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Induction of MHC-mismatched Mouse Lung Allograft Acceptance with Combined Donor Bone Marrow: Lung Transplant using a 12-Hour Nonmyeloablative Conditioning Regimen

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Abstract

Background—Despite broad and intense conventional immunosuppression, long-term survival after lung transplantation lags behind that for other solid organ transplants, primarily because of allograft rejection. Therefore, new strategies to promote lung allograft acceptance are urgently needed. The purpose of the present study was to induce allograft tolerance with a protocol compatible with deceased donor organ utilization.

Methods—Using the MHC-mismatched mouse orthotopic lung transplant model, we investigated a conditioning regimen consisting of pretransplant T cell depletion, low dose total body irradiation and posttransplant (donor) bone marrow and splenocyte infusion followed by posttransplantation cyclophosphamide (PTTT-PTB/PTCy).

Results—Our results show that C57BL/6 recipients of BALB/c lung allografts undergoing this complete short-duration nonmyeloablative conditioning regimen had durable lung allograft acceptance. Mice that lacked 1 or more components of this regimen exhibited significant graft loss. Mechanistically, animals with lung allograft acceptance had established higher levels of donor chimerism, lymphocyte responses which were attenuated to donor antigens but maintained to third-party antigens, and clonal deletion of donor-reactive host Vβ T cells. Frequencies of $Foxp3^+$ T regulatory cells were comparable in both surviving and rejected allografts implying that

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Additional methods are described in supplemental information.

their perturbation was not a dominant cell-regulatory mechanism. Donor chimerism was indispensable for sustained tolerance, as evidenced by acute rejection of allografts in established chimeric recipients of PTTT-PTB/PTCy following a chimerism-ablating secondary recipient lymphocyte infusion.

Conclusion—Together, these data provide proof-of-concept for establishing lung allograft tolerance with tandem donor bone marrow transplantation (BMT) using a short-duration nonmyeloablative conditioning regimen and PTCy.

Introduction

Lung transplantation is the final therapeutic option for select patients with end-stage lung disease. Unfortunately, the longevity of transplanted lungs continues to be shorter than that of other solid organ allografts. Despite the use of broad immunosuppression therapies, episodes of acute and chronic cellular rejection are quite common[1,2]. Therefore, new strategies are needed to limit alloreactivity and promote lung allograft tolerance.

The establishment of stable multi-lineage donor chimerism has been shown to contribute to tolerance induction of transplanted solid organs[3-5]. The clinical translation of this strategy has been further stimulated by the growing success of nonmyeloablative regimens in patients with hematologic malignancies[6]. While early clinical attempts with nonmyeloablative allografting using HLA-mismatched donors were associated with a high risk of rejection, more recent studies suggest that modifications such as use of posttransplant cyclophosphamide (PTCy) can result in engraftment of HLA-mismatched, related BM (haploidentical) with low nonrelapse mortality and acceptable rates of acute and chronic GVHD[7,8]. Furthermore, using HLA-mismatched haploidentical donors after nonmyeloablative conditioning has shown that PTCy is an essential component of the strategy for the treatment of sickle cell disease and for successful combined BM and kidney transplantation[9,10]. While there is growing experience with combined BMT and solid organ transplantation, it is limited to either living related donors[11] or haplo-identical[12] donors. This is impractical for broad application in lung transplantation which mostly relies on the cadaveric donors[4,12].

In this study, we used a mouse orthotopic left lung transplant model[13,14] to test whether a conditioning regimen beginning 12 hours prior to lung transplantation would induce lung allograft tolerance. The nonmyeloablative conditioning strategy. consisting of pretransplantation total body irradiation and T cell depletion, simultaneous lung and bone marrow transplantation, and post transplantation cyclophosphamide administered 72 hours later (PTTT-PTB/PTCy), is based on our previously developed nonmyeloablative regimens[15,16]. We report that MHC-mismatched allograft recipients undergoing PTTT-PTB/PTCy showed significantly improved allograft acceptance. Our data suggests that a 12-hour, nonmyeloablative conditioning regimen prior to tandem MHC-disparate lung and bone marrow transplantation followed by PTCy has the potential for establishing functional lung acceptance.

Materials and Methods

Mice

C57BL/6 (C57BL/6, H-2^b) and BALB/c (H-2^d) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed under specific pathogen-free conditions before surgery, and open access conditions after surgery. The Johns Hopkins University Animal Care and Use Committee approved all animal care protocols.

Nonmyeloablative conditioning

Total body irradiation (TBI; 250 cGy, ^{137}Cs irradiator, Gammacell 40; Atomic Energy of Canada) and T cell depletion with 2 mg of pan-T cell–depleting monoclonal antibody (anti-Thy1.2 mAb, intraperitoneal [i.p]., Bio X Cell; clone 30H12) were initiated 8-12 hours before lung implantation. HSCs comprised of murine bone marrow (BM; 2.5×10^7) cells supplemented with 3×10^7 unfractionated splenocytes were administered after orthotopic lung transplantation, unless otherwise indicated. BM cells were flushed from the hind-limb bones of BALB/c donors with RPMI 1640 containing 5% heat-inactivated fetal calf serum and 2mM EDTA. Spleens were mashed and passed through 70-μm cell strainers as a single-cell suspension. BM and spleen cells were combined and administered as a HSC infusion 2-6 h after completion of orthotopic lung transplantation. Cyclophosphamide (Baxter Healthcare, Deersfield, IL; 200 mg/kg) was administered i.p. 72h after completion of the lung transplantation[15,16]. Allogeneic lung transplantations were performed in the BALB/c to C57BL/6 strain combination.

Mouse orthotopic lung transplant and GVHD monitoring

Lungs were transplanted with a cuffed technique[13,14,17]. Donor mice were sedated with etomidate (1 mg, i.p.), intubated, and maintained on inhaled isoflurane until euthanized. Recipients were both initially sedated and maintained on inhaled isoflurane. Mice received subcutaneous buprenorphine (0.03-0.05 mg/kg) before extubation and every 6 h thereafter as needed. Unless otherwise specified, animals were sacrificed for analysis at 40-60 days posttransplant. GVHD monitoring were performed as previously described[15,18].

Post-surgical care

Lung recipients were maintained in individual cages with food and water *ad libitum*. Cages were changed regularly, and animals were checked twice weekly. The vivarium allows open access and provides unfiltered air.

Results

Assessment of protocol conditioning components required for lung allograft acceptance

We hypothesized that successful induction of donor chimerism would promote acceptance of the lung allograft. We sought to establish lung allograft acceptance using a protocol where recipient conditioning was initiated 12 hours prior to lung implantation. Based on previous murine BMT studies[15,19,20], we hypothesized that host preconditioning with T cell depletion in combination with low-dose TBI, administration of a clinically achievable dose of HSCs and PTCy would induce sufficient macrochimerism to promote orthotopic donor lung

acceptance. Low dose TBI was administered to promote donor myeloid cell engraftment by creating space within the BM for donor cells to engraft[15,16]. Additionally, we depleted host T cells with anti-T cell antibody (anti-Thy 1.2 mAb) to reduce the total number of host-reactive T cells and protect against early rejection[19,20]. The donor HSC inoculum was comprised of BM and spleen cells as mouse BM, unlike human BM, is comparatively deficient in T cells. Figures 1A and S1 summarize the allograft survival results along with corresponding gross photographs of experiments where C57BL/6 mice treated with various conditioning regimens underwent donor BALB/c orthotopic lung transplantation. A regimen consisting of TBI conditioning, anti-Thy1.2, and PTCy without donor BALB/c HSC was insufficient to induce allograft survival (Figure 1A, Group 1). However, the addition of donor HSC inoculum to a TBI/anti-Thy1.2/PTCy regimen resulted in survival of 28/33 (85%) lung allografts (Figure 1A, Group 4). Interestingly, administration of either donor splenocytes alone or BM alone was insufficient to achieve allograft survival in more than 50% of mice (Figure 1A, Groups 2 and 3, respectively). This likely reflects the importance of cell dose and HSC inoculum composition for induction of tolerance with PTCy[15,16,19,21]. Consistent with previous work showing a critical role of PTCy, no allografts survived in mice that received TBI, anti-Thy1.2, and HSC without PTCy (Figure 1A, Group 5). We also explored whether post-surgical conditioning could induce stable allograft acceptance. Allografts survived in only 1 of 5 chimeras that received conditioning post-operatively (Figure S1), implying a critical requirement for preconditioning to be performed 8-12 hours prior to surgery in our model. Based on these results, Figure 1B schematically depicts the optimal regimen we used in all subsequent experiments.

Lung allograft survival is associated with lack of histological signs of rejection but requires sustained donor chimerism

We next sought to determine: 1) the correlation between visual graft acceptance and histological findings; 2) the robustness of graft acceptance; and 3) the relationship between graft acceptance and donor chimerism. Histologic evaluation of lung allografts (BALB/c → C57BL/6) from mice conditioned with different experimental regimens are shown in Figure 2. On microscopic inspection, lung allografts from hosts conditioned with PTTT-PTB/PTCy showed only mild peribronchial, perivascular, and/or interstitial inflammation that was reflected in their pathohistologic scores being comparable to those of the native lungs (Figure 2A-B). It is important to note that while our results show a dramatic attenuation of allograft injury, we cannot exclude statistically insignificant differences between allograft and native lung. Allografts from mice that received TBI, T cell depletion, and PTCy, but no HSCs exhibited massive central hemorrhagic necrosis with some fibrosis, and a pathohistologic score significantly worse than that of allografts from mice receiving our complete regimen (Figure 2A-B). With either treatment, the native lung exhibited only mild peribronchial and/or perivascular inflammation (Figure 2A-B). Notably, all lung allografts harvested between 35 and 44 days after transplantation survived. This was also the case with allografts harvested 65-74 and 75-88 days after transplantation. When lung allografts did not survive, despite use of our full protocol, graft loss was most frequently observed at days 45-54 (71% survival), followed by days 55-64 (86% survival). Overall allograft survival was 28/33 (85%).To determine whether the level of donor chimerism was associated with lung allograft survival, we evaluated individual mice for the relationship between lung allograft

survival/ nonsurvival state and the percentage of donor chimerism in peripheral blood at the time of lung harvest (Figure 2C). Compared with surviving lung allografts, nonsurviving lung allografts exhibited a significantly lower level of peripheral blood donor chimerism at the time of allograft harvest. To validate the causal relationship between donor chimerism and allograft survival, 1 group of PTTT-PTB/PTCy-treated mice with established chimerism 4 weeks following transplantation was given a chimerism-ablating secondary recipient lymphocyte infusion (RLI) comprised of host splenocytes. Two weeks post-RLI administration, the chimerism was ablated (data not shown) and the allografts were rejected (Figure S2 A-B). Cellular analyses revealed elevated levels of cytokine-producing host $CD4^+$ T cells in the allograft (Figure S2 C-D). These results suggest there is a threshold level of donor chimerism that must be established and maintained to achieve durable lung allograft survival with our protocol.

Tolerant mice demonstrate donor-specific loss of alloreactivity and clonal deletion of reactive T cells

Next, mice with viable lung allografts were then evaluated for donor- and third party-alloreactivity in vitro (Figure 3A). Splenic $CD4^+$ T cells from naïve C57BL/6 mice as well as HSC-excluded PTTT-PTB/PTCy-treated recipients of BALB/c lung allograft proliferated robustly when cultured with T cell–depleted splenocytes (stimulators) from donor (BALB/c) or third party (FVB/N) mice. By contrast, $CD4^+$ T cells from PTTT-PTB/PTCy-treated C57BL/6 recipients with surviving BALB/c lung allografts proliferated poorly only with donor alloantigens but with T cell–depleted splenocytes from donor (BALB/c) mice but did retain their proliferative response to stimulators from third-party (FVB/N) mice. These findings suggest that PTTT-PTB/PTCy tolerizes recipient mice by preferentially targeting donor-antigen-specific T cells without otherwise compromising immune competence.

We [15] and others [22,23] have shown that clonal deletion contributes to the tolerance induced by other cyclophosphamide-based nonmyeloablative regimens. $V\beta11^+$ and $V\beta5.1/5.2^+$ T cells are known to be clonally deleted in BALB/c mice, but not in C57BL/6 mice because of the presence of an endogenous, retrovirally encoded superantigen [22,24], whereas $V\beta8.1^+$ T cells are maintained in both of these strains. Therefore, we evaluated whether clonal deletion was associated with PTTT-PTB/PTCy-induced tolerance. As shown in Figure 3B, we confirmed the clonal deletion of $V\beta11^+$ and $V\beta5.1/5.2^+$ families in $CD4^+$ T cells of control donor BALB/c mice and the retention of these clones in T cells of naïve control C57BL/6 mice while both strains expressed the $V\beta8.1^+$ clone. In contrast, both $V\beta11^+$ and $V\beta5.1/5.2^+$ H-$2K^{b+}$ (host) $CD4^+$ T cells were significantly reduced in the peripheral blood of chimeric C57BL/6 mice that received tandem BMT and lung allografts from BALB/c mice. We also examined the role of $CD4^+$ $Foxp3^+$ T regulatory cells (Tregs) in tolerance-induction by PTTT-PTB/PTCy by examining their composition in chimeras on day 60 posttransplant. Interestingly, both surviving allografts from PTTT-PTB/PTCy treated mice and rejected allografts from PTTT-PTB/PTCy minus HSC treated mice, the frequencies of Tregs in host $CD4^+$ T cells from allograft, native lungs and spleen were comparable (Figure 3C). This suggests that major Treg-number perturbation is not a dominant mechanism of organ acceptance in this model. Additional studies are required to fully dissect the importance of

both clonal deletion of donor-reactive host T cells and early expansion of Foxp3$^+$ Tregs seen in alloBMT models (39) in tolerance induction with PTTT-PTB/PTCy regimen.

Discussion

Here, we show for the first time the establishment of long-term lung allograft acceptance across major MHC barriers, using a protocol compatible with deceased donor organ use. Our studies suggest that all components (low-dose TBI, T cell depletion, donor HSCs plus splenocytes, and PTCy) of our protocol (PTTT-PTB/PTCy) are required to establish allograft tolerance, as experiments lacking any of these components resulted in lung rejection. Stable chimerism appears to be essential for long-term lung acceptance, suggesting that transient engraftment is not sufficient in this model. Our results suggest that the capacity of PTCy to modulate alloreactivity and promote bone marrow tolerance as part of a nonmyeloablative conditioning regimen can be used as a tool to achieve acceptance of transplanted solid organs through the establishment of chimerism. Additionally, our experimental results indicate that this protocol can be implemented within the short timeframe of lung transplant and achieve durable acceptance out to 3 months of follow-up.

Our results support the concept that cytotoxic destruction of alloreactive T-effectors by the addition of PTCy[25], plays an important role in establishing hematopoietic chimerism and tolerance to the lung allograft. Cyclophosphamide is a pro-drug[26] whose cytotoxicity is dependent on conversion to aldophosphamide. Aldophosphamide is, in turn, inactivated by aldehyde dehydrogenase (ALDH)[27]. Because ALDH is absent in activated, replicating effector T cells[28-30], alloreactive lymphocytes are highly susceptible to deletion[8,31]. In contrast, stem cells and regulatory T cells with higher ALDH levels are resistant[30,32]. Finally, PTCy allows the preservation of the nonalloantigen responsive and nonproliferative, pathogen-specific memory T cells which are important for host defense[33].

A key aspect of functional lung allograft acceptance that has emerged from our model is that the maintenance of donor chimerism represents a potential biomarker of a functional allograft. Our data also suggest that a potential threshold level of donor hematopoietic chimerism may be important, as lung allografts were rejected both in mice that had lower levels of donor chimerism as in mice whose established chimerism was ablated with RLI. These findings are consistent with previous studies that have shown that a substantial level of donor hematopoietic chimerism is required for functional allograft acceptance[34,35]. In our model, stable donor chimerism appears to be essential for maintaining functional lung acceptance. This is in contrast with kidney allografts, where loss of donor chimerism after combined allografting does not lead to compromise in allograft function[36,37] .

Our study demonstrates that lung allograft acceptance is also associated with unresponsiveness to donor allo-antigens. Encouragingly, transplanted animals maintained functional responsiveness to third party allo-antigens, implying that broad immunologic competence was not affected. Further analysis revealed a reduction of specific Vβ populations in C57BL/6 recipients of BALB/c allografts, suggesting that clonal deletion is a major mechanism of tolerance to donor antigens. Taken together, these findings suggest that the establishment of donor chimerism, along with clonal deletion leading to reduced host

alloreactivity, is critical for long-term lung allograft viability. However, further studies are needed to fully comprehend the role of Foxp3^{+} Tregs especially in early phases of tolerance induction using this regimen.

There are several caveats to our studies. First, because we used nonspecific T cell depletion, the extent to which host and donor T cell depletion with anti-Thy mAb plays a role is unclear. The addition of ATG in the clinic was essential for engraftment in sickle cell patients[10], thus some level of pan-T cell depletion may be essential. The use of T cell depletion could potentially increase the risk of viral infection. One therefore needs to balance the potential benefit of pan-T cell depletion on engraftment with the increased risk of infection when translating these therapeutic concepts to humans. Second, PTCy may not be sufficient as a single agent to prevent allograft rejection in the clinic after HLA-mismatched allografting. We have previously shown, in a murine BMT model, that sirolimus may be synergistic with PTCy in promoting donor chimerism[38]. This synergy was not seen when sirolimus was replaced with cyclosporine. Finally, we acknowledge that other mechanisms may be important for a PTCy-based regimen to establish tolerance. For example, T-regulatory cells appear to be resilient and contribute to tolerance via expression of high protective levels of ALDH[18,32]. Although the role of Tregs in this model appears to be limited, they may very well contribute synergistically to the induction of antigen-specific tolerance early after allografting. Third, although chimerism was low in our model, various modifications, such as adding pretransplant fludarabine or achieving more myeloablation through addition of busulfan, can be applied to increase donor hematopoietic chimerism[8,15,35].

In summary, we have developed a 12-hour protocol that induces lung allograft tolerance across stringent MHC barriers. However, significant modifications to this approach are required prior to its clinical translation specifically in relation to the timing of conditioning administration, deceased donor lung procurement and allografting. Furthermore, the potential toxicity of conditioning on patients with end stage lung-disease would need to be strongly considered. Encouragingly, these same regimens are now routinely used in patients with hematological malignancies up to the 7th decade[39]. Additionally, new strategies for allograft preservation in a transportable, warm-perfused, ventilated system that extend the time between lung harvest and implantation up to 12 hours without deleterious sequelae are being developed[40]. Although we have demonstrated efficacy of PTTT-PTB/PTCy in experimental orthotopic lung transplantation, additional studies, preferentially in large animal models, are needed to further define the optimal timing of pretransplant T cell depletion and total body irradiation and ultimately human translation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BM	bone marrow
BMT	bone marrow transplantation
CFSE	carboxyfluorescein succinimidyl ester
GVHD	graft-versus-host disease
HSC	hematopoietic stem cell
PTCy	posttransplant cyclophosphamide
TBI	total body irradiation

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A

	TCD IP	TBI	Donor bone marrow cells IV, day 0 (2.5×10^7)	Donor splenocytes IV, day 0 (3×10^7)	Cy (200 mg/kg) IP, day 3	Surviving allografts / total transplants
1	Day -1	Day -1	-	-	Yes	0/7
2	Day -1	Day -1	Yes	-	Yes	2/7
3	Day -1	Day -1	-	Yes	Yes	2/8
4	Day -1	Day -1	Yes	Yes	Yes	28/33
5	Day -1	Day -1	Yes	Yes	-	0/5

B

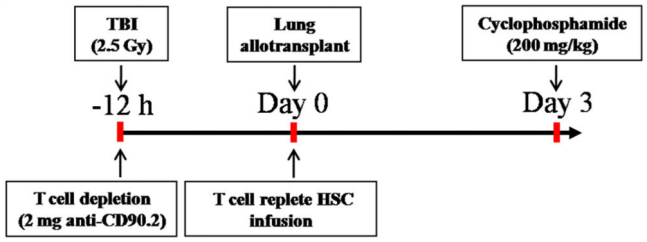

Figure 1.

(A) Allograft survival requires T cell–replete hematopoietic stem cells (HSC) and posttransplant cyclophosphamide (PTCy). Representative gross pathology of left lung allograft corresponding to different experimental conditioning regimens with/without donor bone marrow, splenocytes and posttransplant cyclophosphamide. (B) Regimen for combined BM and orthotopic lung allograft transplantation (PTTT-PTB/PTCy). Recipient mice received 250 cGy total body irradiation (TBI) followed 15 minutes later by T cell depletion with anti-Thy 1.2 monoclonal antibody 8-12 h before lung transplantation. Two to 6 hours following lung implantation, they received 2.5×10^7 donor strain bone marrow cells and 3×10^7 spleen cells (donor T cell–replete hematopoietic stem cells [HSC]) by intravenously followed by intraperitoneal administration of cyclophosphamide (200 mg/kg) 72 h after completion of lung implantation

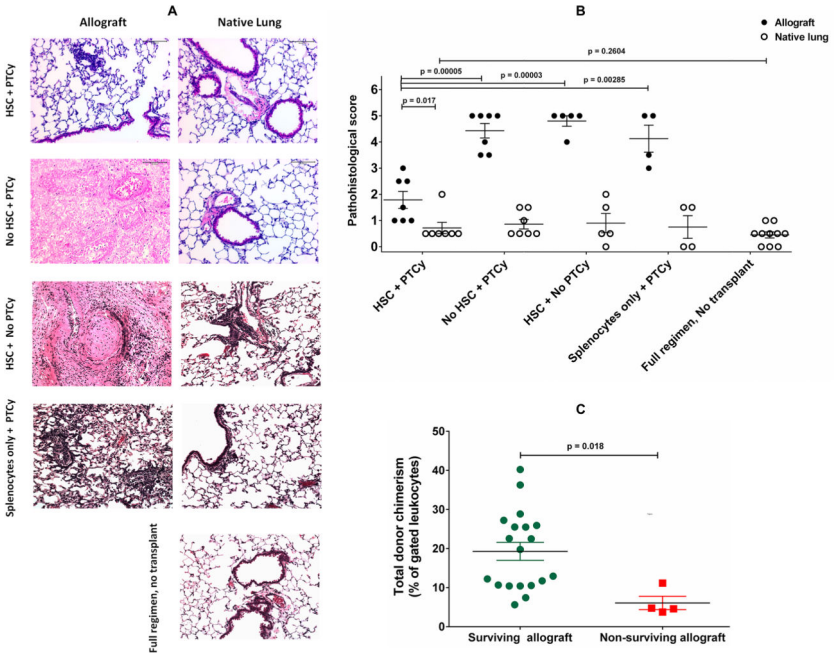

Figure 2.

(A-B) Effect on day 60 histology of including T cell–replete hematopoietic stem cells (HSC) in conditioning protocol. (A) Representative lung histology with H&E-staining (20x) at day 60 and (B) comparison of mean pathohistologic scores (n = 4-10 per group) of lung allografts versus native lungs from BALB/c -> C57BL/6 transplants as well as preconditioned untransplanted mice. Mice received pretransplant irradiation, T cell depletion, and posttransplantation cyclophosphamide (PTCy) with or without donor T cell–replete donor hematopoietic stem cell (HSC) infusion. (C) Relationship between lung allograft acceptance and level of peripheral donor chimerism following PTTT-PTB/PTCy. Comparison of total donor chimerism as a percentage of gated leukocytes at time of harvest in surviving (n=19) and nonsurviving (n=4) lung allografts from recipients treated with PTTT-PTB/PTCy. Bars indicate mean values ± SEM.

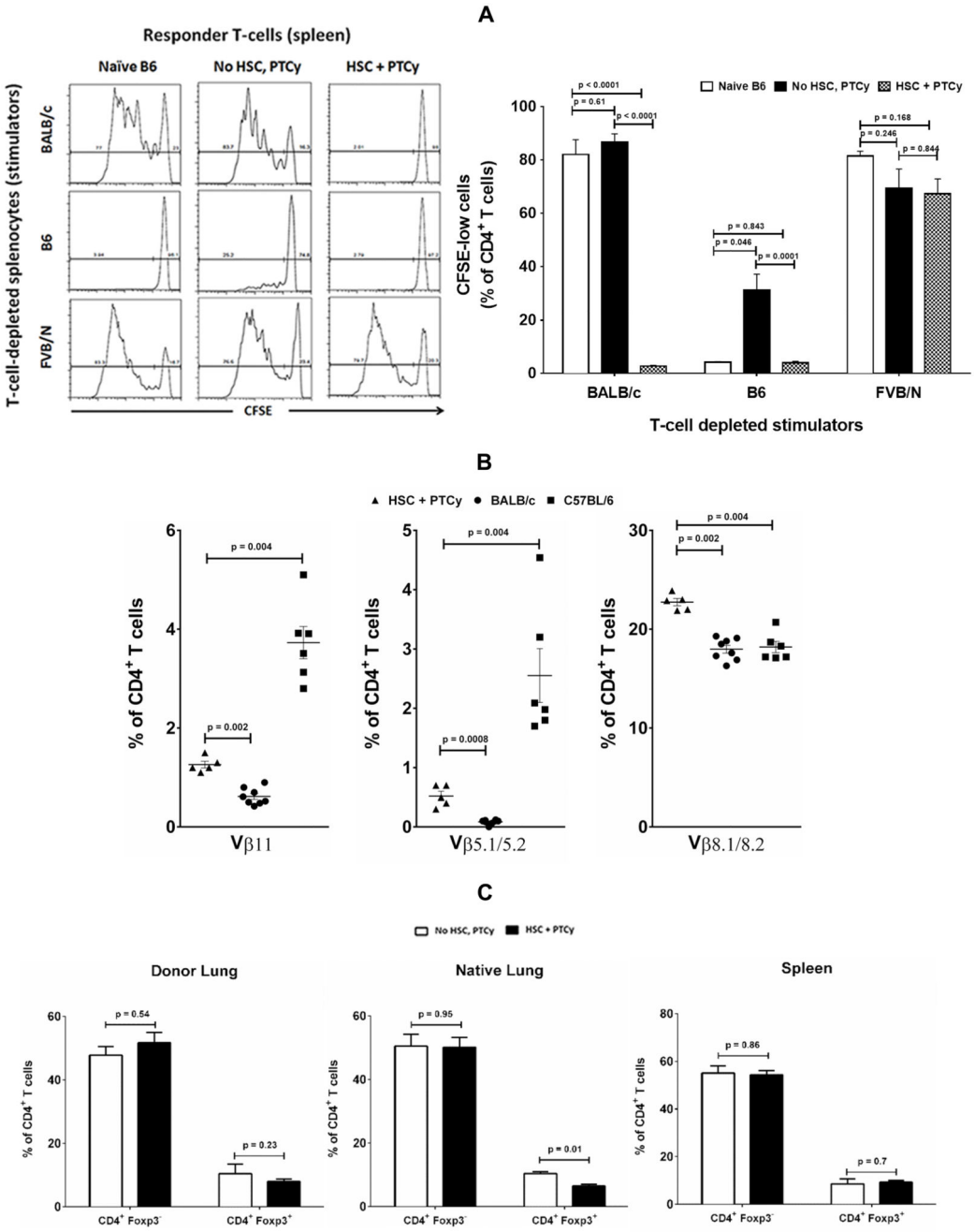

Figure 3.

(A) Donor-specific loss of alloreactivity in lung allograft-accepting mixed chimeras. Lung allograft-tolerant mixed chimeras were evaluated for donor specificity of their tolerance in an in vitro mixed lymphocyte reaction. Splenic $CD4^+$ T cells were isolated from hosts that received either no prior treatment (C57BL/6 untransplanted) or prior lung transplantation (BALBc->C57BL/6) and PTTT-PTB/PTCy with or without T cell–replete donor hematopoietic stem cells (HSC). The splenic $CD4^+$ T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) and co-cultured with T cell–depleted splenocytes from naïve C57BL/6 mice, naïve BALB/c mice, or naive FVB/N mice.

Proliferation patterns, indicated by progressive dilution of CFSE fluorescence intensity with every cell division, were evaluated. Data are representative of 3 independent experiments. Proliferation was quantified in terms of CFSE-low (divided) cell frequency within the gated $CD4^+$ subset and expressed as mean ± SEM.

(B) Deletion of donor-reactive Vβ11 and Vβ5.1/5.2 $CD4^+$ T cells with preservation of Vβ8.1/8.2 $CD4^+$ T cells in peripheral blood at harvest in lung allograft-tolerant C57BL/6 mice that underwent PTTT-PTB/PTCy. Expression of Vβ11, Vβ5.1/5.2, and Vβ8.1/8.2 in peripheral $CD4^+$ T cells of naïve donor (BALB/c), naïve recipient (C57BL/6), and the H-$2K^{b+}$ (host) subset of BALB/c->C57BL/6 lung allograft–accepting mice that received PTTT-PTB/PTCy. Vβ staining was analyzed by flow cytometry. Data are means ± SEM of 5-8 separate experiments in each group.

(C) Comparable H-$2K^{b+}$ $CD4^+$ effector ($Foxp3^-$) and T regulatory ($Foxp3^+$) frequencies in mice with surviving and rejected allografts. Lung allograft, native lung and spleen from PTTT-PTB/PTCy or PTTT-PTB/PTCy minus HSC groups were dissociated into single cell suspensions. Cells were surface-stained for H-$2K^b$, CD3 and CD4, fixed and permeabilized before staining for intranuclear Foxp3. Data are means ± SEM of 5-6 separate experiments in each group.