Published in final edited form as:

Transplantation. 2010 December 27; 90(12): 1321–1327. doi:10.1097/TP.0b013e3181ff8772.

Ex vivo-expanded human regulatory T cells prevent the rejection of skin allografts in a humanised mouse model¹

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Abstract

Background—Composite tissue transplantation effectively reconstructs the most complex defects, but its use is limited due to harmful immunosuppression and the high susceptibility of skin to rejection. Development of tolerance is an ideal solution, and protocols using regulatory T cells (Tregs) to achieve this have been promising in experimental animal models. The aim of this study was to investigate the ability of human Tregs to regulate immune responses to a human skin allograft *in vivo*.

Methods—We isolated and expanded naturally-occurring CD127^{lo}CD4⁺CD25⁺ human Tregs from peripheral blood mononuclear cells (PBMCs) *ex vivo* and examined their phenotype and suppressive activity *in vitro*. Using a clinically relevant chimeric humanised mouse system, we transplanted mice with human skin grafts followed by allogeneic populations of PBMCs with or without Tregs derived from the same PBMC donor.

Results—*Ex vivo*-expanded Tregs maintain the appropriate Treg markers and retain suppressive activity against allostimulated and polyclonally stimulated autologous PBMCs *in vitro*. Mice receiving allogeneic PBMCs alone consistently reject human skin grafts, whereas those also receiving Tregs display stable long-term human skin transplant survival along with a reduction in the CD8⁺ human cellular graft infiltrate.

Conclusions—We show for the first time the unique ability of human Tregs to prevent the rejection of a skin allograft *in vivo*, highlighting the therapeutic potential of these cells clinically.

Keywords

Regulatory T cell (Treg); tolerance; humanised mouse; rejection; skin transplantation

¹Author Contributions: F.I., J.W. and K.J.W. designed experiments. F.I., J.W. and R.G. performed the experiments and analysed the data. S.N.N. and T.E.G. provided expertise, T.G. provided human tissue, F.I., J.W. and K.J.W. wrote the manuscript.

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Introduction

Transplantation of composite tissue allografts (CTAs) for the reconstruction of complex tissue defects is a major advance in reconstructive surgery (1). Results are cosmetically and functionally superior to those obtained by traditional plastic surgical techniques, and in many cases reconstructive transplantation (RT) is the only option for functional reconstruction, such as for whole limb amputations (2).

RT is limited by the same constraints as solid organ transplantation (SOT): the need for chronic global immunosuppressive therapy in the face of acute and chronic rejection. For this reason there is scepticism within the field of plastic surgery as to the utility of RT for anything other than the most complex reconstructive cases. Despite immunosuppressive therapy, acute rejection is common: 85% of patients experience at least one episode in the first year, with multiple episodes in 56% of patients (2). The most problematic component of the CTA is skin, which is always the first, if not the only tissue, to reject (3). This is highlighted by the observation that a higher level of immunosuppressive drug therapy is required for CTAs that contain skin compared to those that do not (4). Importantly, rejection of even one component of a CTA may result in dysfunction of the entire allograft. Furthermore, data from SOT have emphasised that the development of acute rejection is a significant predictor of chronic dysfunction, which is a principal cause of graft loss (5). Tolerance to skin allografts is notoriously difficult to achieve experimentally compared to other tissues (6, 7) and various immunomodulatory protocols have only been able to achieve tolerance to the non-skin components of a CTA (8-10).

Over recent years there has been an increased interest in Treg cellular therapy as a method to regulate specific allogeneic responses *in vivo* (11). We have previously demonstrated the ability of *ex vivo*-expanded CD25⁺CD4⁺ naturally-occurring human regulatory T cells (Tregs) to prevent the development of transplant arteriosclerosis (TA) in a chimeric humanised mouse system (12). In this model, allogeneic peripheral blood mononuclear cells (PBMCs) elicit neointimal expansion within transplanted human arterial segments mimicking TA of chronic allograft dysfunction. With the addition of Tregs selected on the basis of a low expression of CD127 (the α -chain of the IL-7 receptor, CD127^{lo}) there is a marked attenuation of TA. We have also demonstrated the ability of Tregs to promote long-term survival of allogeneic skin grafts in murine models (13-15), but whether this is possible in human skin is unknown. We thus wished to study whether *ex vivo*-expanded human Tregs were able to control the acute rejection of a human skin allograft. We hypothesised that PBMCs allogeneic to a human skin graft would induce its rejection in a humanised mouse system and that rejection could be prevented by *ex vivo*-expanded CD127^{lo}CD25⁺CD4⁺ human Tregs.

Results

Human PBMCs induce the rejection of human skin allografts *in vivo*

We first hypothesised that allogeneic PBMCs would induce the rejection of a human skin graft. We therefore transplanted BALB/c Rag2^{-/-};Il2r γ ^{-/-} mice, which are deficient in T, B and NK cells (16), with a fresh 1×1 cm human skin graft procured from patients undergoing plastic surgery procedures. 35 days later mice received an intraperitoneal injection of 5×10⁶ allogeneic PBMCs to reconstitute their immune systems, or no cells (Fig. 1a). Skin and PBMC donors were tissue-typed to ensure a human leukocyte antigen (HLA) mismatch (median mismatches=5 across HLA-A, B and DR β 1). Grafts were monitored until rejection, defined as complete macroscopic loss. In mice reconstituted with allogeneic PBMCs (defined as >1% splenic human leukocyte chimerism (12, 17)), skin allografts underwent intense inflammation and were lost with a median survival time (MST) of 40 days (Figs. 1b

and c). Mice not receiving an adoptive transfer of human PBMCs maintained normal human skin grafts for over 100 days ($p=0.0027$). We measured the proportion of human CD45⁺ cells in the peripheral blood of mice receiving a human skin graft and allogeneic PBMCs at weekly intervals for 6 weeks by flow cytometry (Fig. 1d). The percentage of human chimerism was initially low but increased with time. At 6 weeks, the point at which all grafts are rejected, human leukocyte chimerism in the peripheral blood averaged 11.6% (SEM 5.7). At the point of rejection, human chimerism was detectable in the spleen, peripheral blood, and skin graft-draining and contralateral lymph nodes of reconstituted mice (Fig 1e). Histologically, skin grafts procured from mice 21 days after injection of allogeneic PBMCs displayed features consistent with human skin rejection: a diffuse leukocytic infiltration of the epidermis and dermis with human CD45⁺, CD4⁺ and CD8⁺ cells, focal dermal-epidermal separation and areas of necrosis of the epidermis (Fig 1f). In healed-in skin grafts procured at the same time point from mice not receiving human cells, skin architecture was grossly maintained with an intact epidermis and dermis containing a population of human CD45⁺ 'passenger' leucocytes (Fig 1g). Skin grafts in these mice closely resembled the human donor skin with only mild contraction (Fig 1c). In a recently reported humanised mouse model of skin transplantation, a significant GR-1⁺ mouse cellular infiltrate was observed in skin grafts transplanted onto non-obese diabetic (NOD)-*scid-IL2r^{null}* (NSG) mice that did not receive human cells (18). In the study reported here, we did not observe this phenomenon, which is likely recipient mouse strain-specific (data not shown). Compared with skin grafts from mice receiving allogeneic PBMCs, the intragraft human CD45⁺ population in skin grafts from mice not receiving cells was considerably smaller ($p<0.0001$, Fig 1h). In order to control for the effects of homeostatic proliferation, we transplanted mice with human skin grafts as above and 35 days later injected them with either 5×10^6 allogeneic or 5×10^6 syngeneic PBMCs (derived from the peripheral blood of the skin graft donor). In mice receiving allogeneic PBMCs, skin grafts were lost as expected with a MST of 34 days, whereas long-term survival (>100 days MST) was observed in mice receiving PBMCs syngeneic to the skin graft, indicating that the immune response directed towards the human skin transplant is allospecific in nature ($p=0.0084$, Fig. 1i). Furthermore, in mice receiving allogeneic PBMCs, a larger number of human CD45⁺ cells was present in the skin graft-draining lymph node compared to the contralateral lymph node at day 21 post-adoptive transfer ($p=0.0418$, Fig 1j). This difference was not apparent in mice receiving syngeneic PBMCs, indicating a preferential aggregation of alloantigen-responding human cells at the site of the alloantigen source (Fig 1j).

Ex vivo-expanded human regulatory T cells maintain suppressive activity *in vitro*

Having developed a human skin rejection system, we then aimed to investigate the potential of *ex vivo*-expanded human Tregs to suppress alloimmune responses both *in vitro* and *in vivo*. We hypothesised that *ex vivo* expanded Tregs would maintain suppressive activity *in vitro*. We therefore FACS-sorted human Tregs from healthy donor PBMCs into CD127^{lo}CD25⁺CD4⁺ cells to a purity of greater than 94% and expanded the cells more than 600-fold *in vitro* with recombinant human IL-2 and α CD3/ α CD28-coated microbeads (Fig 2a). After expansion, these cells retained the expression of Treg markers such as CD25, forkhead box P3 (FoxP3), and CD27 while maintaining a low expression of CD127 (Fig. 2b). The majority of cells expressed CD62L, CCR7 or both (Fig. 2b). The presence of the highly conserved homing receptor CD62L has been demonstrated to be important for the function of Tregs, presumably by allowing homing to lymphoid tissues for induction of peripheral suppression (19). CCR7 is another crucial lymphoid homing receptor for T cells. *In vitro*, expanded human Tregs displayed potent suppressive capability towards polyclonally-stimulated autologous PBMCs as well as to alloantigen-stimulated autologous PBMCs (Fig. 2c). Proliferating polyclonally-stimulated PBMCs were not exhausted in the culture period of the assay as indicated by carboxy-fluorescein diacetate, succinimidyl ester

(CFSE) staining of PBMCs over the same time frame (Supp Fig. 1). Furthermore, the suppression of polyclonally-stimulated PBMCs by Tregs pre-expansion was similar to the levels of suppression attained after *in vitro* expansion, indicating a lack of a significant impact of the expansion process on Treg suppressive potency (Fig. 2d).

Ex vivo*-expanded human regulatory T cells prevent rejection of human skin allografts *in vivo

We then proceeded to test the functional activity of CD127^{lo}CD25⁺CD4⁺ expanded Tregs *in vivo*. BALB/c Rag2^{-/-};Il2r γ ^{-/-} mice were transplanted with a human skin graft and 35 days later injected with human cells as in Fig 1a. Mice received either 5×10⁶ allogeneic PBMCs alone or 5×10⁶ allogeneic PBMCs with *ex vivo* expanded Tregs from the same blood donor at a 1:1 ratio. Mice receiving only PBMCs rejected allografts with a MST of 35 days, whereas those also receiving Tregs at a 1:1 ratio displayed long-term engraftment of the human skin graft to over 100 days ($p=0.0065$, Fig. 3a). Skin grafts in Treg-treated mice maintained a normal appearance with minimal contraction (Fig 3b). The microscopic architecture of these skin grafts at day 100 post-adoptive transfer was also largely maintained with an intact epidermis and dermis, although infiltration with human CD45⁺ cells was still present (Fig 3c). Further histological assessment revealed a reduction in the number of infiltrating CD8⁺ but not CD45⁺ or CD4⁺ human cells in skin allografts of mice receiving Tregs compared with those receiving PBMCs alone (Fig 3d). In rejecting allografts, infiltration by human CD4⁺ and CD8⁺ cells was almost equivalent (histological cell count: 195.6 ± 30.80 for CD4⁺ versus 242.8 ± 32.63 for CD8⁺ cells), an observation which is consistent with clinical data (20).

Discussion

We demonstrate for the first time the distinctive ability of *ex vivo*-expanded human Tregs to prevent the rejection of human skin allografts *in vivo*. This is especially important given the challenges of regulating alloimmune responses to skin. Taken together with our previously published data, we now demonstrate the ability of *ex vivo* expanded CD127^{lo}CD25⁺CD4⁺ Tregs to control immune responses in multiple types of tissue in models of both acute and chronic rejection (12). The low expression of CD127 and high expression of CD27 on Tregs in this study is indicative of a highly suppressive subpopulation that primarily act in the allograft and draining lymph node (12, 21), whilst the expression of CD62L and CCR7 are important for Treg homing to peripheral lymphoid tissues. We have previously shown that Tregs can prevent rejection of skin grafts in a mouse model by attenuating the priming of donor-reactive naïve CD8⁺ T cells in the peripheral lymphoid tissues to prevent their infiltration into the allograft (22). In line with this, we demonstrate in the current study a marked reduction in human CD8⁺ T cell graft infiltration in long-term surviving transplants. We have found the prolongation of survival achieved with Treg therapy to be dose-dependent, as the number of cells may be titrated down with measurable effects on graft survival (data not shown).

Regulation of skin rejection clinically with the use of *ex vivo*-expanded Tregs as an adoptive cellular therapy is a promising prospect. Whilst the data we have presented here are highly encouraging in this regard, they must be interpreted with care. The injection of Tregs did lead to a reduction in the graft cellular infiltrate of CD8⁺ cells, but there remained a substantial number of total human infiltrating cells. We therefore predict the mechanisms of suppression to be complex and active at multiple levels. The effect of Tregs on the level and rate of engraftment of human cells in the mouse host is not fully clear, although previous data from our laboratory have shown that there is no impact of Tregs on immune reconstitution in the absence of an allograft (12).

Over the past decade Tregs have attracted interest as a potential adjunctive cellular therapy and we are closer than ever to the first clinical trials in SOT (11, 23). In this regard, chimeric humanised mouse models such as the one used in the current study are proving to be invaluable in bridging the gap between experimental work and clinical studies (18, 24, 25). Our findings indicate that regulation of human skin rejection in this model is possible with the use of adoptive Treg cellular therapy. Further work will need to focus on identifying more potent Treg populations that may be used effectively in lower numbers, as well as investigating whether Tregs may be used as 'rescue' therapy, and most importantly the safety and stability of these cells *in vivo*.

Methods

Mice

BALB/c Rag2^{-/-};IL2r γ ^{-/-} (H2d) mice were housed under specific pathogen-free conditions in the Biomedical Services Unit of the John Radcliffe Hospital (Oxford, UK). All experiments were performed using protocols approved by the Committee on Animal Care and Ethical Review at the University of Oxford and in accordance with the UK Animals (Scientific Procedures) Act 1986. Mice between the ages of 6 and 12 weeks at the time of the first experimental procedure were used. Over 90% of mice reconstituted with human PBMCs displayed >1% splenic engraftment. The late development of human anti-mouse responses manifesting as graft-versus-host disease (GVHD) has long been recognised in human PBMC engrafted immunodeficient mice (26). We have observed this phenomenon in PBMC engrafted Balb/c Rag2^{-/-};IL2R γ ^{-/-} mice, however, without exception this occurs at a point after skin graft rejection. Mice treated with Treg therapy in our study displayed a lower incidence of GVHD, consistent with many experimental and clinical studies (27, 28). Any mice developing signs of GVHD were euthanized according to local animal welfare regulations.

Procurement of human skin

Split-thickness human skin at between 8-10/1000 inches thick was obtained with the use of an air-driven dermatome from live donors undergoing plastic surgery procedures. Tissue was stored in Ringer's solution on ice and used within 12 hours. Skin was obtained with full patient consent and ethical approval from the Oxfordshire Research Ethics Committee, study number 07/H0605/130. Blood samples were obtained with consent from skin donors for HLA-typing and autologous leukocyte isolation.

Skin grafting

Skin grafting procedures were performed with full sterile precautions. Mice were placed prone and a 1×1cm piece of skin removed from the left dorsal thorax over the costal margin. A 1×1cm piece of human skin was then fashioned and its edges sutured to the mouse recipient skin with a non-absorbable 8-0 Prolene suture (Ethicon, UK). Grafts were fenestrated and covered with a povidone-iodine mesh and pressure dressing, secured with circumferential tape. Bandages were left in place for seven days then removed under general anaesthetic. Skin grafts were monitored every 1-2 days until complete loss.

Procurement of human PBMC

Fresh buffy coats were obtained from the National Blood Bank (Bristol, UK) and PBMC isolated using standard Ficoll separation techniques. PBMCs were obtained from random donors and the data pooled. For autologous PBMC isolation, Ficoll separation was performed on blood obtained from the respective skin donor.

Sorting and expansion of human CD127^{lo} regulatory T cells

Isolation and expansion of T cells was performed as previously described (12). PBMC were isolated from buffy coats obtained from healthy blood donors (National Blood Service), followed by CD4⁺ cell enrichment with a CD4⁺ T cell isolation kit II (Miltenyi Biotec). CD127^{lo}CD25⁺CD4⁺ cells were sorted using a BD FACS Aria cell sorter, using CD127 PE, CD25 APC and CD4 PerCP antibodies. Cells were sorted to over 94% purity and subsequently expanded *in vitro* with 1000U/ml of recombinant human IL-2 (Chiron) and α CD3/ α CD28 beads (Invitrogen) in a 1:2 cell to bead ratio over two 7 day rounds of expansion, followed by 2 days of silencing in a reduced amount of IL-2 (200U/ml) and α CD3/ α CD28 bead removal. Cells were cultured in RPMI 1640 medium supplemented with L-glutamine, penicillin-streptomycin (all PAA Laboratories), sodium pyruvate (Gibco) and 10% human AB pooled serum (National Blood Service). After expansion *in vitro* suppressive capacity and expression of Treg markers were assessed. Expanded cells were cryopreserved for future use.

Adoptive transfer of human cells

Adoptive transfer was performed as previously described (12).

In vitro suppression tests

Treg *in vitro* suppressive activity was assessed by measuring inhibition of proliferation of autologous PBMC stimulated with alloantigen or polyclonally stimulated with α CD3/ α CD28 beads as previously described (12). Briefly, PBMCs (1×10^5) were incubated with irradiated allogeneic PBMCs (1×10^5) and serial dilutions of Tregs. For the bead assay, PBMCs (2×10^4) were incubated with α CD3/ α CD28 beads (2×10^3) and varying numbers of *in vitro* expanded Tregs. In both assays, proliferation was measured after 7 days by addition of ³H-thymidine (Perkin Elmer) for the last 16 hours of culture. For assays using CFSE labelled cells, PBMC were labelled using a Cell Trace CFSE Proliferation Kit (Invitrogen, UK) and labelling confirmed with flow cytometry.

Flow cytometry

Fluorochrome-coupled antibodies specific for 7-AAD (eBioscience), CD45 (BD), CD3 (eBioscience), CD4 (Beckman Coulter), CD8 (BD), CD19 (BD), CD25 (BD), CD127 (BD), Foxp3 (eBioscience) CD27 (BD), CCR7 (BD), and CD62L (Invitrogen) were used to phenotypically profile cells. Flow cytometric data were acquired using a FACS Aria (BD Biosciences, UK) and analysed using FACSDiva software (BD Biosciences, UK). A mouse was considered reconstituted if of the monocyte population, >0.1% of cells within the peripheral blood or >1% of cells within the spleen were viable human CD45⁺ cells (17). A similar analysis was performed on PBMCs isolated from buffy coats and expanded Tregs prior to injection into mice.

Tissue Typing

Blood obtained from skin donors and buffy coats was analysed at the Oxford Transplant Centre Histocompatibility and Genetics Laboratory. A full tissue typing was performed for HLA-A, -B, -Cw, -DR and -DQ.

Immunohistochemistry

Histology and immunohistochemistry was performed as previously described (12). Briefly, snap-frozen specimens were sectioned at 8 μ m and allowed to air-dry before immunohistochemical staining using biotinylated human antigen-specific antibodies (eBioscience) and an avidin-biotinylated enzyme Complex (VECTASTAIN ABC Elite,

Vector Laboratories, USA) followed by haematoxylin counterstaining. For infiltrating cell quantification, positive cells were counted at 40× magnification in three random fields of each of four separate sections for each antibody type.

Statistical Analyses

Statistical evaluations were performed with Graphpad Prism software. Student's *t* tests were applied on grouped data. Survival data was analysed using logrank tests. *p* values under 0.05 were taken as significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank the staff of the plastic surgery theatres and especially Mr. A. Pay and Mr. O. Cassell for their assistance with the procurement of human skin, the staff of the Biomedical Services Unit at the John Radcliffe Hospital for their expert animal care, the Oxford Transplant Centre for molecular human leukocyte antigen typing, A. Bushell, N. Jones and S. Heidt for valuable advice. This work was supported by grants from The Wellcome Trust, the European Union Integrated Project, RISE, Medical Research Council UK, the Royal College of Surgeons of England, the Dunhill Medical Trust and the Oxford Health Services Research Committee Charitable Trust.

Abbreviations

CFSE	Carboxy-fluorescein diacetate, succinimidyl ester
CTA	Composite tissue allograft
FACS	Fluorescence-activated cell sorting
HLA	Human leukocyte antigen
MST	Median survival time
OCT	Optimal cutting temperature
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
RT	Reconstructive transplantation
SD	Standard deviation
SEM	Standard error of the mean
SOT	Solid organ transplantation
TA	Transplant arteriosclerosis
Treg	Regulatory T cell

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(g)

(h)

Figure 1. Skin rejection is mediated by allogeneic human PBMCs *in vivo*

(a) Schematic representation of the chimeric humanised mouse model. BALB/c Rag2^{-/-};Il2rγ^{-/-} mice receive a 1×1cm human skin transplant which is allowed to heal for 35 days before adoptive transfer of 5×10⁶ PBMCs intraperitoneally (i.p.). Skin grafts are monitored until the point of rejection, defined as complete loss. (b) Mice received a skin graft and 5×10⁶ allogeneic PBMCs (n=7 mice) or no cells (n=4 mice). Data are pooled from two separate PBMC/skin donor combinations. (c) Representative photographs of skin grafts at day 100 (no cells) or rejection (PBMCs). (d) The proportion of CD45⁺ human leukocytes of the total monocyte population (% huCD45⁺) was measured in the peripheral blood of mice by flow cytometry weekly for 6 weeks post-adoptive transfer of PBMCs (n=9 mice).

across three independent experiments, data are represented as mean $\pm$ SEM). (e) At the point of rejection, the proportion of human CD45⁺ cells of the total monocyte population (%huCD45⁺) was measured in the spleen, peripheral blood, and draining and contralateral lymph nodes of mice receiving an adoptive transfer of human PBMCs (n=4 mice, data are represented as mean $\pm$ SEM). (f) Representative photomicrographs (40 $\times$ magnification) of sections from human skin grafts harvested from mice 21 days after adoptive transfer of human PBMCs and stained for human CD45, CD4 and CD8. (g) Representative photomicrograph of a section from a human skin graft allowed to heal for 56 days and subsequently harvested from a mouse not receiving human cells, stained for human CD45. (h) The number of graft-infiltrating CD45⁺ human leukocytes (huCD45⁺ cells) was quantified by immunohistochemistry in mice receiving human PBMCs (n=3 mice) or no cells (n=3 mice). (i) Mice received a skin graft and 5×10^6 allogeneic PBMCs (n=4 mice) or 5×10^6 PBMCs syngeneic to the skin graft (n=3 mice). One mouse in the syngeneic PBMC group required euthanasia at day 76 post-cellular adoptive transfer due to the onset of GVHD (*see methods section*). Data are from one skin donor/PBMC combination. (j) Analysis of the number of human CD45⁺ cells of the total monocyte population (huCD45⁺) was analysed by FACS in the skin graft-draining axillary and contralateral axillary lymph nodes in mice 21 days after receiving 5×10^6 syngeneic (n=3 mice) or allogeneic (n=4 mice) PBMCs.

Figure 2. Human Tregs retain suppressive capacity after *ex vivo* expansion

(a) Schematic representation of the Treg sorting and expansion protocol.

CD127^{lo}CD25⁺CD4⁺ cells were FACS-sorted and expanded *in vitro* with recombinant human IL-2 and α CD3/ α CD28-microbeads. (b) Representative flow cytometry plots of the phenotypic analysis of *ex vivo*-expanded human Tregs. Numbers on dot plots are representative percentages in each quadrant. (c) *In vitro* suppression assay of expanded human Tregs. PBMCs were stimulated with α CD3/ α CD28 beads (left panel) or irradiated allogeneic PBMCs (right panel) and cultured in the presence of expanded Tregs for 7 days. ³H-thymidine was added for the last 16 hours of culture (n=4-6 independent assays). (d) *In vitro* ³H-thymidine incorporation assay of PBMCs polyclonally-stimulated with α CD3/ α CD28 beads by autologous unexpanded or expanded human Tregs.

(b)

PBMC + Treg

(c)

Figure 3. Human Tregs promote the long-term survival of allogeneic human skin grafts
 (a) Mice received an adoptive transfer of 5×10^6 PBMCs with (n=4) or without (n=5) *ex vivo*-expanded Tregs at a 1:1 ratio. Data shown are pooled from two separate PBMC/skin donor combinations. (b) Representative photograph of a skin graft from a mouse receiving PBMCs with Tregs at day 100 post-cellular adoptive transfer. (c) Representative photomicrograph of a section from a skin graft procured at day 100 post-adoptive transfer from a mouse receiving PBMCs with Tregs, stained for human CD45. (d) The number of graft-infiltrating human leukocytes was quantified by immunohistochemistry for CD45⁺, CD4⁺ and CD8⁺ cells in samples from mice receiving PBMCs (procured at the point of rejection, n=5) or PBMCs with Tregs (procured at 100 days post-adoptive transfer, n=4). Data are represented as mean $\pm$ SD.