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Human HLA-DR+CD27+ regulatory T cells show enhanced antigen-specific suppressive function

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Abstract

Regulatory T cells (Treg) have potential for the treatment of autoimmune diseases and graft rejection. Antigen-specificity and functional stability are considered to be critical for their therapeutic efficacy. In this study, expansion of human Treg in the presence of porcine PBMC (Xn-Treg) allowed the selection of a distinct Treg subset, co-expressing the activation/memory surface markers HLA-DR and CD27 with enhanced proportion of FOXP3+Helios+ Tregs. Compared to their unsorted and HLA-DA+CD27+ double positive (DP) cell depleted Xn-Treg counterparts, HLA-DR+CD27+ DP-Enriched Xn-Treg expressed upregulated Treg function markers CD95 and ICOS with enhanced suppression of xenogeneic but not polyclonal MLR. They also had less methylated Treg-specific demethylated region (TSDR) of FOXP3 and were more resistant to conversion to effector cells under inflammatory conditions. Adoptive transfer of porcine islet recipient NOD-SCID IL2 receptor γ-/- (NSG) mice with HLA-DR+CD27+ DP-Enriched Xn-Treg in a humanized mouse model inhibited porcine islet graft rejection mediated by 25-fold more human effector cells. The prolonged graft survival was associated with enhanced accumulation of FOXP3+ Treg and upregulated expression of Treg functional genes, IL10 and CTLA4, but downregulated expression of effector Th1, Th2 and Th17 cytokine genes, within surviving grafts. Collectively, human HLA-DR+CD27+ DP-Enriched Xn-Treg expressed a specific regulatory signature that enabled identification and isolation of antigen-specific and functionally stable Treg with potential as a Treg-based therapy.
Introduction

Regulatory T cells (Treg) are an essential component of immune homeostasis. Initially Treg were characterized as CD4+CD25+ T cells in mouse models of autoimmune disease and make up 5% to 10% of peripheral CD4+ T cells (1, 2). FOXP3 was the key transcription factor that characterized this population of thymically derived Tregs (2, 3). In humans, mutation of the FOXP3 gene leads to severe autoimmune disease demonstrating the importance of this cell subset in suppressing unwanted inflammatory responses to self-antigens (4). In addition to their role in preventing autoimmunity CD4+CD25+ T cells that express FOXP3 have been shown to be important at suppressing alloimmunity and for the induction and maintenance of allograft tolerance (5, 6). In models of transplantation, dominant tolerance is characterized by indefinite graft survival after an initial brief period of induction therapy in the presence of an otherwise intact immune system. In other words, this non-responsiveness is antigen specific. Given their potential to reduce the requirement for immunosuppression, CD4+CD25+FOXP3+ Treg have been pursued for their therapeutic potential to suppress autoimmunity and to reduce or eliminate the requirement for immunosuppression after transplantation. Trials of naïve Treg in kidney or liver transplantation have been disappointing in that the results have been modest and the relatively large number of Tregs required have provided regulatory, cost and production challenges (7-9).

Animal studies of transplant tolerance have shown that both the induction and maintenance of tolerance is dependent on the development of antigen specific Treg, as
deletion of Foxp3+ Treg leads to prompt rejection (10, 11). Also, a relatively small number of cells can transfer graft specific tolerance to a naïve host as has been shown by numerous adoptive transfer studies (12-14). The observations are equally true for autoimmunity as they are for all immunity. For example, in models of autoimmune type 1 diabetes, antigen-specific Treg, which were isolated from pancreatic lymph nodes or pulsed with islet antigen, were superior to polyclonal Treg at preventing or curing the disease (15, 16). Similarly, alloantigen-specific Treg, enriched by alloantigen-stimulated expansion in vitro or engineered to express a T cell receptor (TCR) transgene, were more effective than polyclonal Treg at preventing rejection of organ and tissue grafts. Studies in humanized mouse models have shown similar results: alloantigen-expanded human Treg were more potent suppressors of skin graft rejection than are their polyclonal counterparts (14), and human alloantigen-specific Treg generated with an HLA-A2-specific chimeric antigen receptor (A2-CAR) were superior to polyclonal Treg at preventing xenogeneic GVHD caused by HLA-A2+ T cells (17). An important advantage of enhanced Treg antigen specificity is that their suppression is targeted to the graft, hence avoiding potential opportunistic infection and malignancy that may result from non-specific suppression as a result of the application of polyclonal Treg.

One of the problems in developing in vitro expanded Treg has been identifying markers that would facilitate their selection after stimulation. Intracellular FOXP3 expression, enhanced Helios expression and demethylation of a Treg-specific demethylated region (TSDR) within the FOXP3 locus represent the gold standard for estimating the fraction of stable Treg within a population. However, it does not allow
for sorting a specific subset that could be used therapeutically. The lack of
discriminative markers also affects systematic functional optimization of *in vitro*
generated Treg, such as genetically engineered Treg with transgenic TCR or CAR
constructs. In order to identify the characteristics of an antigen activated subset, we
have used a model of Treg development to xenoantigens such as porcine peripheral
blood mononuclear cells (PBMC) or neonatal porcine islet cell clusters (NICC) (18, 19).
Due to the phylogenetic distance between pig and humans, these provide a large
antigen load and hence provide a larger pool of antigen specific Tregs available for
study. For instance, we have also reported that human Treg expanded *ex-vivo* with
xenoantigen stimulation are more potent than polyclonal Treg at suppressing
xenoreactive effector cell proliferation in a xenogeneic mixed lymphocyte reaction
(MLR), although they are equally suppressive as polyclonal Treg in an allogeneic or
polyclonal MLR xenogeneic response, indicating acquisition of xenoantigen-
specificity after xenoantigen stimulation (18).

A number of Treg activation-induced surface markers, such as HLA-DR (20),
CD27 (21), CD45RO (22), and ICOS (23), have been described to identify activated
and/or memory Treg (24). Among those, HLA-DR+ Treg has been reported to express
higher levels of Treg-associated activation markers and produced lower levels of
effector cytokines (20, 24). HLA-DR+ Tregs are present in human peripheral blood,
thymus and umbilical cord blood and are more suppressive than HLA-DR- Treg *in vitro*
(20). CD27 expression has also been associated with increased Treg suppressive
function (21, 25). It has been shown that a CD4+CD127−/lowCD25+CD45RA− Treg
subpopulation cultured in vitro with tacrolimus lost their Treg TSDR demethylation phenotype which correlated with a reduction of CD27 expression, suggesting an association of CD27 expression with Treg stability (26). Thus, given that human Treg expanded with xenoantigen have been demonstrated already to express upregulated levels of activated/effector markers, HLA-DR, ICOS and CD45RO, that were associated with their enhanced suppression (18) it is feasible that one or a combination of surface activation markers could be identified as a specific signature for the isolation of a stable, antigen-specific Treg subset that could be suitable for clinical immunotherapy.

Based on previous findings, we wished to test the hypothesis that human Treg expanded in the presence of antigen and expressing the surface markers of HLA-DR and CD27 would represent a stable antigen specific Treg population that is more potent than their unsorted counterparts at suppressing a graft immune response. To test this hypothesis we used the human T cell response to porcine PBMC in vitro and NICC in vivo as a proof of concept study, where a human HLA-DR+CD27+ double positive Treg subset from xenoantigen expanded Treg was compared in efficacy and stability to their unsorted, HLA-DR+CD27+ double positive depleted Treg, and polyclonal Treg counterparts at protecting islet xenografts from rejection mediated by human T cells.

Results

Xenoantigen expanded Treg expressed upregulated levels of Treg activation/memory markers HLA-DR and CD27. Tregs (CD4+CD25+CD127^{-}/low) were isolated from
human PBMC and stimulated either with anti-CD3/CD28 beads alone (polyclonally stimulated Treg), or with anti-CD3/CD28 dynabeads combined with irradiated porcine PBMCs (xenoantigen expanded Treg) for three rounds (7 days/round). Consistent with our previous study (18) xenoantigen expanded Treg (Xn-Treg) demonstrated similar levels of purity to their freshly isolated Treg (Fresh-Treg) and polyclonally stimulated Treg (Pc-Treg) counterparts (96% vs. 96.2% vs. 89.6% of CD4+CD25+ cells of Xn-Treg vs. Pc-Treg vs. Fresh-Treg), with high-level expression of FOXP3, cytotoxic T-lymphocyte antigen 4 (CTLA4), glucocorticoid-induced tumor necrosis factor receptor (GITR) and CD62L (Figure 1A). Moreover, in addition to their upregulated expression of the activation marker CD62L (98.0% vs. 62.0% of CD4+CD25+CD62L+ of Xn-Treg vs. Fresh-Treg), there was no change in CD127 expression between the two ex vivo expanded Treg subsets and Fresh-Treg (Figure 1A). Next, the transcription factor Helios, a marker for Treg stability (27) and co-expression of FOXP3 were assessed. A similar proportion of FOXP3+Helios+ Treg was seen in xenoantigen expanded Treg (61.3% ± 24.3%) compared to Fresh-Treg (62.5% ± 14.7%), while polyclonally stimulated Treg showed a decreased proportion of FOXP3+Helios+ Treg (41.1% ± 26.3%) (Figure 1B and Supplemental Figure 1), suggesting that Xn-Treg were a more stable Treg subset than Pc-Treg. The xenoantigen expanded Treg also expressed higher levels of the Treg activation/memory surface markers HLA-DR and CD27 than polyclonal stimulated Treg as evidenced by mean fluorescence intensity (MFI) (HLA-DR: 5848 vs. 3242.8 and CD27: 6447.8 vs. 2415.4 of Xn-Treg vs. Pc-Treg) while both types of expanded Tregs had similar levels of CD25 (MFI: 6996 vs. 8513.4 of Xn-Treg
vs. Pc-Treg) and FOXP3 expression (MFI: 2489.8 vs. 2589 of Xn-Treg vs. Pc-Treg) (Supplemental Figure 2). Therefore, xenoantigen expanded Treg showed a greater proportion of cells co-expressing HLA-DR and CD27 when compared to freshly isolated and polyclonally expanded Treg (52% ± 11.0% vs. 24.3% ± 13.8% vs. 9.6% ± 8.0% of Xn-Treg vs. Pc-Treg vs. Fresh-Treg) (Figure 1C), suggesting that a Treg subset co-expressing HLA-DR and CD27 was selectively enriched in Xn-Treg.

We further analyzed the phenotype of the HLA-DR+CD27+ double positive (DP) Treg subset of Xn-Treg (DP-Enriched Xn-Treg) by flow cytometry, demonstrating that significantly enhanced intracellular expression of Treg function molecule CD95 (Fas) (28) was detected in HLA-DR+CD27+ DP-Enriched Xn-Treg compared to either HLA-DR+CD27+ DP-depleted Xn-Treg (DP-Depleted Xn-Treg) or total Xn-Treg, as evidenced by MFI (83708.2 ± 5328.8 vs. 44843.2 ± 5879.9 vs. 56270.4 ± 6725.8 of DP-Enriched Xn-Treg vs. DP-Depleted Xn-Treg vs. total Xn-Treg) (Figure 1D). The enhanced CD95 expression was associated with enhanced HLA-DR and CD27 expression (Supplemental Figure 3A). Moreover, HLA-DR+CD27+ DP-Enriched Xn-Treg expressed a significantly higher level of the Treg activation marker ICOS than DP-depleted Xn-Treg (MFI: 6854.4 ± 498.5 vs. 4790 ± 393.6 of DP-enriched Xn-Treg vs. DP-Depleted Xn-Treg), and there was an increasing trend of ICOS expression when compared to total Xn-Treg (Figure 1D). The enhanced ICOS was also associated with HLA-DR expression (Supplemental Figure 3B). Although not significant, higher expression levels of the Treg activation marker CTLA4 (24), and the transcription factors, Helios and FOXP3, were seen in HLA-DR+CD27+ DP-Enriched Xn-Treg,
when compared to their DP-Depleted Xn-Treg or total Xn-Treg (Figure 1D). Furthermore, HLA-DR+CD27+ DP-Enriched Xn-Treg had significantly increased FOXP3 expression when compared to either HLA-DR-CD27- or HLA-DR+CD27- Xn-Treg subsets, indicating enhanced FOXP3 expression tracked with CD27 expression (Supplemental Figure 3C).

Next, we explored the expression of CD27 and HLA-DR within Xn-Treg across different stages of expansion. For the first 2 rounds of stimulation, the proportion of CD27+ Xn-Treg was decreased slightly when compared to fresh-Treg. However, by round 3 of stimulation, the proportion of CD27+ Xn-Treg was increased (compared to round 2) (Figure 2A). By contrast, the proportion of HLA-DR+ Xn-Treg increased incrementally after each round of stimulation (Figure 2A). As a consequence, co-expression of CD27 and HLA-DR increased from round 1 to round 3 of stimulation, while the other three subsets either decreased (HLA-DR-CD27+ subset) or remained unchanged (HLA-DR+CD27- and HLA-DR-CD27- subsets) (Figure 2B). Consistent with this observation, CD27+HLA-DR+ DP-Enriched Xn-Treg had a higher proportion of FOXP3+Helios+ cells than total Xn-Treg or Fresh-Treg, and this higher proportion of FOXP3+Helios+ cells was observed across different expansion stages, suggesting stability of the HLA-DR+CD27+ DP-Enriched Xn-Treg subset (Figure 2C). Collectively, these findings suggested that 3 rounds of stimulation and expansion in the presence of antigen resulted in a highly stable HLA-DR+CD27+ DP-Enriched Xn-Treg subset.

*HLA-DR+CD27+ DP-Enriched Xn-Treg subset was more suppressive and*
xenoantigen-specific. In order to test this hypothesis, we undertook cell sorting to isolate HLA-DR+CD27+ Treg from xenoantigen expanded Treg. Xn-Treg were sorted into HLA-DR+CD27+ DP-Enriched Xn-Treg and DP-Depleted Xn-Treg subsets after undergoing a series of sequential cell gating (Supplemental Figure 4). Treg suppressive capacity was then assessed by MLR. Effector cells were stimulated with irradiated xenogeneic or allogeneic PBMC, or with polyclonally anti-CD3/CD28 dynabeads for xenogeneic, allogeneic or polyclonal MLR assay, respectively. Treg were added to the assay at predetermined suppressor to effector ratios to determine their efficacy at suppressing the respective MLR. All Treg subsets tested, including Pc-Treg, unsorted Xn-Treg, DP-Depleted Xn-Treg, and HLA-DR+CD27+ DP-Enriched Xn-Treg showed similar potency in inhibition of polyclonal or allogeneic MLR in a Treg number-dependent manner (Figure 3A). However, consistent with our previous study (18), unsorted Xn-Treg showed stronger suppressive capacity in the xenostimulated MLR than Pc-Treg at lower Treg:responder ratios of 1:16 through to 1:256, and this stronger potency in suppressing the xenogeneic but not polyclonal or allogeneic response was further enhanced by replacing unsorted Xn-Treg with the sorted HLA-DR+CD27+ DP-Enriched Xn-Treg subset in the xeno MLR, showing that even at the lowest Treg:responder ratio tested (1:256) a 43.5% suppression of xenoreactive cell proliferation was still detected which was not seen with other Treg subsets (43.5% vs. 1.97% vs. 15.1% vs. 6% of suppression by HLA-DR+CD27+ DP-Enriched Xn-Treg vs. Pc-Treg vs. total Xn-Treg vs. DP-Depleted Xn-Treg) (Figure 3A). The higher suppressive potency and xenoantigen stimulation-dependent suppression by HLA-
DR+CD27+ DP-Enriched Xn-Treg was confirmed by depletion of HLA-DR+CD27+ double positive cells from Xn-Treg resulting in impaired Treg suppressive capacity and xenoantigen-specificity as assessed by the xeno MLR (Figure 3A). Using the same protocol of Xn-Treg expansion, alloantigen expanded Treg (Al-Treg) were generated from CD4+CD25+CD127-/low Treg isolated from PBMC stimulated with irradiated alloantigen PBMC for 3 rounds. HLA-DR+CD27+ DP-Enriched Al-Treg was equally suppressive of the xenostimulated MLR as HLA-DR+CD27+ DP-Depleted Al-Treg or total Al-Treg, thereby suggesting that the antigen specificity and enhanced suppression was a feature of their xeno-antigen stimulation and not solely the result of HLA-DR+ and CD27 co-expression (Figure 3B).

This finding was consistent with ICOS expression in the different expanded Treg populations. ICOS expression in HLA-DR+CD27+ DP-Enriched Xn-Treg was significantly higher than that seen in DP-Depleted Xn-Treg ($P < 0.05$) (Figure 1D), whereas there was no significant difference in ICOS expression in HLA-DR+CD27+ DP-Enriched Pc-Treg (2248 ± 772.9), HLA-DR+CD27+ DP-Depleted Pc-Treg (1686.4 ± 288.8) and total Pc-Treg (1960.8 ± 551.2) (Supplemental Figure 5). This suggests that enhanced ICOS expression in HLA-DR+CD27+ DP-Enriched Xn-Treg was feature of xenoantigen simulation.

In addition to their higher capacity to specifically suppress the proliferating xenoreactive effector cells, HLA-DR+CD27+ DP-Enriched Xn-Treg were also more capable of suppressing IFNγ secretion in the xeno MLR cultures, where the biggest reduction in IFNγ secretion was in the presence of HLA-DR+CD27+ DP-Enriched Xn-
Treg even at the lower Treg:responder ratios of 1:16 through to 1:256 (Figure 3C).

Together, these results demonstrated that the HLA-DR+CD27+ DP-Enriched Xn-Tregs subset led to increased suppression of the xenogeneic response, including xenoreactive cell proliferation and effector cytokine secretion, and this suppression was antigen-specific. Alternately, depletion of HLA-DR+CD27+ cells from Xn-Treg impaired their suppressive capacity and antigen-specificity in vitro.

**HLA-DR+CD27+ DP-Enriched Xn-Treg were functionally stable in vitro.** Since stable FOXP3 expression reflected by demethylation of TSDR within the FOXP3 gene is a prerequisite for their suppressive function, the functional stability of the HLA-DR+CD27+ DP-Enriched Xn-Treg subset was evaluated by TSDR assay. The results showed that HLA-DR+CD27+ DP-Enriched Xn-Treg retained a demethylation phenotype with no significant difference when compared to freshly isolated Treg (10.6% ± 4.9% vs. 14.4% ± 3.7% of methylation in HLA-DR+CD27+ DP-Enriched Xn-Treg vs. Fresh-Treg), and were also less methylated within their FOXP3 gene than total Xn-Treg and DP-Depleted Xn-Treg counterparts, (10.6% ± 4.9% vs. 17.1% ± 6.1% vs. 24.9% ± 11.9% of methylation in HLA-DR+CD27+ DP-Enriched Xn-Treg vs. total Xn-Treg vs. DP-Depleted Xn-Treg) (Figure 4A). This confirms that the demethylation of the FOXP3 gene in expanded HLA-DR+CD27+ DP-Enriched Xn-Treg, was similar to that of fresh naïve/rested unexpanded Treg and like naïve Treg are functionally stable and unlikely to revert to T-effector cells. The functional stability of HLA-DR+CD27+ DP-Enriched Xn-Treg was further assessed under pro-inflammatory conditions to test their plasticity towards an effector Th17 or Th1 cell phenotype. Tregs were stimulated with
a combination of pro-inflammatory cytokines for 6 days prior to detecting the proportion of CD4+FOXP3+ Tregs co-expressing IL17 or IFNγ. After stimulation, no significant change in proportion of IL17 co-expressing cells was observed within both HLA-DR+CD27+ DP-Enriched Xn-Treg and Pc-Treg (Figure 4B and Supplemental Figure 6). In contrast, a considerably increased proportion of IL17 co-expressing cells was detected after inflammatory stimulation within total Xn-Treg or DP-Depleted Xn-Treg, showing a significant difference from that seen within HLA-DR+CD27+DP-Enriched Xn-Treg (Figure 4B). Moreover, while all other Treg subsets tested demonstrated substantially increased IFNγ co-expressing cells upon inflammatory stimulation, HLA-DR+CD27+ DP-Enriched Xn-Treg showed a significantly reduced response to the stimulation, with a slight increase in IFNγ co-expressing cells (Figure 4B and Supplemental Figure 6). Taken together, these results indicated that HLA-DR+CD27+ DP-Enriched Xn-Treg were functionally stable in association with the expression of both HLA-DR and CD27 activation/memory markers.

*HLA-DR+CD27+ DP-Enriched Xn-Treg were more capable of suppressing islet xenograft rejection.* In order to study HLA-DR+CD27+ DP-Enriched Xn-Treg function in vivo, NOD-SCID IL2 receptor γ−/− (NSG) mice were transplanted with NICC xenografts and, 3 days after transplantation, reconstituted with or without 10⁷ human PBMC (depleted of CD4+CD25+CD127−/low Treg) as indicated in Supplemental Figure 7. Human PBMC engraftment was confirmed by flow cytometry at week 5 post islet transplantation with 67.0 ± 11.9% of cells in spleen being human CD45+ cells, with 27.1 ± 14.1 % CD4+ and 61.9 ± 13.7% CD8+ T cells, (Figure 5A). Whilst NICC
grafts survived for at least 90 days in non-reconstituted recipients, mice with adoptively transferred engrafted human PBMC rejected their xenografts completely within 35 days with no visible insulin positive-staining cells in the rejecting xenografts compared with intact and insulin positive-staining NICC grafts detected in non-reconstituted recipients (Figure 5B). Rejection was confirmed by porcine C-peptide assay with no detectable porcine C-peptide in reconstituted NICC graft recipients (<10 pmol/L) (Figure 5C). A large infiltrate of human CD4+ and CD8+ cells were detected in the grafts of human PBMC-reconstituted recipients at the graft-rejection time point (Figure 5B) similar to that seen in our previous study (29). Next, we tested the in vivo suppressive capability of the three groups of ex vivo expanded human Treg subsets. NSG were transplanted with NICC xenografts and 3 days later injected with $1 \times 10^7$ PBMC (CD4+CD25+CD127−/low Treg depleted) and $4 \times 10^5$ of the different Treg subsets at a ratio of PBMC:Treg of 25:1 (Supplemental Figure 7). All 3 groups of recipient mice demonstrated a similar large number of human CD45+ and CD4+ and CD8+ T cells in mouse spleen determined by flow cytometry 60 days after transfer (Figure 5A). This confirmed successful human leukocyte engraftment in these recipient mice, thereby ensuring that graft survival in the following experiments was the result of Treg-mediated suppression and not a failure of human leukocyte engraftment. In our previous study we showed adoptive transfer of NICC xenograft recipient mice with $2 \times 10^6$ polyclonally expanded human Treg was sufficient to suppress rejection induced by $1 \times 10^7$ (5:1 of PBMC:Treg) human PBMC-with NICC xenograft surviving beyond 100 days after human PBMC rechallenge (29). In the study reported here transplanted mice,
adoptively transferred with 5-fold less Pc-Treg ($4 \times 10^5$ at a ratio of 25:1 of PBMC:Treg) could not prevent NICC xenografts rejection with all grafts rejected by day 60 post human cell transfer. Graft histology showed only a few single insulin-positive cells with a large infiltrate of human CD4+ and CD8+ T cells (Figure 5D). Reconstitution of mice with human PBMC and Xn-Treg at the same ratio of PBMC:Treg (25:1) resulted in a little better islet xenograft survival with the presence of small islet clusters with insulin and glucagon positive staining within a large infiltrate of human T cells 60 days after human cell transfer (Figure 5D). However, DP-Depleted Xn-Treg were unable to achieve the same NICC xenograft survival to that shown by their Xn-Treg counterparts, when co-transferred at the same ratio of PBMC, NICC grafts were rejected in a similar fashion to that found in PBMC and Pc-Treg co-transferred mice (Figure 5D). These results demonstrated that adoptive transfer to NICC recipients of non-selected Treg at a ratio of 25:1 of PBMC:Treg was not sufficient or potent enough to effectively protect against human PBMC-mediated islet xenograft rejection in this model. In contrast, mice co-transferred with human PBMC and HLA-DR+CD27+ DP-Enriched Xn-Treg at the same ratio of 25:1 prolonged NICC xenograft survival to at least 60 days after human cell transfer, as shown by intact surviving grafts containing endocrine-secreting cells including insulin, glucagon, and somatostatin positive staining cells, which were surrounded but not infiltrated by a small number of human CD4+ and CD8+ cells (Figure 5D). This suggests that HLA-DR+CD27+ DP-Enriched Xn-Treg with xenoantigen-specificity, were substantially more potent at suppressing the xenogeneic response mediated by human PBMC, thereby leading to superior islet xenograft
survival in recipient mice. Graft survival beyond 60 days was difficult to assess as mice transferred with HLA-DR+CD27+ DP-Enriched Xn-Treg succumbed to GVHD beyond 60 days with intact NICC grafts, which again confirmed the relative antigen specificity for the anti-pig T cell response. In the one surviving recipient mouse the graft remained intact at day 90 after cell transfer (data not shown). This suggests that this protection mediated by HLA-DR+CD27+ DP-Enriched Treg was xeno-antigen specific as it was protective of NICC xenograft rejection but not protective of human to mouse GVHD. To confirm these histological findings, the in vivo function of the surviving grafts was further confirmed by porcine C-peptide assay. Consistent with the immunohistochemical findings, the highest levels of porcine C-peptide were detected in the serum from HLA-DR+CD27+ Treg co-transferred mice (465.5 ± 170.1 pmol/l) 60 days after human cell transfer compared with that seen in mice co-transferred with Xn-Treg (180.7 ± 149.6 pmol/l) or DP-Depleted Xn-Treg (97.1 ± 61.9 pmol/l) or Pc-Treg (129.5 ± 63.7 pmol/l) (Figure 5C). Similar to the immunohistochemistry results there was no significant difference in porcine C-peptide levels among mice co-transferred with Pc-Treg or Xn-Treg or DP-Depleted Xn-Treg subset (Figure 5C).

*HLA-DR+CD27+ Treg were able to selectively migrate to the graft after adoptive transfer to NSG recipients of NICC xenografts.* In order to determine whether the potency shown by HLA-DR+CD27+ DP-Enriched Xn-Treg in suppressing islet xenograft rejection was associated with an increased accumulation of FOXP3+Treg within the graft we performed a triple immunofluorescence staining of NICC xenografts with anti-human CD4 and FOXP3 antibodies and anti-porcine insulin antibody to look
simultaneously for the presence of intact NICC grafts and intra-graft human CD4+ T cells co-expressing FOXP3. Consistent with the results seen in Figure 5, HLA-DR+CD27+ DP-Enriched Treg co-transferred mice had large and intact insulin secreting NICC xenografts (Figure 6A). Associated with these intact functioning islet xenografts was an increased number of CD4 positive staining cells co-expressing FOXP3 and surrounding the intact islet xenografts (Figure 6A). In mice co-transferred with human PBMC plus Xn-Treg, Pc-Treg or DP-Depleted Xn-Treg, only small fragments of NICC xenografts or single islet cells were seen. As expected, there were fewer intragraft FOXP3+CD4+ cells found in these mice (Figure 6A). The proportion of intragraft CD4+FOXP3+ cells was further analyzed by flow cytometry. The results revealed that 20.6% ± 5.5% of intragraft CD4+ T cells in HLA-DR+CD27+ Treg co-transferred mice co-expressed Foxp3 (Fig. 5A), and this CD4+FOXP3+ cell proportion was significantly higher than that found in mice transferred with other Treg subsets (20.9% ± 2.8% vs. 10.5% ± 2.9% vs. 8.0% ± 3.6% vs. 4.4% ±1.9%, for DP-enriched Xn-Treg vs. Xn-Treg vs. Pc-Treg vs. DP-Depleted Xn-Treg, respectively) (Figure 6B). These results suggest that more potent suppression of islet xenografts in HLA-DR+CD27+ DP-Enriched Xn-Treg co-transferred mice was associated with a larger intra-graft accumulation of functioning Treg which were functionally stable with in vitro and in vivo evidence of antigen specificity.

Adoptive transfer of HLA-DR+CD27+ DP-Enriched Xn-Treg and PBMC led to a gene profile consistent with enhanced Treg function within islet xenografts. To determine if grafts protected by the infusion of HLA-DR+CD27+ DP-Enriched Xn-
Treg had a distinctive anti-inflammatory genetic profile compared to grafts taken from mice infused with other Treg populations, NICC grafts were taken from recipient mice at 60 days after PBMC:Treg transfer and at 35 days in recipient mice that received PBMC alone, and intra-graft RNA was evaluated using the TaqMan human immune panel consisting of 96 target genes from immune system functions, including those associated with Treg and effector T cell function. Differentially expressed genes in surviving islet xenografts from mice receiving HLA-DR+CD27+ DP-Enriched Xn-Treg were compared with rejecting xenografts from mice that received human PBMC alone. The heatmap displayed a picture of predominant Treg function from the islet xenografts of mice that received PBMC and HLA-DR+CD27+ DP-Enriched Treg, with down-regulated expression of effector Th1, Th2 and Th17 cytokine genes along with elevated intra-graft expression levels of two key Treg function genes, IL10 and CTLA4 (Figure 7). By contrast, in the rejected NICC grafts from mice given PBMC alone or from mice co-transferred with any other Treg subset there was expression of comparatively low levels of IL10 and CTLA4 and higher levels of gene expression of all effector T cell cytokines examined (Figure 7). In addition to the differences in the IL10 and CTLA4 gene expression there were significant differences in gene expression associated with other functional T cell subsets among the different groups of mice and these results were shown in Table 1. Compared with mice receiving both human PBMC and Pc-Treg or Xn-Treg or DP-Depleted Xn-Treg, mice co-transferred with human PBMC and HLADR+CD27+ DP-Enriched Treg had the largest down regulation of effector Th1 (IFNG and LTA), Th2 (IL4, IL9 and IL13) and Th17 (IL17) cytokine gene
expression, as well as the greatest up-regulation of Treg associated gene expression (IL10 and CTLA4) (Table 1). This confirms the more powerful suppressive function of the HLA-DR+CD27+ DP-Enriched Xn-Treg subset and their resistance to being converted to effector Th cells in the presence of an inflammatory response. Taken together, these findings suggest that HLA-DR+CD27+ DP-Enriched Xn-Treg were able to selectively target the NICC graft and exert an effective and stable suppressive function that regulates the human effector Th cell initiated xenogeneic immune response in vivo.

**Discussion**

Given their role in regulating immunity, infusion of Treg after ex vivo manipulation has been proposed as a treatment for autoimmune diseases and/or tolerance induction in transplantation. Numerous preclinical studies have indicated that antigen-specific Treg cells are more potent than polyclonal Treg cells in the control of immune responses in autoimmune diseases and transplantation (6). They have the ability migrate towards the site of antigen presentation (30), thereby leading to more efficient and localized control of inflammation without the risks of broad immunosuppression and associated adverse events. The enhanced trafficking of antigen-specific Treg to target tissues may allow the administration of fewer Treg than currently used in trials of polyclonal Treg, which should allow for more efficient and cost effective in vitro expansion protocols for Treg adoptive cell therapy. Generation of antigen-specific Treg in vitro has been reported by induction of antigen-specific effector T cells into cells with suppressive capacity (31,
32), expansion of Treg with allogenic DC or B cells (16, 33), or by genetic engineering of TCR or CAR-Treg (17, 34-36). However, as it is for polyclonal Treg, antigen specific Treg will need to demonstrate prolonged survival, and stability and lack of plasticity in vivo (37).

In this study, we described a simple and effective approach to produce antigen-specific Treg that meet the requirements for adoptive Treg therapy. By culturing naïve/resting human Treg in the presence of porcine PBMC we selectively expanded a distinct subset of Treg co-expressing activation/memory surface markers HLA-DR and CD27. We show that compared to their unsorted and HLA-DR and CD27 double positive cell-depleted counterparts, HLA-DR+CD27+ DP-Enriched Xn-Treg were antigen-specific with enhanced suppression of the xenogeneic response in association with upregulated expression of Treg function markers CD95 and ICOS. Moreover, HLA-DA+CD27+ DP-Enriched Treg are more stable in their phenotype and function and are resistant to conversion to effector Th1 and Th17 cells under inflammatory conditions. This is confirmed by their demethylation TSDR state and high level of Helios expression that was similar to that of unexpanded naïve/resting Treg. Adoptive transfer of porcine islet graft recipient NSG mice with even a small number of HLA-DR+CD27+ DP-Enriched Xn-Treg efficiently inhibited porcine islet graft rejection mediated by 25-fold more human effector cells in a humanized mouse model. The prolonged graft survival was associated with downregulated intra-graft expression of effector Th1, Th2 and Th17 cytokine genes, enhanced accumulation of FOXP3+ Treg within surviving grafts and upregulated intra-graft expression of Treg functional genes
$IL10$ and $CTLA4$. These findings confirmed their functional stability and the capacity of adoptively transferred HLA-DR+CD27+ DP-Enriched Treg to migrate to the site of the transplanted graft. In previous studies using unselected expanded human Treg in higher doses we demonstrated effective suppression of porcine islet xenograft rejection and graft versus host disease in NSG recipients reconstituted with human PBMC (29). The observation that HLA-DR+CD27+ DP-Enriched Xn-Treg at much lower Treg to PBMC ratios could prevent porcine islet xenograft rejection without suppression of graft versus host disease, further highlights their enhanced antigen-specificity.

Taken together, these findings indicate that $in$ $vitro$ antigen stimulated HLA-DR+CD27+ DP-Enriched Xn-Treg acquired the properties of activated phenotype, potent suppression and antigen-specificity. CD27 is a member of the tumor necrosis factor superfamily of costimulatory receptors associated with the regulation of immune responses. For example, CD27 expression has been shown to identify highly suppressive and antigen specific Treg, distinguishing them from activated CD25+CD4+ effectors (21, 38-41). Moreover, CD27 expression has been reported to be inversely correlated with Treg IL17 production in the skin of patients with psoriasis and hidradenitis suppurativa, suggesting the role of CD27 in regulating Treg plasticity in inflammatory tissue (42). HLA-DR expression has been associated with active rather than resting Treg and HLA-DR+ Treg are more suppressive than HLA-DR-counterparts (20, 24). Studies have also shown that alloantigen-induced regulatory CD3+CD4+HLA-DR+ T cells express the regulatory and activation markers CD25, CTLA4, CD62L, PD1, and TNFRII (43). Autologous PBMC-simulated CD8+HLA-
DR+ Treg revealed similar phenotypic and functional features to CD4+FOXP3+ Treg, with highly antigen-specific suppression of responder CD8+ T cells (44). Thus, co-expression of HLA-DR and CD27 allows us to identify and sort a highly suppressive and antigen-specific Treg subset with stable features, from unstable subsets in xenoantigen-expanded human Treg populations. HLA-DR+CD27+ Treg that are expanded by xenoantigen stimulation have the potential to meet the proposed criterion of potent suppressive activity, antigen-specificity, functional stability and capacity to localize at the site of the transplanted graft. However, it should be noted that identification of a molecular signature and profiling of TCR clonotypes in HLA-DR+CD27+ Treg would help us to further characterize their antigen-specificity and functional stability.

Treg mediate their immunosuppressive effects via a variety of mechanisms, including immunosuppressive cytokines, such as IL10, TGFβ and IL35, the consumption of IL2, production of immunosuppressive adenosine by ectoenzymes CD39 and CD73, modulation of antigen-presenting cells (APC) by CTLA4, LAG3 and ICOS, and granzyme and perforin mediated cytolysis (45-49). Treg can also target effector T cells directly by Fas/Fas-mediated apoptosis (28, 50, 51). Moreover, the Treg activation/memory marker ICOS (23, 24) not only stabilizes FOXP3 function but also increases IL10 production by Treg, thereby leading to enhanced Treg suppressive potency (52-56). In this study, HLA-DR+CD27+ DP-Enriched Xn-Treg were more suppressive than other Treg subpopulations and this correlated with their upregulated expression of Fas and ICOS and a trend of increasing expression of CTLA4, FOXP3
and Helios, indicating the contribution of these suppressive mechanisms to their enhanced suppression potency \textit{in vitro}. Moreover, islet xenograft recipients adoptively transferred with HLA-DR+CD27+ DP-Enriched Xn-Treg showed prolonged graft survival, whilst maintaining enhanced FOXP3 expression and elevated levels of \textit{IL10} and \textit{CTLA4}, further supporting the finding that HLA-DR+CD27+ DP-Enriched Xn-Treg retained their suppressive phenotype long term.

Although this study demonstrates the potential of \textit{in vitro} antigen primed HLA-DR+CD27+ Treg to suppress T cell-mediated effector function, its findings should be interpreted within the limitations of the model. Although HLA-DR+CD27+ DP-Enriched Xn-Treg had enhanced expression of CD95 and ICOS and increased proportion of FOXP3+Helios+ Tregs the precise mechanisms for the enhanced suppression remain unclear. Although, their enhanced regulatory function and antigen specificity was confirmed \textit{in vivo} after their infusion in NSG recipients of porcine islet grafts reconstituted with human PBMC, this model does not result in full immune reconstitution. In particular, there are limited B cell and NK cell populations. Whilst these cells populations can contribute to the rejection response, B cells have also been implicated in the development of tolerance in both mouse and human studies, making the \textit{in vivo} findings here even more remarkable (57). Despite these limitations, this study has identified a sub-population of Treg cells that are enriched for antigen specific Treg with enhanced suppressive function. Upon in vitro expansion, HLA-DR+CD27+ expression emerges for the first time as a specific Treg activation signature allowing the identification and isolation of an epigenetically stable antigen-activated Treg subset.
and provides the means and essential knowledge for the design of improved Treg-base cell therapy and could form the basis for more personalized therapy in immunosuppression.

**Methods**

*Animals.* Newborn and adult Landrace pigs from local farms were used as donors for NICC and PBMC, respectively. NSG mice were housed under specific pathogen-free conditions in the Biological Services Facility of The Westmead Institute for Medical Research. Mice between the ages of 6-8 weeks were used for NICC transplantation.

*Peripheral blood mononuclear cells isolation and expansion of human Treg.* Human PBMC were obtained from healthy donor buffy coat samples provided by NSW Red Cross, Australia. CD4+CD25+CD127-/low Treg were isolated from PBMC as described previously (18). Porcine PBMC from Landrace pigs and human PBMC from a single donor were used as xenogeneic and allogeneic stimulator cells, respectively. The resulting CD4+CD25+CD127-/low Treg were cultured in 96-well round-bottom plates (5 × 10⁴/well) in RPMI 1640 media (GIBCO, Carlsbad, CA), supplemented with 10% human AB serum (Invitrogen, San Diego, CA), 2 mmol/L glutamine, 25 mmol/L HEPES, 50 U/mL penicillin, 50 µg/mL streptomycin, 50 µM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), and 100 nmol/L rapamycin (Sigma-Aldrich) at 37°C and 5% CO2, in the presence of 400 U/mL IL2 (Chiron, Emeryville, CA) either stimulated with anti-CD3/CD28 dynabeads (Invitrogen, Carlsbad, CA) at a 1:1 ratio of Treg:beads and designated as polyclonal stimulated Treg, or stimulated with anti-
CD3/CD28 dynabeads combined with irradiated porcine PBMC (30 Gy) at a 4:1 ratio of porcine PBMC: Treg (4 × 10^5 porcine PBMC: 1 × 10^5 Treg as xenoantigen expanded Treg), respectively. After 7 days, cells were counted and split every 3 days, supplied with fresh RPMI 1640 media containing IL2, rapamycin and anti-CD3/CD28 dynabeads, and restimulated with irradiated pig PBMC at day 7 and 14 as described above. Expanded Treg were continuously counted at day 14 and 21 and harvested at day 21 for subsequent experiments (18).

Alloantigen expanded Treg were produced from CD4+CD25+CD127^-/low Treg, isolated from PBMC and expanded in the presence of irradiated alloantigen PBMC, as described for the production of xenoantigen expanded Treg.

**Flow cytometry and cell sorting.** Flow cytometric analysis of Treg phenotype, human leukocyte engraftment, and graft-infiltrating human leukocytes, was performed as described previously (18, 29) by staining in different combinations with the following antibodies according to manufacturer’s recommendations: CD4-APC-H7, CD4-PE-Cy7, CD127-PE, CD27-BV711, HLA-DR-FITC, CD62L-PE, 7AAD, CTLA-4-PE, ICOS-BV421, IL-17A-BV421, IFN-γ-BV711 and Foxp3-PECF594 from BD Bioscience (Carlsbad, USA), CD25-APC, Foxp3-PE from eBioscience (San Diego, CA); CTLA-4-BV605, Helios-pacific blue, and CD95-BV421 from Biolegend; GITR-PE from R&D systems (Minneapolis, MN, USA). Intracellular cytokine staining was performed using the BD Cytofix/Cytoperm™ Fixation and Permeabilization Solution, and intracellular staining for FOXP3, Helios and CD95 was performed using the Foxp3 Buffer Set from eBioscience, all according to manufacturers’ protocols. The used
antibodies are detailed in Supplemental Table 1. All data were acquired on BD LSR II, LSR Fortessa or Symphony (BD Bioscience) and analyzed using FlowJo (TreeStar, Inc, Ashaland, OR, USA). Cell sorting was performed on a BD FACSAria III (BD) by the following gating strategy. Xn-Treg and Al-Treg stained with CD4-APC-H7, CD25-APC, CD127-PE, CD27-BV711, HLA-DR-FITC and 7AAD were firstly gated on human lymphocytes and 7AAD- alive cells to deplete residual irradiated porcine cells and dead cells, followed by gating on CD4+ cells then CD25+CD127- cells (Supplemental Figure 4) which were finally sorted into HLA-DR+CD27+ DP-enriched Xn-Treg and DP-depleted Xn-Treg subsets, respectively. Pc-Treg also went through the cell sorter to depleted dead cells. The purity of the resulting HLA-DR+CD27+ DP-enriched Xn-Treg and DP-depleted Xn-Treg subsets were all greater than 98%, respectively. The isolated cells were used after an overnight rest at 37°C in the same Treg culture medium as used during expansion.

Suppression assays. The suppressive capacity of Treg was assessed by MLR assays. Carboxy-fluorescein diacetate succinimidyl ester (CFSE; Invitrogen)-labeled 1 \times 10^5 responder cells (autologous PBMC without CD4+CD25+Cd127- Tregs) were either incubated with anti-CD3/CD28 dynabeads at a 1:3 ratio of cell:beads for polyclonally stimulated MLR assays (Poly MLR), or cocultured with 3 \times 10^5 irradiated (30 Gy) xenogeneic or allogeneic PBMCs for xenogeneic (Xeno MLR) or allogeneic (Allo MLR) MLR, respectively. Treg were titrated into different MLR cultures at different ratios. After 3 days culture for Poly MLR, or 7 days for Xeno and Allo MLR, proliferation of responder cells (CFSE-positive cells) was evaluated based on the
percent proliferating responder cells cultured in the absence of Treg compared with the percent proliferating responder cells cultured in the presence of Treg. The percent proliferating responder cells in the absence of Treg was considered as 100% of proliferation and 0% of suppression.

*Cytokine analysis.* IFNγ was measured by ELISA (Human IFNγ ELISA Kit, Invitrogen) in supernatants collected from the above xenogeneic MLR assays according to the manufacture’s recommendation.

*TSDR analysis.* DNA samples were extracted from Fresh-Treg (no expansion), Pc-Treg, Xn-Treg, HLA-DR+CD27+ DP-Enriched Xn-Treg, DP-Depleted Xn-Treg and using AllPrep DNA/RNA Mini Kit (Qiagen) for TSDR detection. Purified DNA (200ng) was subjected to sodium bisulfite conversion with an EZ DNA Methylation-Gold Kit (Zymo Research). For detection of methylated CpGs, bisulfite converted genomic DNA was subjected to qPCR using HEX and FAM-labelled probes that recognize methylated and demethylated CpG sites, respectively. The methylation-qPCR 5’ primer is ATTTGGGTTTTGTTGTTATAGTTTT and 3’ primer is AAAATATCTACCCCTCTTCTTCCCTC. The probes include 6FAM/Zen-ATGGGTGGTTGGATGTGTTGGGTT-lBFQ and HEX/Zen-ATGGCGGGTCTGGATTCGTGGTGTTGGGTT-lBFQ. Methylation was calculated using the formula:

\[
\% \text{methylation} = 100/[1 + 2^{\Delta Ct(\text{methylated- unmethylated})}]
\]

*Inflammatory stimulation.* Treg were collected after sorting and placed immediately into culture media (90% RPMI 1640(Invitrogen), 10% FBS(Invitrogen), 50mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), 100nM Rapamycin
(Sigma-Aldrich) containing 10IU/mL IL2, 10ng/mL IL1β, 5ng/mL IL6, 25ng/mL IL-21, 25ng/mL IL23, and 5ng/mL TGFβ (all cytokines from R&D Systems) for 6 days for inflammatory cytokine producing cell induction. Cells cultured in the same medium containing 10IU/mL IL2 only were used as non-induction controls (non-stimulated). After 6 days stimulation, cells were further incubated with 50ng/mL Phorbol 12-Myristate 13-Acetate (PMA, Sigma-Aldrich), 1ug/mL ionomycin (Sigma-Aldrich) and BD GolgiStop protein transport inhibitor (BD Bioscience) for a further 5 hours prior to cytometric analysis of percent of CD4+ cells co-expressing IL17 or IFNγ, respectively.

Porcine islet isolation and transplantation. NICC were isolated from the pancreases of 1-3 days old piglets and propagated in culture for 6 days as described previously (59). 4000 NICC islet equivalents (IEQ) were transplanted into NSG mice under the renal capsule of both kidneys.

Adoptive transfer of human cells. $1 \times 10^7$ human PBMC that were depleted of CD4+CD25+CD127-/low Treg, were injected intravenously (i.v.) alone or together with $4 \times 10^5$ ex-vivo expanded autologous Treg subsets into NSG mice 3 days after NICC transplantation. Peripheral blood, serum, spleen and grafts were collected from recipient mice at predetermined time points after human cell transfer to assess human leukocyte engraftment and NICC xenograft survival. Graft rejection was defined as no visible intact graft observed by histological examination (29).

Immunohistochemistry and immunofluorescence. Histology and immunohistochemistry of cryostat sections (6 to 8 um) and paraffin (4 um) was undertaken as described previously (29). Porcine endocrine cells were detected using
guinea pig anti-porcine insulin (Dako Laboratories, Mississauga, Ontario, Canada),
guinea pig anti-porcine glucagon (Linco Research, St. Charles, MO, USA), and goat
anti-human somatostatin (Santa Cruz Biotechnology, Santa Cruz, CA, USA),
antibodies and the Universal ABC Kit (Vector Laboratories, Burlingame, CA, USA).
Graft infiltrating human leucocytes were stained using rabbit or mouse anti-human CD4
(Abcam), CD8 (Dako) antibodies, followed by incubation with horseradish peroxidase-
conjugated secondary goat anti-mouse antibody (Abcam). Sections were visualized
with diaminobenzidine (Dako). Triple immunofluorescence staining of human CD4 and
FOXP3, and porcine insulin was undertaken with rabbit anti-human CD4 polyclone Ab
and mouse anti-human Foxp3 mAb (Abcam) and Guinea pig anti-porcine insulin,
followed by secondary incubation with Alex488 conjugated-goat anti-mouse Abs and
Alex562 conjugated-goat anti-rabbit (Abcam). The sections were then stained with
DAPI (Sigma, St. Louis, USA). The used antibodies are detailed in Supplemental Table
2. The sections were viewed under an Olympus FV1000 (Olympus, Japan).

Porcine C-peptide assay. Porcine C-peptide in NICC recipient mouse serum was
measured using Mercodia Porcine C-peptide ELISA kit (Mercodia AB, Sylveniusgatan,
Uppsala, Sweden) according to the manufacturer’s instructions in a Victor X3
multilabel plate reader (Perkin Elmer, Waltham, Massachusetts, USA).

Taqman human immune panel array. Islet xenografts were harvested from both
kidneys of recipient mice at predetermined time points as indicated. One graft from
each mouse was fixed in 4% formalin for histological studies and the other one was
cryopreserved for immunohistology and RNA extraction using the RNA extraction kit
RNA quality and quantity were determined using Nano drop (Thermo Fisher). RNA was reverse transcribed into cDNA using SuperScript™ IV VILO™ (SSIV VILO) Master Mix with ezDNase enzyme (QIAGEN). Gene expression assay was performed using Taqman Human Immune Panel (Applied Biosystems) according to the manufacturer’s instruction by QuantStudio 12K Flex realtime PCR system (ThermoFisher Scientific, USA) The mean expression of GUSB and GAPDH was used for normalization and the results were analyzed by ExpressionSuit software. The interested and differentially expressed genes were selected using the volcano plot for PBMC vs. HLA-DR+CD27+ DP-Enriched Xn-Treg comparison with false discovery rate (FDR) < 0.05 and a greater than a two-fold change. Heat maps were generated with Multiple Experiment Viewer software and unsupervised hierarchical clustering was performed using Euclidean Correlation.

Statistical Analysis. Results involving multiple groups were evaluated using one way or two-way ANOVA with the Tukey multiple comparison test, or Kruskal-Wallis multiple comparison test (nonparametric) (Graphpad prism software version 8.0). For differences between two Treg expansion protocols or two types of Treg subsets the paired comparison t test (2-tailed) was used. Data was presented as mean ± SD for all results except MFI data that was presented as mean ± SEM. P<0.05 was considered as statistically significant.

Study approval. The study was approved by the Westmead Area Health Service Human and Animal Ethics Committees and conducted in compliance with State Government Legislation and NH&MRC Animal Research Guidelines.
Data availability. All data needed to evaluate the conclusions in the paper are present in the paper or the supplemental materials. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Author contributions
XM, LC and MR as co-first authors performed and interpreted the results of experiments, analyzed data, prepared figures, and drafted the manuscript. XM was responsible for the establishment of transplant model and related experiments and her name appears first because of her role in the initiation of the project; LC and MR was responsible for the phenotypic and functional experiments in vitro. QC performed animal experiment and analyzed data. HW and YZ performed some Treg isolation and flow cytometric analysis experiments. NGB and GN performed TSDR analysis. LCH reviewed and revised the manuscript. WJH isolated and provided pig islets and performed pig-to-mouse islet transplantation experiments. MH designed the research, interpreted the results of experiments, analyzed data, edited the manuscript and finalized the revised version of the manuscript. SY and PJO conceived and designed the research, interpreted the results of experiments, analyzed data, and revised the manuscript. All authors approved the final version of the manuscript. PJO is the guarantor of this work. MH, SY and PJO had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Acknowledgments

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Table 1. Genes with significant fold change in expression within islet xenografts of recipient mice co-transferred with PBMC and individual Treg subsets compared to that detected in PBMC transferred recipient mice.

<table>
<thead>
<tr>
<th>Log2FC</th>
<th>Pc-Treg</th>
<th>Xn-Treg</th>
<th>DP-depleted Treg</th>
<th>DP-enriched Treg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Th1</strong></td>
<td><strong>LTA</strong></td>
<td>-0.79±0.54****</td>
<td>-1.18±0.58**</td>
<td>-2.30±0.48</td>
</tr>
<tr>
<td></td>
<td><strong>IFNG</strong></td>
<td>-0.44±0.55</td>
<td>-0.28±1.12</td>
<td>0.99±0.62**</td>
</tr>
<tr>
<td><strong>Th2</strong></td>
<td><strong>IL4</strong></td>
<td>-1.22±0.94****</td>
<td>-1.13±1.90****</td>
<td>-2.46±1.10****</td>
</tr>
<tr>
<td></td>
<td><strong>IL9</strong></td>
<td>-2.61±0.45****</td>
<td>-3.58±1.55****</td>
<td>-3.96±1.15****</td>
</tr>
<tr>
<td></td>
<td><strong>IL13</strong></td>
<td>-0.22±0.42****</td>
<td>-0.71±0.63 ****</td>
<td>-2.63±0.52</td>
</tr>
<tr>
<td><strong>Th17</strong></td>
<td><strong>IL17</strong></td>
<td>-2.93±3.38***</td>
<td>-2.99±3.97***</td>
<td>-1.03±3.51****</td>
</tr>
<tr>
<td><strong>Treg</strong></td>
<td><strong>IL10</strong></td>
<td>-0.26±2.29****</td>
<td>-1.36±0.33****</td>
<td>-3.07±0.87****</td>
</tr>
<tr>
<td></td>
<td><strong>CTLA4</strong></td>
<td>0.75±1.01*</td>
<td>-0.44±1.31***</td>
<td>-0.52±0.52***</td>
</tr>
</tbody>
</table>

FC, fold change. Data are presented as mean ± SD of four individual mice of each group.

Statistical analysis was using two-way ANOVA with the Tukey multiple comparison

*P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 compared with HLADR+CD27+

DP-Enriched Treg group.

LTA, Lymphotoxin alpha.
Figure 1. Phenotypical characterization of ex-vivo expanded human Treg.

Representative flow cytometric plots of CD4+CD25+CD127-/low Treg phenotypes isolated from human PBMC (Fresh-Treg), Treg expanded with anti-CD3/CD28 dynabeads (Pc-Treg), and stimulation in presence of irradiated porcine PBMC (Xn-
Treg) after three cycles (weeks) of stimulation. (A) Gates were set on CD4+ T cells. FOXP3 and other cell surface marker expression are shown as the percentage of CD4+ T cells co-expressing individual Treg markers (CD4+CD25+, CD25+FOXP3+, CD25+CTLA-4+, CD127-CD25+, CD62L+CD25+, CD25+GITR+). (B) The proportion of Treg co-expressing FOXP3 and Helios on Fresh-Treg, Pc-Treg, Xn-Treg and negative control effector T cells are shown. The gating strategies are shown in Supplemental Figure 1. (C) The proportion of Treg co-expressing HLA-DR and CD27 after gating on CD4+CD25+ cells. (D) Phenotyping of Xn-Treg. Representative histograms of CD95 expression (surface and intracellular staining), ICOS (surface staining), CTLA-4 (surface staining), FOXP3 (intracellular staining) and Helios (intracellular staining) on HLA-DR+CD27+ double positive enriched Xn-Treg (DP-Enriched; red line), total Xn-Treg (Total; green line) and Xn-Treg depleted of HLA-DR+CD27+ double positive cells (DP-Depleted; blue line). Expression of CD95, ICOS, CTLA4, FOXP3 and Helios in different Treg subsets is also shown by the mean fluorescence intensity (MFI). Numbers in brackets in each plot are the ranges of the percentage of individual Treg markers detected in four independent experiments with Treg from four individual donors for (A) and five individual donors for (B); and 7 independent experiments with 9 individual donors for (C). Data represents 4 independent experiments with Xn-Treg from 5 individual donors for (D). Error bars indicate the mean ± SD for (A-C) and mean ± SEM for (D). P value (1-way ANOVA): *P<0.05, **P<0.01 and ****P<0.0001).
Figure 2. HLA-DR and/or CD27 expression within Xn-Treg at different stimulation times and their FOXP3 and Helios expression. (A) The proportion of Xn-Treg expressing HLA-DR or CD27 after gating on CD4+ cells after round (week) 1, 2, and 3 of stimulation. (B) The proportions of HLA-DR+CD27-, HLA-DR+CD27+, HLA-DR-CD27+, HLA-DR-CD27- subsets within Xn-Tregs (after gating on CD4+ cells) following round 1, 2, and 3 of stimulation. (C) The representative flow cytometric
plots and the percentage of FOXP3+Helios+ cells on HLA-DR+CD27+ DP-Enriched Xn-Tregs following round 1, 2, and 3 of stimulation (after gating on HLA-DR+CD27+ cells) and the proportion of FOXP3+Helios+ cells in Fresh-Tregs, total Xn-Tregs and HLA-DR+CD27+ DP-Enriched Xn-Tregs. Data represent 3 independent experiments with Treg from 5 individual donors. Error bars indicate the mean ± SD. $P$ value (1-way ANOVA): *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. 
Figure 3. *In vitro* suppression assay of HLA-DR+CD27+ DP-Enriched Xn-Treg.

(A) MLR assay of Xn-Treg suppressive capacity compared to Pc-Treg. CFSE-labeled autologous human PBMC (CD4+CD25+CD127-/low depleted) were stimulated with irradiated xenogeneic porcine (Xn-MLR) or allogeneic (Allo-MLR) PBMC or anti-CD3/CD28 dynabeads (Poly-MLR), in the presence or absence of serial dilutions of unsorted xenoantigen expanded Treg (Xn-Treg) or HLA-DR+CD27+ DP-Enriched Xn-
Treg or HLA-DR+CD27+ DP-Depleted Xn-Treg or anti-CD3/CD28 dynabead expanded Pc-Treg for 7 days, prior to measurement of PBMC proliferation by CFSE dilution. **(B)** Alloantigen expanded Treg (Al-Treg) suppressive capacity in Xeno-MLR. CFSE-labeled autologous human PBMC were stimulated with irradiated xenogeneic porcine (Xn-MLR), in the presence or absence of serial dilutions of unsorted Al-Treg or HLA-DR+CD27+ DP-Enriched Al-Treg or HLA-DR+CD27+ DP-Depleted Al-Treg or Pc-Treg for 7 days, prior to measurement of PBMC proliferation by CFSE dilution. 

**(C)** Assessment of IFNγ concentration in supernatants of the Xn-MLR assay of Xn-Treg suppression. IFNγ secretion in supernatants collected from xenogeneic MLR assay as described in (A) was measured by ELISA. Data are presented as mean ± SD of four independent experiments with Treg from four individual donors for (A) except in Poly-MLR with six individual donors for Xn-Treg, DP-Depleted Xn-Treg and DP-Enriched Xn-Treg, from three individual donors for (C), and six independent experiments with Treg from six individual donors for (B). *P value (2-way ANOVA)* *P<0.05, * **P<0.01; ***P<0.001; ****P<0.0001.
Figure 4. Evaluation of Treg functional stability. (A) TSDR assay. The stability of Treg master function marker FOXP3 was evaluated by measurement of the status of demethylation of TSDR within FOXP3 in all Treg subsets examined. Data are mean ± SD of independent experiments with Treg from 5 individual donors. (B) Test of Treg plasticity and stability under pro-inflammatory conditions. The multiple types of Tregs were stimulated with a combination of pro-inflammatory cytokines (IL1β, IL6, IL21, IL23, TGFβ) and IL2 for 6 days (Stimulated) (details in methods) prior to flow
cytometric analysis of proportions of these cultured Treg co-expressing IL17 or IFNγ. Control samples were Treg subsets with IL2 only (Non-stimulated). Data presented are mean ± SD of five independent experiments with Treg from five individual donors. $P$ value [paired T test comparison (2-tailed) between DP-enriched Xn-Treg and DP-Depleted Xn-Treg or Xn-Treg for (A) and 2-way ANOVA for (B)]: *$P < 0.05$, **$P < 0.01$***$P < 0.001$ and ****$P < 0.0001$. 
Figure 5. Evaluation of NICC xenograft survival and function in vivo.

HLADR+CD27+ DP-Enriched Xn-Treg are more capable of suppressing islet xenograft injection. (A) Flow cytometric measurement of percentage of human leukocytes (CD45+ cells, CD4+ and CD8 T cells) in the spleen of mice receiving human
PBMC (CD4+CD25+CD127-/low depleted) alone at 35 days, and different types of human expanded Treg combined with PBMC at 60 days after human cell transfer. Data are shown as mean ± SD of at least three independent experiments (n ≥ 8 mice of each group). (B) Representative immunohistochemical examination for human CD4, CD8 and porcine insulin of graft samples from mice receiving no cells (NICC alone day 90 post-transplantation) or human PBMC (NICC + PBMC day 35 post-NICC transplantation). Original magnification x 200. (C) Porcine c-peptide was measured at day 60 post transplantation, and control PBMC group at day 30. Data are represented as mean ± SD (n ≥ 8 mice of each group, except control PBMC group with n = 5). (D) Representative immunohistochemical staining of graft samples from the same mice as in (A) for human CD4, and CD8 and porcine insulin, glucagon, and somatostatin. Original magnification x 200. P value [1-way ANOVA with Tukey multiple comparison test for (A) and Kruskal-Wallis test for (C)]: *P < 0.05; **P < 0.01, ****P<0.0001.
Figure 6. The graft infiltrating CD4+FOXP3+ Treg. (A) Representative triple immunofluorescence staining for human CD4, FOXP3 and porcine insulin of graft samples. NICC xenografts from mice receiving human PBMC and different individual Treg subsets, at day 60 after human cell transfer, were stained for human CD4 in green, FOXP3 in red and porcine insulin in purple. Original magnification × 200 or × 400. (B)
Representative flow cytometric plots and the proportion of CD4+FOXP3+ cells within graft infiltrating human CD45+CD4+ cells by flow cytometric analysis. Data are the mean ± SD of six graft samples from each human Treg group, co-transferred with human PBMC. $P$ value (1-way ANOVA): **$P<0.01$ and ****$P<0.0001$. 
Figure 7. Examination of intragraft immune gene profiles in humanized recipients of NICC grafts. The heatmap represents normalized and color-coded relative expression values of differentially expressed genes (log\textsubscript{2}FC $>$2.0; FDR <0.05, HLADR+CD27+ DP-Enriched Xn-Treg vs. PBMC) (n≥4 individual mice from each group) in islet xenograft from recipient mice co-transferred with PBMC and different Treg subsets at day 60 and the PBMC alone rechallenge group at day 35. The genes relating to Treg and T effector cell function are shown in the red and blue boxes, respectively. The distance metric is based on Euclidean distance. Red values indicate up-regulated, and green values indicate down-regulated.