

Ex Vivo Expanded Human Regulatory T Cells Can Prolong Survival of a Human Islet Allograft in a Humanized Mouse Model

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Background. Human regulatory T cells (Treg) offer an attractive adjunctive therapy to reduce current reliance on lifelong, nonspecific immunosuppression after transplantation. Here, we evaluated the ability of ex vivo expanded human Treg to prevent the rejection of islets of Langerhans in a humanized mouse model and examined the mechanisms involved.

Methods. We engrafted human pancreatic islets of Langerhans into the renal subcapsular space of immunodeficient BALB/c.*rag2*^{-/-}.*cy*^{-/-} mice, previously rendered diabetic via injection of the β -cell toxin streptozocin. After the establishment of stable euglycemia, mice were reconstituted with allogeneic human peripheral blood mononuclear cells (PBMC) and the resultant alloreactive response studied. Ex vivo expanded CD25^{high}CD4⁺ human Treg, which expressed FoxP3, CTLA-4, and CD62L and remained CD127^{low}, were then cotransferred together with human PBMC and islet allografts and monitored for evidence of rejection.

Results. Human islets transplanted into diabetic immunodeficient mice reversed diabetes but were rejected rapidly after the mice were reconstituted with allogeneic human PBMC. Cotransfer of purified, ex vivo expanded human Treg prolonged islet allograft survival resulting in the accumulation of Treg in the peripheral lymphoid tissue and suppression of proliferation and interferon- γ production by T cells. In vitro, Treg suppressed activation of signal transducers and activators of transcription and inhibited the effector differentiation of responder T cells.

Conclusions. Ex vivo expanded Treg retain regulatory activity in vivo, can protect a human islet allograft from rejection by suppressing signal transducers and activators of transcription activation and inhibiting T-cell differentiation, and have clinical potential as an adjunctive cellular therapy.

Keywords: Regulatory T cells, Islet transplantation, Allograft, Humanized mouse model, Cellular therapy.

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Type 1 diabetes mellitus is a cell-mediated autoimmune disease that causes the destruction of pancreatic β cells and results in lifelong insulin dependency. Currently, exogenous

insulin therapy is the most popular therapeutic modality, and rigorous glycemic control improves glycosylated hemoglobin and can protect against secondary complications (1). Unfortunately, these benefits are concurrent with an increased risk

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D.C.W., J.H., and K.J.W. were involved in all aspects of research and experimental design and article preparation. S.N.N. was involved in all aspects of research and experimental design. W.Z. was involved in the

development and optimization of ex vivo expansion protocols of human Treg. P.T. was involved in the development and optimization of ex vivo expansion protocols of human Treg, development of humanized mouse model, and flow cytometric analysis. S.H. provided human islet tissue used in experiments. D.G. and P.J. provided considerable intellectual contribution toward research and experimental design and provided human islet tissue used in experiments.

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of serious hypoglycemic events such as recurrent seizures and coma (2). In search for a more physiologic approach to glycemic control, pancreas transplantation and pancreatic islet transplantation have been shown to be viable options in the treatment of type 1 diabetes mellitus (3). However, although advances in immunosuppressive therapy have facilitated excellent short-term graft outcomes after islet transplantation, prolonged graft function remains elusive (4). In addition, the requirement for lifelong immunosuppression after transplantation and its associated adverse effects precludes the widespread adoption of islet transplantation, especially in younger patients.

In lieu of toxic immunosuppressive drugs, an alternative or adjunctive therapy to prevent rejection of islet allografts, as well as other cell or organ transplants, may lie in harnessing the potential of regulatory T cells (Treg). The powerful, dominant immunomodulatory capabilities of Treg has been demonstrated in various settings including the prevention of autoimmunity (5) and rejection after cell and organ transplantation (6). In human peripheral blood, Treg were initially isolated based on the expression of CD4 and high levels of CD25 (7–11). Regulatory activity is associated with sustained expression of FoxP3 and low levels of CD127, the interleukin (IL)-7 receptor (12, 13). Treg can be expanded *ex vivo* via the cross-linking of CD3 and CD28 and the addition of exogenous IL-2 while still maintaining their suppressive capacity *in vitro* (8, 14, 15).

One of the next steps toward applying Treg as an immunotherapy lies in testing their ability to retain regulatory capacity after *ex vivo* expansion in relevant *in vivo* models (16, 17). Here, BALB/c.*rag2*^{-/-}.*cy*^{-/-} mice deficient in T, B, and NK cells were used to analyze the rejection response of human leukocytes against a human islet allograft and to assess the impact and mode of action of *ex vivo* expanded human Treg in modulating this response.

RESULTS

Characterization of Ex Vivo Expanded Human Treg

Human CD25^{high}CD4⁺ T cells were purified by cell sorting and *ex vivo* expanded (Fig. 1A). After two rounds of expansion, cells retained the expression of CD25, FoxP3, CTLA-4, GITR, CD27, and CD62L, whereas CD127 expression remained low (Fig. 1B). More detailed analysis across several cell donors demonstrated that, on average, 80% and 90% of expanded cells expressed FoxP3 and CD25, respectively (Fig. 1C). The expression of Treg-associated markers CD27, CD62L, and GITR varied between approximately 20% and 90% of expanded cells, whereas, on average, less than 20% of cells expressed CD45RA, CD57, and CD127 (Fig. 1C). Importantly, on average, 75% of Treg were CD25⁺CD127^{lo} after expansion (see Figure S1, SDC, <http://links.lww.com/TP/A848>). *Ex vivo* expanded Treg were highly suppressive *in vitro* (Fig. 1D), and importantly, Treg phenotype postexpansion correlated with their *in vitro* suppressive activity. In particular, FoxP3 mean fluorescence intensity (MFI) and the frequency of cells expressing CD62L correlated positively with the ability of Treg to suppress proliferation of responder cells (Fig. 1E), whereas CD127 expression correlated negatively (Fig. 1E).

Human Islets Reverse Diabetes in Immunodeficient Mice

To determine critical mass of human islets able to establish stable, long-term normoglycemia in diabetic (streptozocin-induced) BALB/c.*rag2*^{-/-}.*cy*^{-/-} mice, we transplanted 2500 to 10,000 islet equivalents (IEQ) into the renal subcapsular space and monitored blood glucose levels. Approximately 7500 to 10,000 IEQ could establish stable normoglycemia (see Figure S2, SDC, <http://links.lww.com/TP/A848>); therefore, 8000 IEQ were used subsequently. Implantation of human islets (8000 IEQ) resulted in immediate and stable establishment of normoglycemia over an observation period of 60 days (Fig. 2A). At the end of the observation period, human islet grafts were removed by unilateral nephrectomy resulting in all mice returning to a hyperglycemic state within 2 days (blood glucose ≥ 14.5 mM) (Fig. 2A). Immunofluorescence analysis of the excised human islet grafts revealed intensely staining, densely packed insulin-positive islet tissue in the renal subcapsular space (Fig. 2B).

Allogeneic Human Leukocytes Trigger Islet Allograft Rejection

To analyze the immunologic reaction between human peripheral blood mononuclear cells (PBMC) and a human islet allograft, mice with a functional human islet graft (stable normoglycemia maintained for a period of 14 days) were reconstituted with 40×10^6 allogeneic human PBMC. Acute islet allograft rejection, as measured by a rise in blood glucose above 14.5 mM, was observed in all animals that successfully reconstituted with human PBMC (>1% human CD45⁺ cells in the spleen) (Fig. 2C). Histologic analysis of rejected grafts confirmed obliteration of insulin-positive islet tissue in the renal subcapsular space (compare Fig. 2D vs. Fig. 2B) and showed significant infiltration of the graft site by human CD4⁺ and CD8⁺ T cells (Fig. 2E). Leukocyte infiltration was specifically restricted to the graft site, as neighboring kidney tissue was relatively devoid of human cells.

Ex Vivo Expanded Treg Prevent Human Islet Allograft Rejection

Next, we assessed the effect of Treg cotransfer on human leukocyte reconstitution in the immunodeficient mice. *Ex vivo* expanded human Treg were adoptively transferred simultaneously with human PBMC from the same blood donor (1:1 ratio) into mice that had maintained stable function with a human islet graft for 14 days or more. Control groups received either PBMC alone or no human cells (to confirm the long-term function of the islet allograft). Two (of 15) mice in each group did not reconstitute and were excluded from further analysis. The reconstituted mice were characterized by high level of reconstitution with an average of 30% to 40% of human leukocytes (mainly T lymphocytes) in the spleen and Treg cotransfer did not affect leukocyte reconstitution (Fig. 3A). Similarly, there was no difference in the frequency or number of CD4⁺ and CD8⁺ T cells (Fig. 3A,B; data not shown).

Next, we investigated the impact of Treg on the modulation of rejection of human islet allografts. The cotransfer of Treg at a 1:1 ratio resulted in a prolongation of islet allograft survival compared with the transfer of PBMC alone (median survival time >45 vs. 23 days in mice receiving PBMC alone) (Fig. 3C). Blood glucose levels of mice receiving

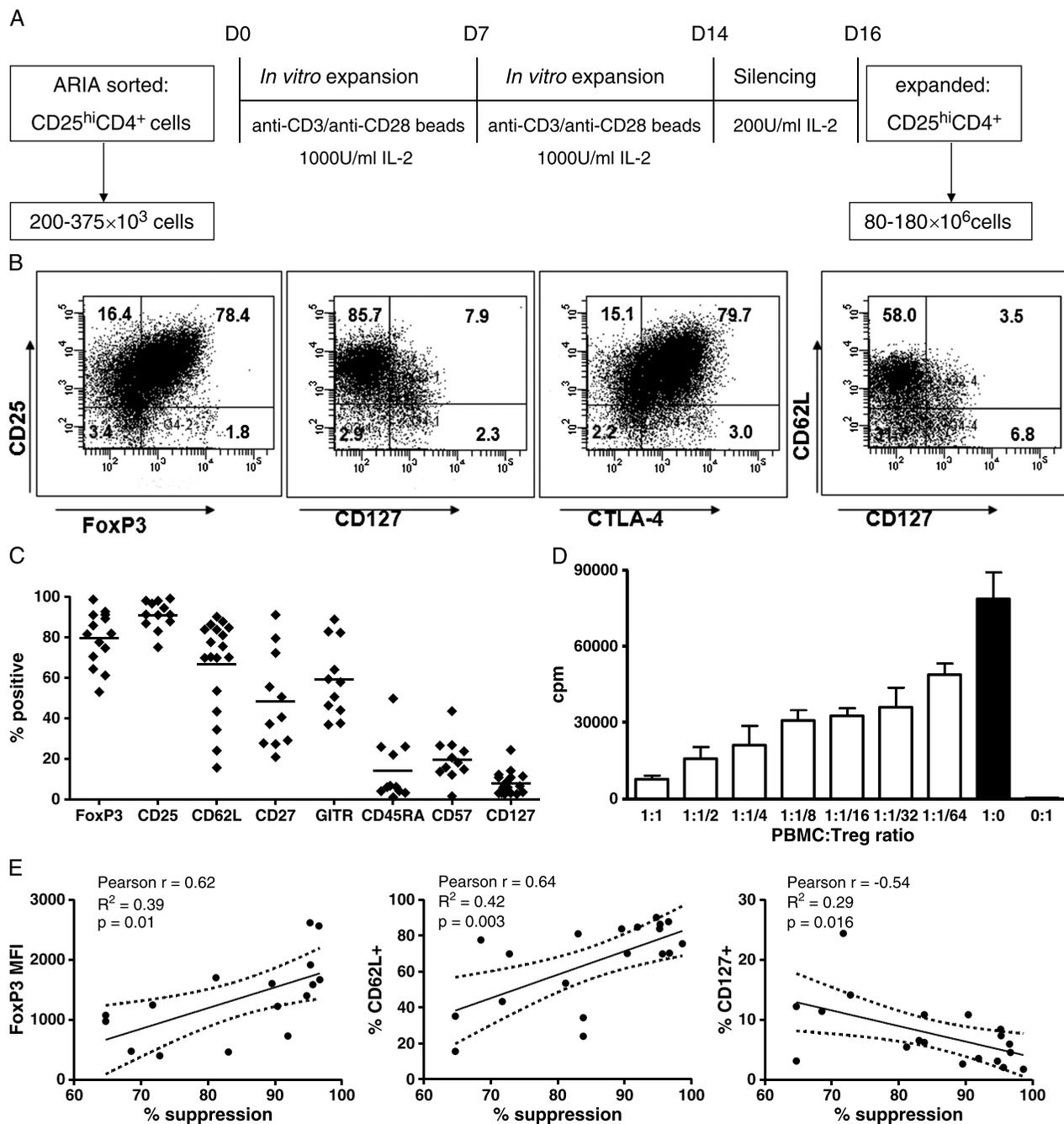


FIGURE 1. Characterization of ex vivo expanded human Treg. A, expansion protocol: Human CD25^{high}CD4⁺ cells purified by FACSARIA cell sorting were expanded in vitro in the presence of anti-CD3/anti-CD28 beads and 1000 U/mL recombinant human IL-2. After two rounds of expansion (7 days each), the beads were removed and cells were cultured for an additional 2 days before functional analysis in vitro and in vivo. B, representative dot plots demonstrating the expression of CD25, FoxP3, and CTLA-4; CD127 and CD62L on expanded Treg. C, expression of indicated markers on expanded Treg sorted from different blood donors. D, ex vivo expanded Treg cells suppress the proliferation of syngeneic PBMC in vitro in response to alloantigen stimulation. Serial dilutions of expanded Treg were added to cultures of 1 × 10⁵ autologous PBMC stimulated with an equal number of irradiated allogeneic PBMC and incubated for 7 days. ³H-thymidine was added for the final 16 hr of the culture. Error bars reflect mean ± SD of triplicate cultures. E, correlation between FoxP3 MFI or frequency of CD62L or CD127 expressing Treg and their in vitro suppressive capacity at 1:1 Treg/PBMC. Phenotypic data (FoxP3 MFI, CD62L, and CD127 expression) was assessed post-expansion, before the suppression assay. Data are from multiple separate donors and independent expansions.

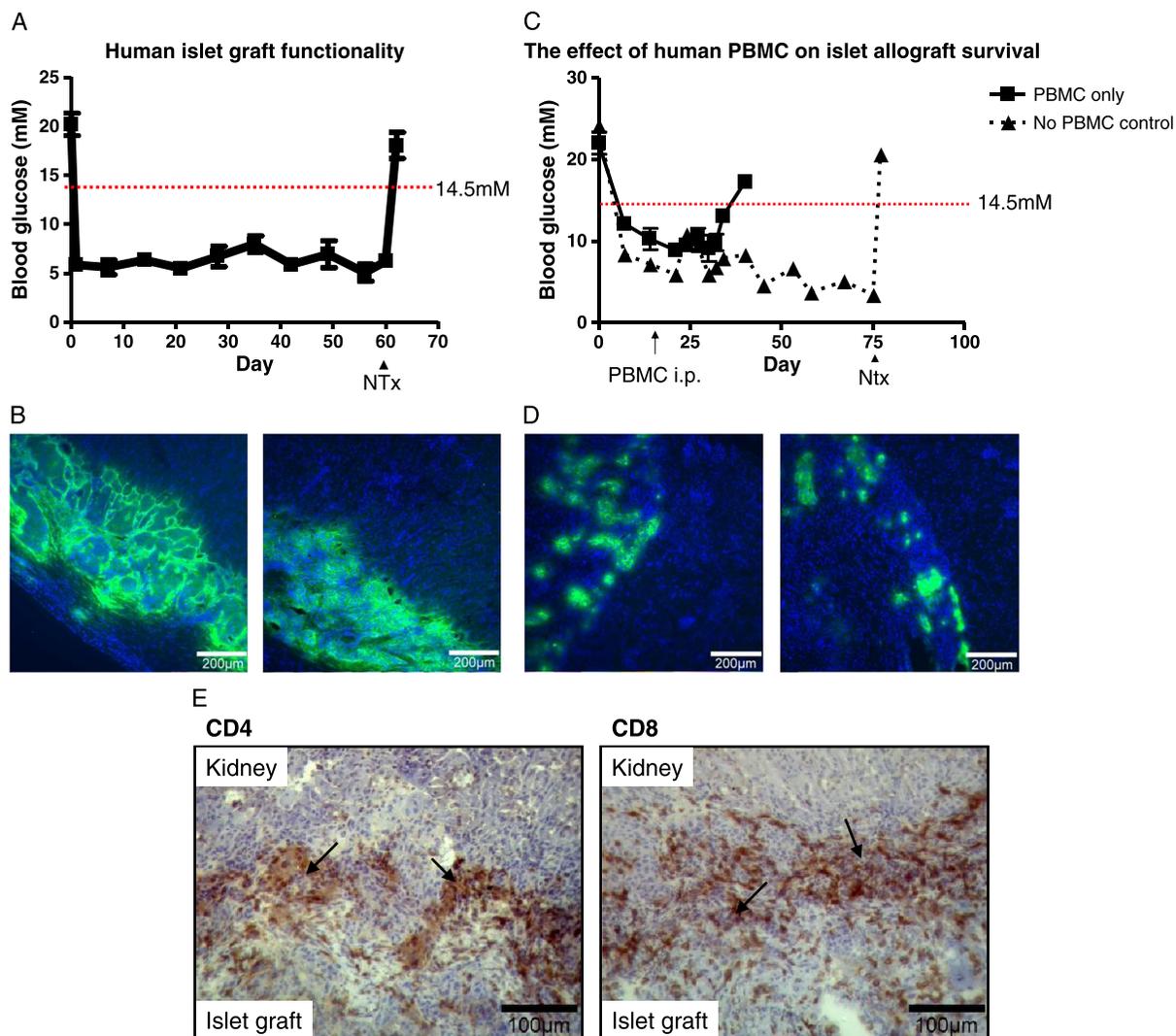


FIGURE 2. Human PBMC that reconstitute *BALB/c.rag2^{-/-}.common- γ c^{-/-}* mice function to reject a human islet allograft. **A**, human islets function long-term in *BALB/c.rag2^{-/-}.common- γ c^{-/-}* mice. Human islets (8000 IEQ) were transplanted underneath the kidney capsule of streptozocin-induced diabetic mice and blood glucose was measured at regular intervals as indicated (60 days after transplantation, blood glucose <14.5 mM). On day 60, the islet grafts were removed via unilateral nephrectomy. All mice returned rapidly to a hyperglycemic state (n=6). **B**, insulin expression in excised human islet grafts. Immunofluorescence staining for insulin (green; nuclei were counterstained with 4',6-diamidino-2-phenylindole [DAPI; blue]). The photomicrographs are from two separate human islet grafts and are representative of grafts taken from all control mice (n=9), that is, mice that did not receive any human PBMC. **C**, human PBMC reject a human islet allograft. Human PBMC (40×10^6) were injected intraperitoneally after successful transplantation of human islets (stable blood glucose of <14.5 mM maintained for 14 days after transplantation. Blood glucose was monitored regularly as indicated. All mice that successfully reconstituted with human PBMC (>1% human CD45⁺ cells in the spleen at the time of analysis) rejected the human islet allografts acutely with a median survival time of 23 days (blood glucose >14.5 mM). Control animals that did not receive PBMC maintained excellent graft function throughout the observation period. Data shown are from a single experiment (n=5 mice reconstituted with human PBMC and n=1 control), where PBMC and islets were prepared from two unrelated individuals and are representative of a series of experiments using three different human islet isolations and four PBMC donors. **D**, insulin expression in rejected human islet grafts. Islet allografts from mice with a blood glucose of more than 14.5 mM were excised and analyzed by immunofluorescence staining for insulin (green; nuclei were counterstained with DAPI [blue]). The photomicrographs are from two separate human islet grafts and are representative of grafts from all animals (n=13) that successfully reconstituted with human PBMC (>1% human CD45⁺ cells detectable in the spleen at the time of the analysis). **E**, CD4⁺ and CD8⁺ T cells infiltrate rejected human islet grafts. Immunoperoxidase staining of islet allografts from mice with a blood glucose of more than 14.5 mM showed infiltration by human CD4⁺ (left) and CD8⁺ (right) leukocytes. The photomicrographs are from one human islet graft and are representative of grafts from all animals (n=13) that successfully reconstituted with human PBMC (>1% human CD45⁺ cells detectable in the spleen at the time of the analysis). The arrows represent human CD4⁺ and CD8⁺ leukocytes forming infiltrate at the graft site, whereas neighboring kidney tissue is relatively devoid of human cells.

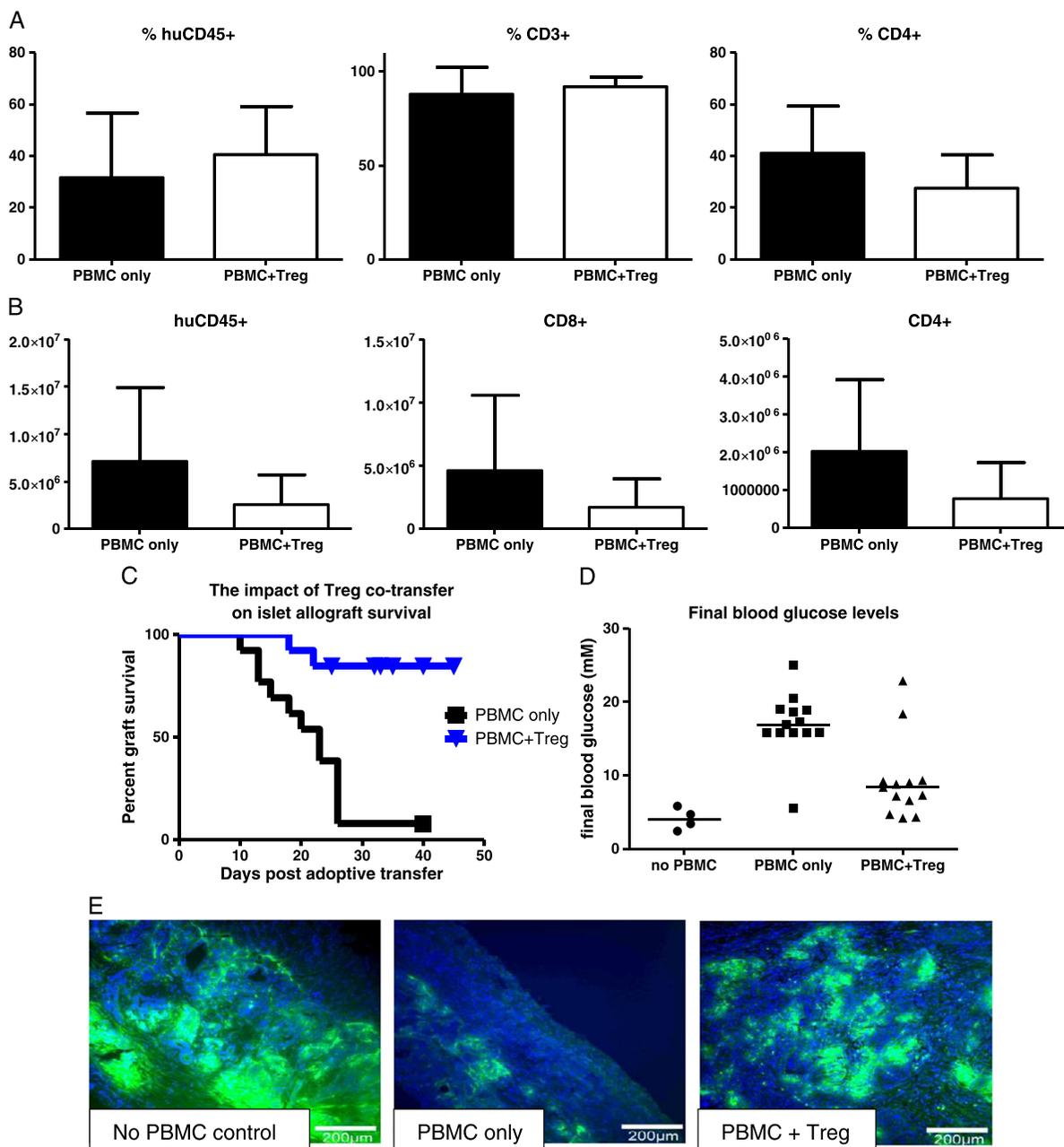


FIGURE 3. Ex vivo expanded human Treg protect a human islet allograft from rejection. A and B, Treg cotransfer does not affect mice reconstitution with human leukocytes. Mice with functioning human islet allografts (blood glucose <14.5 mM for a minimum of 14 days) received either 40×10^6 allogeneic human PBMC alone or with cotransfer of 40×10^6 ex vivo expanded human Treg cells. At the time of harvest, splenic reconstitution with human leukocytes was measured. A, percentage of human CD45⁺ cells within leukocyte gate, the percentage of CD3⁺ cells within human leukocytes (CD45⁺ cells), and the percentage of CD4⁺ cells within T-cell gate. B, absolute numbers of human CD45⁺, CD8⁺, and CD4⁺ cells. C, human Treg prevent human islet graft rejection. Mice with functioning human islet allografts were either left unreconstituted or received either 40×10^6 allogeneic human PBMC alone (n=13) or with cotransfer of 40×10^6 ex vivo expanded human Treg cells (n=13). Data pooled from three experiments with three unique islet preparations and four unique human cells donors. Graft survival was determined by regular blood glucose measurements, with graft failure indicated by a blood glucose of more than 14.5 mM. D, final blood glucose measurement at the time of harvest. Mice were treated as in C. E, differential insulin expression in rejected and protected human islet grafts. Islet grafts from mice that did not receive human PBMC, received either 40×10^6 allogeneic human PBMC alone, or received 40×10^6 allogeneic human PBMC with cotransfer of 40×10^6 ex vivo expanded human Treg cells were excised and analyzed by immunofluorescence staining for insulin (green; nuclei were counterstained with DAPI [blue]). Grafts above were analyzed on days 75, 37, and 39 after transplantation. The photomicrographs are from a single experiment using the same PBMC and islet preparations and are representative of all of the independent experiments performed (n=4).

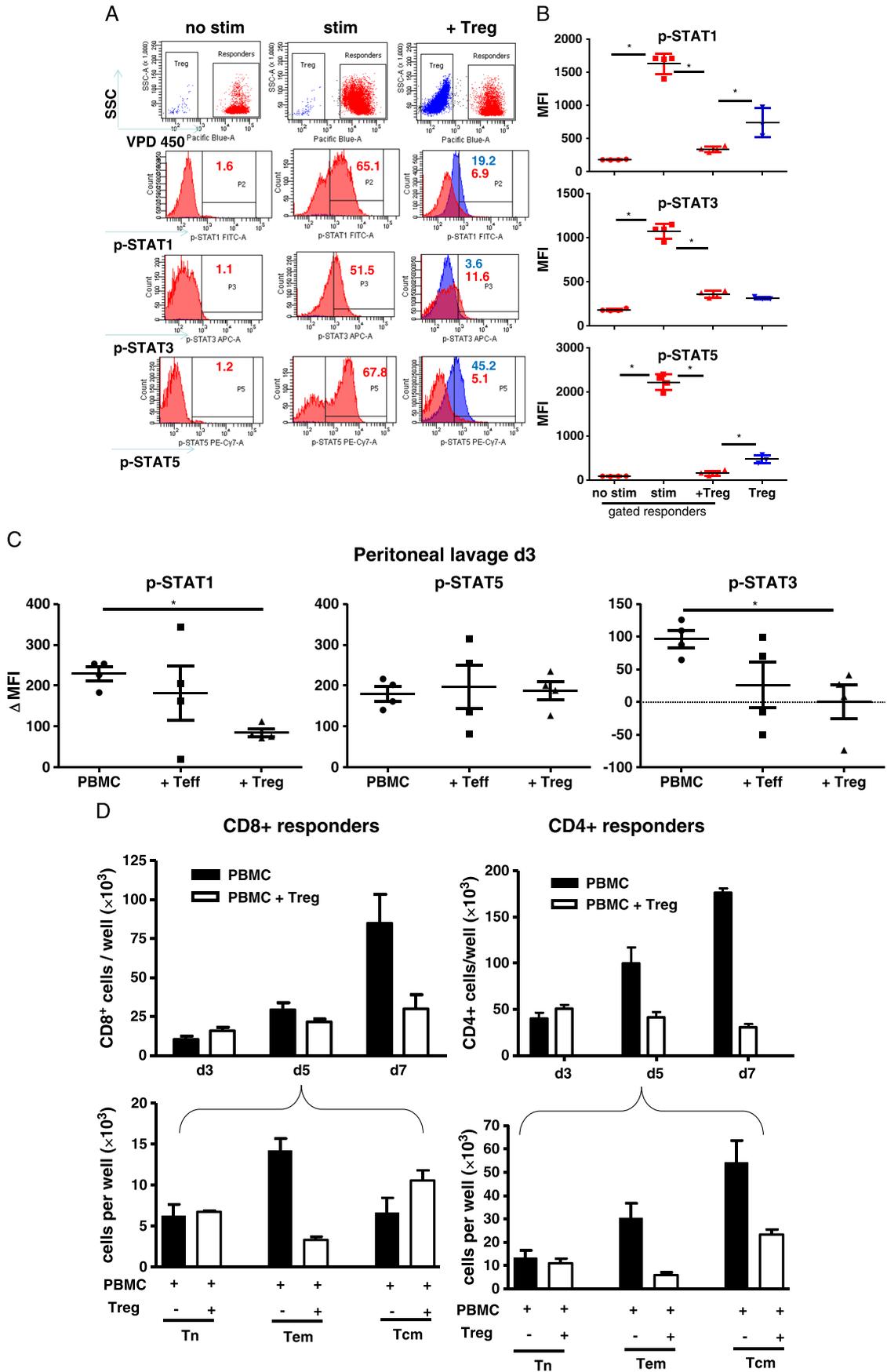


FIGURE 4. (Legend on next page).

the Treg and PBMC were maintained at similar levels to those in mice that were not reconstituted with human PBMC (<14.5 mM glucose; data not shown) and only two mice receiving Treg rejected the grafts (>14.5 mM glucose) (Fig. 3D). Islet allografts from mice that received Treg exhibited markedly increased levels of insulin-positive tissue compared with islet allografts from mice reconstituted with allogeneic PBMC alone (Fig. 3E).

Ex Vivo Expanded Human Treg Suppress Cytokine Signaling and T Lymphocyte Proliferation

To investigate the molecular mechanisms of Treg-mediated suppression, we analyzed the effect of coculture with Treg on activation of intracellular signaling pathways, especially signal transducers and activators of transcription (STATs) engaged in mediating cytokine signaling. We cocultured PBMC and Treg and measured phosphorylation of interferon (IFN)- γ -signaling STAT1, IL-6-signaling STAT3, and IL-2-signaling STAT5. Stimulation of PBMC with anti-CD3/anti-CD28 beads resulted in activation of all investigated STAT proteins, whereas addition of Treg resulted in inhibition of phosphorylation to nearly basal levels (Fig. 4A,B), suggesting inhibition of cytokine production in cocultures. Interestingly, Treg themselves (Fig. 4A, blue population) had higher activation levels of STAT1 and STAT5 than suppressed responders, suggesting possible consumption of remaining IFN- γ and IL-2 by Treg (Fig. 4A,B). To test whether Treg-mediated inhibition of STAT activation occurs in vivo, we injected BALB/c.rag2^{-/-}.cy^{-/-} mice intraperitoneally with carboxyfluorescein succinimidyl ester (CFSE)-labeled PBMC in the presence or absence of VPD450-labeled Treg. Coinjection of Treg resulted in suppression of STAT1 and STAT3 activation in PBMC (Fig. 4C), whereas cotransfer of T effector cells from the same donor did not affect STAT activation (Fig. 4C), demonstrating the suppressive effect is Treg specific.

Having demonstrated defective cytokine signaling in responder leukocytes under coculture with Treg, we hypothesized that inhibited cytokine production and signaling would affect proliferation and effector lymphocyte differentiation. To test this hypothesis, HLA-A2⁺ Treg were cocultured with HLA-A2⁻ responder PBMC in the presence of anti-CD3/anti-CD28 beads. On days 5 and 7, there was a decrease in number of HLA-A2⁻ CD4⁺ and CD8⁺ T cells in the presence of Treg, especially manifested by the suppression of effector memory T-cell development (Fig. 4D).

Ex Vivo Expanded Human Treg Suppress Leukocyte Proliferation in vivo

Having established the protective effect of ex vivo expanded human Treg on a human islet allograft and demonstrated ability of Treg to inhibit proliferation of effector T cells in vitro, we next used CFSE division profiling to assess the mechanism of Treg action in the in vivo model. Flow cytometric analysis of cells in the peritoneal lavage on day 5 after administration of CFSE-labeled human PBMC in the presence and absence of Treg (1:1 ratio, 20 \times 10⁶ PBMC/20 \times 10⁶ Treg) revealed that cotransfer of Treg resulted in the suppression of CD8⁺ T-cell proliferation (see Figure S3, SDC, <http://links.lww.com/TP/A848>). By day 10, live human cells could also be detected in the spleen and mesenteric lymph nodes. Again, analysis of absolute cell numbers showed a marked suppression of CD8⁺ T-cell proliferation in both spleen and mesenteric lymph nodes (see Figure S3, SDC, <http://links.lww.com/TP/A848>).

Ex Vivo Expanded Human Treg Accumulate and Suppress IFN- γ Production In vivo

At the time of rejection, or at the endpoint of the observation period (day 45), draining lymphoid tissue from mice reconstituted with either PBMC alone or PBMC and human Treg was harvested and analyzed by flow cytometry. Increased numbers of CD25⁺CD4⁺FoxP3⁺ T cells were found to accumulate in the draining lymph nodes of mice reconstituted with PBMC and Treg, but not in mice receiving PBMC alone (Fig. 5A); however, there was no difference in Treg accumulation in the spleen (Fig. 5A). Moreover, IFN- γ production was suppressed in both CD4⁺ and CD8⁺ human T cells when Treg were cotransferred (Fig. 5B), but the total number of CD4⁺ and CD8⁺ T cells was not affected (Fig. 3B). Taken together, these data suggest that ex vivo expanded human Treg are able to home to the peripheral lymphoid tissues and suppress the proliferation, activation, and production of effector cytokines by human PBMC that, when present alone, have the potential to reject the islet allograft.

DISCUSSION

In this study, we show that ex vivo expanded human Treg generated by a routine and reproducible method retain their regulatory activity in vivo and can prevent the rejection of a human islet allograft in a humanized mouse model. Functional studies demonstrated that Treg could home to the lymphoid tissue draining the graft site and inhibit the priming

FIGURE 4 (Continued). Expanded human Treg cells suppress cytokine signaling and inhibit expansion of conventional CD4⁺ and CD8⁺. A, 1 \times 10⁵ VPD450-labeled PBMC (red population) were stimulated with 2 \times 10⁴ anti-CD3/anti-CD28 beads and cultured for 2 days with or without 1 \times 10⁵ Treg (blue population). Gating strategy and percentage of PBMC (red) and Treg (blue) expressing pSTAT1, pSTAT3, and pSTAT5 is shown. B, cells were prepared and gated like in A. MFI of pSTAT1, pSTAT3, and pSTAT5 in PBMC (red) and Treg (blue) are shown. **P*<0.05. C, reduced STAT1 and STAT3 activation in PBMC cotransferred with Treg. CFSE-labeled PBMC and VPD450-labeled expanded Treg (CD25^{hi}CD4⁺) or expanded Teff (CD25⁻CD4⁺) were injected intraperitoneally at 1:1 ratio. Cells were recovered from peritoneum 3 days later and STAT activation was measured in CFSE⁺ cells. Results are displayed as Δ MFI, calculated as a difference between MFI of specifically stained and unstained samples. n=4 mice per group, each stained as one to four repeats. **P*<0.05. D, 1 \times 10⁵ HLA-A2⁻ responder PBMC were incubated with or without 1 \times 10⁵ mismatched HLA-A2⁺ ex vivo expanded human Treg cells in the presence of 1 \times 10⁴ anti-CD3/anti-CD28 beads for 3, 5, or 7 days. Top, number of CD8⁺ responders (left) and CD4⁺ responders; bottom, number of naïve (Tn: CD45RA⁺CD62L⁺) and memory (central memory Tcm: CD45RA⁻CD62L⁺ and effector/effector memory Tem: CD45RA⁻CD62L⁻) responding cells on day 5 of culture.

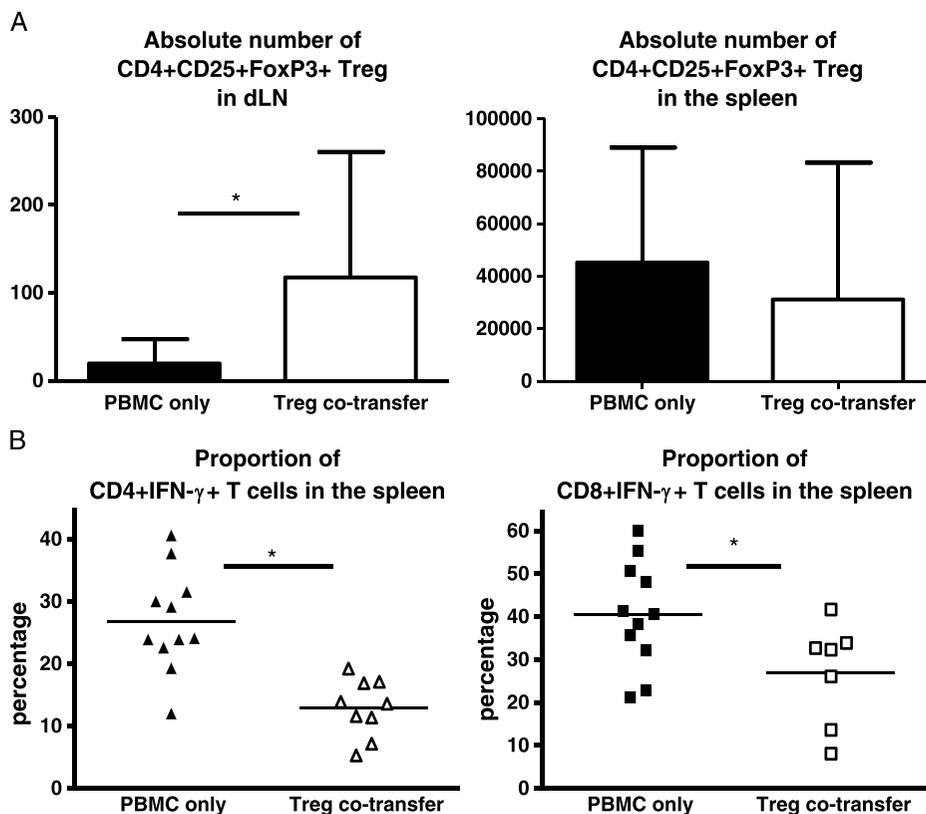


FIGURE 5. Ex vivo expanded human Treg cells suppress the proliferation and activation of T cells responding to alloantigens in vivo. **A**, accumulation of human Treg in the draining lymph nodes. Draining lymph nodes and spleens were removed from mice that had been transplanted with a human islet allograft and reconstituted either 40×10^6 human PBMC alone ($n=10$) or together with 40×10^6 ex vivo expanded Treg cells ($n=8$). Single-cell suspensions were prepared at the time of rejection (PBMC only group) or at the end of the observation period (PBMC+Treg), and the absolute number of $CD25^+$ $CD4^+$ $FoxP3^+$ cells present was determined by flow cytometry (mean \pm SE; $P=0.0484$, Student's t test). **B**, reduced IFN- γ production by $CD4^+$ and $CD8^+$ T cells in the presence of human Treg. IFN- γ production by $CD4^+$ (right) and $CD8^+$ (left) T cells isolated from mice that had been transplanted with a human islet allograft and reconstituted with either 40×10^6 human PBMC alone ($CD4^+$ $n=11$ and $CD8^+$ $n=9$) or together with 40×10^6 ex vivo expanded Treg cells ($CD4^+$ $n=9$ and $CD8^+$ $n=7$) was analyzed by flow cytometry. Mice reconstituted with PBMC alone were analyzed at the time of rejection; mice reconstituted with PBMC and Treg cells were analyzed at the end of the observation period (mean \pm SE; $CD4^+$ $P=0.0003$ and $CD8^+$ $P=0.0354$, Student's t test).

of both $CD4^+$ and $CD8^+$ T cells, thus preventing the generation of potential effector T cells that could elicit graft rejection.

In an elegant recent article, Yi et al. have demonstrated that expanded human Treg can prevent rejection of porcine islet xenograft (18). We present here a more clinically relevant model in which we test the functional survival of human allogeneic islets in humanized mice. By rendering mice diabetic before islet transplantation, we have been able to measure the functional outcome of Treg cotransfer on rejection mediated by allogeneic leukocytes by measuring blood glucose levels. In our model, a relatively high number of PBMC (40×10^6) was used to reconstitute mice to ensure robust islet allograft rejection. Therefore, high number of Treg was required to control PBMC. Such cell numbers may appear to be high when attempted to directly scale-up to human; however, it needs pointing out that, in aorta (16, 19) and skin (17) transplantation models, where a lower number of PBMC is being used (10×10^6 and 5×10^6 , respectively), accordingly lower number of Treg is required

to regulate graft rejection. Importantly, combining Treg cellular therapy with Treg-promoting immunosuppression allows a further decrease in the Treg numbers allowed (19). Therefore, we can speculate that the best time to use Treg in patients receiving islet transplants would be within the first 2 months after transplantation after alemtuzumab (Campath-1H) or antithymocyte globulin depletion of lymphocytes, when T cells levels are low. Taking into account a recent study which demonstrated the clinical advantage of intensive lymphocyte-depleting induction therapy in promoting long-term insulin independence after islet transplantation (20), delayed Treg infusion would benefit patients, both by tempering the impact of surgery-induced inflammation on regulatory cells themselves and by reducing the number of autoreactive and alloreactive T cells Treg need to regulate. Our model, where human PBMC are adoptively transferred into immunodeficient mice, creates a useful model to study immunologic responses in a lymphopenic host undergoing immunoreconstitution. However, it needs to

be stressed that whereas the current model provides reliable reconstitution of human T lymphocytes, some components of the human immune response, especially innate immune cells, are either missing or significantly under-represented.

Current experimental data and the findings reported here demonstrate that Treg infused *in vivo* can migrate to the relevant microenvironments and are not systemically distributed (21, 22). Moreover, functionality of Treg *in vivo* is dependent on their ability to effectively home to the relevant immunologic compartment (23–25). Importantly, a recent study demonstrated that human Treg can infiltrate porcine islet xenograft (18). These data corroborate our current findings of accumulation of CD25⁺CD4⁺ FoxP3⁺ T cells in the lymph nodes that drain the site of the islet allografts, together with the reduced priming of potentially alloreactive T cells when Treg are present. These findings were further strengthened by the demonstration that Treg can inhibit the differentiation of effector/effector memory T cells possibly by defective cytokine production and STAT signaling. In our model, ability of CD4⁺ T cells to produce IFN- γ was significantly inhibited by Treg administration and *in vitro* studies demonstrated reduced IFN- γ signaling in responding cells cultured in the presence of Treg. Interestingly, we demonstrated here increased STAT1 signaling in Treg compared with suppressed responders. These data corroborate recent studies demonstrating the ability of Treg to differentiate into specialized populations, expressing transcriptional profiles similar to their effector cell counterparts (26–28). Treg expression of T-bet is important for limiting Th1 responses (26), and IFN- γ and IL-27 have been shown to promote the development of Th1-controlling Treg population (29, 30). Future studies will determine if Th1-like Treg cells are generated *in vivo* in this model and if administration of enriched Th1-like Treg cells could provide better control of islet allograft rejection.

In summary, this study establishes the potential of *ex vivo* expanded human Treg to modulate the rejection response against an allogeneic human pancreatic islet graft *in vivo* and provides insight into their mechanism of action both *in vitro* and *in vivo*. These data, in combination with previous work in a variety of models of both acute and chronic rejection (16, 17), provide additional support for the use of *ex vivo* expanded human Treg as an adjunctive therapy to current immunosuppression in clinical transplantation and for the treatment of autoimmune disorders. They offer an approach to immunomodulation, which may enable immunosuppressive drugs to be tailored to the needs of the individual, thus reducing the deleterious impact of lifelong, nonspecific immunosuppression.

MATERIALS AND METHODS

Animals

BALB/c.*rag2*^{-/-}.*cy*^{-/-} double knockout mice (Charles River Laboratories, Wilmington, MA) were housed under specific pathogen-free conditions and used between the ages of 6 and 12 weeks. Principles of laboratory animal care were followed and all experiments were performed according to institutional regulations and the UK Animals (Scientific Procedures) Act 1986.

Sorting and Expansion of Human Treg Cells

Isolation, expansion, and testing of suppressive activity of Treg was performed as previously (16).

Coculture Experiments

HLA-A2⁺ responder PBMC (10⁵) were incubated with or without 10⁵ mismatched HLA-A2⁺ *ex vivo* expanded human Treg cells in the presence of 10⁴ anti-CD3/anti-CD28 beads. On days 3, 5, and 7, cells were collected and stained with monoclonal antibodies against CD8, CD45RA, CD62L (all BD, East Rutherford, NJ), CD4 (Beckman Coulter, Brea, CA), CD3 (eBioscience, San Diego, CA), HLA-A2 (Serotec, Raleigh, NC), and 7-aminoactinomycin D (BD). All culture conditions were performed in triplicates. Data were collected on FACS Aria and analyzed using Diva software.

Phosflow Staining

For the *in vitro* experiment, PBMC were stained with 1 μ M VPD450 (BD), stimulated with anti-CD3/anti-CD28 beads (Invitrogen) at 1:5 bead/cells ratio, and cultured for 2 days with or without Treg at 1:1 Treg/PBMC ratio. For Phosflow staining, BD Protocol III with minor modifications was used with anti-STAT1 (pY701) Alexa Fluor 488, anti-STAT5 (pY694) PE-Cy7, and anti-STAT3 (pY705) Alexa Fluor 647 antibodies (all BD). For the *in vivo* experiment, PBMC were stained with 10 μ M CFSE, whereas expanded Treg (CD25^{hi}CD4⁺) or expanded Teff (CD25^{lo}CD4⁺) were labeled with 1 μ M VPD450. PBMC were injected intraperitoneally alone or at 1:1 ratio with either Treg or Teff. Cells were recovered from peritoneum on day 3 and stained following BD Protocol III using anti-STAT1 (pY701) PerCP-Cy5.5, anti-STAT5 (pY694) PE-Cy7, and anti-STAT3 (pY705) Alexa Fluor 647 antibodies (all BD).

Transplantation of Human Islets of Langerhans

Human pancreases were retrieved from deceased donors with informed consent and appropriate ethical approval, and islets of Langerhans were isolated at the Oxford Diabetes Research and Wellness Foundation Human Islet Isolation Facility using routine methods. All islets were maintained in CMRL 1066 medium supplemented with 10% human albumin, 1% L-glutamine, 1% penicillin/streptomycin, 1 mM nicotinamide, and 1% ITS supplement at 37°C in an atmosphere of 95% air 5% carbon dioxide for 48 hr before transplantation. Islet viability, assessed by fluorescein diacetate staining, was 60% to 70% and islet cell purity, assessed by dithizone staining, was 70% to 90%. The islet number was converted into IEQ, being the total number of islets with an average diameter of 150 μ m. Then, 8000 human IEQ were transplanted into the renal subcapsular space of BALB/c.*rag2*^{-/-}.*cy*^{-/-} double-knockout mice, which had been previously rendered diabetic (blood glucose >14.5 mM) by a single intravenous injection of streptozocin (250 mg/kg). Nonfasting blood glucose levels were monitored regularly and animals demonstrating normoglycemia (blood glucose <14.5 mM) for 2 weeks were selected for subsequent human PBMC transfer.

Adoptive Transfer of Human Leukocytes

In all experiments, human PBMC were obtained from random donors and the data pooled. Then, 40 \times 10⁶ human PBMC were injected intraperitoneally into recipient mice. Alternatively, human PBMC were adoptively transferred with an equal number of *ex vivo* expanded human T cells. After cell transfer, blood glucose and body weight were monitored on a regular basis. Only mice with more than 1% engraftment of human CD45⁺ cells in the spleen were included in the study (16).

Nephrectomy

Selected mice underwent unilateral left nephrectomy to evaluate a return to hyperglycemic states after removal of a functioning human islet graft. Anesthetized mice were placed in a lateral decubitus position and a 2 cm incision was made over the left flank. Lateral wall muscles were dissected and the kidney was extracorporealized. Hilar vessels, along with the ureter, were ligated with 7.0 nylon suture and muscle/skin layers were closed with 4.0 vicryl running suture. All animals were monitored closely postoperatively.

Immunohistochemistry

Upon islet graft rejection, or at the endpoint of the experiment, islet grafts were removed, frozen in OCT embedding medium, and sectioned at a thickness of 8 to 10 μ m. After drying and fixing in 100% acetone for 15 min, all sections were blocked with 10% bovine serum albumin for 30 min. Primary antibodies mouse anti-human CD4 and CD8 (BD) and guinea pig anti-swine insulin (DAKO, Carpinteria, CA) were then applied. After several washes,

either horseradish peroxidase or fluorescein isothiocyanate-conjugated secondary antibody (DAKO) was applied, and sections were visualized with diaminobenzidine (Sigma, St. Louis, MO) or under fluorescence microscopy.

Flow Cytometry of Lymphoid Tissue

Single-cell suspensions from spleen and lymph nodes were stained with antibodies against CD3 and FoxP3 (eBioscience), CD4 (Caltag), and CD45, CD8, CD16, and CD19 (BD). For cytokine analysis, cells were restimulated for 5 hr with phorbol 12-myristate 13-acetate (50 ng/mL; Sigma), ionomycin (1 μ g/mL; Sigma), and monensin (GolgiStop, 1 μ L/mL; BD) before being stained for intracellular IFN- γ (BD).

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism software using the unpaired Student's *t* test for grouped data, log-rank test for survival data, and linear regression module for correlation analysis.

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