Pretransplant Infusion of Donor B Cells Enhances Donor-Specific Skin Allograft Survival

Julia Gao¹,², Megan S. Ford McIntyre¹,³, Cheryl A. D’Souza¹, Li Zhang¹,²,³*

¹ University of Toronto Transplantation Institute, Toronto General Research Institute, University Health Network, Toronto, Ontario, Canada, ² Department of Immunology, University of Toronto, Toronto, Ontario, Canada, ³ Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada

Abstract

Pretransplant donor lymphocyte infusion (DLI) has been shown to enhance donor-specific allograft survival in rodents, primates and humans. However, the cell subset that is critical for the DLI effect and the mechanisms involved remain elusive. In this study, we monitored donor cell subsets after DLI in a murine MHC class I L5-mismatched skin transplantation model. We found that donor B cells, but not DCs, are the major surviving donor APCs in recipients following DLI. Infusing donor B, but not non-B, cells resulted in significantly enhanced donor-specific skin allograft survival. Furthermore, mice that had received donor B cells showed higher expression of Ly6A and CD62L on antigen-specific TCRαβ⁺CD3⁺CD4⁺CD8⁻NK1.1⁻ double negative (DN) regulatory T cells (Tregs). B cells presented alloantigen to DN Tregs and primed their proliferation in an antigen-specific fashion. Importantly, DN Tregs, activated by donor B cells, showed increased cytotoxicity toward anti-donor CD8⁺ T cells. These data demonstrate that donor B cells can enhance skin allograft survival, at least partially, by increasing recipient DN Treg-mediated killing of anti-donor CD8⁺ T cells. These findings provide novel insights into the mechanisms underlying DLI-induced transplant tolerance and suggest that DN Tregs have great potential as an antigen-specific immune therapy to enhance allograft survival.

Introduction

Pretransplant donor specific transfusion or donor lymphocyte infusion (DLI) has been used either alone or in combination with other treatments to prolong graft survival in various animal models and in clinical settings [1–6]. However, the mechanism by which DLI induces donor-specific transplantation tolerance is poorly defined. DLI-induced graft survival has been shown to be directly correlated with the infused lymphocytes in the recipients [7]. Nevertheless, which subsets of donor cells are critical for tolerance induction remains controversial [7–10].

B cells have long been considered as positive regulators in immune responses contributing to pathogenesis in a variety of immune disorders because of their ability to generate antibodies. However, evidence that B lymphocytes are able to regulate immune responses is accumulating. Convincing data has demonstrated that B cells can be tolerogenic rather than immunogenic in several immune-related diseases [11,12]. As B cells have been shown to play critical roles in both graft rejection and tolerance, further understanding the role of B cells in transplantation will facilitate the development of novel B cell directed strategies as well as modify previous B cell therapies to achieve donor-specific transplant tolerance [13,14].

As a subset of regulatory T cells (Tregs), TCRαβ⁺CD3⁺CD4⁺CD8⁻NK1.1⁻ double negative regulatory T cells (DN Tregs) comprise 1–3% of peripheral T lymphocytes in mice and humans [15,16]. Accumulating evidence has demonstrated that DN Tregs can function as critical immunoregulators in various diseases [17,18]. It has been shown that DN Tregs can inhibit type 1 diabetes [19,20], suppress antigen-specific allo- /xeno-reactive syngeneic T cells and induce long-term skin, cardiac and islet graft survival [21–23]. Previous studies have demonstrated that DLI activates recipient DN Tregs which are important for suppressing anti-donor T cells and maintaining long-term donor-specific transplantation tolerance [23,24]. However, the subset of donor cells that is critical for activating DN Tregs and the underlying mechanisms remain obscure.

In this study, we monitored infused donor cells and found that donor B cells, but not DCs, are the major surviving donor APCs in recipients following DLI. Interestingly, infusing purified donor B cells resulted in significantly enhanced donor-specific skin allograft survival. Donor B cells were able to present alloantigen to DN Tregs, induce their proliferation and enhance DN Treg-mediated elimination of anti-donor CD8⁺ T cells. These findings provide novel insights into the role of donor B cells in DLI-induced donor-specific transplant tolerance, and open a new window for using B cells to enhance DN Treg function and allograft survival.

Materials and Methods

Ethics Statement

Animals were housed in the Toronto Medical Discovery Tower under specific pathogen-free conditions. The animal use protocol was approved by the University Health Network Animal Care Committee. Animal care was conducted in accordance with the
policies and guidelines of the Canadian Council on Animal Care and the Province of Ontario’s Animals for Research Act.

Mice

2G (H-2\(^b\), expressing the 1B2\(^+\) anti-L\(^d\) transgenic TCR) breeders on C57BL/6 (B6) background were kindly provided by Dr. D.H. Loh (Nippon Research Centre, Japan). Dm2 mice, a BALB/c L\(^d\)-loss mutant, (H-2\(^d\), K\(^d\), L\(^d\)) were bred with 2G mice to create 2C\(^{F1}\) mice (anti-L\(^d\) TCR, H-2\(^b/d\), L\(^d\)) or with B6 mice to create (B6\(\times\)dm2)\(^F1\) (H-2\(^b/d\), L\(^d\)) mice. B6, GFP\(^+\)B6, BALB/c and SJL (H-2\(^d\)) mice were purchased from the Jackson Laboratory. CBy and GFP\(^+\)CBy (H-2\(^b/d\), L\(^d\)) mice were crossed by B6 or GFP\(^+\)B6 (H-2\(^b\)) to BALB/c (H-2\(^b\)) mice. All mice were housed in specific pathogen-free conditions at the University Health Network (Toronto, ON). All experiments were approved by the University Health Network animal care committee.

Antibodies and reagents

Anti-L\(^d\) monoclonal antibody (mAb) and 1B2 mAb that recognizes the 2C\(^{F1}\)-TCR were produced and purified in our lab as described previously [16]. Other mAbs include anti-CD3, anti-CD4, anti-CD8, anti-CD19, anti-CD62L, anti-DX5, anti-CD25, anti-CD44, anti-Ly6A, and the reagents used to identify non-B cells and resting B cells were collected and used as iDCs. To induce DC maturation, 2C\(^{F1}\) recipients were incubated with 30 g/ml LPS was added into the culture. The non-adherent cells were gated and the CD11c\(^+\) cell population, cells from spleen and LN of 2C\(^{F1}\) mice were collected and red blood cells were lysed as described before. Cells were incubated at 4°C with anti-CD8 (FITC) mAb for 10 minutes, washed and incubated with anti-FITC MACS beads for 15 minutes, then washed and passed through the AutoMACS to positively select the CD19\(^+\) cells for 4 days. The remaining CD4/CD8/NK1.1\(^+\) cells had greater than 90% purity and viability.

To purify the CD8\(^+\) T cell population, cells from spleen and LN of 2C\(^{F1}\) mice were collected and red blood cells were lysed as described before. Cells were incubated at 4°C with anti-CD8 (FITC) mAb for 10 minutes, washed and incubated with anti-FITC MACS beads for 15 minutes, then washed and passed through the AutoMACS to positively select the CD8\(^+\) population following the manufacturer’s directions (Milenyi Biotech, Auburn, CA). The purity and viability of CD8\(^+\) T cells used in the experiments were more than 95%.

APC cell culture

LPS activated-B cells were obtained by culturing CBy-L\(^d\) splenocytes in the presence of 10 μg/ml LPS (E. coli, Sigma-Aldrich, Oakville, ON) for 24 hours. Cultures were found to be greater than 90% CD19\(^+\). BM derived mature DCs (mDCs) were obtained as previously described [25]. On day 8–9, non-adherent cells were collected and used as iDCs. To induce DC maturation, 0.1 μg/ml LPS was added into the culture. The non-adherent cells were harvested the next day and were used as mDCs. Cell surface marker expression was analyzed by flow cytometry (Cytomics\(^\text{TM}\) FC 500, Beckman Coulter). Over 90% of the DCs used in experiments were CD11c\(^+\) cells. Viability of DGs was >95%, as determined by staining with PI.

Proliferation Assay

Primary DN Tregs were stimulated in vitro by purified B cells in the presence of either 10 μM QL9 (QLSPFPFDL) or P1A (LPYLGLWVVF) peptides. Cell proliferation was determined by pulsing with 5 μCi/ml "H-TdR for 18 hrs on day 3 of culture. Cells were harvested onto 96 well filter plates and "H-TdR labelling was counted on a TOPCOUNT plate reader (Perkin Elmer Life and Analytical Science, Woodbridge, ON).

Cytotoxicity Assay

CD8\(^+\) T cells purified from naive (B6\(\times\)dm2)\(^F1\) mice were used to either irradiated CBy (L\(^d\), donor-specific) or SJL (H-2\(^b/d\), third party) splenocytes for 4 days and used as targets. DN Tregs purified from spleen and LNs of 2C\(^{F1}\) mice 4 days after receiving purified B or non-B lymphocyte infusion, were stimulated by irradiated L\(^d\) B or non-B cells ex vivo for 4 days, purified and co-incubated with CD8\(^+\) T cell targets at varying ratios for 24 hours. Cells were then stained with anti-CD8 mAb followed by Annexin V and PI. CD8\(^+\) cells were gated and the percentages of Annexin V\(^+\) and/or PI\(^+\) cells were determined by flow cytometry.

B Cell Infusion Enhances Donor-Specific Tolerance

PLOS ONE | www.plosone.org 2 October 2013 | Volume 8 | Issue 10 | e77761
Pretransplant infusion of donor cells induces long-term single MHC locus-mismatched donor-specific skin allograft survival

Pretransplant infusion of donor cells induces long-term single MHC locus-mismatched donor-specific skin allograft survival

Pretransplant infusion of donor spleen and lymph node (LN) cells, either alone or together with other treatments such as anti-CD4 monoclonal antibody (mAb), has been shown to benefit donor-specific allo- and xeno-graft survival [23,24]. To identify the beneficial donor cell subsets, we used pan-GFP+ mice as lymphocyte donors to track donor cells in GFP recipients, GFP-2C111 mice (H-2d, Ld+) which express a transgenic TCR that specifically recognizes Ld. Before infusion, donor cell composition was examined and is shown in Fig. 1A-B. As expected, CD19+ B cells were the major subset of donor cells, which was followed by CD4+ and CD8+ T cells. DCs, NK cells and macrophages were also present but comprised much smaller proportions. One week after infusion, all mice were transplanted with skin allografts from both the lymphocyte donor strain, CBy, and a third-party strain, SJL. Graft survival was monitored by visual inspection daily for the first 2 weeks and twice a week thereafter. Representative data are shown from at least three independent experiments.

doi:10.1371/journal.pone.0077761.g001

Figure 1. Pretransplant infusion of donor cells induces long-term single MHC locus-mismatched donor-specific skin allograft survival. (A–B) Cells from the LNs and spleen of naïve GFP+CBy mice were stained with mAbs against CD4, CD8, CD11c, Mac1, Lin (lineage markers, including CD3, CD19 and DX5) and NK1.1. Percent (A) and total number (B) of different subsets of donor cells were assessed by flow cytometry. (C) 2G11 (anti-Ld.2C-TCR, H-2d, Ld+) recipient mice were i.v. injected with 3×106 spleen and LN cells (■, ▽) from GFP+CBy (H-2d, Ld+) donors. Seven days later, recipient mice were transplanted with sex-matched CBy (Ld+, ■, n = 15) or SJL (H-2d, ▽, n = 15) skin grafts. CBy mice that were transplanted with CBy skin grafts without pretransplant DLI were used as control (▲, no DLI, n = 15). Graft survival was monitored by visual inspection daily for the first 2 weeks and twice a week thereafter. Representative data are shown from at least three independent experiments.
Figure 2. Tracking donor cells in recipient mice. 2C31 mice were infused with $6 \times 10^7$ allogeneic GFP$^+$ CBy (L$^{d+}$) cells. Two or seven days later, cells were harvested from recipient thymus, bone marrow (BM), spleen and LNs. (A) Numbers in the graphs indicate % GFP$^+$ donor cells in each organ.
Donor B cells are more effective at enhancing donor-specific skin allograft survival than non-B cells

Accumulating evidence suggests that B cells can promote immune tolerance in various disease models [12], albeit their role in transplantation tolerance has not been well defined. To determine whether donor B cells are important for enhancing donor-specific skin allograft survival, lymphocytes from naïve CBy (Ld<sup>+</sup>) mice were sorted into CD19<sup>+</sup> and CD19<sup>−</sup> populations and infused into 2C<sub>F1</sub> (Ld<sup>−</sup>) mice. One week later, all mice were transplanted with a syngeneic skin graft (negative control) together with a skin allograft from either lymphocyte donor CBy mice or 3<sup>rd</sup> party SJL mice. As shown in Fig. 3, mice that did not receive DLI rejected CBy skin allografts within 12 days with a Median Survival Time (MST) of 10 days. All 3<sup>rd</sup>-party SJL skin grafts were rejected within 8 days regardless of the type of donor cells they received prior to transplantation. Infusing CD19<sup>−</sup> CBy lymphocytes slightly prolonged the survival of donor specific skin grafts compared to the no DLI group, but the difference was not statistically significant (p = 0.7301). Interestingly, 2C<sub>F1</sub> mice that received CBy CD19<sup>+</sup> B cells showed significant enhancement of donor specific skin graft survival (MST = 61 days) compared to those that received CBy CD19<sup>−</sup> cells (MST = 22 days, p = 0.0004). Three out of nine mice accepted donor specific skin allografts for over 100 days (Fig.3). These data indicate that infusion of CD19<sup>+</sup>, but not CD19<sup>−</sup> donor cells, can enhance donor-specific, but not third party, skin allograft survival.

In the above experiment, we used anti-CD19 mAb coated magnetic beads to positively select CD19<sup>+</sup> cells for i.v. injection. To exclude the potential effect of this selection procedure on skin graft survival, untouched naïve B cells were purified by depleting CD4<sup>+</sup>CD4<sup>+</sup>Ter119<sup>+</sup> cells and i.v. injected into 2C<sub>F1</sub> mice. The recipients were then transplanted with either a CBy or an SJL skin allograft together with a syngeneic skin graft. As shown in Fig. 3, mice receiving untouched naïve B cells showed similar CBy skin graft survival as mice receiving B cells purified by positive selection of CD19<sup>+</sup> cells. 4 out of 10 mice which received negatively selected B cells had their CBy skin grafts survive for 100 days. Taken together, these data demonstrate that pretransplant infusion of donor B lymphocytes is significantly more effective at enhancing donor-specific skin allograft survival than infusion of non-B donor lymphocytes. However, B cells alone were not able to induce complete tolerance as observed in Fig. 1C.

DN Tregs can be activated by allogeneic donor B cells in an antigen-specific manner and preferentially acquire alloantigen from B cells

It has been shown previously that DN Tregs play a key role in DLI-induced donor-specific transplantation tolerance [23,24]. In order to understand the mechanisms by which infusion of donor B cells prolonged donor-specific allograft survival, we first examined whether donor B cells can function as APCs to activate recipient DN Tregs. Although both B and non-B cell infusion resulted in similar increased expression of CD25 and CD44 on recipient DN Tregs (data not shown), CD62L<sup>+</sup> and Ly6A<sup>+</sup> expression on recipient DN Tregs were significantly increased in mice that received donor B cells compared to those that received non-B cells (Fig. 4A-B). We next determined whether donor B cells could directly prime DN Tregs in an antigen-specific manner. As shown in Fig. 4C, 2C<sub>F1</sub> DN Tregs proliferated significantly in response to B cells which had been pulsed with 2C TCR specific peptide QL9. However, no proliferation of DN Tregs was observed when they were stimulated with B cells pulsed with TCR non-specific P1A peptides (Fig. 4C, p<0.0001). This data demonstrates that B cells
Figure 4. DN Tregs can be activated by allogeneic donor B cells in an antigen-specific manner and preferentially acquire alloantigen from B cells. (A–B) 2CF1 mice were infused with 3 × 10^7 purified B or non-B cells from CBv mice as described in Fig. 3. Cells were
can present alloantigen and prime DN Treg proliferation in an antigen-specific fashion.

Previous studies have shown that acquisition of alloantigens is required for both human and mouse DN Treg-mediated antigen-specific immune suppression [15,16]. We found that activated donor B cells expressed higher levels of alloantigen Ld on their surface than mature DCs (mDCs) (Fig. 4D). To determine whether higher Ld expression would facilitate DN Treg acquisition of alloantigen from B cells, we compared acquisition levels achieved by 1B2DN Tregs in the presence of either activated B cells or mDCs from allogeneic Ld mice. The DN Tregs that were cocultured with B cells showed significantly increased expression of Ld on their surface when compared to those cocultured with mDCs (Fig. 4E–F). These data suggest that although DN Tregs can acquire alloantigens from both mDCs and activated B cells, interaction with the latter resulted in higher acquisition and expression of Ld by DN Tregs.

Recipient DN Tregs activated by donor B cells show higher toxicity towards anti-donor CD8+ T cells in an antigen-specific fashion

Ly6A has been shown to be required for DN Treg-mediated cytotoxicity toward CD8+ T cells [26]. As Ly6A expression on DN Tregs was significantly increased in mice that received donor B cell infusion (Fig. 4A–B), we addressed the question of whether infusion of donor B cells would increase the ability of DN Tregs to kill syngeneic anti-donor CD8+ T cells in vivo. To this end, 2C11 mice were injected with either naïve B or non-B lymphocytes from CBBy mice and sacrificed 7 days later. We found that mice that had received B cell infusion showed a significant decrease in 1B2CD8+ T cells, concomitant with an increase in 1B2DN Tregs, leading to significantly increased antigen-specific DN to CD8+ T cell ratio (Fig. 5A–B). These findings further suggest that DN Tregs that were activated by donor B cells may possess enhanced cytotoxicity toward anti-donor CD8+ T cells. To directly test this hypothesis, DN Tregs isolated from spleen and LN6 4 days after infusion of either donor B or non-B cells were stimulated ex vivo by either irradiated Ld+ B cells or non-B cells before being used as putative effectors. CD8+ T cells purified from naïve (B6.xdm2y121Ld) mice were stimulated by irradiated Ld+ splenocytes and used as targets. As shown in Fig. 5C–D, both non-B cell and B cell activated DN Tregs were able to kill CD8+ T cells effectively, however, donor B cell primed DN Tregs showed significantly higher cytotoxicity toward anti-donor CD8+ T cells in an antigen-specific manner than those primed with donor non-B cells (p<0.001). Furthermore, the DN Tregs activated by either CBBy or non-B cells showed no cytotoxicity to either B or non-B SJL lymphocyte-activated CD8+ T cells. Collectively, these findings suggest that infusion of donor B cells may facilitate donor-specific skin allograft survival by activating recipient antigen-specific DN Tregs which then kill anti-donor CD8+ T cells in an antigen-specific manner.

Discussion

Numerous reports have shown that pretransplant exposure to donor antigens through injection of donor leukocytes or blood transfusion either alone or in combination with other treatments can facilitate donor-specific graft survival. To dissect the mechanisms involved in this phenomenon, we used GFP+ mice as donors to investigate the spacial and temporal kinetics of infused donor cells in recipients. Consistent with a previous report using GFP mice [16], pretransplant infusion of GFP+ MHC class I Ld-mismatched lymphocytes induced permanent acceptance of donor-specific skin allografts in all recipients (Fig. 1C). Since all non-DLL-treated mice rejected Ld skin grafts and all mice that were treated with GFP+1B2 DLI rejected their 3rd-party SJL skin allografts within 2 weeks (Fig. 1C), these results indicate that pretransplant infusion of unfraccionated donor lymphocytes can induce donor-specific transplantation tolerance in this MHC class I Ld-mismatched model.

Several studies have investigated the subsets of donor cells that might be important for inducing donor-specific transplantation tolerance in minor antigen-mismatched models and the results remain controversial. Johnson reported that infusion of donor T cells alone was not sufficient to achieve tolerance to male H-Y antigens [8]. Sheng et al. found that infusion of donor T cells together with Mac-1+ cells induced tolerance toward male antigens [10]. On the other hand, Fuchs and Matzinger reported that infusion of resting donor B cells could induce long term survival of male H-Y+ skin grafts in female mice [27]. The interaction of FasL, expressed on the infused B cells and Fas expressed on the recipient T cells was essential in DLL-induced tolerance to H-Y antigens [28]. Whether infusion of donor B cells alone is necessary or sufficient to attain MHC-mismatched donor-specific allograft survival was unknown. We found that a single injection of naïve donor B cells, purified by either positive or negative selection, at 7 days prior to transplantation resulted in significantly prolonged donor-specific skin allograft survival (MST = 61 days) compared with infusion of non-B cells (MST = 22 days, p = 0.0004) (Fig. 3). In both treatment groups, 3rd party skin grafts were rejected within 2 weeks. These data provide direct evidence supporting an important role of donor B cells in enhancing donor-specific skin allograft survival in an MHC class I mismatched model.

Interestingly, while inducing non-fraccionated splenocytes induced permanent donor-specific allograft acceptance (Fig. 1C), infusion of the same number of B cells by two different purification methods could only prolong graft survival as the majority of donor skin allografts were eventually rejected (Fig. 3). This was not due to insufficient number of B cells nor donor antigens as even when 60×10^6/mouse purified B cells were infused, complete tolerance was not achieved (data not shown). Furthermore, infusion of non-B
cells was also able to enhance allograft survival compared to no DLI, however, the difference was not statistically significant (Fig. 3). Taken together, these data suggest that although B cells are superior to non-B cells in enhancing donor-specific skin allograft survival, infusion of B cells alone is not sufficient to induce permanent skin allograft acceptance. The type of cells in the non-B cell portion that can facilitate B cell tolerogenic function requires further investigation.

Figure 5. Recipient DN Tregs activated by donor B cells show higher toxicity towards anti-donor CD8⁺ T cells in an antigen-specific fashion. (A, B) 2C-F1 mice were i.v. injected with 3 × 10⁷ B or non-B cells isolated from CBy mice. Cells were harvested from spleen and LNs on day 7, stained with anti-CD4, anti-CD8, anti-NK1.1 and anti-1B2 mAbs, then examined by flow cytometry. (A) Donor-specific DN Tregs and CD8⁺ T cells were gated on 1B2⁺ population. (B) Based on the flow cytometry data, 1B2⁺DN Treg to 1B2⁺CD8⁺ ratio from a total of 8 mice from 3 independent experiments was calculated. (C) DN Tregs were isolated from 2C-F1 mice 7 days after infusion of either CBy B or non-B cells followed by restimulation ex vivo of either CBy or SJL B or non-B cells before being used as effectors. Activated anti-L10 or anti-H-2D⁺ CD8⁺ T cells were used as targets. Effectors and targets were cultured either alone or together at varying ratios as indicated. After 24 hrs, cultures were stained with anti-1B2, anti-CD8 mAbs, Annexin V and PI. Percentages of Annexin V⁺ and PI⁺ in 1B2⁺CD8⁺ gated cells are shown in (C). Data shown in (D) are from 3 independent experiments. Two-way ANOVA with Bonferroni’s post-test was used to analyze the data. The asterisk symbol (*) shows significance between B (CBy) and Non-B (CBy) graphs. The hash symbol (#) shows significance between B (CBy) and B (SJL) graphs. *p<0.05, **p<0.01, ***p<0.001 doi:10.1371/journal.pone.0077761.g005
In searching for the mechanisms by which donor B cells may contribute to enhanced donor-specific skin allograft survival, we found that donor B cells, but not DCs, were the major surviving donor APCs following DLI. Large numbers of donor B cells were found in the spleen and LN of the recipients both on day 2 and day 7 following DLI (Fig. 2C-E). In contrast, almost no donor CD11c⁺CD3⁺CD19⁺DX5⁻ DCs were detected in recipient LNs and spleen (Fig. 2C-E). In addition, recipient DN Tregs showed higher expression of CD62L and Ly6A when recipients were infused with donor B cells compared to when they received non-B cells (Fig. 4A-B). While Ly6A has been shown to be required for DN Treg-mediated cytotoxicity toward CD8⁺ T cells [26], expression of cell adhesion molecule CD62L may be required for their efficient recirculation to facilitate their regulatory function [29]. Furthermore, B cells are able to induce DN Treg proliferation in an antigen-specific fashion (Fig. 4C). These findings suggest that infused donor B cells may facilitate recipient DN Treg activation.

Antigen-activated DN Tregs have been shown to play important roles in inducing donor-specific transplantation tolerance in various models [21,24,30]. Furthermore, DN Tregs are able to suppress B cells and NK cells through the perforin/granzyme pathway for tolerance achievement [31–33]. Recently DN Tregs were found to express high levels of CTLA4 which could down regulate CD80/CD86 on mDCs [25]. DN Tregs could also kill mDCs and activated B cells through the Fas-FasL or the perforin pathway [25,31]. So far the immune suppression mediated by mouse, rat and human DN Tregs were found to be antigen-specific [17]. Recent evidence indicates that DN Tregs are able to acquire MHC-peptides from APCs through trogocytosis, and that expression of acquired allo-MHC-peptides on the surface of DN Tregs is critical for their cytokinesis of antigen-specific CD8⁺ effector cells [34]. This may be one of the mechanisms by which DN Tregs convey antigen-specific suppression. Here we found that DN Tregs can acquire and express higher levels of donor antigens from B cells than from DCs (Fig. 4E–F). In addition, recipient DN Tregs activated by donor B cells were able to kill donor-specific CD8⁺ T cells more effectively than those activated by non-B cells or by 3rd-party B cells (Fig. 5C–D). These data further support the notion that donor B cells may program recipient DN Tregs to enhance their cytotoxicity toward antidonor CD8⁺ T cells leading to a higher DN Treg to CD8⁺ T cell ratio following DLI (Fig. 5A–B), which could ultimately result in enhancing donor-specific skin allograft survival as observed in this animal model. Consistent with our findings, a recent study also suggested that a higher DN Treg to CD8 ratio in bone marrow transplant patients correlated with a reduced severity of graft versus host disease [35].

Taken together, we demonstrate that pretransplant infusion of donor B cells can prolong skin allograft survival in an antigen-specific fashion, although this treatment is not sufficient to induce complete tolerance. The infused donor B cells may interact with donor-specific DN Tregs, which results in an increased DN Treg-mediated killing of CD8⁺ T cells and contributes to the enhancement of donor-specific skin allograft survival. These findings provide new insights into the mechanisms underlying DLI-induced transplant tolerance and suggest that DN Tregs have great potential as an antigen-specific immune therapy to enhance allograft survival.

Acknowledgments

We thank Dr. Jinbo Zhao for critical comments on the manuscript.

Author Contributions

Conceived and designed the experiments: JG MSFM LZ. Performed the experiments: JG MSFM. Analyzed the data: JG MSFM CAD. Wrote the paper: JG CAD LZ. Performed experiments required for resubmission: CAD.

References

1. Prigozhina TB, Elkin G, Slavin S (2007) Deletion of donor-reactive cells as a new conditioning regimen for allogeneic bone marrow transplantation. Transplant Proc 39: 678–684.
2. Liu B, Hao J, Pan Y, Luo B, Westgard B, et al. (2006) Increasing donor chimerism and inducing tolerance to allografts by post-transplant donor lymphocyte infusion. Am J Transplant 6: 933–946.
3. Rademaker J, Canolino L, Crespi S, Lenti LM, Lillo E, et al. (2006) [Immunoablation] after combined spleen and kidney transplantation in swine. Minerva Chir 61: 85–94.
4. Urakami H, Ostanin DV, Hunig T, Grinham MB (2006) Combination of donor-specific blood transfusion with anti-CD28 antibody synergizes to prolong graft survival in rat liver transplantation. Transplant Proc 38: 3244–3246.
5. Hoerbelt R, Johnston DR, Shoji T, Houser SL, Hase RS, et al. (2005) Combination treatment with donor-specific transfusions and cyclosporine a induces long-term survival of cardiac allografts in miniature swine. Transplantation 80: 1275–1281.
6. Faye MW, Burton K, Mohanakumar T, Brennan D, Keller C, et al. (1995) Donor-specific transfusions have long-term beneficial effects for human renal allografts. Transplantation 60: 1395–1401.
7. Sheng-Tanner X, Miller RG (1992) Correlation between lymphocyte-induced donor-specific tolerance and donor cell recirculation. J Exp Med 176: 407–413.
8. Johnson LL (1988) Properties of intravenously infused donor cells that prolong life in mice. J Immunol 140: 2214–2220.
9. Johnson LL (1988) Properties of intravenously infused donor cells that prolong life in mice. J Immunol 140: 2214–2220.
10. Sheng B, McCormack WT, Smith RT (1999) Purified donor T cells alone induce transplantation immunity to the male antigen but induce tolerance in combination with MHC+ donor cells. Transplantation 68: 1024–1029.
11. Klinker MW, Lundy SK (2012) Multiple mechanisms of immune suppression by B lymphocytes. Mol Med 18: 123–137.
12. Mauri C, Bosma A (2012) Immune regulatory function of B cells. Annu Rev Immunol 30: 221–241.
13. Redfield RR III, Rodriguez E, Parsons R, Vivek K, Mustafa MM, et al. (2011) Essential role for B cells in transplantation tolerance. Curr Opin Immunol 23: 651–659.
14. Kirk AD, Turgon NA, Ivakoshi NN (2010) B cells and transplantation tolerance. Nat Rev Nephrol 6: 584–593.
15. Fischer K, Voelkl S, Heymann J, Przybylski GK, Mondal K, et al. (2005) Isolation and characterization of human antigen-specific TCR alpha beta+ CD4-/CD8- double-negative regulatory T cells. Blood 105: 2829–2835.
16. Zhang ZX, Yang L, Young KJ, DuTemple B, Zhang L (2000) Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression. Nat Med 6: 782–789.
17. Hillhouse EE, Lesage S (2012) A comprehensive review of the phenotype and function of antigen-specific immunoregulatory double negative T cells. J Autoimmun.
18. Juvel SC, Zhang L (2012) Double negative regulatory T cells in transplantation and autoimmunity: recent progress and future directions. J Mol Cell Biol 4: 46–58.
19. Dugas V, Beauchamp C, Chabot-Roy G, Hillhouse EE, Lesage S (2010) Implication of the CD47 pathway in autoimmune diabetes. J Autoimmun 35: 23–32.
20. Ford MS, Chen W, Wong S, Li C, Vanama R, et al. (2007) Peptide-activated double-negative T cells can prevent autoimmune type-1 diabetes development. Eur J Immunol 37: 2234–2241.
21. Zhang D, Yang W, Dejagne N, Tian Y, Mikita A, et al. (2007) New differentiation pathway for double-negative regulatory T cells that regulates the magnitude of immune responses. Blood 109: 4071–4079.
22. Chen W, Ford MS, Young KJ, Zhang L (2003) Infusion of in vitro-generated DN T regulatory cells induces permanent cardiac allograft survival in mice. Transplant Proc 35: 2479–2483.
23. Young KJ, Yang L, Philips MJ, Zhang L (2002) Donor-lymphocyte infusion induces transplantation tolerance by activating systemic and graft-infiltrating double-negative regulatory T cells. Blood 100: 3408–3414.
24. Chen W, Zhou D, Torrealba JR, Waddell TK, Grant D, et al. (2005) Donor lymphocyte infusion induces long-term donor-specific cardiac xenograft survival through activation of recipient double-negative regulatory T cells. J Immunol 175: 3409–3416.
25. Gao JF, McIntyre MS, Juvet SC, Diao J, Li X, et al. (2011) Regulation of antigen-expressing dendritic cells by double negative regulatory T cells. *Eur J Immunol* 41: 2699–2708.

26. Zhang ZX, Stanford WL, Zhang L (2002) Ly-6A is critical for the function of double negative regulatory T cells. *Eur J Immunol* 32: 1384–1392.

27. Fuchs EJ, Matzinger P (1992) B cells turn off virgin but not memory T cells. *Science* 258: 1156–1159.

28. Minagawa R, Okano S, Tomita Y, Kishihara K, Yamada H, et al. (2004) The critical role of Fas-Fas ligand interaction in donor-specific transfusion-induced tolerance to H-Y antigen. *Transplantation* 78: 799–806.

29. Hengel RL, Thaker V, Pavlick MV, Metcalf JA, Dennis G Jr, et al. (2003) Cutting edge: L-selectin (CD62L) expression distinguishes small resting memory CD4+ T cells that preferentially respond to recall antigen. *J Immunol* 170: 28–32.

30. Ford MS, Young KJ, Zhang Z, Ohashi PS, Zhang L (2002) The immune regulatory function of lymphoproliferative double negative T cells in vitro and in vivo. *J Exp Med* 196: 261–267.

31. Ford McIntyre MS, Gao JF, Li X, Nacizi BM, Zhang L (2011) Consequences of double negative regulatory T cell and antigen presenting cell interaction on immune response suppression. *Int Immunopharmacol* 11: 597–603.

32. He KM, Ma Y, Wang S, Min WP, Zheng R, et al. (2007) Donor double-negative Treg promote allogeneic mixed chimerism and tolerance. *Eur J Immunol* 37: 3455–3466.

33. Zhang ZX, Ma Y, Wang H, Arp J, Jiang J, et al. (2006) Double-negative T cells, activated by xenoantigen, lyse autologous B and T cells using a perforin/granzyme-dependent, Fas-Fas ligand-independent pathway. *J Immunol* 177: 6920–6929.

34. Ford McIntyre MS, Young KJ, Gao J, Joe B, Zhang L (2008) Cutting edge: in vivo trogocytosis as a mechanism of double negative regulatory T cell-mediated antigen-specific suppression. *J Immunol* 181: 2271–2275.

35. McIver Z, Serio B, Dunbar A, O'Keefe CL, Powers J, et al. (2008) Double-negative regulatory T cells induce allotolerance when expanded after allogeneic haematopoietic stem cell transplantation. *Br J Haematol* 141: 170–178.