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ORIGINAL ARTICLE

CD8$^+$ $\gamma\delta$ T regulatory cells mediate kidney allograft prolongation after oral exposure to alloantigen

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Summary

The long term goal of immunological therapy for transplantation is to induce antigen specific unresponsiveness. One approach of significant current interest is the induction of T regulatory (Treg) cells that downregulate immune responses in an antigen specific manner. In this study, we examined the nature of the immunological regulation initiated by oral exposure to alloantigen. We previously demonstrated that feeding of allogeneic donor splenocytes significantly prolongs kidney allograft survival in rats. Purified CD8$^+$ graft infiltrating cells (GIC), but not CD4$^+$ GIC transfer graft prolongation to naïve animals demonstrating the presence of a CD8$^+$ Treg population in the graft. In this study, we provide evidence that is consistent with a hypothesis that the CD8$^+$ Treg generated by oral exposure to alloantigen is an IL-10 secreting, $\gamma\delta$ TCR$^+$ T cell.

Introduction

The long term goal of immunological therapy for transplantation is to induce antigen specific unresponsiveness, thus freeing the patient from the side effects of nonspecific immunosuppressive drugs. One approach of significant current interest is the induction of T regulatory (Treg) cells that downregulate immune responses in an antigen-specific manner. These cells are of clinical importance as there is evidence that Treg cells play a role in the regulation rejection responses in the clinical setting. For example, increased numbers of Treg cells have been recorded in rejection-free patients [1], whereas a lack of Treg cells in patients is often associated with early acute rejection episodes [1,2]. In animal models, Treg cells have been implicated in long-term allograft survival and several phenotypes of Treg cells have been demonstrated in transplantation tolerance [3,4]. The most extensively studied Treg cells are CD4$^+$ CD25$^+$ T cells that naturally occur in humans and rodents [5,6]. CD8$^+$ and double negative (CD4$^-$CD8$^-$) populations of Treg cells have also been described [4,7,8]. Recently, attention has turned to $\gamma\delta$ TCR$^+$ T cells as a potential source for Treg activity [9–11]. These relatively rare non $\alpha\beta$ TCR cells play a variety of roles in immune activation and effector function.

In this study, we examined the nature of the immunological regulation initiated by oral exposure to alloantigen. We previously demonstrated that feeding of allogeneic donor splenocytes significantly prolongs kidney allograft survival in rats [12]. This prolongation is characterized by an influx of CD8$^+$ T cells into the surviving grafts. These CD8$^+$ T cells not only express cytotoxic mediators (Granzyme and FasL) but also express cytokines (IL-4 and TGF-β) linked to T regulatory cell activity. Graft prolongation can be transferred to naïve animals by transferring purified CD8$^+$ graft infiltrating cells (GIC), but not CD4$^+$ GIC, suggesting the presence of a CD8$^+$ (but not CD4$^+$) Treg cell population in the graft [13]. In this study, we examined the phenotype of CD8$^+$ Treg cells that mediate graft prolongation in our experimental model. Our data are consistent with a

hypothesis that the CD8$^+$ Treg generated by oral exposure to alloantigen is an IL-10 secreting, $\gamma\delta$ TCR$^+$ T cell.

Materials and methods

Animals

Male Brown Norway (BN, RT1^n) and Lewis rats (LEW, RT1^1) purchased from Harlan Sprague Dawley (Indianapolis, IN, USA) were used as donors and recipients, respectively. This strain combination is fully disparate at both major and minor histocompatibility complex loci. Animals were maintained in the Dalhousie University Faculty of Medicine animal care facility and provided with water and rodent chow *ad libitum*. All animal experimentation was undertaken in compliance with the guidelines of the Canadian Council on Animal Care.

Single cell suspensions

Lymphocytes were isolated from the spleen and single cell suspensions prepared following standard protocols (as we have described previously, [13]). Cells were prepared, washed and used for *in vitro* experiments in RPMI 1640 medium (Gibco BRL, Life Technologies, Burlington, ON, Canada) supplemented with 20 mM HEPES (USB, Cleveland, OH, USA), 100 U/ml Penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (Gibco BRL). Spleen cells were purged of red blood cells (RBC) by lysis for 5 min with ACK erythrocyte lysing buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4). After lysis of RBC, spleen cells were washed in RPMI.

Oral exposure to alloantigen

Lewis rats were fed without anesthetic by intragastric intubation using a 3 1/2 Fr premature human infant feeding tube (Sherwood Industries, St Louis, MO, USA). All rats were fed 1×10^8 BN splenocytes suspended in 300 μl phosphate buffered saline (PBS; 0.15 M NaCl, 0.05 M Na_2PO4, pH 7.4) on days −14, −13, −12, −11, −10, and −1, with the day of kidney transplantation being day 0. In the sham fed and unfed control groups, Lewis rats were fed with 300 μl PBS or were not fed, respectively.

Kidney transplantation

Kidney transplantation was performed as described previously [13]. Briefly, BN rats were anaesthetized with sodium pentobarbital. After ligation of the abdominal aorta and vena cava proximal and distal to the renal artery and vein, the left kidney was perfused *in situ* with cold (4 °C) heparinized 0.9% saline and removed with a cuff of the aorta and the renal vein, as well as a long piece of the ureter. The donor kidney was kept in cold saline during the preparation of the Lewis recipient. Following left nephrectomy of the recipient, the donor kidney was transplanted in an orthotopic position by end to side anastomosis of the cuff of renal artery to the abdominal aorta, and the renal vein was connected to the inferior vena cava. The ureter was attached by end to end anastomosis. Three days after the transplant, the right native kidney was removed, leaving rat survival dependent on the transplanted kidney.

Isolation of graft infiltrating cells

Five days after kidney transplant, the grafts were flushed *in situ* with heparinized (100 U/ml) 0.9% saline and were removed from Lewis rats. The grafts were minced and digested with a 50 μg/ml solution of a broad spectrum collagenase (from Clostridium; catalogue number 17018-029, Gibco BRL). To clear the debris including dead tubular epithelial cells, the cell suspensions were rapidly passed down a loosely packed glass wool column (300 mg sterile glass wool in a 10 ml syringe), then mixed with isotonic Percoll (Pharmacia, Piscatawa, NJ, USA) to a concentration of 30% Percoll and centrifuged at 460 *g* for 20 min at 10 °C. The pellet was resuspended in RPMI, then loaded on top of a cushion of 70% Percoll (in RPMI) and centrifuged as above. The cells overlying the cushion were removed for GIC examination.

Purification of CD8$^+$

CD8$^+$ T cells were positively selected from GIC or splenocytes by MACS columns using anti-CD8 magnetic microbeads (Miltenyi Biotech, Auburn, CA, USA) according to manufacturer's recommendations. Purity of the CD8$^+$ cell populations was verified by flow cytometric analysis using a monoclonal antibody to CD8a (BD Pharmingen, Mississauga, ON, Canada). We showed that the purity of CD8$^+$ cell from total GIC is greater than 95% and the purity of CD8$^+$ cell from total isolated splenocytes is greater than 97% (data not shown).

Flow cytometry

CD8$^+$ GIC or splenocytes were incubated with 10% heat inactivated normal rat serum in FACS buffer (1% heat inactivated fetal bovine serum, 0.09% NaN_3 in BPS) for 20 min on ice (4 °C) to prevent nonspecific antibody binding by blocking Fc receptors. Then the cell suspensions were centrifuged and re-suspended in FACS buffer (1×10^6cells/100 μl) and incubated with the following monoclonal antibodies: OX-8 PerCP-

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conjugated anti-rat CD8a (BD Biosciences, Mississauga, ON, Canada); R73 FITC-conjugated anti-rat αβ TCR (BD Biosciences, Raleigh, NC, USA); V65 FITC-conjugated anti-rat γδ TCR (AbD Serotec); OX-39 FITC-conjugated anti-rat CD25 (CALTAG™ distributed by Invitrogen, Burlington, ON, Canada); JJ319 FITC-conjugated anti-rat CD28 (AbD Serotec) at a concentration of 10 μg/ml in FACS. After incubation, the cells were washed twice with FACS buffer and fixed with 1% Formalin in FACS buffer and examined on a BD-FACSCalibur. WinList™ 5.0 (Verity Software House, Inc. Topsham, ME, USA) was used to analyze the fluorescence data. Gates were set on the lymphocyte population relative to the appropriate isotype controls.

Intracellular cytokine staining

CD8$^+$ GIC isolated from alloantigen pre-fed animals and nonfed control animals 5 days post kidney transplant were analyzed for expression of the cytokines IL-4 or IL-10 by intracellular flow cytometric analysis according to the protocol provided in the BD Biosciences Cytofix/Cytoperm™ Plus Fixation/Permeabilization Kit. Briefly, CD8$^+$ GIC were stimulated with immobilized anti-CD3 (25 μg/ml, clone: G4.18, BD Biosciences) and soluble anti-CD28 (2 μg/ml, clone: JJ319, BD Biosciences) antibodies in the presence of a protein transport inhibitor (BD GolgiStop™, BD Biosciences) for 5 h at 37 °C. Cells obtained from culture were incubated with 10% normal rat serum in FACS buffer for 20 min on ice (4 °C) to block Fc receptors for prevention of nonspecific antibody binding. Cells were then fixed and permeabilized by 20 min incubation at 4 °C in BD Fixation/Permeabilization solution (1×10^6 cells/250 μl) in 5 ml polystyrene round-bottom tubes. Cells were washed twice in 1x BD Perm/Wash™ (BD Biosciences) buffer (1 ml/tube) prior to staining with fluorescent antibodies.

For intracellular cytokine staining, 1×10^6 cells were incubated with PE-conjugated mouse anti-rat IL-4 (OX-81) or anti-rat IL-10 (A5-4) monoclonal antibody or the appropriate isotype control (BD Biosciences) in 50 μl of BD Perm/Wash™ buffer at 4 °C for 30 min in the dark. After antibody incubation, cells were washed twice with 1x BD Perm/Wash™ buffer and stored at 4 °C until flow cytometric analysis. Rick-2 cells (Rat intracellular cytokine positive control cells, BD Biosciences) were used as positive cytokine-secreting cell controls for initial antibody titration.

Cell transfer experiments

Cell transfer experiments were performed by intravenous transfer of whole GIC, of CD8$^+$ GIC, or of GIC depleted or enriched for γδ cells. Cells isolated from one kidney graft were transferred into naïve Lewis rats that had received a kidney transplant 1 day before the GIC injection. γδ cells were depleted or enriched from whole GIC populations by MACS depletion (LD) or separation (LS) columns, respectively, using anti-γδ TCR magnetic microbeads (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's recommendations. In the depletion columns, γδ cells were depleted using the anti-γδ TCR magnetic microbeads. Enrichment was achieved by positive selection using the same bead technology. Cell transfer experiments were also performed by adoptive transfer of 2×10^8 splenocytes from fed and unfed control animals into naïve Lewis rats that had received a kidney transplant 1 day after injection. Three days after kidney transplantation, the recipients native right kidney was removed, leaving animal survival dependent only on the transplanted kidney.

Results

Graft survival after transfer of CD8$^+$ graft infiltrating cells

We previously demonstrated that oral administration of alloantigen prolongs kidney allograft survival. This prolongation can be transferred to syngeneic naïve animals by the transfer of CD8$^+$ T cells but not CD4$^+$ T cells isolated from the grafts of fed animals. Third party experiments confirmed that this is an antigen specific phenomenon [13]. In our previous experiments, CD8$^+$ and CD4$^+$ cells were obtained by negative selection on immunoaffinity columns. In this study, we confirmed the role of CD8$^+$ T cells in graft prolongation using positive selection from total GIC by magnetic selection on MACS columns. As shown in Table 1, the mean survival of the grafts in the control animals ($n = 4$) was 9.5 days with the longest survival being 12 days. In contrast, the mean survival of kidneys in the animals receiving CD8$^+$ GIC Treg cells ($n = 3$) was 38.7 days with the longest survival being 65 days. These data are consistent with our previous observations and demonstrate a significant ($P = 0.019$) improvement in graft survival in these naive rats by the transfer of CD8$^+$ Treg cells. These data confirm that a population of CD8$^+$ intra-graft regulatory T cells are present in the kidney allografts of animals orally exposed to alloantigen.

Table 1. Graft survival after transfer of CD8$^+$ GIC from grafts of fed and unfed control rats.

Cell transfer	Survival days	Mean
CD8$^+$ GIC from grafts of control rats	8, 9, 9, 12	9.5
CD8$^+$ GIC from grafts of fed rats	17, 29, 65	38.7

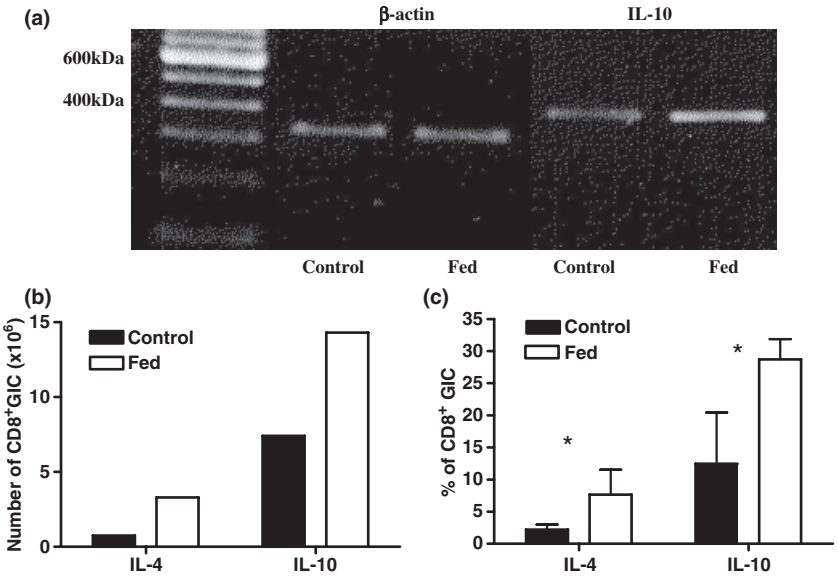

Figure 1 (a) mRNA expression of β-actin and IL-10 by $CD8^+$ GIC isolated from alloantigen fed (Fed) and nonfed (Control) animals 5 days post kidney transplant. (b) Number of IL-4 and IL-10 positive $CD8^+$ GIC isolated from a representative graft from fed or unfed animals. These data are representative of three experiments. (c) Mean (±SD) percentage $CD8^+$ GIC expressing IL-4 or IL-10 from grafts harvested from fed or unfed animals. $CD8^+$ GIC were stained intracellularly with OX-81 (anti-rat IL-4) or A5-4 (anti-rat IL-10) and analyzed by flow cytometry. ($n = 3$, $^*P < 0.05$. Wilcoxon Rank Sum).

Cytokine production by $CD8^+$ GIC

We previously demonstrated that $CD8^+$ GIC harvested from grafts of fed animals express high levels of IL-4 mRNA [13]. In this study, we demonstrated that they also express high levels of IL-10 mRNA (Fig. 1a). In contrast, $CD8^+$ T cells harvested from grafts of control animals expressed much lower levels of this cytokine (Fig. 1a). Cytokine protein production was confirmed using intracellular immuno-staining for IL-4 and IL-10 and flow cytometry. In Fig. 1b, we demonstrate the number of IL-4 or IL-10 expressing $CD8^+$ GIC that were harvested from kidneys in a single paired experiment of an alloantigen fed rat or from a nonfed control rat. This pattern of data is representative of all three experiments we performed, although absolute numbers varied from experiment to experiment. We show an increased number of IL-4 and IL-10 secreting cells in the $CD8^+$ GIC from fed animals. In addition, in Fig. 1c we express the percentage (rather than the number) of the $CD8^+$ GIC that express IL-4 or IL-10. There is a significantly ($P < 0.05$, $n = 3$) increased percentage of IL-4 and IL-10 secreting cells in the $CD8^+$ GIC from grafts of fed animals in comparison with nonfed control animals.

Surface markers on $CD8^+$ GIC

Given our demonstration of Treg activity in this model and our data suggesting IL-4 and IL-10 production as a potential mechanism of action, we examined the surface expression of various markers (CD25, CD28, αβ TCR and γδ TCR) to ascertain whether this $CD8^+$ T-cell generated in our system was similar to previously described Treg cells. As shown in Fig. 2, flow cytometric analysis revealed that neither CD25 nor CD28 expression was relevant markers for Treg activity in that there was no significant

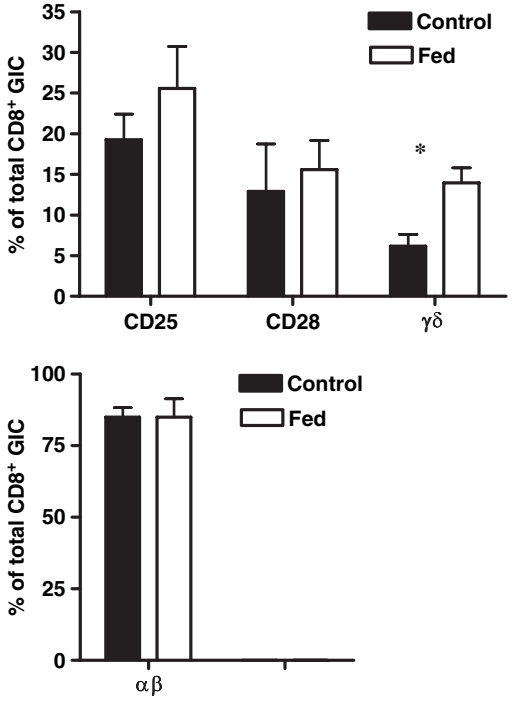

Figure 2 Flow cytometric analysis of surface markers on $CD8^+$ GIC isolated from grafts of alloantigen fed (Fed) and nonfed (Control) animals 5 days post kidney transplant. $CD8^+$ GIC were labeled with FITC conjugated mouse anti-rat monoclonal antibodies: OX-39 (anti-CD25), JJ319 (anti-CD28), R73 (anti-αβ TCR) or V65 (anti-γδ TCR) and analyzed by flow cytometry ($n = 7$, fed versus control for CD25: $P = 0.23$, CD28: $P = 0.71$, αβ TCR: $P = 0.95$, γδ TCR $^*P = 0.014$. Mann–Whitney).

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difference in the expression of these markers between control CD8 GIC and GIC harvested from the kidneys of orally exposed animals ($P = 0.23$ for CD25; $P = 0.71$ for CD28).

Of very significant interest was the finding that cells expressing the $\gamma\delta$ TCR were significantly ($P = 0.014$) increased in grafts harvested from alloantigen fed animals compared to nonfed control animals (Fig. 2a). The data show a two fold increase in the presence of $\gamma\delta$ TCR$^+$ T cells in the animals previously exposed to alloantigen by the oral route. No such increase in the presence of $\alpha\beta$ TCR$^+$ cells was observed (Fig. 2b).

Role of $\gamma\delta$ T cells in graft prolongation

The data above was suggestive that $\gamma\delta$ T cells may play a role in the prolongation induced by oral exposure to alloantigen. To confirm such a role of $\gamma\delta$ T cells in graft prolongation we performed adoptive cell transfer experiments using $\gamma\delta$ depleted GIC populations. The data obtained (Fig. 3) show an ablation of prolongation when $\gamma\delta$ T cells are removed from the transferring population. Kaplan–Meier survival analysis shows a significantly ($P = 0.02$) increased survival of animals receiving adoptive transfers from GIC populations containing $\gamma\delta$ T cells compared to GIC populations lacking $\gamma\delta$ T cells. No significant prolongation of survival was seen in the $\gamma\delta$ depleted population compared to the expected survival in nontreated animals ($n = 35$ historical controls).

$\gamma\delta$ T-cell accumulation in the spleen

In the next stage of the study, we examined the origin of the $\gamma\delta$ T-cell population responsible for the prolongation

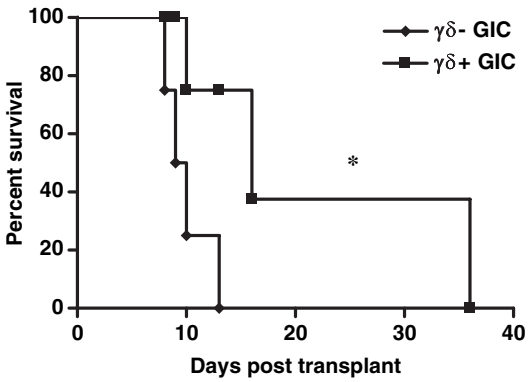

Figure 3 Kidney allograft survival after adoptive transfer of $\gamma\delta$ enriched ($\gamma\delta$+) or $\gamma\delta$ depleted ($\gamma\delta$-) GIC isolated from the rats that had been orally exposed to alloantigen into naïve Lewis recipients 1 days post kidney transplant (*P = 0.02, Kaplan–Meier).

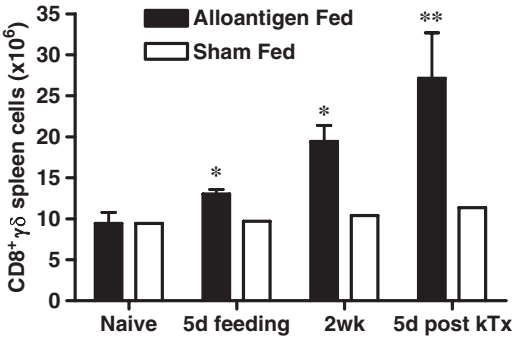

Figure 4 The number of CD8$^+$ $\gamma\delta$ T cells in the spleen of alloantigen fed animals in comparison with sham fed control animals at various time points after oral exposure of alloantigen. 0 days postfeeding (naïve), 5 days postfeeding (5 days feeding), 2 weeks postfeeding (2 weeks) and 5 days post kidney transplant (5 days post kTx). ($n = 5$, *$P < 0.05$; **$P < 0.01$ Mann–Whitney).

of survival. We hypothesized that the $\gamma\delta$ cells infiltrating the graft may have come from the spleen. To obtain evidence to support or refute this hypothesis, we first assessed the presence of CD8$^+$ $\gamma\delta$ T cells in the spleen at various time points throughout the feeding/transplantation regimen. As shown in Fig. 4, there is a significant increase in the number of CD8$^+$ $\gamma\delta$ T cells in the spleen at 5 days post alloantigen feeding ($P = 0.046$) and 2 weeks postfeeding ($P = 0.011$) compared to levels of CD8$^+$ $\gamma\delta$ T cells in sham fed animals. The numbers of CD8$^+$ $\gamma\delta$ T cells in the spleen were also significantly ($P = 0.0098$) increased 5 days post-transplant when compared to sham fed transplanted animals. These findings are consistent with a hypothesis that the $\gamma\delta$ T cells come from the spleen.

Graft survival after transfer of splenocytes

Given the increase in $\gamma\delta$ T cells in the spleen of animals orally exposed to alloantigen and the data above implicating $\gamma\delta$ T cells in graft prolongation, we tested the hypothesis that the spleen cells from orally exposed animals would transfer graft prolongation to syngeneic naive rats. As shown in Fig. 5, Kaplan–Meier survival analysis demonstrated that survival of the grafts in the animals receiving splenocytes from the animals orally exposed to alloantigen was significantly ($P = 0.012$) increased when compared to survival of grafts in the animals receiving splenocytes from control animals.

Discussion

Our previous data indicate that oral exposure to alloantigen significantly prolongs kidney allograft survival [12]. This prolongation can be transferred to syngeneic naive

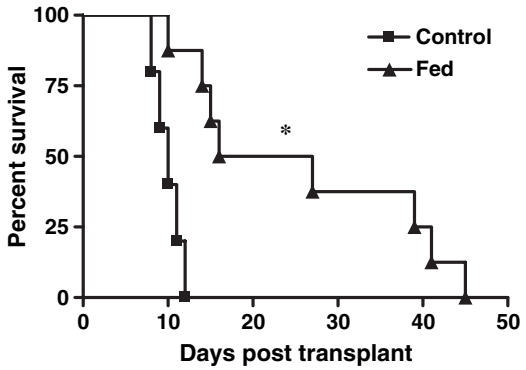

Figure 5 Kidney allograft survival after adoptive transfer of splenocytes isolated from naïve Lewis rats or from the rats that were orally exposed to alloantigen 2 weeks after the first feeding (*P = 0.012, Kaplan–Meier).

animals by transfer of $CD8^+$T cells but not $CD4^+$ T cells, implicating a $CD8^+$ Treg cell mediating the observed prolongation [13]. Third party experiments have demonstrated that this is an antigen specific phenomenon. The $CD8^+$ T cells harvested from these surviving grafts not only express cytotoxic mediators but also express regulatory cytokines such as IL-4 and TGF-β [13]. To characterize further the T-cell population responsible for this observed regulatory event, we examined cytokine secretion, surface marker expression and TCR phenotype associated with prolongation.

We demonstrate that graft infiltrating $CD8^+$ T cells in this model express both IL-4 and IL-10. Both these cytokines have been implicated in the activities of a variety of Treg cells [14,15]. Tr1 cells, for example, are characterized by a strong production of IL-10, which is essential for their regulatory activity [16]. These cells have been implicated in prevention or amelioration of graft-versus-host disease and allograft rejection [17,18].

IL-4 and/or IL-10 secreting T cells have been suggested to play a role in oral tolerance [19–22] and portal tolerance [23] by mediating the down-regulation of inflammatory Th1 responses dependent on IFN-γ. Current evidence supports a hypothesis that such $CD4^+$ Th1 regulated inflammatory responses are the major effector mechanisms responsible for allograft rejection. In contrast, Type 2 (IL-4 and IL-10) responses are thought to favor allograft survival [24,25]. Changes in overall cytokine profile from a predominantly type-1 IFN-γ dominant response to a type-2 response have been reported to prolong allograft survival in several experimental models [26–29]. The presence of increased levels of IL-4 and IL-10 in the $CD8^+$ GIC from allografts of fed animals suggested that the observed graft prolongation was mediated by a $CD8^+$ type-2 T cell, potentially a Tc2 cell [30,31].

CD25 has emerged as an important marker for certain types of Treg cells [32]. $CD8^+CD25^+$ T cells are naturally present in the human thymus [8] and in mouse lymph nodes [3,33]. We found no evidence to support a hypothesis that the Treg cells we observed were $CD25^+$. The percentage of $CD8^+$ T cells expressing CD25 was not significantly different between the GIC populations harvested from the kidney grafts of animals exposed orally to alloantigen and GIC from kidney grafts of control animals. These data demonstrate that oral exposure of alloantigen does not increase the percentage of $CD25^+$ T cells in the graft infiltrating population. Likewise, we found no correlation between graft prolongation and the accumulation of $CD8^+$ $CD28^-$ Treg cells in the graft.

In contrast, we have demonstrated a role for $CD8^+$ γδ T cells in the graft prolongation initiated by oral exposure to alloantigen. Our data demonstrate an increased percentage of γδ T cells in the $CD8^+$ kidney GIC population after oral exposure to alloantigen. Moreover, adoptive cell transfer experiments yielded data consistent with a hypothesis that γδ T cells are essential to the Treg activity of the GIC harvested from kidneys from animals orally exposed to alloantigen. When γδ T cells were depleted from the GIC population before cell transfer, graft prolongation was ablated. In contrast, GIC containing γδ T cells transferred graft prolongation. As we have shown that only $CD8^+$not $CD4^+$ GIC are able to prolong graft survival [13], it follows that the γδ T cells that mediate graft prolongation are $CD8^+$ T cells.

γδ T cells have been demonstrated to possess Treg activity in several animal models, including in oral tolerance [34–36], transfusion tolerance [37], portal transplantation tolerance [11], ocular immune privilege [38] and autoimmune diseases [39]. Of significant interest, antigen specific oral tolerance cannot be generated in γδ knockout mice [34,40]. In clinical transplantation, it has been found that an increased percentage of γδ T cells is associated with stable renal grafts and a decreased percentage of γδ T cells is associated with rejection [41]. In addition, γδ T cells also play a regulatory role in graft-versus-host disease [42]. A recent study demonstrated that $CD8^+$ γδT cells play a role in the maintenance of peripheral tolerance induced by oral tolerance in a neonatal thymectomy model in NOD mice [43].

Our current working hypothesis is that oral exposure to alloantigen generates γδ Treg cells, which leave the intestinal site and/or local draining nodes, then accumulate in the spleen and subsequently transit to the kidney. Our evidence that graft prolongation can be adoptively transferred to naïve animals by transfer of spleen cells or graft infiltrating cells supports such a hypothesis. However, labeling experiments using splenocytes from orally tolerized animals to track the transit would be required

to confirm this hypothesis. Our hypothesis is supported by evidence that IEL are able to migrate from the intestine to the spleen [44]. Moreover, others have found that in response to intestinal infection, $\gamma\delta^+$ IEL levels in the gut fall, but rise in the spleen, suggesting migration from the enteral site to the spleen [45].

Our hypothesis that CD8$^+$ $\gamma\delta$ T cells are responsible for allograft prolongation induced by oral alloantigen exposure is supported by the work that CD8$^+$ $\gamma\delta$ TCR$^+$ Treg cells can be identified in the spleen and are responsible for the transfer of tolerance in a mucosal tolerance model [46]. Moreover, in a portal tolerance model, adoptive transfer of $\gamma\delta$ T cells can transfer unresponsiveness to allogeneic skin grafts [47] and infusion of anti-$\gamma\delta$ TCR monoclonal antibody into transplant recipients blocks allograft enhancement [11].

The mechanism by which $\gamma\delta$ Treg cells prolong survival is still unclear. $\gamma\delta$ Treg cells have been suggested to downregulate immune responses by secretion of Th2 cytokines, such as IL-4 and/or IL-10 [11,37,47,48] or by their cytotoxic activity through FasL expression [39]. Moreover, $\gamma\delta$ T cells may regulate the function of $\alpha\beta$ T cells [49] and suppress CTL activity [50,51]. These activities may be because of the secretion of inhibitory cytokines IL-10 and TGF-β. Results from this study are consistent with a hypothesis that oral administration of alloantigen generates CD8$^+$ $\gamma\delta$ Treg cells, which mediate graft prolongation by the secretion of type 2 cytokines.

Authorship

JZ: designed and performed the research and wrote the paper. SEA: performed the research, collected and analyzed data and contributed to writing the paper. AS: contributed idea for the research. TDGL: designed the research and contributed substantially to writing the paper. BAPN: designed the research and provided guidance.

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