Xenotransplantation 2008: 15: 56–63
Printed in Singapore. All rights reserved
doi: 10.1111/j.1399-3089.2008.00444.x

XENOTRANSPLANTATION

Adoptive transfer of double negative T regulatory cells induces B-cell death in vivo and alters rejection pattern of rat-to-mouse heart transplantation

Ma Y, He KM, Garcia B, Min W, Jevnikar A, Zhang Z-X. Adoptive transfer of double negative T regulatory cells induces B-cell death in vivo and alters rejection pattern of rat-to-mouse heart transplantation. Xenotransplantation 2008; 15: 56–63. © 2008 Blackwell Munksgaard

Abstract: Background: Antibody-mediated hyperacute and acute graft rejection are major obstacles in achieving long-term graft survival in xenotransplantation. It is well documented that regulatory T (Treg) cells play a very important role in regulating immune responses to self and non-self antigens. Our previous studies have shown that $TCR\alpha\beta^+CD3^+CD4^-CD8^-$ (double negative, DN)-Treg cells can suppress anti-donor T-cell responses and prolong graft survival in allo- and xenotransplantation models. We have demonstrated that DN-Treg cells can induce B-cell apoptosis in vitro through a perforin-dependent pathway.
Methods: B6 mice received rat heart grafts, followed by 14 days of LF15-0195 treatment. Some mice received Lewis rat cell activated DN-Treg cells after LF treatment. DN-Treg cells, purified from perforin$^{-/-}$ mice and from B6 mice pre-immunized with third party rat cells, were used as controls.
Results: In this study, we investigated the possibility that adoptive transfer of xenoreactive DN-Treg cells could suppress B cells in vivo, thus prolonging xenograft survival. We found that apoptotic death of B cells significantly increased after adoptive transfer of DN-Treg cells. In addition, anti-donor IgG subtypes were significantly inhibited in the DN-Treg cell-treated group, in which the rejection pattern was altered towards cellular-mediated rejection rather than antibody-mediated acute vascular rejection. However, perforin-deficient DN-Treg cells failed to induce B-cell death and to prolong heart graft survival, indicating a perforin-dependent mechanism contributes to B-cell death in vivo.
Conclusions: This study suggests that adoptive transfer of xenoreactive DN-Treg cells can inhibit B-cell responses in vivo. DN-Treg cells may be valuable in controlling B-cell responses in xenotransplantation.

Yuexia Ma,[1] Kathy M. He,[1] Bertha Garcia,[1,2] Weiping Min,[1,2,3,4] Anthony Jevnikar[1,2,3,4] and Zhu-Xu Zhang[1,2,4]

[1]Immunology and Transplantation, Lawson Health Research Institute, [2]Department of Pathology, University of Western Ontario, [3]Department of Surgery, University of Western Ontario, [4]Department of Medicine, University of Western Ontario, London, ON, Canada

Key words: antibody – B cells – cytotoxicity – T cells – xenotransplantation

Abbreviations: AVR, acute vascular rejection; DN-Treg, $CD4^-CD8^-$ double negative regulatory T; FasL, Fas ligand; LF, LF15-0195; $PFN^{-/-}$, perforin deficient; TCR, T-cell receptor.

Address reprint requests to Zhu-Xu Zhang, Ph.D., C9-116, Multi-Organ Transplant Program, London Health Sciences Centre, 339 Windermere Road, London, Ontario N6A 5A5, Canada (E-mail: zhuxu.zhang@lhsc.on.ca)

Received 8 January 2008;
Accepted 9 January 2008

Introduction

Xenotransplantation has the potential to provide a virtually unlimited source of donor organs for patients with life-threatening organ failure. Antibody-mediated rejection is the major challenge in xenotransplantation. The mechanism of xenograft rejection in discordant models, such as pig to non-human primate, involves pre-existing, naturally occurring antibodies that bind to antigens on the surface of vascular endothelial cells. This results in vascular injury and hyperacute graft rejection [1–4]. In a concordant animal model, such as rat to mouse, where natural antibodies are not present,

graft-induced antibodies also appear to be responsible for acute vascular rejection (AVR). Further supporting this notion, it has been shown that B-cell deficiency allows rat heart grafts to survive for an extended time [5]. At present, no immunosuppressive drug can prevent antibody-mediated vascular xenograft rejection in the pig-to-non-human primate model. Thus, new therapeutic approaches are needed to enable successful xenotransplantation.

Current studies strongly suggest that transplant tolerance might be achievable via the manipulation of regulatory T (Treg) cells [6,7]. Those studies have focused only on allotransplantation; the role of Treg cells in xenotransplantation needs to be explored directly. A few investigators have proposed using Treg cells to enhance xenograft survival, and a recent study has shown that human CD4 Treg cells can suppress anti-porcine xenogeneic T-cell responses ex vivo [8]. Previous studies also have implied that $CD4^+$ T cells are involved in tolerance when a regimen of CD4 and/or CD8 depletion therapy was used following rat-to-mouse xenotransplantation [9,10]. It has been reported that $CD4^+$ Treg cells can directly suppress B cells in lymphoid tissue [11] and lyse antigen-presenting B cells through either Fas ligand (FasL)–Fas interactions [12] or the perforin pathway [13,14]. These studies suggest the possibility of using Treg cells to control B-cell responses.

In recent studies, we have discovered that T-cell receptor (TCR)$\alpha\beta^+CD3^+CD4^-CD8^-$ (double negative, DN)-Treg cells possess immune regulatory functions, and they play an important role in the development of tolerance after transplantation [15–18]. We have demonstrated that mouse DN-Treg cells specifically can eliminate activated syngeneic anti-donor $CD4^+$ T cells and $CD8^+$ T cells [15,16,18,19]. Furthermore, transfusion of DN-Treg cells leads to significant prolongation of donor-specific skin and heart allografts in a single major histocompatibility complex (MHC)-mismatched model [15,16,20]. In support of our findings, others have reported that DN-Treg cells are highly potent in suppressing alloimmunity both in vitro and in vivo in an antigen-specific manner [21]. DN-Treg cells can down-regulate $CD8^+$ T-cell-mediated immune responses in autoimmune and infectious disease models [22,23]. Our recent study has shown that DN-Treg cells, activated by xenoantigen, can kill syngeneic B cells through a perforin/granzyme-dependent pathway in vitro [24]. In this study, we investigate whether adoptive transfer of DN-Treg cells can induce B-cell death in vivo, thereby modulating the pattern of rat-to-mouse xenograft rejection.

Materials and methods

Animals

C57BL/6 (B6, H-2^b) and B6 perforin-deficient ($PFN^{-/-}$) mice as well as Brown Norway ($RT1^n$) and Lewis rats ($RT1^1$) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and Charles River Laboratories (Wilmington, MA, USA). Animals were maintained in the animal facility at the University of Western Ontario using approved protocols and procedures.

Antibodies and reagents

At various time points after activation, DN-Treg cells were characterized with fluorescent-conjugated monoclonal antibodies that specifically recognize the αβ-TCR, CD3, CD4, CD8, CD25, CD28, NK1.1, and γδ-TCR (eBioscience, San Diego, CA, USA). Data were acquired and analyzed on a Cytomics FC500 flow cytometer (Beckman Coulter, Fullerton, CA, USA). Isotype controls included mouse IgG1 (anti-rat, 1C7) and total mouse IgG1 (BD Pharmingen, San Diego, CA, USA). IgG subtype antibodies were purchased from Caltage (San Diego, CA, USA).

Isolation of DN-Treg cells

B6 mice were immunized twice (−10 and −1 days) with Lewis or Brown Norway rat spleen cells (3×10^7) before isolating DN-Treg cells. Spleen and lymph node cells were treated with anti-CD4 and anti-CD8 MACS beads (MiltenyiBiotec, Auburn, CA, USA) to deplete $CD4^+$ and $CD8^+$ T cells. The remaining cells were added to anti-CD90 (Thy-1)-coated MACS beads (MiltenyiBiotec) to purify $CD4^-CD8^-$ T cells. Viability and purity of the cells were monitored by flow cytometry. The purity of naïve DN-Treg cells was monitored by flow cytometry and $TCR\gamma\delta^-CD3^+CD4^-CD8^-NK1.1^-$ cells were > 92% pure. Further purification was performed if necessary.

Heart transplantation

Intra-abdominal heterotopic cardiac transplantation was performed using 2-week-old Lewis rats (25 to 30 g) as donors. Recipient mice were treated with the immunosuppressive analog of 15-deoxyspergualin, LF15-0195 (LF provided by Fournier Laboratories, Daix, France), at 2 mg/kg (dissolved in 0.9% sodium chloride) subcutaneously from day 0 to postoperative day 14. DN-Treg cells were purified from B6 mice that had been immunized with either Lewis or Brown

Norway rat spleen cells (3×10^7) 7 to 10 days earlier and boosted 1 day before purification. A total of 5×10^6 DN-Treg cells were purified from immunized B6 mice, and then intravenously adoptively transferred into a transplanted mouse. DN-Treg cells were transferred on days 16, 25, and 35 or days 16, 20, and 25 after transplantation.

Quantitation analysis of anti-donor Ig type

When cardiac impulses were no longer palpable, the graft and blood were removed for ex vivo studies. The circulating donor-specific Ig levels in recipient sera (1 : 100 dilution) were evaluated by flow cytometry, using donor spleen cells as targeting cells. The increase fold of mean fluorescent intensity was calculated according to serum obtained from naïve B6 mice before transplantation. The detecting anti-mouse IgM, IgG, IgG1, IgG2a, IgG2b, and IgG3-FITC were purchased from Caltag Laboratories (Burlingame, CA, USA).

Immunohistochemistry

A portion of the graft was collected on day of rejection, embedded in O.C.T. compound, and snap frozen in liquid nitrogen. Cryostat sections were fixed in acetone and stained for graft-infiltrating CD4 and CD8 as well as deposition of IgG using a standard protocol. All slides were stained by a strepavidin-biotin immunoperoxidase and its substrate method as described in our previous studies [5,25,26]. IgG deposition and infiltrating T cells were further quantified by calculating the positive stained area using Image-Pro Plus software (MediaCybernetics, Bethesda, MD, USA). At least six fields were calculated per slide.

Statistical analysis

The obtained data were compared using Student's *t*-test. P-value less than 0.05 were considered significant.

Results

Adoptive transfer of DN-Treg cells induces B-cell death in vivo while prolonging rat-to-mouse heart graft survival

In our previous study, we demonstrated that DN-Treg cells can lyse B cells in vitro through a perforin/granzyme-dependent pathway [24]. In this study, we assessed the possibility of eliminating B cells in vivo through adoptive transfer of DN-Treg cells.

We treated B6 mice recipients receiving Lewis rat cardiac xenografts with 2 mg/kg LF from day 0 to day 14. DN-Treg cells were purified from B6 mice that had been pre-immunized with Lewis or Brown Norway rat spleen cells (3×10^7) 7 to 10 days earlier. A total of 5×10^6 DN-Treg cells were then adoptively transferred into each transplanted mouse on days 16, 25, and 35 or days 16, 20, and 25. As summarized in Table 1, Lewis rat cells and short-term LF treatment significantly prolonged Lewis heart xenograft survival (62.1 ± 13.9 days vs. 32.3 ± 6.0 days in LF-treated alone group, $P < 0.001$, Student's *t*-test). Interestingly, adoptive transfer of DN-Treg cells, purified from mice immunized with Brown Norway rat spleen cells, also prolonged Lewis rat heart xenograft survival (43.4 ± 7.0 days vs. 32.3 ± 6.0 days in LF-treated alone group, $P < 0.05$, Student's *t*-test). Although Brown Norway specific DN-Treg cells were not as effective as Lewis specific DN-Treg cells (43.4 ± 7.0 days vs. 62.1 ± 13.9 days), these results indicate that the effect of DN-Treg cells in prolonging xenografts is not xenoantigen specific.

Prolongation of xenogeneic heart grafts may be a result of two possibilities: DN-Treg cells suppress T-cell responses, subsequently limiting T-dependent B-cell responses [27,28]; second, DN-Treg cells directly attack B cells in vivo. We assessed the latter possibility in our study. Spleen cells were administered on various days as listed in Table 1. Apoptotic death of B cells was measured by staining with anti-CD19 and 7-AAD. As shown in Fig. 1A, adoptive transfer of DN-Treg cells elicited substantial B-cell apoptotic death (by percentage) even though absolute B-cell numbers varied among mice. Adoptive transfer of DN-Treg cells significantly induced apoptotic death of B cells as compared with groups treated with LF-alone or $PFN^{-/-}$ DN-Treg cells (mean

Table 1. Lewis rat-to-mouse heart xenografts

Treat treatment	Individual survival (days)	MST ± SD (days)
None	14, 15, 16, 16 17, 18	16.0 ± 1.4
LF alone	22, 29, 32, 32, 33, 33, 34, 44	32.3 ± 6.0
LF, $PFN^{-/-}$DN-Treg	30, 31, 32, 40, 42, 44	36.5 ± 6.2
LF, B6 DN-Treg[a]	37, 42, 42, 60, 64,70, 81, 84	62.1 ± 13.9
LF, 3rd party DN-Treg[b]	35[c], 40, 44, 44, 54	43.4 ± 7.0

LF: 2mg/kg, 0 to 14 days, DN-Treg cell transfusion: days 16, 25, 35 or 16, 20, 25 after xenograft heart transplantation.
[a]$P < 0.001$ vs. LF-alone. Student's *t*-test.
[b]$P < 0.05$ vs. LF-alone. Student's *t*-test. Mice received LF + third party (BN rat) DN-Treg cells.
[c]The mouse died with a beating heart.

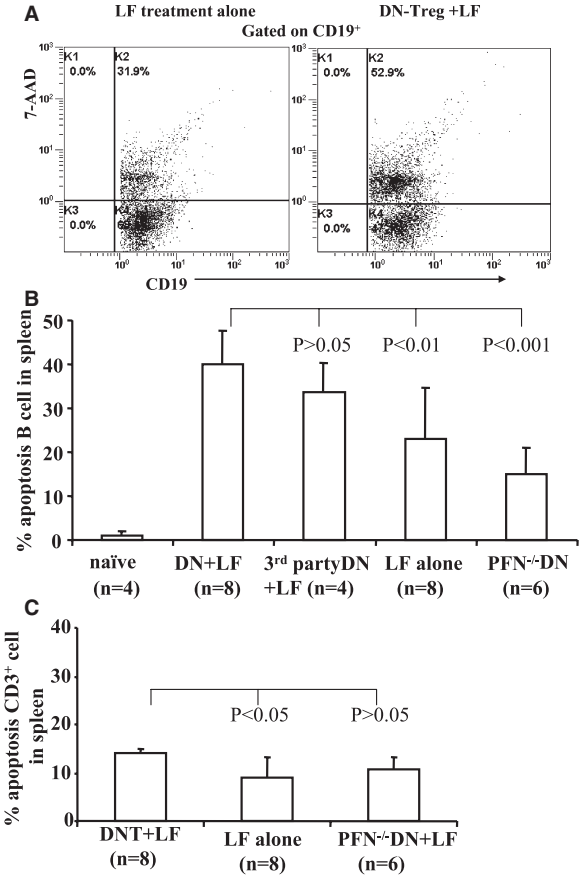

Fig. 1. DN-Treg cells induce apoptotic death of B cells in vivo. B6 mice received Lewis rat heart grafts and were treated with 2 mg/kg LF from day 0 to day 14. DN-Treg cells (5×10^6) were purified from B6 or $PFN^{-/-}$ mice that had been immunized with either Lewis or Brown Norway rat spleen cells (3×10^7) 10 days previously and then boosted 1 day before purification, before being intravenously adoptively transferred into transplanted mice. Multiple DN-Treg cell transfers were performed on days 16, 25, and 35 or days 16, 20, and 25 after transplantation. 7-AAD staining was performed to determine the percentage of apoptotic B and T cells in spleens of recipient mice by gating on the $CD19^+$ or $CD3^+$ cells. (A) Phenotype analysis of B cells in one of the recipient spleens. (B) Pooled $CD19^+$ and $7\text{-}AAD^+$ staining results from each group. (C) Pooled $CD3^+$ and $7\text{-}AAD^+$ staining results from each group. P-value, calculated by Student's *t*-test, was compared with DN-Treg + LF co-treated groups in all experiments.

apoptosis: 39.9% vs. 21.8% and 15.5%; Fig. 1B). Adoptive transfer of third party DN-Treg cells also induced B-cell apoptosis, indicating an antigen non-specific function of DN-Treg cells. Hence, this data indicates that adoptive transfer of DN-Treg cells can control B-cell responses in vivo by PFN-dependent and antigen-independent mechanisms, which correlates with the survival results presented (Table 1).

Interestingly, we found that not only B cells, but also $CD3^+$ T cells, became apoptotic after DN-Treg cell treatment (Fig. 1C). Although the level of apoptotic T cells was much lower than B cells, this result supports the notion that DN-Treg cells can suppress anti-donor $CD4^+$ and $CD8^+$ T-cell responses [27].

Adoptive transfer of DN-Treg cells inhibits anti-donor antibody production

Next, we studied if an increased B-cell apoptosis level after DN-Treg cell treatment is correlated with production of anti-donor antibody.

Sera, collected on days of rejection, were used for antibody subtyping. The anti-donor total IgG and IgM levels were significantly inhibited in mice receiving DN-Treg cells + LF treatment (Fig. 2A, IgG: $P < 0.05$, IgM: $P < 0.01$, compared to LF-alone, Student's *t*-test). Interestingly, all mice generated dominant IgG1 and IgG2b subtypes against donor antigens in LF-alone group. The level of IgG1, not IgG2b, was significantly inhibited after DN-Treg cell transfer (Fig. 2A). However, transfer of DN-Treg cells from $PFN^{-/-}$ mice did not affect anti-donor IgG and IgG subtypes production (Fig. 2A), lending support to our previous finding that DN-Treg cells mediate B-cell death in a PFN-dependent manner.

We further assessed direct evidence of antibody inhibition in the grafts using immunohistochemistry analysis. As shown in Fig. 2B, IgG deposition was reduced after DN-Treg cell treatment. We quantified the antibody deposition area by image analysis (Image-Pro; MediaCybernetics) to estimate the IgG-stained positive area. The pooled statistical data indicate that the IgG positive area was significantly reduced after DN-Treg cell treatment vs. LF-alone treatment (27.1% vs. 10.0%, Fig. 2C, $P < 0.01$, Student's *t*-test). Hence, our results indicate that adoptive transfer of DN-Treg cells can limit anti-donor B-cell responses as well as reduce antibody production and deposition in the donor graft.

Adoptive transfer of DN-Treg cells alters immunohistochemistry pattern of AVR in heart xenograft

Although adoptive transfer of DN-Treg cells prolonged survival of xenografts, all grafts were eventually rejected (Table 1). Because the above data indicate that adoptive transfer of DN-Treg cells can inhibit B-cell-mediated xenograft rejection, we used immunohistochemistry analysis to further assess direct evidence in the grafts. LF-treated grafts showed strong vasculitis and hemorrhage. Heart graft vessels had been severely damaged (Fig. 3A, H &E staining), indicating

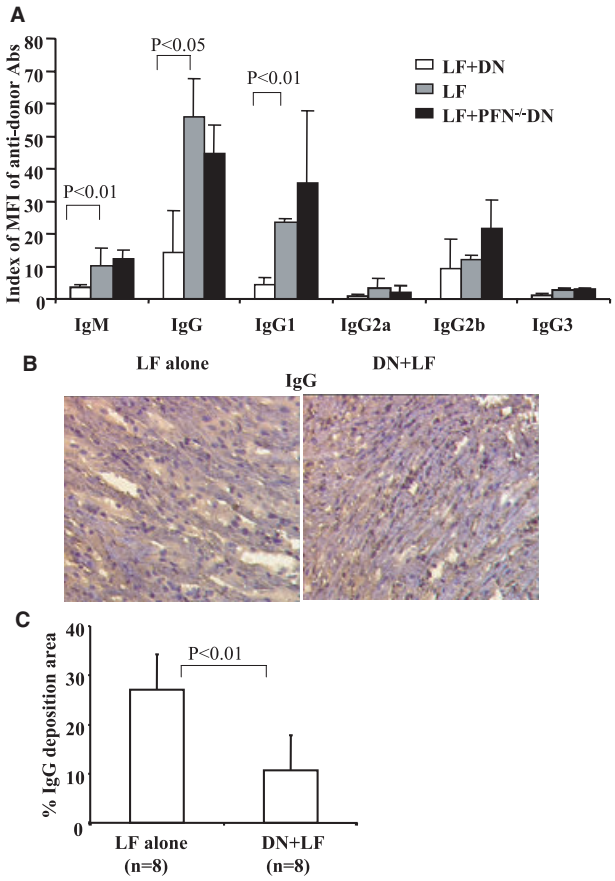

Fig. 2. Adoptive transfer of DN-Treg cells inhibits anti-donor antibody production and graft deposition. Sera were collected from mice with rejected grafts. The anti-donor IgM, IgG, IgG1, IgG2a, IgG2b, and IgG3 were measured against donor spleen cells as described in the Materials and methods. Increase ratio of anti-donor mean fluorescence intensity (MFI) was calculated according to sera obtained from naïve B6 mice before transplantation (A). Immunohistochemistry was performed according to a standard protocol. Graft-deposition total IgG of one mouse from each treatment group was presented (B). IgG deposition was quantified by image analysis (Image-Pro, MediaCybernetics) to estimate the IgG stained positive area (magnification ×200). Six random areas were calculated from each slide. The statistical data were pooled from eight mice from each treatment group (C). P-value was calculated by Student's *t*-test.

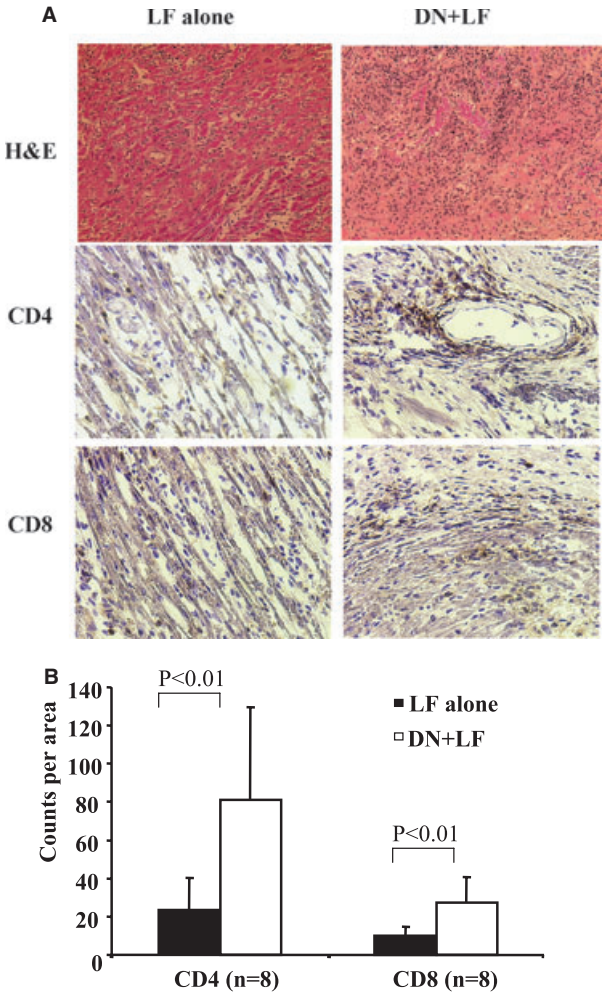

Fig. 3. Pathology analysis of heart xenografts. Routine pathology and immunohistochemistry were performed according to standard protocols. H&E staining and graft-infiltrating $CD4^+$ and $CD8^+$ T cells from one mouse in each treatment group (A). Graft-infiltrating $CD4^+$ and $CD8^+$ T cells were quantified using Image-Pro Plus software (Media-Cybernetics). Six random areas were calculated from each slide. Statistical data were pooled from all mice from each treatment group (B). P-value was calculated by Student's *t*-test.

antibody-mediated vascular graft rejection. Interestingly, although tissue damage was also seen in DN + LF co-treated mice, a high level of graft-infiltrating cells was present in these mice compared to the LF-alone mice (Fig. 3A, H &E staining). Furthermore, increased levels of $CD4^+$ and $CD8^+$ T cells were observed in grafts (Fig. 3A). We further quantified infiltrating T cells using image analysis software (Image-Pro Plus, Media Cybernetics). As shown in Fig. 3B, graft-infiltrating $CD4^+$ T cells were significantly increased after co-treatment with DN-Treg cells and LF, compared to LF-alone treatment ($P < 0.01$, Student's *t*-test). Although low levels of infiltrating $CD8^+$ T cells were found in both groups, similar to the pattern of $CD4^+$ T cells, the mice treated with DN-Treg cells had more infiltrating $CD8^+$ T cells compared to LF-alone group ($P < 0.01$, Student's *t*-test). Hence, co-treatment using DN-Treg cells and LF may suppress B-cell responses and control AVR, but this is not efficient to control later cell-mediated xenograft rejection. Taken together, our immunohistochemistry data suggest that adoptive transfer of DN-Treg cells can alter the xenograft rejection pattern from AVR to one of cell-mediated rejection.

Discussion

Because the major barrier to xenotransplantation is antibody-mediated AVR, the first goal to achieving its success is the establishment of B-cell tolerance, either by eliminating or regulating anti-donor B-cell responses. Currently, conventional immunosuppressive drugs, which are successful in allotransplantation, cannot prevent AVR after xenograft heart transplantation. In this study, we demonstrated that xenoantigen-activated DN-Treg cells can eliminate syngeneic B cells in vivo while prolonging rat-to-mouse heart xenograft survival. We have identified a mechanism of xenoreactive DN-Treg cell-mediated B-cell apoptotic death in vivo that is dependent on perforin/granzymes. Together, our data indicate that DN-Treg cells can mediate immune regulation in xenotransplantation by targeting B cells.

Although adoptive transfer of DN-Treg cells prolonged xenograft survival, we found that Brown Norway-specific DN-Treg cells could also prolong Lewis rat heart xenograft survival (Table 1), indicating the effect of DN-Treg cells could be xenoantigen non-specific. This result supports our previous in vitro finding that DN-Treg cells do not form a classical immunological synapse with target B cells in vitro [24]. The TCR–MHC recognition may not be needed for the killing, however, the engagement of cell adhesion molecules lymphocyte function-associated antigen (LFA)-1 and its ligand intercellular adhesion molecule 1 is necessary [24]. Recent studies have supported the notion that neither the formation of a stable and mature immunological synapse nor complete signaling are required for T-cell cytotoxicity [29], and $CD8^+$ T cells can form an antigen-independent ring junction with APC [30]. Our in vitro and in vivo results indicate that DN-Treg cells can suppress B cells through a bystand mechanism.

The suppressive function of $CD4^+$ Treg cells has been previously shown to be mediated by IL-10, CTLA-4, and/or membrane-bound TGF-β [31]. In addition, recent studies have suggested that chemokine metabolism [32], indirect tryptophan metabolism [33], FasL [12], and granzymes [34] could also be involved in the function of Treg cells. In previous studies, alloreactive DN-Treg cells suppressed $CD8^+$ T cells through the Fas–FasL interaction [15,16,22]. However, DN-Treg cells can also suppress $CD4^+$ T cells through a Fas-independent mechanism involving the inhibition of IL-2 production in $CD4^+$ T cells [35]. Interestingly, in our previous in vitro study, the xenoantigen-activated DN-Treg cells target both B and T cells by a perforin/granzyme-dependent pathway, rather than FasL-dependent pathway [24]. In this study, we have further shown that DN-Treg cells can induce B-cell apoptotic death in vivo, and this is perforin dependent (Fig. 1). Our finding that DN-Treg cells eliminate B cells in vivo and in vitro via a perforin-dependent mechanism indicates that DN-Treg cells, activated by xenoantigen, are different from alloantigen-activated ones. This is worthy of further investigation in terms of TCR/xeno MHC and co-stimulator interactions.

A recent study has reported that $CD4^+$ Treg cells can directly suppress B cells in lymphoid tissue [11]. In this study, we found a high percentage of apoptotic B cells in recipient spleens (Fig. 1B), in which 9.6% of DN-Treg cells were found among $CD3^+$ T cells (data not shown). Furthermore, the levels of anti-donor IgM and IgG were significantly inhibited after DN-Treg cell transfer (Fig. 2A). A similar pattern was also seen in immunohistochemistry in staining (Fig. 2B and data not shown). Quantification of antibody deposition by image analysis indicates that DN-Treg cell treatment significantly inhibited anti-donor antibody levels in the graft (Fig. 2C), indicating that DN-Treg cells are capable of attacking activated B cells after xenotransplantation.

The finding that DN-Treg cells can significantly delay xenoheart rejection through suppression of anti-donor antibodies is supported by immunohistopathological data in which DN-Treg mice showed much less deposition of IgG in the graft (Fig. 2B,C). Although adoptive transfer of DN-Treg cells could prolong graft survival, and one mouse had no signs of AVR with fewer infiltrating cells after 50 days (data not shown) [24], all grafts were eventually rejected. Immunohistochemistry data indicate that graft-infiltrating $CD4^+$ and $CD8^+$ T cells significantly increased in DN-Treg cell-treated group (Fig. 3), suggesting the rejection pattern has been altered towards cellular-mediated rejection. Although we found that T-cell apoptosis occurs after DN-Treg cell treatment (Fig. 1C), the effect of DN-Treg cells was not efficient or sustained, and the grafts were all rejected (Table 1). Hence, it is necessary to develop a new strategy that will increase the in vivo efficacy of DN-Treg cells over the long term as adoptive transfer of DN-Treg cells did not induce tolerance in these recipients. Further investigations are needed to optimize the conditions for maintaining DN-Treg cells in vivo as this may play a vital role in the ultimate success of achieving tolerance after xenotransplantation.

In summary, we have shown that adoptive transfer of DN-Treg cells can induce B-cell apoptotic death in vivo and can limit anti-donor antibody responses, thereby altering the rejection

pattern from AVR to cell-mediated rejection. Further studies should assess whether DN-Treg cells could be combined with currently available immunosuppressive protocols to prevent antibody-mediated rejection in a large animal model.

Acknowledgments

The authors acknowledge Jifu Jiang and Winnie Liu for laboratory assistance and Ms. Cate Abbott for editorial assistance. This study has been supported by funds from the Heart and Stroke Foundation of Canada, and Canada Foundation of Innovation to Z-X Zhang.

References

1. Koch CA, Khalpey Z, Platt JL. Humoral immunity in xenotransplantation: B cell tolerance and accommodation. Curr Opin Organ Transplant 2004; 9: 170–175.
2. Cascalho M, Platt JL. The immunological barrier to xenotransplantation. Immunity 2001; 14: 437–446.
3. Ohdan H, Sykes M. B cell tolerance to xenoantigens. Xenotransplantation 2003; 10: 98–106.
4. Lin SS, Weidner BC, Byrne GW et al. The role of antibodies in acute vascular rejection of pig-to-baboon cardiac transplants. J Clin Invest 1998; 101: 1745–1756.
5. Wang H, Devries ME, Deng S et al. The axis of interleukin 12 and gamma interferon regulates acute vascular xenogeneic rejection. Nat Med 2000; 6: 549–555.
6. Bluestone JA. Regulatory T-cell therapy: is it ready for the clinic? Nat Rev Immunol 2005; 5: 343–349.
7. Waldmann H, Cobbold S. Exploiting tolerance processes in transplantation. Science 2004; 305: 209–212.
8. Porter CM, Bloom ET. Human CD4+CD25+ regulatory T cells suppress anti-porcine xenogeneic responses. Am J Transplant 2005; 5: 2052–2057.
9. Chen Z, Cobbold S, Metcalfe S, Waldmann H. Tolerance in the mouse to major histocompatibility complex-mismatched heart allografts, and to rat heart xenografts, using monoclonal antibodies to CD4 and CD8. Eur J Immunol 1992; 22: 805–810.
10. Ikehara Y, Yasunami Y, Kodama S et al. CD4(+) Valpha14 natural killer T cells are essential for acceptance of rat islet xenografts in mice. J Clin Invest 2000; 105: 1761–1767.
11. Lim HW, Hillsamer P, Banham AH, Kim CH. Cutting edge: direct suppression of B cells by CD4+ CD25+ regulatory T cells. J Immunol 2005; 175: 4180–4183.
12. Janssens W, Carlier V, Wu B et al. CD4+CD25+ T cells lyse antigen-presenting B cells by Fas-Fas ligand interaction in an epitope-specific manner. J Immunol 2003; 171: 4604–4612.
13. Grossman WJ, Verbsky JW, Barchet W et al. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. Immunity 2004; 21: 589–601.
14. Zhao DM, Thornton AM, Dipaolo RJ, Shevach EM. Activated CD4+CD25+ T cells selectively kill B lymphocytes. Blood 2006; 107: 3925–3932.
15. Zhang ZX, Yang L, Young KJ, Dutemple B, Zhang L. Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression. Nat Med 2000; 6: 782–789.
16. Ford MS, Young KJ, Zhang Z, Ohashi PS, Zhang L. The immune regulatory function of lymphoproliferative double negative T cells in vitro and in vivo. J Exp Med 2002; 196: 261–267.
17. Young KJ, Yang L, Phillips MJ, Zhang L. Donor-lymphocyte infusion induces transplantation tolerance by activating systemic and graft-infiltrating double-negative regulatory T cells. Blood 2002; 100: 3408–3414.
18. Zhang ZX, Stanford WL, Zhang L. Ly-6A is critical for the function of double negative regulatory T cells. Eur J Immunol 2002; 32: 1584–1592.
19. Zhang ZX, Ford MS, Chen W, Zhang L. Double negative T regulatory cells can develop outside the thymus and do not mature from CD8+ T cell precursors. J Immunol 2006; 177: 2803–2809.
20. Chen W, Ford MS, Young KJ, Zhang L. Infusion of in vitro-generated DN T regulatory cells induces permanent cardiac allograft survival in mice. Transplant Proc 2003; 35: 2479–2480.
21. Zhang D, Yang W, Degauque N et al. New differentiation pathway for double-negative regulatory T-cells that regulates the magnitude of immune responses. Blood 2007; 109: 4071–4079.
22. Priatel JJ, Utting O, Teh HS. TCR/self-antigen interactions drive double-negative T cell peripheral expansion and differentiation into suppressor cells. J Immunol 2001; 167: 6188–6194.
23. Johansson M, Lycke N. A unique population of extrathymically derived alpha beta TCR+CD4-CD8- T cells with regulatory functions dominates the mouse female genital tract. J Immunol 2003; 170: 1659–1666.
24. Zhang ZX, Ma Y, Wang H et al. 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 2006; 177: 6920–6929.
25. Wang H, Hosiawa KA, Garcia B et al. Attenuation of acute xenograft rejection by short-term treatment with LF15-0195 and monoclonal antibody against CD45RB in a rat-to-mouse cardiac transplantation model. Transplantation 2003; 75: 1475–1481.
26. Wang H, Hosiawa KA, Garcia B et al. Treatment with a short course of LF 15-0195 and continuous cyclosporin A attenuates acute xenograft rejection in a rat-to-mouse cardiac transplantation model. Xenotransplantation 2003; 10: 325–336.
27. Chen W, Ford MS, Young KJ, Cybulsky MI, Zhang L. Role of double-negative regulatory T cells in long-term cardiac xenograft survival. J Immunol 2003; 170: 1846–1853.
28. Chen W, Zhou D, Torrealba JR et al. Donor lymphocyte infusion induces long-term donor-specific cardiac xenograft survival through activation of recipient double-negative regulatory T cells. J Immunol 2005; 175: 3409–3416.
29. Purbhoo MA, Irvine DJ, Huppa JB, Davis MM. T cell killing does not require the formation of a stable mature immunological synapse. Nat Immunol 2004; 5: 524–530.
30. Somersalo K, Anikeeva N, Sims TN et al. Cytotoxic T lymphocytes form an antigen-independent ring junction. J Clin Invest 2004; 113: 49–57.
31. Von Boehmer H. Mechanisms of suppression by suppressor T cells. Nat Immunol 2005; 6: 338–344.
32. James MJ, Belaramani L, Prodromidou K et al. Anergic T cells exert antigen-independent inhibition of cell-cell interactions via chemokine metabolism. Blood 2003; 102: 2173–2179.

33. Fallarino F, Grohmann U, Hwang KW et al. Modulation of tryptophan catabolism by regulatory T cells. Nat Immunol 2003; 4: 1206–1212.
34. Grossman WJ, Verbsky JW, Tollefsen BL et al. Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. Blood 2004; 104: 2840–2848.
35. Hamad AR, Mohamood AS, Trujillo CJ et al. B220+ double-negative T cells suppress polyclonal T cell activation by a Fas-independent mechanism that involves inhibition of IL-2 production. J Immunol 2003; 171: 2421–2426.