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B cell-derived IL-1 β and IL-6 drive T cell reconstitution following lymphoablation

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Abstract

Understanding the mechanisms of T cell homeostatic expansion is crucial for clinical applications of lymphoablative therapies. We previously established that T cell recovery in mouse heart allograft recipients treated with anti-thymocyte globulin (mATG) critically depends on B cells and is mediated by B cell-derived soluble factors. B cell production of IL-1 β and IL-6 is markedly up-regulated after heart allotransplantation and lymphoablation. Neutralizing IL-1 β or IL-6 with mAb or the use of recipients lacking mature IL-1 β , IL-6, IL-1R, MyD88, or IL-6R impair CD4⁺ and CD8⁺ T cell recovery and significantly enhance the graft-prolonging efficacy of lymphoablation. Adoptive co-transfer experiments demonstrate a direct effect of IL-6 but not IL-1 β on T lymphocytes. Furthermore, B cells incapable of IL-1 β or IL-6 production have diminished capacity to mediate T cell reconstitution and initiate heart allograft rejection upon adoptive transfer into mATG treated B cell deficient recipients. These findings reveal the essential role of B cell-derived IL-1 β and IL-6 during homeostatic T cell expansion in a clinically relevant model of lymphoablation.

1. Introduction

Antibody-mediated lymphocyte depletion is a common strategy to eliminate donor-reactive T cells in solid organ and stem cell transplantation (1). A large proportion of kidney transplant recipients are treated with polyclonal rabbit anti-thymocyte globulin (ATG) as part of the induction therapy (2). Lymphoablation improves short-term outcomes but has modest effects on long-term graft survival (3–5). Furthermore, memory T cells are less susceptible to depletion and are still detectable after ATG treatment following renal transplantation (6).

Studies in mouse transplant models using a murine analog of ATG (mATG) provided mechanistic insights into the challenges of systemic lymphoablation. Using a mouse cardiac transplantation model we previously showed that following mATG treatment recovering T

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Supporting Information

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cell repertoire is dominated by homeostatically expanding T cells originating from the pre-existing memory compartment. The rapid memory T cell reconstitution resulted in only modest heart allograft prolongation by mATG and did not depend on T cell specificity for donor alloantigens suggesting an important role for posttransplant inflammation in this process (7, 8). Subsequent studies revealed that the mechanisms underlying homeostatic T cell expansion following in situ lymphoablation in organ transplant recipients are quite distinct from those observed after whole body irradiation or adoptive T cell transfer into lymphopenic hosts. Following the treatment with mATG or other polyclonal or monoclonal depleting antibody, the depletion efficiency of CD4⁺ T cells is typically lower than that of CD8⁺ T cells (8, 9). Our group previously reported that the helper signals from depletion-resistant memory CD4⁺ T cells are essential for CD8⁺ T cell homeostatic reconstitution and that B cells activated via CD40 are central mediators of this help (7). In addition to antibody production, B cells can perform other functions in the context of transplantation including antigen presentation, costimulatory signals, and cytokine and chemokine secretion (10–13). Our recent findings indicate that cognate TCR-pMHC interactions between B cells and T cells are dispensable for T cell recovery suggesting the potential role for B cell derived cytokines in the context of mATG lymphoablation. NanoString gene expression analysis revealed that mATG treatment upregulated several cytokines and cytokine receptor genes including IL-27, IL-15, IL-1 β and IL-6 in B cells and that the upregulation was dependent on CD4⁺ T cell help. Unexpectedly, IL-15 was dispensable for CD8⁺ T cell recovery, whereas targeting IL-27 reduced but not completely inhibited CD8⁺ T cell reconstitution suggesting the contribution of other cytokines (14).

IL-1 β and IL-6 are key proinflammatory cytokines that drive local and systemic immune responses (15, 16). The direct effects of these cytokines on T cell survival, expansion and effector functions have been previously reported in several models including autoimmunity, anti-tumor immunity and model antigen immunization (17–24). While B cell production of IL-1 β and IL-6 has been previously described, myeloid and non-hematopoietic cells are typically considered as main producers of these cytokines during inflammation. Both IL-1 β and IL-6 are found at low levels in normal heart tissue, however their local and systemic expression is upregulated following syngeneic or allogeneic transplantation (25). Numerous studies in mouse transplant models have demonstrated a role for IL-1 β and IL-6 in allograft rejection and resistance to graft tolerance (26–36). However, the origin of these cytokines in lymphopenic transplant recipients and their role in T cell homeostatic recovery remain to be elucidated.

In this study, we show that B cells are the major source of IL-1 β and IL-6 following in situ antibody-mediated lymphoablation in heart allograft recipients. B cell-derived IL-1 β and IL-6 mutually regulate each other's production. Most importantly, targeting IL-1 β and IL-6 or their respective receptors delays T cell recovery and enhances the graft prolonging effects of mATG lymphoablation.

2. Methods

2.1. Animals

Male and female C57BL/6J (H-2^b) [B6.WT], BALB/cJ (H-2^d) [BALB/c], SJL/J-Pde6b^{rd1} (H2^s) [SJL], B6.129S2-Ighm^{tm1Cgn}/j (H-2^b) [B6.μMT], B6N.129S2-Casp1^{tm1Flv}/J [caspase-1 KO], B6.129P2(SJL)-Myd88^{tm1.Delf}/J [MyD88 KO], B6.129S7-Il1r1^{tm1Imx}/J [IL-1R1 KO], B6.129S2Il6^{tm1Kopf}/J [IL-6 KO], B6.SJL-Ptprc^a Pepc^b/BoyJ [B6.CD45.1], B6;SJL-Il6ra^{tm1.Drew}/J [Il6ra^{fl}], and Tg(Cd4-cre)1Cwi/BfluJ [CD4^{Cre}] mice aged 6–8 weeks, were purchased from The Jackson Laboratories (Bar Harbor, ME). All animals were maintained and bred in the pathogen-free facility at the Cleveland Clinic. All animal procedures were approved by the Institutional Animal Care and Use Committee.

2.2. Heart transplantation

Vascularized heterotopic cardiac transplants were performed as described previously (37). Rejection was defined as a loss of palpable heartbeat and confirmed by laparotomy.

2.3. mATG preparation and recipient treatment

Rabbit anti-mouse thymocyte serum was generated by the Hybridoma Core at the Cleveland Clinic Lerner Research Institute as previously described (14). Heart allograft recipients were treated with mATG (0.5 mg i.p.) on days 0 and 4 posttransplant. For B cell depletion, we used anti mouse CD20 mAb (250 μg, clone 18B12, Biogen Inc., Cambridge, MA) on days –3 (i.v.) and 11 (i.p.). For IL-1β neutralization, recipients were injected i.p. with 200 μg anti-IL-1β mAb (clone: B122, BioXCell, West Lebanon, NH) or control polyclonal Armenian hamster IgG (BioXCell) on days –1, 2, 5, 8, and 11 posttransplant. For IL-6 neutralization, recipients were injected i.p. with 200 μg anti-IL-6 mAb (clone: MP5–20F3, BioXCell) on d. –1, 2, 5, 8, and 11.

2.4. Adoptive cell transfer

B6.μMT mice were transplanted with BALB/c heart allografts and injected with mATG on days 0 and 4 posttransplant. B220⁺ cells were isolated by EasySep immunomagnetic B cell isolation kit (STEMCELL Technologies, Cambridge, MA) from spleens of either naïve B6 mice, caspase-1 (Casp-1) KO or IL-6 KO mice (> 94% B220⁺ cells) and 30×10⁶ cells were injected i.v. into mATG-treated B6.μMT recipients on day 6 posttransplant (2 d after last mATG injection).

For co-transfer study, CD8⁺ T or CD4⁺ T cells were isolated by EasySep immunomagnetic CD8, CD4 T cell isolation kit (STEMCELL Technologies) from spleens of B6.CD45.1⁺ WT and either IL-1R1 KO, MyD88 KO or Il6ra^{fl/fl} × CD4^{Cre} CD45.2⁺ mice (>93% purity) and were mixed at a 1:1 ratio. 20 × 10⁶ mixed cells were intravenously injected into congenic CD45.1/2 mice (generated by crossing B6.WT and B6.CD45.1⁺ mice) on d. –2 followed by BALB/c heart transplantation and mATG treatment.

2.5. In vitro B cell stimulation

B cells were isolated from naïve WT, Casp-1 KO or IL-6 KO mice by EasySep immunomagnetic kit and incubated for 16 h in complete media (RPMI 1640 supplemented

with 10% FBS and 2mM L-glutamine) with or without the addition of 1 µg/ml LPS (Lipopolysaccharides from *Escherichia coli* O55:B5) (MilliporeSigma, St. Louis, MO). For intracellular cytokine staining, after 16 h of LPS incubation, cells were stimulated with agonistic anti-CD40 mAb (10 µg/ml, clone FGK4.5; BioXcell, West Lebanon, NH), PMA (10 ng/ml, MilliporeSigma) and ionomycin (1 µM, MilliporeSigma) for 4 hours in the presence of 2 µM monensin (Biolegend, San Diego, CA) during the last 2 hours of stimulation to inhibit cytokine release from the Golgi/endoplasmic reticulum complex. After incubation, cells were analyzed by flow cytometry for the expression of IL-1β and IL-6.

2.6. Real-time PCR

RNA samples were prepared from spleen B220⁺ cells isolated by EasySep immunomagnetic B cell isolation kit. Reverse transcription and quantitative real-time PCR were performed as previously described (14). Probes and primers were from Taqman gene expression assay reagents (Applied Biosystems): IL-1β (Mm00434228_m1) and IL-6 (Mm00446190_m1). Data were normalized to Mrpl 32 (Mm00777741-sH) RNA amplification and calculated relative to the expression of the target gene in B220⁺ cells from naive B6 mice.

2.7. Flow cytometry

Fluorochrome-conjugated antibodies were purchased from BD Biosciences (San Diego, CA) or from eBioscience (San Diego, CA) (Supplementary Table 1). Cells were isolated from peripheral blood and spleen and stained as previously described (8, 38). At least 50,000 live events per sample were acquired on a LSR II (BD Bioscience) followed by data analysis using FlowJo software (Tree Star Inc., Ashland, OR).

2.8. ELISPOT assay

IFN-γ ELISPOT assays were performed as described previously using capture and detecting anti-mouse IFN-γ Abs from BD Biosciences (37, 39, 40). Briefly, recipient spleen cells were stimulated with mitomycin C-treated BALB/c or SJL spleen cells for 20–24 h. The resulting spots were analyzed using an ImmunoSpot Series 4 analyzer (Cellular Technology, Cleveland, OH).

2.9. Statistics

All data are expressed as the mean ± SD. The following data analysis tests were used: a twotailed Student's t test for comparisons between two groups, one-way analysis of variance (ANOVA) with Tukey's multiple-comparisons post hoc test for comparisons among three or more groups, multiple t-tests for kinetics of T cell recovery, and Mantel-Cox test for graft survival. Analyses were performed with GraphPad Prism 8 (San Diego, CA). Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Allograft-induced inflammation facilitates T cell recovery

We first investigated the kinetics of T cell depletion and recovery after mATG treatment in naive non-transplanted B6 mice, B6 (H-2^b) recipients of B6 (H-2^b) heart isografts and B6

(H-2^b) recipients of BALB/c (H-2^d) heart allografts. Both circulating CD8⁺ and CD4⁺ T cells were markedly decreased by day 7 and gradually recovered by day 28. T cell expansion occurs faster in heart allograft recipients compared to non-transplanted and isograft transplanted mice in both the peripheral blood (Figure 1A) and spleen (Figure 1B). Consistent with the rapid T cell reconstitution, mATG treated allograft recipients rejected transplanted hearts (MST of 11 days vs > 60 days in isograft recipients, n = 6–8 animals per group) and developed donor-specific T cell responses (Figure 1C). While we have previously reported that post-mATG T cell reconstitution does not depend on T cell specificity for donor alloantigens (8), these current findings raise the possibility that alloimmunity drives T cell recovery by enhancing posttransplant inflammation.

3.2. B cells are the major source of IL-1 β and IL-6 following mATG treatment and allogeneic transplantation

We have previously shown that residual CD4⁺ T cells activate B cells, and that B cells in turn are required to promote CD8⁺ T cell proliferation (7). Transient B cell depletion with anti-human CD20 mAb (Rituximab) temporarily delayed CD8⁺ T cell recovery in mATG treated B6 heart allograft recipients expressing huCD20tg under B220 promoter (7). We confirmed our previous findings using anti-mouse CD20 mAb. Prolonged B cell depletion significantly diminished the reconstitution of both CD4⁺ and CD8⁺ T cells and prolonged survival of BALB/c (H-2^d) heart allografts in B6 (H-2^b) recipients treated with mATG (Figure 2A–C). We also previously reported that the B cell expression of MHC and CD80/86 molecules is dispensable for T cell recovery suggesting the potential role for B cell derived soluble factors. NanoString gene expression assay performed on isolated spleen B cells showed that more than 30 genes are upregulated by mATG in CD4 dependent manner with proinflammatory cytokines IL-1 β and IL6 among the top candidates (14).

We investigated the source of IL-1 β and IL-6 in allograft and isograft recipients. RT-PCR analysis of isolated B cells confirmed the high expression of IL-1 β and IL-6 genes after allotransplantation and mATG treatment (Figure 2D). Intracellular flow cytometry revealed that mATG treatment enhances IL-1 β and IL-6 production in B220⁺, CD11b⁺ and CD11c⁺ cell subsets from isograft recipients. In B cells, the cytokine production is further increased by allograft placement (Figure E–F, Supplementary Figure 1). Recipient deficiency in IL-6 leads to low levels of IL-1 β expression in B cells (Figure 2G). Similarly B cell IL-6 expression is reduced in mATG treated Casp-1 KO allograft recipients that do not produce active IL-1 β (Figure 2H). To address the effects of Casp-1 or IL-6 deficiency specifically in B lymphocytes, B220⁺ cells were isolated from naïve WT, Casp-1 KO or IL-6 KO mice and stimulated with LPS in vitro for 20 h. Intracellular flow cytometry analysis demonstrated that B cells deficient in Casp-1 have diminished capacity to produce IL-6 in response to LPS stimulation. Conversely, IL-6 KO B cells produce markedly lower levels of IL-1 β compared to WT B cells (Figure 2I). Taken together, these results suggest that B cells are the major source of IL-1 β and IL-6 after mATG lymphoablation and that these cytokines mutually regulate each other's production in B cells.

3.3. IL-1 β neutralization or deficiency compromises homeostatic recovery of T cells

To test the role of IL-1 β in T cell homeostatic reconstitution, we used several complementary strategies. Initially, B6 mice were transplanted with BALB/c heart allografts and treated with either mATG alone, with mATG plus anti-IL-1 β mAb or mATG plus control hamster IgG. IL-1 β neutralization inhibited both CD4 $^{+}$ and CD8 $^{+}$ T cell recovery in peripheral blood (Figure 3A) and in the spleen (Figure 3C). Moreover, it modestly but significantly improved the efficacy of mATG in prolonging heart allograft survival (MST of 18 d in mATG + anti-IL-1 β vs 13 d in mATG + control IgG (Figure 3B). The frequencies of donor-reactive IFN- γ producing cells were comparable in both groups at d. 28, after the onset of rejection (Figure 3D).

As a second approach, we used Casp-1 KO recipients that have impaired inflammasome mediated IL-1 β maturation (41). Prior to depletion, Casp-1 KO mice had similar or higher numbers of T cells in circulation and in the spleen (Figure 3E, G). Deficiency of Casp-1 resulted in delayed recovery of both CD4 $^{+}$ and CD8 $^{+}$ T cells in peripheral blood (Figure 3E) and in the spleen (Figure 3G). Consistent with delayed T cell reconstitution, Casp-1 KO mice treated with mATG had prolonged heart allograft survival compared to WT recipients (MST of 20 d in Casp-1 KO vs 11 d in WT) (Figure 3F). The frequencies of donor-specific IFN- γ producing cells were reduced, albeit not significantly, in Casp-1 KO recipients compared to WT mice (Figure 3H). Taken together these results show that IL-1 β is essential for optimal T cell recovery after mATG depletion.

3.4. IL-1R signaling is required for optimal T cell reconstitution after mATG treatment

To investigate the requirement for IL-1 receptor signaling during T cell reconstitution we transplanted BALB/c heart allografts into B6 IL-1R1 KO and MyD88 KO recipients treated with mATG. MyD88 is an adaptor protein in IL-1R and TLR signaling cascade, and all IL-1 β mediated functions are impaired in MyD88 deficient mice (42). Prior to depletion, IL-1R1 KO or MyD88 KO mice had similar or higher numbers of T cells in circulation and in the spleen (Figure 4A, C). However, either IL-1R1 or MyD88 deficiency resulted in the decreased recovery of both CD4 $^{+}$ and CD8 $^{+}$ T cell in peripheral blood (Figure 4A) and in the spleen (Figure 4C). Consistent with the delayed T cell reconstitution, mATG treatment significantly prolonged heart allograft survival in IL-1R1 KO and MyD88 KO mice compared to WT recipients (MST of 18 d in IL-1R1 KO, MST of 22 d in MyD88 KO, MST of 11 d in WT) (Figure 4B). The frequencies of donor-reactive T cells producing IFN- γ were lower in IL-1R1 KO and MyD88 KO recipients compared to WT recipients on day 28 posttransplant (Figure 4D).

Next we tested whether IL-1R1 signaling in T cells is required for their homeostatic recovery. Congenic CD8 $^{+}$ or CD4 $^{+}$ T cells isolated from CD45.1 $^{+}$ WT and CD45.2 $^{+}$ IL-1R1 KO mice were co-transferred into CD45.1 $^{+}$ /CD45.2 $^{+}$ recipients of BALB/c heart allografts treated with mATG so as to be able to distinguish between transferred WT, KO, and endogenous host T cells within the same recipient (Figure 5A). The depletion efficacy at d. 7 posttransplant was similar in both types of transferred CD8 $^{+}$ and CD4 $^{+}$ T cells (Figure 5B, C). Both WT and IL-1R1 KO CD8 $^{+}$ T cells had similar recovery rates at d. 28 posttransplant. While there was a tendency towards a decrease in IL-1R1 KO compared to

WT CD4⁺ T cell numbers within the same recipients, it did not reach statistical significance (Figure 5C). Therefore, IL-1R1 expression by T lymphocytes is not essential for optimal homeostatic expansion in our model. When analogous co-transfer experiments were performed with WT and MyD88 KO CD8⁺ T cells, the reconstitution of MyD88 KO CD8⁺ T cells was significantly impaired compared to WT cells (Figure 5D, E). These results suggest that other MyD88-dependent signaling pathways may directly support CD8⁺ T cell proliferation in lymphopenic hosts.

3.5. B cell-derived IL-1 β promotes CD8⁺ T cell reconstitution after mATG treatment

To further test the role of B cell-derived IL-1 β , we injected either WT or Casp-1 KO B cells into groups of B cell deficient μ MT mice 6 days after the BALB/c heart transplantation (2 days after last mATG treatment) (Figure 6A). Casp-1 deficiency did not compromise B cell engraftment or survival (Figure 6C). However, the accumulation of CD8⁺ T cells in peripheral blood and spleen numbers was markedly increased in recipients injected with WT but not with Casp-1 KO B cells (Figure 6B) demonstrating that B cell IL-1 β production is required for optimal CD8⁺ T cell reconstitution. mATG treatment of B cell deficient μ MT recipients results in significantly prolonged heart allograft survival compared to mATG treated WT recipients (MST of > 30 d. vs 11 d., n = 7–8/group, p < 0.001, Figure 6D and Figure 2C). Consistent with observed differences in T cell recovery, adoptive transfer of WT but not Casp-1 KO B cells into μ MT recipients markedly accelerated heart allograft rejection (MST of 24d. vs 28 d. for WT vs Casp-1 KO B cell transfer, n = 7/group, p = 0.005, Figure 6D).

3.6. B cell-derived IL-6 facilitates homeostatic T cell recovery following mATG treatment.

To investigate the influence of IL-6 on CD8⁺ T cell reconstitution, we administered neutralizing anti IL-6 mAb into B6 recipients of BALB/c heart allografts treated with mATG. Even though IL-6 neutralization had modest effect on T cell recovery in peripheral blood (Figure 7A), it markedly diminished T cell accumulation in the spleen (Figure 7C), and extended heart allograft survival (MST of 19 d in anti-IL-6 treated vs 11 d in non-treated recipients) (Figure 7B). Moreover, the combination of mATG and IL-6 neutralization decreased the numbers of donor-reactive IFN- γ producing spleen cells compared to mATG treatment alone (Figure 7D). In parallel experiments, WT and IL-6 KO mice were transplanted with BALB/c heart allografts and treated with mATG. IL-6 deficiency resulted in reduced CD4⁺ and CD8⁺ T cell recovery after mATG treatment in peripheral blood (Figure 7E) and in the spleen (Figure 7G). The graft-prolonging efficacy of mATG was increased in IL-6 KO mice compared to WT recipients, with the majority of allografts surviving more than 30 days (MST of 28 d in IL-6 KO vs 11 d in WT (Figure 7F). The graft prolongation was associated with decreased frequencies of donor-reactive IFN- γ producing spleen cells in IL-6 KO recipients compared to WT controls (Figure 7H).

To test the requirement for IL-6R signaling in T cells, congenic CD8⁺ or CD4⁺ T cells were isolated from CD45.1⁺ WT and IL-6R α deficient CD45.2⁺ mice (IL6ra^{fl/fl} x CD4^{cre}) and co-injected into CD45.1⁺/CD45.2⁺ recipients of BALB/c heart allografts treated with mATG (Figure 8A). Despite similar depletion rates for WT and IL-6R deficient T cells, the absence

of IL-6R α markedly impaired both CD8⁺ and CD4⁺ T cell reconstitution indicating that IL-6 directly enhances T cell homeostatic proliferation (Figure 8B–D).

To further evaluate the role of B cell-derived IL-6, WT or IL-6 KO B cells were injected into groups of B cell deficient μ MT mice 6 days after the BALB/c heart transplantation (2 days after last mATG treatment) (Figure 8E). At the similar rates of engraftment and survival, the transferred WT B cells were more efficient in inducing CD8⁺ T cell reconstitution compared to IL-6 KO B cells (Figure 8F–G) indicating that B cell-derived IL-6 is necessary for CD8⁺ T cell homeostatic recovery. The decreased CD8⁺ T cell numbers observed upon IL-6 KO B cell transfer were associated with delayed heart allograft rejection compared to μ MT recipients injected with WT B cells (Figure 8H).

4. Discussion

The success of lymphoablative therapies depends on the rate and magnitude of T cell proliferation in lymphopenic hosts. While ongoing immune responses and ensuing inflammation are likely to profoundly influence T cell homeostatic proliferation, the mechanisms of such influence remain to be determined. We have previously reported that CD154/CD40 interactions between depletion-resistant memory CD4⁺ T cells and B cells are critical for CD8⁺ T cells reconstitution, and that limiting CD4⁺ T cell help impairs the recovery of CD8⁺ T cells, increases the efficacy of lymphoablation and prolongs mouse heart allograft survival (7). Here we demonstrate that in the settings of allotransplantation and mATG-induced lymphopenia, B cells become major producers of proinflammatory cytokines IL-1 β and IL-6 thus driving CD8⁺ T cell expansion. IL-1 β and IL-6 regulate each other's production in B cells. Whereas IL-6 directly targets both CD8⁺ and CD4⁺ T cells and enhances their proliferation under lymphopenic conditions, our data indicate that IL-1R1 expression on T lymphocytes is not required for optimal expansion and suggest that IL-1 β affects other cells (such as B cells themselves in a positive feedback loop) and may thus influence CD8⁺ T cell expansion indirectly (Figure 9).

IL-1 β and IL-6 are key proinflammatory cytokines with pleiotropic effects in multiple cell types (41, 43, 44). The effects of these cytokines on T cell differentiation and effector functions are well documented in models of autoimmunity, mucosal inflammation and cancer (17–24). Both IL-1 β and IL-6 were previously reported to augment homeostatic T cell expansion in adoptive transfer models and their effect was dependent on IL-1R or IL-6R expression in T cells (18, 19, 23, 24). However, the sources of proinflammatory cytokines in most of these studies have not been precisely identified and are likely to be model-dependent.

The impaired T cell reconstitution in Casp1-KO or IL-6 KO recipients (Figures 3 and 7) indicates that allograft-derived cytokines are not sufficient to sustain optimal T cell proliferation in mATG-treated recipients and emphasizes the essential role for recipient-derived cytokines. The central observation of our study is that despite many cell types being capable of producing proinflammatory cytokines during lymphoablation and allotransplantation, recipient B lymphocytes are the main source of IL-1 β and IL-6 in mATG treated heart allograft recipients. Up-regulation of these cytokines is not likely to be

restricted to alloantigen-reactive B cells as a large proportion of splenic B cells produce IL-1 β (up to 35%) or IL-6 (up to 15%) at d. 8 posttransplant. B cell derived cytokines have been documented to modulate T cell functions and immune cell recruitment (10–13, 45). Previously described cytokine-producing B cell subsets include Bregs, Beff1 and Beff2 cells secreting IL-10 and/or TGF β , IFN γ and IL-4, respectively (46–49). Most relevant to our study, IL-6 secreting B cells were reported to play an important role in a mouse model of experimental autoimmune encephalomyelitis (EAE) by supporting functions of Th17 cells (50). In contrast, even though IL-1 β production by B cells was observed long time ago (51, 52), the exact functions of IL-1 β secreting B cells remain to be determined.

In our model, B cell IL-1 β or IL-6 production is markedly increased by mATG lymphoablation or by heart allograft placement. Nevertheless, there is no further elevation in heart allograft recipients treated with mATG (Figure 2). Similar trends are observed in CD11b⁺ and CD11c⁺ cells albeit due to their dominant quantity B cells are major spleen cell population secreting IL-1 β and IL-6 in lymphopenic host. The impairment in T cell recovery after B cell depletion was similar to that achieved by systemic deficiency or neutralization of IL-1 β and IL-6 indicating that proinflammatory cytokine production is a predominant B cell function that facilitates T cell proliferation during lymphopenia. The failure of IL-1 β deficient or IL-6 KO B cells to mediate T cell reconstitution and accelerate heart allograft rejection after adoptive transfer into mATG-treated B cell-deficient recipients further supports this scenario.

As temporal separation of lymphoablation and heart transplantation (i.e. mATG administration 7 and 4 days prior to transplantation) significantly delays T cell recovery and prolongs allograft survival (8), our findings suggest that proinflammatory signals must be combined with other lymphopenia-induced cues such as elevated IL-7 levels in order to drive rapid T cell proliferation. Another remaining question is the signals leading to IL-1 β and IL-6 induction in B cells. Our previous data show that CD40 signals by residual CD4⁺ memory T cells are required for IL-1 β and IL-6 up-regulation (7). In addition, massive cell death during lymphoablation and transplant ischemia reperfusion injury releases various types of DAMPs that can augment B cell activation. Ongoing studies in our laboratory investigate the role of transplantation-induced TLR ligands and MyD88 signaling in this process. The initial experiments implicate other sensors of cell death such as C-type lectin receptors (unpublished observations).

Multiple studies demonstrate the involvement of IL-1 β and IL-6 signaling pathways during transplant ischemia/reperfusion injury, activation and functions of alloreactive T cells and allograft rejection (53–55). Donor or recipient IL-1R or MyD88 deficiency or the use of soluble IL-1R antagonist impaired T cell alloimmune responses but had only modest effects on the survival of MHC-mismatched allografts (30–33, 56). Similarly, either IL-6 neutralization or the use of IL-6 deficient recipients mice model resulted in reduced T cell infiltration and improved allograft survival especially when combined with costimulatory blockade (28, 34, 36, 57–59). However, the contribution of IL-1 β or IL-6 in allograft recipients undergoing lymphoablation induction therapy has not been previously assessed. Our data present the first evidence that targeting either of these cytokines has profound effects on homeostatic T cell recovery and improves graft-prolonging efficacy of antibody-

mediated lymphoablation. Even though T cells eventually expand in the absence of IL-1 β or IL-6 signaling, this delayed reconstitution is likely to be the result of thymus activity rather than homeostatic proliferation (60). Such a shift from lymphopenia-induced proliferation of depletion-resistant memory T cells towards thymopoiesis curbs pre-existing alloreactivity and is a highly desirable outcome in transplant recipients undergoing induction therapy. Given the previously demonstrated importance of IL-1 β and IL-6 signaling even in non-lymphopenic allograft recipients, it is possible that the heart allograft prolongation observed in our studies may not be solely due to the effects of these cytokines on memory T cell reconstitution. However this does not undermine the significance of our findings that controlling posttransplant inflammation inhibits the recovery of pathogenic memory T cells.

Collectively, our data demonstrate that allotransplantation-induced inflammation greatly affects the kinetics of T cell homeostatic reconstitution and reveals the novel role for B lymphocytes as producers of proinflammatory cytokines in lymphopenic environment. Most importantly, the results identify B cells, IL-1 β and IL-6 as promising therapeutic targets for enhancing the efficacy of lymphoablation in transplant recipients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Allograft-induced inflammation facilitates T cell recovery.

Naive non-transplanted B6 mice, B6 (H-2^b) recipients of B6 (H-2^b) heart isografts and B6 (H-2^b) recipients of BALB/c (H-2^d) heart allografts were injected with mATG (0.5 mg i.p. on days 0 and 4). (A) The numbers of CD4⁺ and CD8⁺ T cells in 100 μ l of peripheral blood. (B) The numbers of CD4⁺ and CD8⁺ T cells in recipient spleen at d. 28 posttransplant. The T cell numbers in the spleen of naïve non-transplanted B6 mice are indicated by dashed lines. (C) The frequencies of spleen cells secreting IFN- γ in response to BALB/c alloantigens were determined by ELISPOT assay at 8 weeks posttransplant. The frequencies of IFN- γ producing cells after stimulation with third party SJL spleen cells were $< 50 / 1 \times 10^6$ for all samples tested. Results are cumulative of 3 experiments with $n = 4$ mice/group; error bars represent SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 2. B cells are the major source of IL-1 β and IL-6 following MATG treatment and allogeneic transplantation.

(A-B) WT recipients of BALB/c heart allografts were treated with anti-mouse CD20 mAb (250 μ g, d. -3 and 11) or left untreated. B cell deficient μ MT recipients treated with mATG were used as a control. (A) Recovery of CD4 $^{+}$ and CD8 $^{+}$ T cells in the peripheral blood. (B) The kinetics of B cell reconstitution following anti-mouse CD20 mAb treatment. (C) Heart allograft survival. (D) Real-time RT-PCR analysis of RNA from splenic B cells isolated at d. 8 posttransplant. (E-F) Intracellular flow cytometry performed on spleen cells at d. 8 posttransplant showing the frequencies of IL-1 β^{+} (E) and IL-6 $^{+}$ (F) cells among B220 $^{+}$, CD11b $^{+}$, or CD11c $^{+}$ cells. (G-H) Intracellular flow cytometry analysis of IL-1 β (G) and IL-6 (H) expression in B cells isolated on d. 8 posttransplant from MATG treated Casp-1

KO, IL-6 KO and WT heart allograft recipients. (I) Cytokine production by spleen B220⁺ cells isolated from naïve B6 WT, Casp-1 KO or IL-6 KO mice after 20 h of in vitro stimulation with LPS. n = 4–5 animals/group; error bars represent SD. *P < 0.05, **P < 0.01.

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Figure 3. IL-1 β neutralization or deficiency compromises homeostatic recovery of T cells.

(A-D) B6 recipients of BALB/c cardiac allografts were treated with mATG (d. 0 and 4) and either anti-IL-1 β mAb (200 μ g i.p. on days -1, 2, 5, 8, and 11 posttransplant) or control IgG (200 μ g i.p. on days -1, 2, 5, 8, and 11 posttransplant). (A) The numbers of CD4 $^{+}$ and CD8 $^{+}$ T cells in 100 μ l of peripheral blood. (B) Heart allograft survival. (C) The numbers of CD4 $^{+}$ and CD8 $^{+}$ T cells in recipient spleen at d. 28 posttransplant. (D) Frequency of donor-reactive IFN- γ producing cells were measured with ELISPOT assay on d. 28 posttransplant. (E-H) Casp-1 KO and WT recipients of BALB/c heart allografts were treated with mATG. CD4 $^{+}$ and CD8 $^{+}$ T cells were analyzed in peripheral blood (E) and the spleen (G). (F) Heart allograft survival. (H) Frequency of donor-reactive IFN- γ producing cells measured with ELISPOT assay on d. 28 posttransplant. The frequencies of IFN- γ producing cells after

stimulation with third party SJL spleen cells were $< 100 / 1 \times 10^6$ for all samples tested. n = 4–10 mice; error bars represent SD. *P < 0.05, **P < 0.01, ***P < 0.001.

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Figure 4. IL-1R1 signaling is required for T cell reconstitution after mATG treatment. (A–D) IL-1R1 KO, MyD88 KO and WT mice received BALB/c cardiac allografts and mATG treatment. (A) The numbers of CD4⁺ and CD8⁺ T cells in 100 μ l of peripheral blood. (B) Cardiac allografts survival. (C) The numbers of CD4⁺ and CD8⁺ T cells in recipient spleen at d. 28 posttransplant. (D) Donor-specific T cell responses were measured in the spleen by IFN- γ ELISPOT assay at day 28 posttransplant. The frequencies of IFN- γ producing cells after stimulation with third party SJL spleen cells were $< 100 / 1 \times 10^6$ for all samples tested. $n = 4-10$ mice; error bars represent SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Figure 5. The expression of IL-1R on CD8⁺ T cells is not required for optimal reconstitution after mATG treatment.

(A) Experimental design. Splenic CD8⁺ or CD4⁺ T cells were isolated from CD45.1⁺ WT either IL-1R1 KO or MyD88 KO CD45.2⁺ mice and co-injected into CD45.1/2⁺ mice followed by BALB/c heart transplantation and mATG treatment. (B, D) Representative dot plots analyzing transferred WT and IL-1R1 KO (B) or MyD88 KO (D) T cells in peripheral blood or spleen at indicated time points. Data are shown after gating on CD8⁺ cells. (C, E) Absolute numbers of transferred WT, IL-1R1 KO CD8⁺ T cells and CD4⁺ T cells (C) and WT, MyD88 KO CD8⁺ T cells (E) in peripheral blood at day 7 and 28 posttransplant and in the spleen at d. 28 posttransplant. n = 4 mice/group; error bars represent SD. *P < 0.05, **P < 0.01. The experiments were repeated twice with similar results.

Figure 6. B cell derived IL-1 β promotes CD8⁺ T cell reconstitution after mATG treatment.

(A) Experimental design. μ MT mice were transplanted with BALB/c heart allografts, treated with mATG on days 0 and 4, and injected with 30×10^6 of either WT or Casp-1 KO B cells day 6 of posttransplant. (B) Numbers of CD8⁺ T cell in peripheral blood and spleen at d. 28 posttransplant. (C) Numbers of transferred B220⁺ cell in the spleen at d. 28 posttransplant. n = 4 mice/group; error bars represent SD. *P < 0.05. (D) Heart allograft survival. n = 7–8 mice/group.

Figure 7. IL-6 facilitates homeostatic T cell recovery following MATG treatment.

(A-D) B6 mice were transplanted with BALB/c heart allografts and treated with MATG with or without anti-IL-6 mAb, (200 μ g i.p. on days -1, 2, 5, 8, and 11 posttransplant). Numbers of CD4⁺ and CD8⁺ T cells in the peripheral blood (A) and spleen (C) evaluated by flow cytometry. (B) Heart allograft survival. (D) Numbers of donor-reactive spleen cells producing IFN- γ detected by ELISPOT on day 28 posttransplant. (E-H) IL-6 KO and WT mice were transplanted with BALB/c heart allografts and treated with MATG on days 0 and 4 posttransplant. Numbers of CD4⁺ and CD8⁺ T cells were evaluated in peripheral blood (E) and spleen (G). (F) Heart allograft survival. (H) Numbers of donor-reactive T cells producing IFN- γ in the spleens of WT and IL-6 KO recipients on day 28 posttransplant. The frequencies of IFN- γ producing cells after stimulation with third party SJL spleen cells were

$<100 / 1 \times 10^6$ for all samples tested. n = 4–10 mice/group; error bars represent SD. *P < 0.05, **P < 0.01, ***P < 0.001.

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Figure 8. B cell derived IL-6 is required for homeostatic CD8⁺ T cell reconstitution after mATG treatment.

(A) Experimental design. Spleen CD8⁺ T cells were isolated from CD45.1⁺ WT and IL-6Rα deficient CD45.2⁺ mice (IL6ra^{fl/fl} × CD4^{cre}) and co-injected intravenously into CD45.1/2⁺ mice followed by BALB/c heart transplantation and mATG treatment. (B) Representative dot plots analyzing transferred WT and IL-6Rα KO CD8⁺ T cells in peripheral blood at indicated time points. Data are shown after gating on CD8⁺ cells. (C, D) Absolute numbers of transferred WT and IL-6Rα KO CD8⁺ (C) or CD4⁺ (D) T cells in peripheral blood at days 7 and 28 posttransplant and in the spleen at day 28 posttransplant. (E) Experimental design. μMT mice were transplanted with BALB/c heart allografts, treated with mATG on days 0 and 4 and injected with either WT or IL-6 KO B cells on day 6 posttransplant. (F)

Spleen B220⁺ cell numbers at d. 28 posttransplant. **(G)** CD8⁺ T cell numbers in peripheral blood and spleen at d. 28 posttransplant. n = 4 mice/group; error bars represent SD. *P < 0.05, **P < 0.01. **(H)** Heart allograft survival. n = 6–8 mice/group.

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Figure 9. Proposed model.

After receiving CD40-mediated help from depletion-resistant memory CD4⁺ T cells, B cells up-regulate production of IL-1 β and IL-6. Both IL-1 β and IL-6 provide positive feedback to B cells, whereas IL-6 also directly enhances CD4⁺ and CD8⁺ T cell homeostatic reconstitution.