 5’- AGCATCTGGGAGCAGTGATGGA-3’ | 5’- TATCTCAAGCAGTGATGGCCGT-3’ |
| angpt2                                             | 5’- AACACCGAGAAGATGGCAGTGT-3’ | 5’- AGACAAACTCATTGCCCAGCCA-3’ |
| cdh5                                               | 5’- TTCGACCAGGTATTCACCGCA-3’ | 5’- TCATCTGCATCCACTGCTGTCA-3’ |
| flt1                                                | 5’- TGCAGGAAACCACACAGCAGGAA-3’ | 5’- TTCAATGGCGAGCCAGCAT-3’ |
| tie1                                                | 5’- CAGCATGAAACTTCGCAAGCCA-3’ | 5’- TGGGCACTTCAAACCTGTGCTGT-3’ |
| vegfa                                               | 5’- TGCAGGGCTGCTGTAACGATGAA-3’ | 5’- TGCTGTGCTGTAGGAAGCTCAT-3’ |
| tbx21                                               | 5’- TCCAAGTCTCAACCAGCACCAGA-3’ | 5’- TCCACCAAGACCACATCCACAA-3’ |
| rorc                                                | 5’- ACAGCCACTGCAATCCCCATTT-3’ | 5’- TCTCGGAAGGACTTGCAAGCAT-3’ |