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Antigen-specific CD4⁺CD25⁺ T cells induced by locally expressed ICOS-Ig: The role of Foxp3, Perforin, Granzyme B and IL-10.

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COMPETING INTERESTS

The authors declare no conflict of interest.

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ABBREVIATIONS

APC: antigen presenting cell

CTLA4: Cytotoxic T Lymphocyte Antigen 4

Diphtheria toxin: DT

DTH: Delayed Type Hypersensitivity

Foxp3: Forkhead box P3

GzmB: Granzyme B

ICOS: Inducible Co-Stimulator

ICOSL: ICOS ligand

IDO: Indoleamine 2,3-dioxygenase

IFN γ : Interferon γ

IL-10: Interleukin 10

iTreg: induced Treg

MHC II: major histocompatibility complex class II molecules

MST: Median survival time

nTreg: thymic derived Treg

PBS: Phosphate buffered saline

Prf: Perforin

qRT-PCR: quantitative real-time polymerase chain reaction

Treg: regulatory T cells

WT: wild-type

Abstract

Background: We have previously reported that ICOS-Ig expressed locally by a graft induces a perigraft cellular accumulation of CD4⁺CD25⁺Foxp3⁺ T cells and specific xenograft prolongation. In the present study we isolated and purified CD4⁺CD25⁺ T cells from ICOS-Ig secreting grafts to examine their phenotype, and used knockout and mutant mice to examine molecules involved in graft prolongation.

Methods: The mechanism of xenograft survival observed with ICOS-Ig-secreting PIEC grafts was examined using selected knockout mice. CD4⁺CD25⁺ T cells were isolated from xenografts secreting ICOS-Ig for phenotyping by flow cytometry, gene expression analysis by real-time PCR and regulatory function by suppression of xenogeneic or allogeneic primed CD4 T cells *in vivo*.

Results: Graft prolongation is dependent on a pre-existing Foxp3⁺ Treg, IL-10, perforin and granzyme B. CD4⁺CD25⁺Foxp3⁺ T cells isolated from xenografts secreting ICOS-Ig have a phenotype consistent with nTreg but with a higher expression of CD275 (ICOSL), expression of CD278 (ICOS) and MHC II and loss of CD73. Moreover, these cells are functional and specifically suppress primed T cells *in vivo*.

Conclusions: Expression of soluble ICOS-Ig by a xenograft generates antigen specific CD4⁺CD25⁺Foxp3⁺ T cells that mediate specific graft prolongation using at least 3 key mediators: IL-10, perforin and granzyme B.

KEYWORDS

Inducible Co-Stimulator (ICOS), T Cells, IL-10, perforin and granzyme B, xenotransplantation

INTRODUCTION

Immunological tolerance would remove the need for non-specific immunosuppressive therapy and clinically applicable strategies to achieve this goal are rapidly approaching. Allograft, and more recently xenograft protocols for the induction of cellular tolerance are under intense investigation. Harnessing regulatory T cells (Treg) for the purpose of modulating allograft and xenograft immune responses has been one of the key strategies under investigation by a number of laboratories. Defining the diverse Treg subsets and their mechanism(s) of action using multiple molecular markers and genetically modified mice is a continually evolving area of research and an essential step towards the clinical application of immune-therapy using this approach. Treg expressing the transcription factor Foxp3 represent a key cell subtype under investigation [1], and are the focus of our laboratory's studies.

Through significant progress in basic research and clinical approaches, xenotransplantation may finally be ready to fulfill its enormous potential. Indeed, clinical xenotransplantation trials have commenced and continued studies are now critical to establish a platform for progress toward routine clinical application. Recent results from preclinical studies of pig cellular grafts (hepatocytes, neuronal cells, islets and corneas [2] to non-human primates have produced promising results, demonstrating graft survival times of greater than 1 year. Moreover, pig islet grafts reverse diabetes and maintain normoglycaemia over a year in diabetic monkeys [3,4]. These clinically significant outcomes provide the basis for further clinical trials.

Our approach to xenograft tolerance has been modification of the donor tissue with immunomodulatory agents which has been shown to influence the rejection response of host T-cells [5-9]. ICOS-Ig was unique amongst a select group of immunomodulatory molecules (ICOS-Ig, CTLA-4Ig, IDO, CD40-Ig and IL-10) in that xenografts secreting this molecule induced a perigraft cellular accumulation of CD4⁺CD25⁺Foxp3⁺ T cells (Treg phenotype) and an increase in interleukin-10 (IL-10) levels [5]. Moreover, ICOS-Ig secreting xenografts implanted on one side of the recipient animal prolonged survival of a distant wild-type xenograft simultaneously transplanted on the contralateral flank. Importantly the distant wild-type grafts were also associated with perigraft cellular accumulation of CD4⁺CD25⁺Foxp3⁺ T

cells. Strikingly, this prolongation was specific, as allografts or xenografts from a different species were not prolonged [5].

In this study we show that graft prolongation is dependent on pre-existing Foxp3⁺ cells and that graft infiltrating CD4⁺CD25⁺Foxp3⁺ T cells have a distinct phenotype. Purified CD4⁺CD25⁺ T cells isolated from ICOS-Ig secreting grafts specifically suppress xenoantigen primed T cells when transferred *in vivo*. Moreover, IL-10, perforin and granzyme B are pivotal in the mechanism of graft prolongation.

MATERIALS AND METHODS

Cells

PIEC, PIEC-ICOS-Ig [5], or PIEC-IL-10 [7] cell lines were cultured in DMEM (Sigma-Aldrich, St Louis, USA) with 10% foetal bovine serum (Thermo Electron, Melbourne, Australia), 20 µg/ml glutamine (SAFC Biosciences, Lenexa, USA), 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich).

Mice

BALB/c, C57BL/6 or knockout mice were housed in Austin Health under specific pathogen-free conditions. BALB/c, *gld/gld* (B6.C3-Fas^lgld, C57BL/6 background) were obtained from WEHI; IFN γ ^{-/-} (C57BL/6 background), DERE^G (BALB/c Foxp3 DTR, BALB/c background), Prf^{-/-} (C57BL/6 background) and GzmB^{-/-} (B6.GzmBw, C57BL/6 background) mice were obtained from PMCI; IL-10^{-/-} (C57BL/6 background) mice were obtained from Robinson Research Institute, The University of Adelaide.

***In vivo* depletion of Foxp3⁺ Treg**

Foxp3⁺ Treg were depleted from DERE^G mice [10] by injection of 500ng Diphtheria toxin (DT) (List Biological Laboratories, Inc., Campbell, CA) and two days later mice were grafted as described below. Treg depletion was confirmed by analysis of peripheral blood, which typically yields 100% depletion

Antibodies

CD39 (PE-Cy7) (BD Pharmingen, San Jose, USA): CD3e (APC), CD4 (FITC), CD25 (APC), CD127 (PE-Cy7), Foxp3 (PE) and streptavidin-APC (eBiosciences, San Diego, USA): CD4-FITC, CD73 (FITC), CD45RA (FITC), CD45RB (APC), CD101 (APC), CD103 (FITC), CD137 (APC), CD275 (APC) CD278 (FITC), CD304 (APC), FR4 (APC), GARP (APC),

GITR (APC), Foxp3 (APC) and Helios (FITC) (Miltenyi Biotech Inc, Auburn, CA) were used for flow cytometry. For intracellular staining, cells were fixed and permeabilized using the FoxP3 Buffer Set (Miltenyi Biotech Inc).

Subcutaneous Grafts

PIEC, PIEC-ICOS-Ig, or PIEC-IL-10 cells were harvested, washed 3 times in sterile PBS and counted. Five x 10⁶ cells/50µl were injected subcutaneously into the abdomen. Grafts were measured daily with calipers, with rejection defined as when the graft could no longer be measured (<0.02mm²).

CD4⁺CD25⁺ T cell isolation

The Regulatory T Cell Isolation Kit (Miltenyi Biotech) was used to isolate CD4⁺25⁺ T cells from day 7 ICOS-Ig secreting grafts and spleen (either normal mice or mice with ICOS-Ig grafts). This kit uses negative isolation of CD4⁺ T cells, followed by positive isolation of CD25⁺ Treg. Single-cell suspensions were prepared, incubated with antibody followed by the addition of microbeads and separation using a MACS Separator as per the manufacturers instructions. Typically, one graft yields 5 x 10⁴ CD4⁺25⁺ T cells.

RNA preparation and Real time PCR

High quality total RNA was prepared from isolated CD4⁺25⁺ T cells using a Qiagen RNeasy Micro kit. The commercial Mouse T-cell Anergy and Immune Tolerance RT² Profiler PCR array (PAMM-074ZA) and RT²SYBR Green qPCR mastermix were used. Samples were analyzed on an Agilent Technologie Stratagene MX3000P instrument. Analytical screens, detecting 84 pathway-specific genes and appropriate RNA quality controls were performed using three independent RNA preparations from CD4⁺25⁺ T cells isolated from multiple day 7 ICOS-Ig secreting subcutaneous grafts (up to 20 per preparation), from CD4⁺25⁺ T cells isolated from splenocytes retrieved from the same grafted mice or naïve mice. To calculate fold regulation (represented in Fig. 4), firstly the average fold change ($2^{-\Delta\Delta Ct}$) was expressed as the normalized gene expression in the CD4⁺25⁺ T cells isolated from day 7 grafts divided by the normalized gene expression from CD4⁺25⁺ T cells isolated from the spleen of the naive mice. Fold regulation equals fold change for calculated values >1, whereas for values <1 fold regulation is the negative inverse of the fold-change. Several genes shown to be reproducibly upregulated in experimental vs control groups were subsequently analyzed by either flow cytometry and/or *in vivo* subcutaneous grafting experiments.

Inhibition of Delayed type hypersensitivity (DTH)

BALB/c mice were primed by intraperitoneal injection with 5×10^6 PIEC (xenogeneic antigen) or C57BL/6 spleen cells. Seven days after priming mice were challenged by 40 μ l subcutaneous injection of corresponding cell lysates into the hind footpads and swelling responses measured at 24 and 48hr [11]. Cell lysates were prepared by repeated freeze-thawing and sonication of 10^8 cells/ml.

Statistical Analysis

The Log-rank (Mantel-Cox) test was used to analyse the data to determine statistical significance, using GraphPad Prism (GraphPad Software Inc, La Jolla, USA).

RESULTS

Our previous studies demonstrated that secretion of locally expressed ICOS-Ig by porcine endothelial cell line (PIEC) xenografts had highly specific graft prolongation and showed perigraft cellular accumulation of CD4⁺CD25⁺Foxp3⁺ T cells (Treg phenotype), and an increase in interleukin-10 (IL-10) levels [5]. The mechanism of graft prolongation was investigated in detail.

Foxp3⁺ Treg are essential for ICOS-Ig dependent xenograft prolongation

DEREG mice (on both a BALB/c and C57BL/6 background) were used to examine the function of Foxp3⁺ Treg in ICOS-Ig induced graft survival. Without depleting the Treg in the DEREG mice with DT, the observed median survival time (MST) of ICOS-Ig secreting grafts was 28 days (Fig. 1A), which was the same as observed in wild type mice (Fig. 1B) and not significantly different to that previously reported [5]. In contrast, the observed MST following treatment with DT (administered 2 days before grafting) was significantly reduced to 13 days ($p=0.002$). This key observation not only confirms a central role for Foxp3⁺ Treg in our model but also demonstrates that graft prolongation is dependent on pre-existing Foxp3⁺ Treg. As controls, additional experiments were performed by treatment of wild type mice with DT, which had no effect on the survival of ICOS-Ig secreting grafts (Fig. 1B).

Role of IL-10 in ICOS-Ig induced graft prolongation

To test the hypothesis that IL-10 is a key functional molecule in the mechanism of ICOS-Ig induced xenograft prolongation, similar grafts were implanted into IL-10^{-/-} mice. No

significant increase in graft survival was observed with a MST of 15 days for PIEC-ICOS-Ig grafts and 14 days for PIEC (Fig. 2A). These data, when compared to our previous results showing significant graft survival in wild-type mice[5], demonstrate that IL-10 is required for ICOS-Ig induced xenograft prolongation.

Source of IL-10 is critical for xenograft survival

Although the above data supports the absolute requirement for IL-10 in the mechanism of ICOS-Ig induced xenograft prolongation, local expression of IL-10 alone is not sufficient to produce a similar effect. Compared to wild-type grafts, survival of PIEC-IL-10 grafts was significantly prolonged (MST 12 and 20 days respectively, $p < 0.05$) (Fig. 2B), however this was inferior to PIEC-ICOS-Ig grafts (MST 33 days). These data indicate that local expression of ICOS-Ig induces a specific temporal and spatial delivery of IL-10, and/or that additional mechanisms are involved.

IFN γ does not play a role in ICOS-Ig induced xenograft prolongation

IFN γ ^{-/-} mice were used to examine if this cytokine plays a role in xenograft prolongation (Fig 2C). In these knockouts the MST of control PIEC xenografts was 13 days, similar to that observed in wild type mice (MST 12 days, Fig. 2B). A prolongation of PIEC-ICOS-Ig xenografts was observed in the IFN γ ^{-/-} mice, with a MST of 29 days ($p = 0.003$). These data show that IFN γ is not involved in the mechanism of graft prolongation induced by ICOS-Ig.

Phenotype of graft infiltrating CD4⁺CD25⁺ T cells

CD4⁺CD25⁺ T cells were purified from day 7 ICOS-Ig secreting grafts as well as spleen from these mice and naïve spleen, and were analyzed and compared using a panel of Treg markers. We have attempted to purify and characterize CD4⁺CD25⁺ T cells infiltrating wild type grafts several times, but the recovery of these cells is extremely low as both the size of wild type grafts are smaller (55%) and the number of Foxp3⁺ cells are reduced (by 80%), and as such have used cells purified from naïve spleen as controls. Graft infiltrating CD4⁺CD25⁺ T cells expressed lower levels of Foxp3 than CD4⁺CD25⁺ T cells from naïve spleen (nTreg) (Fig. 3B), but similar levels of Helios (Fig. 3C). nTreg were CD127^{low} whereas graft infiltrating CD4⁺CD25⁺ T cells were CD127⁻ (Fig. 3D). Analysis showed that in addition to CD4 and CD25 (Fig. 3A), 10 other cell surface molecules were expressed on both nTreg and graft infiltrating CD4⁺CD25⁺ T cells (CD39, CD86, CD101, CD103, CD137, CD45RB, CD304,

FR4, GITR and GARP). Of the two major nucleotide metabolizing enzymes CD39 and CD73 examined, both nTreg and graft infiltrating CD4⁺CD25⁺ T cells expressed CD39 (Fig. 3E) with a larger proportion of graft infiltrating CD4⁺CD25⁺ T cells expressing this molecule at a higher level. In contrast CD73 was expressed exclusively on nTreg (Fig. 3F). CD45RA was not expressed on either cell type (Fig. 3G). High and intermediate expressing populations of CD45RB were observed with nTreg, whereas only an intermediate population for graft infiltrating CD4⁺CD25⁺ T cells (Fig. 3H). CD86 was expressed at low levels on both cells (Fig. 3J), whereas the other B7 costimulatory molecule CD80 was not detected (Fig. 3I). A modest increase in expression of CD101 (Fig. 3K), CD137 (TNFRSF9) (Fig. 3L) and GARP (Fig. 3M) was observed in graft infiltrating CD4⁺CD25⁺ T cells. Lower levels of CD304 (neuropilin) (Fig. 3N), GITR (Fig. 3O), and FR4 (Fig. 3P) were observed on graft infiltrating CD4⁺CD25⁺ T cells. CD103 was expressed on the majority of graft infiltrating CD4⁺CD25⁺ T cells but only on a subpopulation of nTreg (Fig. 3Q). MHC II (Fig. 3R) and CD278 (ICOS) (Fig. 3S) was only expressed on graft infiltrating CD4⁺CD25⁺ T cells (Fig. 3R). Our analysis also showed that CD275 (ICOSL) was expressed on both cell types, with graft infiltrating CD4⁺CD25⁺ T cells having increased expression (Fig. 3T). Moreover, identical expression profiles were seen from nTreg purified from naïve spleen and spleen cells isolated and purified from mice bearing ICOS-Ig secreting grafts.

Analysis of differential gene expression in graft infiltrating CD4⁺25⁺ T cells

The T cell anergy and tolerance kit (SABiosciences) was used to compare gene expression from CD4⁺25⁺ T cells isolated from multiple day 7 ICOS-Ig secreting grafts with CD4⁺25⁺ T cells isolated from splenocytes of naïve mice. Overall, of the 84 genes in the array, mRNA from 37 (44%) were up regulated more than 2.0 fold, of these 23 (27%) were up regulated more than 5.0 fold, with 20 (24%) being up-regulated more than 9 fold. In contrast, 6 genes (7%) were down regulated more than 2.0 fold and of these only 2 (2%) were down regulated more than 5.0 fold.

If we further breakdown the 84 genes into broad functional groups: cell surface molecules, cytokines, transcription factors and other, a more detailed picture of the differences between CD4⁺25⁺ T cells isolated from multiple day 7 ICOS-Ig secreting grafts and those isolated from naïve mice can be observed. Of the 30 cell surface molecules, mRNA from 3 were up-regulated more than 9 fold; CD278 (ICOS), CD137 (TNFRSF9), CD49A (Integrin 1alpha), and 7 were up regulated more than 2 fold; CD40, ICAM1, CD30 (TNFRSF8), CD210 (IL10R alpha), CD95-L (FasL, TNFRSF6), GITR (TNFRSF18) and

CD134 (TNFRSF4), (Fig. 4A). The mRNA for only one gene was down regulated, Btla (ligand for TNFRSF14) (Fig. 4A). Consistent with these data was the observed increase in the cell surface expression of both CD278 from ICOS-Ig graft infiltrating CD4⁺25⁺ T cells in the phenotypic analysis described above (Fig. 3).

Of the 16 cytokines represented in the array, mRNA from 13 were up-regulated. 6 were up-regulated greater than 100 fold; IL13, Ccl3, IL1a, IL17a, IL6, IL5, 4 were up-regulated more than 10 fold: IL4, Csf2, IL15, IL10, and 3 more than 5 fold; IFN γ , Csf1 and IL31 (Fig. 4B). The observed increase in IL10 is consistent with our previously published data[5]. Only a single cytokine, Lymphotoxin A was down regulated more than 2 fold (Fig. 4B).

Of the 15 transcription factors, mRNA from 1 was up-regulated more than 10 fold; Nhlh27, 6 were upregulated more than 2 fold: cFos, Stat3, Foxp3, Tbet, Irf4, Foxp2 and two were down regulated more than 2 fold: Stat6 and Hdac9 (Fig. 4C).

Of the other genes, Prostaglandin-endoperoxide synthase 2 (Ptgs2, Cox-2) mRNA was the highest up regulated gene (4500 fold). Interestingly, the granule-bound serine protease Granzyme B (Gzmb) and the pore-forming protein perforin (Prf1), which together constitute one of the mechanisms of Treg action (induction of target cell death through apoptosis), were found to be significantly up-regulated 290 and 9-fold respectively. A greater than 100 fold increase was also seen with Chymase 1 (Cma1), the ubiquitin protein ligases Mac-2 (Lgals3) and Leptin (Lep) were up-regulated more than 10 fold, the other ubiquitin ligase, GRAIL (Rnf128) was increased by more than 2 fold (Fig. 4D). Whereas a greater than 2 fold down regulation was seen for Diacylglycerol kinase alpha and zeta.

A fold change of less than 2 was observed for the 2 Cyclin-dependent kinases (Cdk2 and Cdk4), 3 protein kinases (Jak1, Jak3 and Prkcg) or the 6 nuclear factors (Ing4, Nfatc1, Nfatc2, Nfatc3, Nfkb1 and Mef2a).

A role for apoptosis in ICOS-Ig induced graft prolongation

To evaluate the significance of the observed increase in mRNA expression for both perforin and granzyme B in the gene array data described above, ICOS-Ig PIEC and PIEC were grafted into gene-disrupted mice null for the expression of Pfr or GzmB, and graft survival assessed.

No significant increase in graft survival was observed in GzmB knockout mice with the MST of 19 days for PIEC-ICOS-Ig grafts and 20 days for PIEC (Fig. 5A). Similarly, no

significant increase in graft survival was observed in Prf-deficient mice with the MST of 19 days for PIEC-ICOS-Ig grafts and 20 days for PIEC (Fig. 5B). These data demonstrate that both Prf and GzmB are required for ICOS induced xenograft prolongation and are consistent with both pro-apoptotic mediators cooperating to mediate granule-mediated cytotoxicity.

It has been shown that nTregs have the capacity to kill CD8⁺ T cells via FasL-Fas interactions [12]. The role of the Fas/FasL pathway in mediating the ICOS-Ig dependent Treg xenograft prolongation was examined by grafting *gld/gld* mice. These mice are FasL^{-/-}, therefore are unable to kill target cells via this pathway. In these mice the MST of control PIEC xenografts was 17 days (Fig. 5C), not significantly different to that observed in wild type mice (MST 13 days). A prolongation of PIEC-ICOS-Ig xenografts was observed in the *gld/gld* mice, with a MST of 25 days ($p=0.003$) (Fig 5C). Survival of PIEC-ICOS-Ig xenografts in *gld/gld* mice is also reduced compared to survival in C57BL/6 (MST 28 days) mice ($p=0.04$). Therefore, interpretation of the role of FasL in this model is difficult as this pathway could be involved in both graft destruction and graft protection.

CD4⁺25⁺ T cells purified from ICOS-Ig secreting grafts specifically suppress primed T cells *in vivo*

While numerous *in vitro* tests have been developed to assess Treg activity, these do not necessarily reflect the complex *in vivo* microenvironment that may influence Treg function; therefore assessing Treg function *in vivo* is more physiologically relevant. CD4⁺ T cells mediate both DTH and graft rejection and it is considered that these are two different manifestations of the same response [13], therefore DTH represents an excellent model to assay immune responses to alloantigen and xenoantigen. CD4⁺25⁺ T cells were purified from ICOS-Ig secreting grafts and normal spleen, and tested for their ability to suppress xenogeneic and allogeneic primed CD4 T cells *in vivo*. An 80% inhibition of mean change in footpad swelling was observed when antigen was coinjected with purified CD4⁺25⁺ T cells isolated from ICOS-Ig secreting grafts in xenogeneic primed mice ($p=0.003$), in contrast no inhibition was seen in allogeneic primed mice (Fig. 6A). Inhibition of neither xenogeneic nor allogeneic primed mice was observed when antigen was coinjected with CD4⁺CD25⁺ T cells isolated from spleen of naïve mice (Fig. 6B).

DISCUSSION

The literature describes varying characteristics of Tregs including phenotypic markers, nomenclature and biological role of Treg, and contributes to the rapidly changing

transplantation field [14]. The heterogeneity of Treg described in the literature include CD4⁺CD25⁺Foxp3⁺ T cells, CD4⁺Tr1 and Th3 cells characterised by IL-10 and TGFβ production respectively, δγT cells, CD8⁺ T cells, CD8/4 double negative cells and NKT cells [1]. Our studies have focused on CD4⁺CD25⁺Foxp3⁺ Treg, as this phenotype was observed at the graft site of ICOS-Ig secreting grafts[5], and indeed is currently one of the strategies using T cell therapies in clinical trials of transplantation [1].

CD4⁺CD25⁺Foxp3⁺ Treg may suppress both CD4⁺ and CD8⁺ T cells either specifically or non-specifically, and their importance in allotransplantation has been well characterized [1]. For suppressors of alloreactivity, the data indicate that antigen-specific Treg acquire their activity by activation via DC expressing the relevant antigens [15]. Furthermore, *in vivo*, this involves processing and presentation of donor antigens via the indirect pathway by host-derived Antigen-Presenting Cells (APCs). CD4⁺CD25⁺Foxp3⁺ Treg either originate from thymus in response to self-antigens (nTreg) or in the periphery in response to foreign antigens (iTreg) [16,17]. The latter originate from CD4⁺ T cells and are induced to express Foxp3 [16,17]. Expansion of alloantigen-specific Treg from nTreg *in vitro* that suppressed rejection *in vivo* has been reported [18-20]. Our data shows that graft prolongation is dependant on a pre-existing Foxp3⁺ Treg (Fig. 1), but does not exclude a contribution of iTreg. Adoptive transfer experiments using relevant purified cell populations into RAG^{-/-} mice form part of our future studies to delineate that relative contribution of nTreg and iTreg in ICOS-Ig induced graft prolongation.

IL-10 has been associated with both induction, and as a suppressor mechanism of certain Treg. Both natural and expanded Treg (CD4⁺CD25⁺ or CD4⁺CD25⁺Foxp3⁺) have been shown to suppress human xenogeneic responses to porcine cells *in vitro* (reviewed in [21]) with IL-10 playing a pivotal role [21-23]. Our data demonstrates that IL-10 is required for ICOS induced xenograft prolongation (Fig. 2), however whether IL-10 is specifically involved in the activation and/or function of the xenospecific Treg is unclear. Future studies using conditional knockout mice or adoptive transfer experiments are required to determine the precise source of IL-10.

The phenotype of graft infiltrating CD4⁺CD25⁺ T cells is consistent with an activated antigen specific Treg: activated Treg show increased expression of CD137 [24] and GARP [25], in addition antigen specific activated Treg show increased CD101 [26], CD103 [27,28] and lower expression of CD45RB [29]. The major phenotypic differences we observed between nTreg and graft infiltrating CD4⁺CD25⁺ T cells is that CD73 is expressed

exclusively on nTreg (Fig. 3) and CD278 (ICOS) and MHC II was only expressed on graft infiltrating CD4⁺CD25⁺ T cells (Fig. 3). In addition, CD275 (ICOSL) was expressed on both cell types, with graft infiltrating having greater expression (Fig. 3). CD39 and CD73 are nucleotide metabolizing enzymes expressed on the cell surface membrane of many cells that metabolizes ATP/ADP to AMP (CD39) and then to adenosine (CD73). In the mouse, Treg can co-express both molecules with production of adenosine contributing to suppression [30,31]. Although CD4⁺CD25⁺CD39⁺CD73⁻ T cells have been described in humans [32], to our knowledge this is the first report of such cells in the mouse.

ICOS induced costimulation has been shown to increase proliferation of Foxp3⁺ cells and secretion of IL-10 *in vitro* [33]. *In vivo*, ICOS costimulation is associated with IL-10 producing CD4⁺CD25⁺ Treg in autoimmune diabetes [34]. Furthermore, the importance of the ICOS/ICOSL mediated costimulation pathway in Treg is highlighted by the fact that ICOS^{-/-} mice have decreased Foxp3⁺ Treg numbers and impaired suppressive function *in vitro* [35]. ICOSL^{-/-} mice also have decreased numbers of Foxp3⁺ Treg and reduced IL-10 production [35]. ICOS may also be important in the generation of Treg. In allergen-induced airway hyper-reactivity ICOS:ICOSL interaction is critical for the development of antigen specific Treg [36]. ICOS has also been shown to mediate the generation and suppressive function of CD4⁺CD25⁺ Foxp3⁺ Treg that mediate respiratory tolerance [37]. Furthermore, ICOS is a marker for highly suppressive antigen-specific CD4⁺CD25⁺ Foxp3⁺ Treg in a model of contact hypersensitivity [38].

In humans, a functionally distinct Treg population involved in contact-dependent *in vitro* suppression has been shown to express MHC II by Tregs [39], to our knowledge this is the first report of MHC II expression on Treg in the mouse.

Our observation of increased ICOSL expression on CD4⁺CD25⁺ T cells infiltrating ICOS-Ig grafts (Fig. 3) is consistent with the report that a subpopulation of T cells express ICOSL [40]. Soluble ICOS-Ig can deliver an immunogenic signal by crosslinking ICOSL on APC and promote an increase in phagocytosis and antigen-presentation, in addition to an increase in MHC II expression [41].

The mechanism for the prolonged allograft survival mediated by anti-ICOS antibody and ICOS-Ig fusion protein is considered to be suppression of intragraft T cell activation and cytokine expression [42]. A more intriguing hypothesis is that the crosslinking of ICOSL on the Treg delivers a reverse signal that activates/modulates this cell. In keeping with this hypothesis is the observed increase in the expression of both ICOSL and MHC II on CD4⁺CD25⁺ T cells infiltrating ICOS-Ig grafts (Fig. 3).

Killing of target cells via Perforin/Granzyme B induced apoptosis is a mechanism used by both human and mouse Treg [43]. In addition, a Perforin-independent, Granzyme B-dependent mechanism has also been reported in mice [44]. Our data shows that both Perforin and Granzyme B are components of the suppressor mechanism of the soluble ICOS-Ig dependent antigen specific Treg (Fig. 4), although it is unclear if the Treg kill directly or act indirectly via other cytotoxic cells. The functional role of Galectin 3 is also unclear but may have a role in promoting cell-to-cell adhesion by Tregs to activated T cells and/or promote apoptosis if expressed in the extracellular space, however intracellular Galectin 3 has been shown to promote cell survival and may aid in prolonging Treg function[45,46]. The role of GRAIL (mfl28), E3 ubiquitin ligase, is also interesting as it potentially induces anergy in activated T cells through the inhibition of activation induced IL2 and IL4[47]. Future investigations to define the involvement of these molecules will include adoptive transfer experiments using knockout mice and are part of our future studies.

This study shows graft protection by local secretion of ICOS-Ig is feasible and effective. However, the strategy requires refinement since graft modification results in prolongation but not indefinite survival. To achieve this, one possibility is the addition of low dose immunosuppression using of drugs that allow expansion of Tregs while inhibiting pathogenic T cells (eg mTOR inhibitors).

In summary, this study significantly extends our previous observation that locally expressed ICOS-Ig induces a perigraft cellular accumulation of CD4⁺CD25⁺Foxp3⁺ T cells and specific graft prolongation [5] to conclusively show requirement of pre-existing Foxp3⁺ Treg, IL-10, Prf and GzmB. Purified CD4⁺CD25⁺ T cells from ICOS-Ig grafts have a phenotype consistent with nTreg, but with increased expression of CD275 (ICOSL), expression of CD278 (ICOS) and MHC II and loss of CD73. Furthermore these cells suppress a DTH response mediated by primed T cells with the same specificity as that reported for graft prolongation. Ongoing studies are directed towards identifying the cell source of each biological mediator (IL-10, Prf and GzmB).

AUTHORS' CONTRIBUTIONS

DC, RH, VRS, JAT, FLI and MSS participated in research design; DC, RH, VRS, JAT, FLI and MSS participated in the writing of the paper; DC, EM, RH, VRS, FLI and MSS participated in the performance of the research; DC, VRS, JAT, FLI and MSS participated in data analysis.

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FIGURE LEGENDS

FIGURE 1. Prolonged survival of ICOS-Ig secreting xenografts requires pre-existing Foxp3⁺ cells. Kaplan Meier survival plots of (A) PIEC-ICOS-Ig in C57BL/6 DREG mice with DT treatment were significantly reduced (n=5, —) (p=0.002) compared to PBS control (n=5, ---), and PIEC alone in C57BL/6 DREG mice (n=5,). (B) PIEC-ICOS-Ig in C57BL/6 mice with DT treatment (n=5, —) or PBS control (n=5, ---), and PIEC alone in C57BL/6 mice (n=5,). Data in each panel are representative of 3 independent experiments.

FIGURE 2. Prolonged survival of ICOS-Ig secreting xenografts requires IL-10 but not Interferon- γ . Kaplan Meier survival plots of (A) PIEC-ICOS-Ig (n=5, —) or PIEC (n=5, ---), in IL-10^{-/-} mice; (B) PIEC-ICOS-Ig (n=5, —), PIEC-IL-10 (n=5, ---) or PIEC (n=5, ----), in BALB/c mice and (C) PIEC-ICOS-Ig (n=5, —) or PIEC (n=5, ---), in IFN γ ^{-/-} mice. Data in each panel are representative of 3 independent experiments. Compared to wild-type grafts, survival of PIEC-IL-10 grafts was significantly prolonged (MST 12 and 20 days respectively, p<0.05). Prolongation of PIEC-ICOS-Ig xenografts was observed in the IFN γ ^{-/-} mice, with a MST of 29 days (p=0.003).

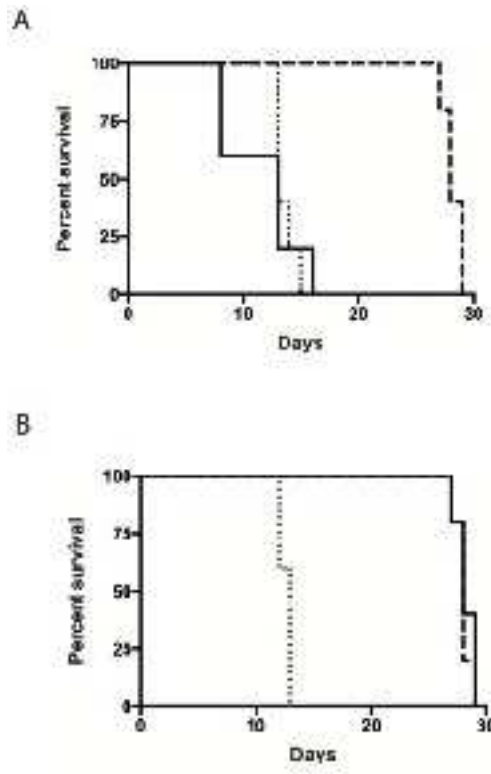
FIGURE 3. Cytofluorographic analysis of CD4⁺25⁺ T cells. Left panels of each pair represents day 7 ICOS-Ig graft infiltrating CD4⁺25⁺ T cells and the right panels CD4⁺25⁺ T cells isolated from spleen; panels B-T: solid line specific antibody, dotted line isotype control. (A) Contour plot of CD4 vs CD25; (B) Foxp3; (C) Helios; (D) CD127; (E) CD39; (F) CD73; (G) CD45RA; (H) CD45RB; (I) CD80; (J) CD86; (K) CD101; (L) CD137; (M) GARP; (N) CD304; (O) GITR; (P) FR4; (Q) CD103; (R) MHC II; (S) CD278; (T) CD275. Data are representative of 3 independent experiments.

FIGURE 4. Comparative gene expression from CD4⁺25⁺ T cell mRNA. Fold regulation of day 7 ICOS-Ig graft infiltrating CD4⁺25⁺ T cells compared to CD4⁺25⁺ T cells isolated from naïve spleen. Genes grouped into: (A) Cell surface; (B) Cytokines; (C) Transcription factors and (D) Other.

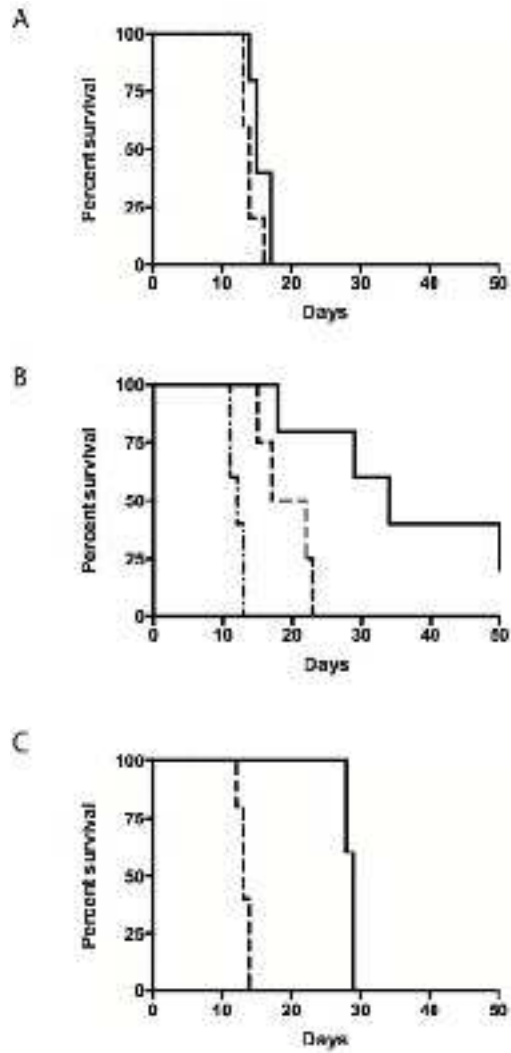
FIGURE 5. Prolonged survival of ICOS-Ig secreting xenografts requires perforin and granzyme B but not Fas/FasL. Kaplan Meier survival plots of (A) PIEC-ICOS-Ig (n=5, —)

or PIEC (n=5, ---), in *GzmB*^{-/-} mice; (B) PIEC-ICOS-Ig (n=5, —), PIEC-IL-10 (n=5, ---) or PIEC (n=5, ---), in *Prf*^{-/-} mice and (C) PIEC-ICOS-Ig (n=5, —) or PIEC (n=5, ---), in *FasL*^{-/-} mice. Data in each panel are representative of 3 independent experiments. Graft survival was not significantly increased in *GzmB* knockout mice with the MST of 19 days for PIEC-ICOS-Ig grafts compared to 20 days for PIEC. Similarly, no significant increase in graft survival was observed in *Prf*-deficient mice with the MST of 19 days for PIEC-ICOS-Ig grafts compared to 20 days for PIEC. Using *gld/gld* mice (*FasL*^{-/-}) the MST of control PIEC xenografts (17 days) was not significantly different to that observed in wild type mice (MST 13 days). Prolongation of PIEC-ICOS-Ig xenografts was observed in the *gld/gld* mice, with a MST of 25 days (p=0.003). Graft survival of PIEC-ICOS-Ig xenografts in *gld/gld* mice is also reduced compared to survival in C57BL/6 (MST 28 days) mice (p=0.04).

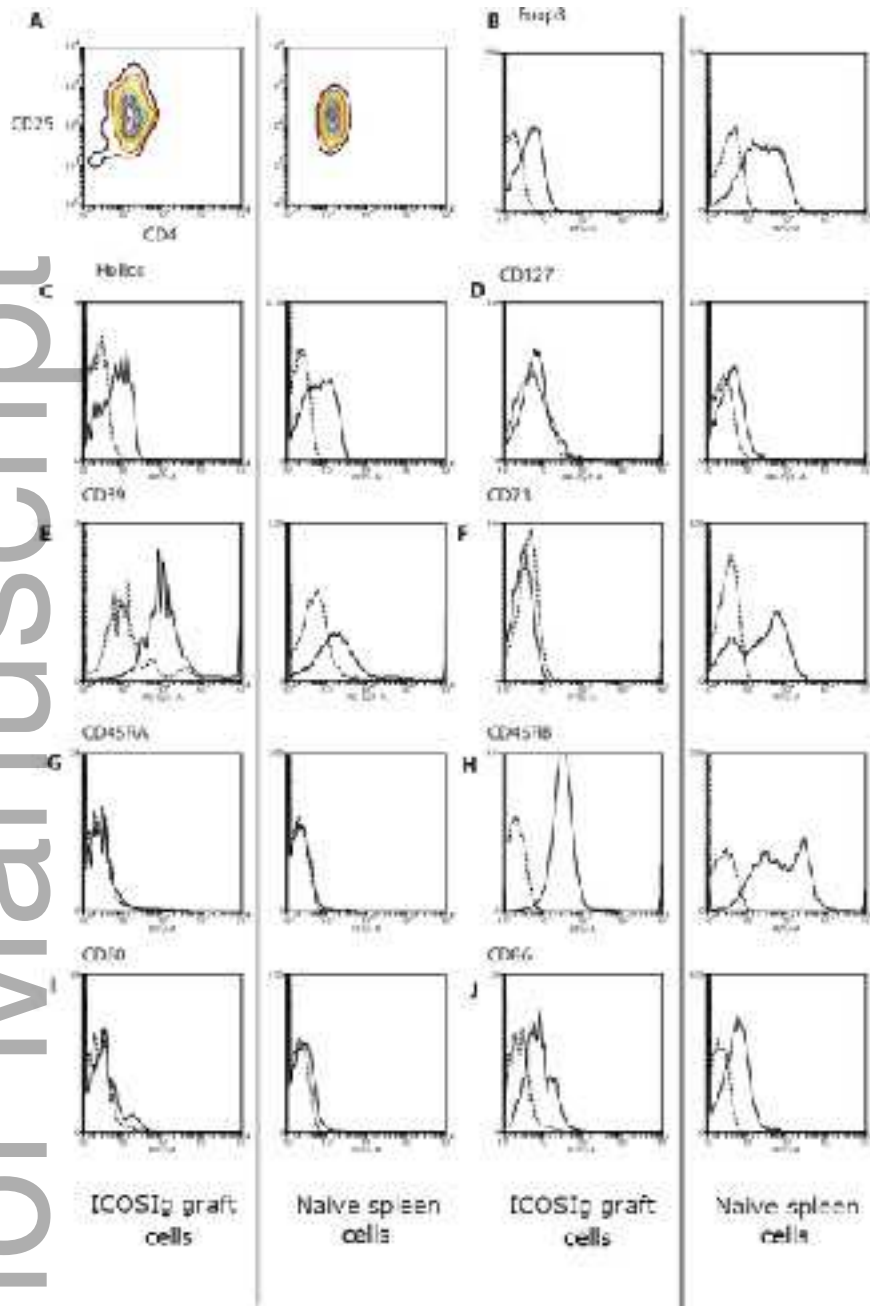
FIGURE 6. Specific inhibition of DTH by purified graft infiltrating CD4⁺25⁺ T cells. Seven days after priming BALB/c mice (n=3 per group), DTH (foot pad swelling) was measured at 24hr from mice challenged with antigen mixed with or without CD4⁺25⁺ T cells, purified from (A) day 7 ICOS-Ig graft infiltrating cells; (B) spleens from naïve mice. For all panels, the right footpads received 10µg of cell lysate alone (white bars) and the left footpads received either PBS or 10µg of appropriate cell lysate together with 7x10⁴ purified CD4⁺25⁺ T cells (Treg) (black bars). Data are representative of 2 independent experiments.



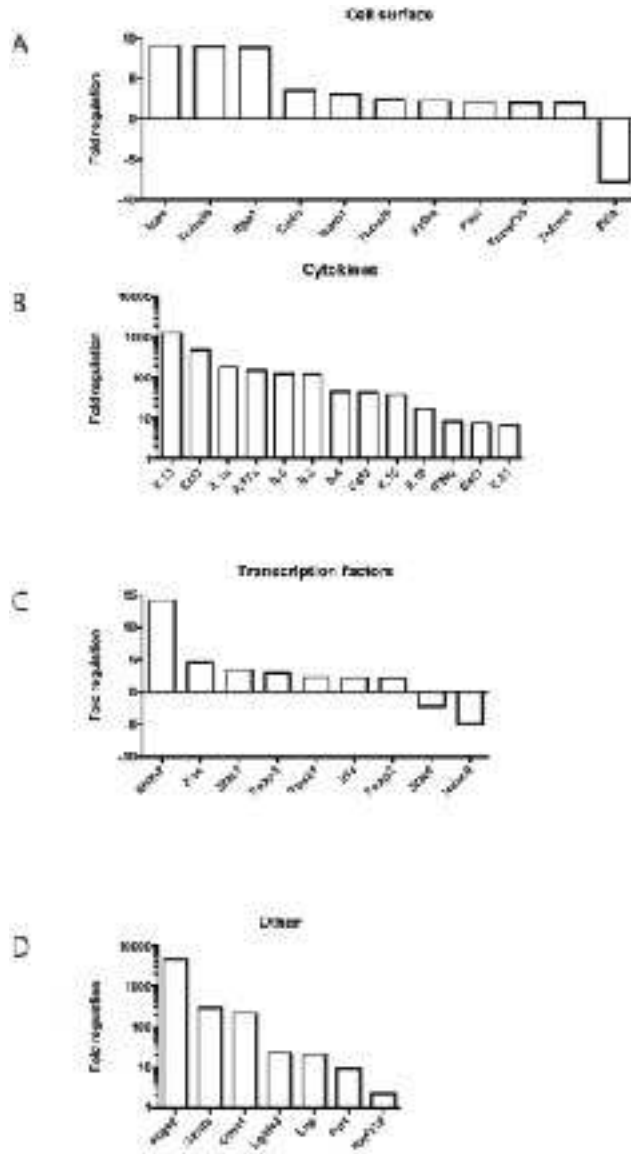
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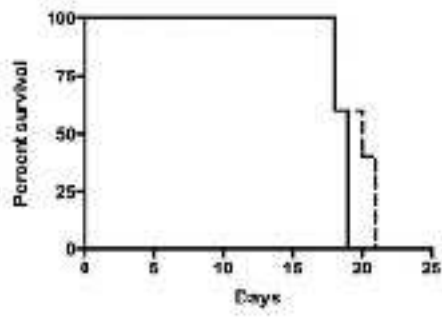


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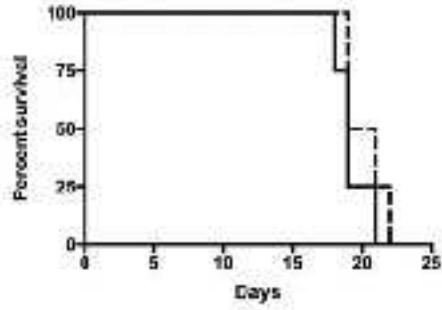


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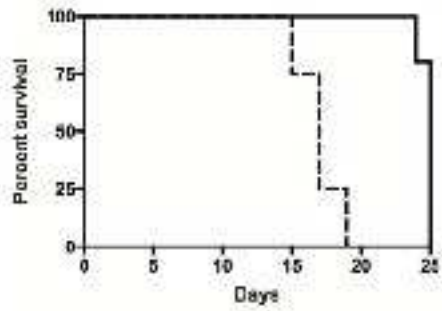
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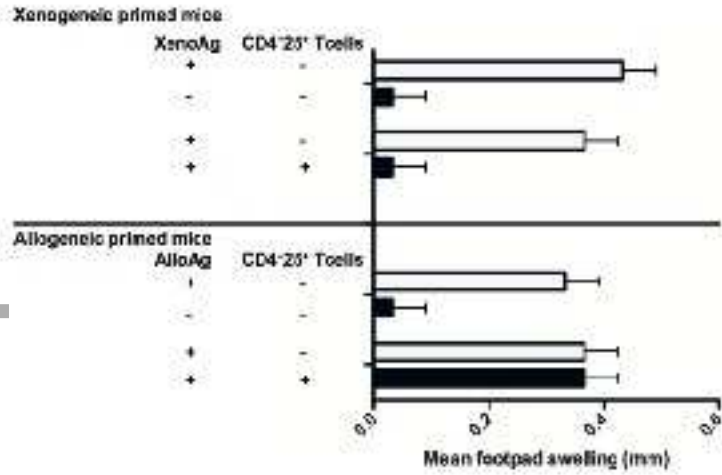


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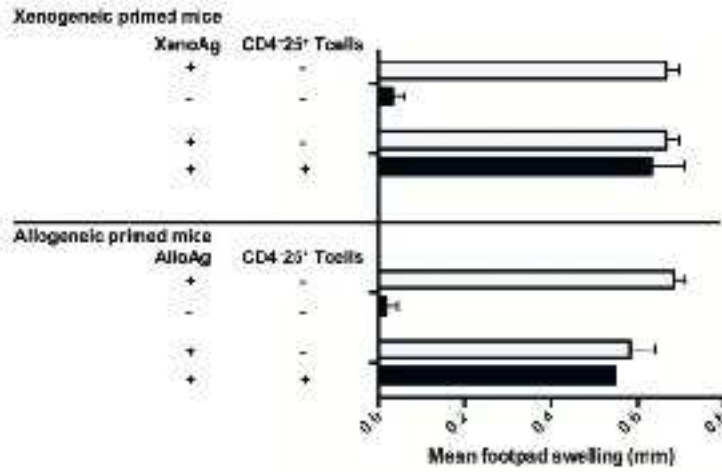


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A. CD4⁺25⁺ T cells isolated from ICOS-Ig grafts



B. CD4⁺25⁺ T cells isolated from spleens of naïve mice



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