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Impaired CD8⁺ T cell immunity after allogeneic bone marrow transplantation leads to persistent and severe respiratory viral infection

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Abstract

Rationale—Bone marrow transplant (BMT) recipients experience frequent and severe respiratory viral infections (RVI). However, the immunological mechanisms predisposing to RVIs are uncertain. Therefore, we hypothesized that antiviral T cell immunity is impaired as a consequence of allogeneic BMT, independent of pharmacologic immunosuppression, and is responsible for increased susceptibility to RVI.

Methods—Bone marrow and splenocytes from C57BL/6(H2^b) mice were transplanted into B10.BR(H2^k) (Allo) or C57BL/6(H2^b) (Syn) recipients. Five weeks after transplantation, these mice were inoculated intranasally with mouse parainfluenza virus type 1 (mPIV-1), commonly known as Sendai virus (SeV), and monitored for relevant immunological and disease endpoints.

Main Results—Severe and persistent airway inflammation, epithelial injury, and enhanced mortality are found after viral infection in Allo mice but not in control Syn and non-transplanted mice. In addition, viral clearance is delayed in Allo mice as evidenced by prolonged detection of viral transcripts at Day 15 post-inoculation (p.i.) but not in control mice. In concert with these events, we also detected decreased levels of total and virus-specific CD8⁺ T cells, as well as increased T cell-expression of inhibitory receptor programmed death-1 (PD-1), in the lungs of Allo mice at Day 8 p.i. Adoptive transfer of CD8⁺ T cells from non-transplanted mice recovered

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from SeV infection into Allo mice at Day 8 p.i. restored normal levels of viral clearance, epithelial repair and lung inflammation.

Conclusions—Taken together these results indicate that allogeneic BMT results in more severe RVI based on the failure to develop an appropriate pulmonary CD8⁺ T cell response, providing an important potential mechanism to target in improving outcomes of RVI after BMT.

Keywords

Bone marrow transplant; Respiratory virus; lung; CD8⁺T cells

1. INTRODUCTION

Approximately 50,000 patients undergo bone marrow transplantation (BMT) each year for treatment of both malignant and non-malignant disorders [1–4]. Pulmonary complications, mostly infectious, occur in up to 60% of transplant recipients [5]. Respiratory viral infections (RVIs) with respiratory syncytial virus (RSV), parainfluenza virus (PIV), or influenza A virus (IAV) are among the most common pulmonary complications after BMT [6, 7]. BMT recipients experience more frequent, persistent, and severe complications of RVIs [8–10]. Consistent with these data, experimental models of allogeneic (AlloBMT) and syngeneic BMT (SynBMT) demonstrate increased pulmonary susceptibility and more severe infection to non-RVI pathogens such as bacteria or herpes viruses [11–13].

Mouse parainfluenza virus type 1 (mPIV-1), or Sendai virus (SeV), is a murine paramyxovirus virus that causes acute respiratory disease in mice similar to parainfluenza infection in humans [14]. Previous studies suggest that SeV immunity is a multifaceted response involving both the innate and adaptive immune systems [15]. One of the known adaptive immune responses in SeV infection involves T cells that are activated in the lymph nodes and migrate to the site of inflammation in the lungs to limit the extent of infection [16]. The primary T cells in this response appear to be CD8⁺ cytotoxic T cells, which have a central role in the clearance of SeV from the lungs of infected mice [17]. Under normal circumstances, effector CD8⁺ cytotoxic T cells are first detectable in the lungs on day 7 post infection and peak around day 9 or 10 [17–20]. The appearance of effector CD8⁺ cytotoxic T cells during respiratory viral infection correlates with clearance of the virus by day 9 or 10 that is dependent on either perforin or Fas related mechanisms [21, 22].

The clearance of viruses by CD8⁺ T cells can be affected by their functional state. For example, human metapneumovirus (hMPV) or IAV can impair CD8⁺ T cell function through upregulation of inhibitory receptors such as programmed death-1 (PD-1) [23]. PD-1 is a transmembrane protein that is a member of the CD28 family and is expressed on activated T, B, and myeloid cells after prolonged antigen stimulation. PD-1 functions as an immunoreceptor tyrosine-based inhibition motif (ITIM) to downregulate T cell activation and converts effector CD8⁺ T cells into “exhausted” CD8⁺ T cells that manifest impaired function during infection [24, 25].

Given the serious consequences of RVI in patients undergoing BMT, we sought to better understand the effect of AlloBMT on the immune response to RVI. Specifically, we

assessed if SeV clearance, lung pathology, or mortality is exacerbated after AlloBMT as compared to SynBMT or non-transplanted control conditions in a well-established mouse model of BMT that uses whole body lethal irradiation but no pharmacologic immunosuppression. We hypothesized that AlloBMT mice infected with SeV would manifest increased viral levels linked to increased severity of lung injury and mortality and that these events would be associated with CD8⁺ T cell deficiency and exhaustion. Furthermore, we hypothesized that viral clearance could be restored through transfer of functional T cells to AlloSeV mice, thereby providing T cell-dependent mechanism and a therapeutic strategy for impaired immunity to viral infection in this setting.

2. OBJECTIVE

RVIs are a major cause of morbidity and mortality in BMT recipients. However, the mechanisms that lead to increased severity of these types of infections are uncertain. Therefore, the objective of this study is to determine if antiviral T cell immunity is impaired as a consequence of allogeneic BMT and whether it is responsible for increased susceptibility to RVI.

3. MATERIALS AND METHODS

Mice

Animals were handled in the Regional Biocontainment BSL2 Laboratory at Duke University in accordance with the Institutional Animal Care and Use Committee (IACUC) of Duke University and Duke University Medical Center (all studies in this manuscript were approved by IACUC of Duke by protocol number A056–09–02) and strictly followed the National Institutes of Health recommendations cited in the Guide for the Care and Use of Laboratory Animals. All potentially painful procedures were performed under isoflurane anesthesia and all efforts were made to minimize suffering. All animals were housed in a pathogen-free facility at Duke University on LPS-free bedding (*Alpha Dri* bedding, Shepherd Specialty Papers Inc., Kalamazoo, MI) with a 12 hour light/dark cycle. Male 8-week-old C57BL/6J(H2^b) and B10.BR-*H2^kH2-T18^a*/SgSnJ(H2^k) mice (Jackson Laboratories, Bar Harbor, ME), approximately 20–25 grams in weight, received irradiated food (pellet and mash; Purina, Richmond, IN) and antibiotic water (Sulfamethoxazole/Trimethoprim 1.2/0.24mg/mL) after BMT. Mice were group housed with no more than 4 mice per cage. Mice were monitored and weighed daily by laboratory staff after infection. Once body weight dropped below 20% of starting weight or the animal exhibited any sign of disease or illness, the animal was euthanized using CO₂.

Murine bone marrow transplantation

BMT was performed as previously described [26]. Briefly, recipient B10.BR (Allo) and C57BL/6J (Syn) mice were lethally irradiated using a Cesium irradiator (8 and 10.25 Gy respectively) and injected with 4×10⁶ bone marrow cells and 1×10⁶ splenocytes from donor mice (C57BL/6J). All mice used were >95% engrafted 4 weeks post BMT per engraftment analysis Engraftment of CD3⁺, CD19⁺, CD11b⁺, and CD11c⁺ (eBioscience, San Diego, CA)

peripheral blood cells was evaluated 4 weeks post-transplantation using anti-H2D^b-FITC and anti-H2K^k-PE (BD Biosciences).

Viral inoculation

5 weeks post BMT, mice were inoculated intranasally (i.n.) with 1×10^5 PFU of Sendai virus (SeV; Fushimi strain) or saline. Mice were weighed daily after inoculation and necropsied at Days 1, 4, 8, and 15 post-inoculation (p.i.).

Bronchoalveolar lavage and analysis

The bronchoalveolar lavage fluid (BALF) was collected as previously described [26] and supernatant was analyzed using mouse Group I 23-plex cytokine assay (including IFN- γ) (Bio-Rad Laboratories, Hercules, CA) as well as by ELISA for Granzyme B (R&D systems, Minneapolis, MN) and IFN- β (PBL Interferon Source, Piscataway, NJ).

Immunohistochemistry

The left lung was fixed in 10% formalin, paraffin-embedded, and sectioned (5- μ m) for immunohistochemical analysis. Lung sections were stained with hematoxylin and eosin (H&E). Additional 5- μ m lung sections were blocked with 5% BSA in phosphate-buffered saline (PBS) for non-specific antigen reactivity following citrate buffer retrieval. Sections were stained with goat anti-rat-secretoglobulin 1A1 (Scgb1a1) (1:15,000; a generous gift from Dr. Barry Stripp), goat anti-parainfluenza virus type 1 (1:500; Gene Tex, Irvine, CA). Secondary antibodies used were Alexa Fluor 594 Donkey anti-Rabbit IgG, Alexa Fluor 488 Goat anti-mouse IgG2b (1:500, Invitrogen, Carlsbad, CA). Slides were cover-slipped with DAPI (Sigma) in Fluoromount G mounting media (Southern Biotech, Birmingham, AL). Images were obtained using an Olympus Provis AX70 microscope (Center Valley, PA) equipped with a digital camera and processed using Image-Pro Plus (Media Cybernetics, Silver Spring, MD).

RNA analysis

After necropsy, total RNA was extracted from lung tissue and cDNA transcribed (Applied Biosystems, Foster City, CA). 50ng of cDNA was used for qPCR for SeV nucleocapsid protein RNA (nt 519–587 in GenBank accession M30202; Applied Biosystems), Scgb1a1 (Mm00442046_m1; Applied Biosystems), and endogenous β -actin (4352933) (Applied Biosystems). Ct values were determined using ABI 7500 real-time PCR System with SDS software 1.3.1. Change in expression was calculated using the 2^{-Ct} method after normalization to β -actin expression.

Lung flow cytometry

Lungs were digested with collagenase-A (Roche Diagnostics, Mannheim, Germany) and DNase-I (Sigma-Aldrich), filtered through a 70- μ m filter (BD Biosciences), red blood cells lysed, washed, and resuspended in FACS buffer. Live cells were counted using 0.4% Trypan-Blue (Sigma-Aldrich) dead-cell exclusion. Cells were blocked using 5% normal mouse serum and 5% normal rat serum (Jackson ImmunoResearch Laboratories Inc, West Grove, PA), and 1% Fc-receptor-block (anti-mouse CD16/32; eBioscience, San Diego, CA),

and then stained with anti-mouse antibodies for anti-CD3-FITC, anti-CD4-PE-Cy7, anti-CD62L-APC, anti-CD8-APC-Cy7, anti-PD-1-PE (clone DX5), and anti-CD44-PerCP-Cy5.5 (eBioscience). Viral specific SeV⁺ T cells were detected using tetrameric MHC-peptide reagents for SeV nucleoprotein (NP₃₂₄₋₃₃₂) complexed with K^b conjugated to PE provided by the National Institute of Allergy and Infectious Disease Tetramer Core Facility. Fluorescence was measured (FACSCantoII flow cytometer) and analyzed using FlowJo software (Tree Star Inc, Ashland, OR).

Adoptive transfer of CD8⁺ T cells

Donor mice (C57BL/6J) were inoculated with 1×10^5 PFU of SeV and necropsied at Day 15 p.i. Single-cell suspensions were prepared from spleens and CD8⁺ T cells were purified using magnetic beads for mouse CD8a (Ly-2) (Miltenyi Biotec, Auburn, CA). The CD8⁺ T cell population was >93% pure as determined by flow cytometry. CD8⁺ T cells were administered retro-orbitally (3.5×10^6 /mouse) to Allo and Syn naïve or Day 8 p.i. mice. Recipient mice were necropsied at Day 15 p.i.

Statistical analysis

Data are expressed as mean $\pm$ SEM. Comparisons were performed using a one-way ANOVA in GraphPad Prism Software (Version 5.03, La Jolla, CA). P-values of <0.05 were considered significant.

4. RESULTS

Pulmonary viral clearance is impaired after allogeneic BMT

After allogeneic (Allo) or syngeneic (Syn) BMT, mice recovered for 5 weeks. Sendai virus (1×10^5 PFU) was administered to Allo (AlloSeV), Syn (SynSeV), and non-transplanted (NTSeV) mice. Infected mice as well as time-matched uninfected controls were necropsied at Days 1, 4, 8, and 15 p.i. (Figure 1A–G). In NTSeV or SynSeV mice, viral burden peaks at Day 4 p.i. and clears by Day 8 p.i. with no detectable viral load at Day 15 p.i. (Figure 1A, B, D, and F). After AlloBMT, viral transcripts and immunohistochemical staining for SeV are significantly less at Day 4 p.i. but significantly higher at Day 8 and 15 p.i. when compared to NT or Syn controls (Figure 1A, C, E, and G). These data suggest that viral clearance of SeV is impaired in lungs after AlloBMT.

AlloSeV mice have decreased survival

SeV infection causes an approximate 15% reduction in body weight at Day 8 p.i. in NT mice, with body weight recovering by Day 10 p.i. [27]. This same trend was noted in SynSeV mice (Figure 2A). AlloSeV mice have a 10% loss in body weight by Day 4 p.i. that increases to a 15% loss by Day 8 and persists to 15 Days p.i. (Figure 2A). There also is significantly worse survival in AlloSeV mice, with less than 20% of mice alive at Day 21 p.i. when compared to SynSeV (Figure 2B). Uninfected Syn and Allo mice have no differences in body weight or survival (Figure 2).

AlloSeV mice have persistent perivascular and peribronchiolar pulmonary inflammation and increased epithelial injury

We were interested to determine if impaired viral clearance in AlloSeV mice is associated with worsened pulmonary pathology. Unexposed NT, Syn, and Allo mice without viral infection display minimal peribronchiolar and perivascular infiltrates 5 weeks post transplantation (Figure 3A, B, and C). At Day 4 p.i., NTSeV, SynSeV, and AlloSeV mice have evidence of significant perivascular and peribronchiolar lymphocytic inflammation (Figure 3D, E, and F). This inflammation resolves by Day 15 p.i. in the NTSeV and SynSeV mice (Figure 3J and K) but not in the AlloSeV mice (Figure 3L), which have persistent inflammation, severe epithelial sloughing and flattening (Figure 3F, I, and L) that is more pronounced than unexposed AlloBMT (Figure 3C). In addition to these pulmonary histological findings, reduced expression of club (previously referred to as Clara) cells is observed in AlloBMT alone when compared to SynBMT, indicating baseline epithelial injury as result of allotransplantation (Supplemental Figure 1A). SeV infection is associated with more persistent epithelial injury in Allo mice with significantly reduced secretoglobin 1A1 (Scgb1a1, the gene for the Club cell secretory protein) levels at all timepoints when compared to uninfected Allo mice. This transcript data was confirmed with immunostaining of airways of SynSeV and AlloSeV mice (Supplemental Figure 1B–G), illustrating that post-BMT epithelial cell injury is exacerbated after RVI. Since viral infections have been associated with increased alloimmunity [28–30], we systematically assessed the mice for any signs of GVHD. Besides weight loss and lung inflammation, no other signs that could be ascribed to GVHD were noted in the gastrointestinal tract (such as dilation of the colon or small intestine) or skin (such as scaly skin or hair loss) at the time of necropsy (data not shown).

Impaired viral clearance in AlloSeV is not associated with decreased Type I Interferon expression

Type I Interferons (IFN) are secreted primarily by the respiratory epithelium in the early days of viral infection to elicit an innate antiviral response including recruitment and activation of inflammatory cells as well as control of viral replication [31]. Given the previous results, we wanted to determine if impaired viral clearance after AlloBMT is a result of decreased lung Type I IFN levels (Figure 4A). Allogeneic or syngeneic transplantation alone did not affect IFN- β production, although production is increased with virus infection at Day 4 and 8 p.i. in both groups. At Day 15 p.i. AlloSeV mice have a significant increase in IFN- β in BALF when compared to SynSeV and uninfected controls (Figure 4A). A similar trend is seen in the case of IFN- β , a type II IFN, with increased BALF levels in AlloSeV mice compared to SynSeV controls (Figure 4B). These data suggest that the lack of respiratory viral clearance after AlloBMT is not associated with a decrease in Type I or II IFN production.

Impaired viral clearance in AlloSeV is associated with decreased pulmonary CD8⁺ T cells and granzyme B

Since T cells are essential for the clearance of pulmonary viral infections in the normal lung [31, 32], it was of interest to identify if alloBMT alters the pulmonary T cell populations in

response to viral infection. The number of pulmonary CD4⁺ T cells after AlloBMT, without infection, is higher after transplant when compared to Syn (Figure 5A). Once infected with SeV, Allo mice have no significant increase in CD4⁺ T cells in lungs at Day 4 p.i. when compared to SynSeV mice or uninfected controls. By Day 8 p.i., SynSeV mice have an influx of CD4⁺ T cells to the lungs that recedes back to baseline levels by Day 15 p.i. AlloSeV mice have less CD4⁺ T cells at Day 8 p.i. but significantly more at Day 15 p.i. Similar to the CD4⁺ response, SynSeV mice have a significant influx of CD8⁺ T cells into the lung at Day 8 and 15 p.i., but AlloSeV mice have no increases in CD8⁺ T cells at either timepoint (Figure 5B). Consistent with the lack of CD8⁺ T cell recruitment, reduced BALF levels of Granzyme B are observed in AlloSeV mice as compared to SynSeV at Day 4 and 8 p.i. (Figure 5C). Of note, only minimal changes are observed in spleen CD4⁺ and CD8⁺ T cell populations after SeV infection suggesting that the reduced numbers of CD8⁺ T cells after AlloSeV, as compared to SynSeV, reflects a lung-specific difference in the antiviral response (data not shown).

Impaired viral clearance in AlloSeV is associated with a lack of viral-specific CD8⁺ T cells in the lung

CD8⁺ viral specific T cells in the lungs of AlloSeV mice were further examined by flow cytometry using MHC I tetramers specific for the nucleocapsid protein of SeV (NP⁺) [33]. No NP⁺CD8⁺ T cells are noted in the lungs of uninfected controls (Figure 6B). SynSeV have an increase in the number of viral-specific CD8⁺ T cells at Day 8 and 15 p.i. (Figure 6A and B). This population is almost absent in the lungs of AlloSeV mice at all timepoints p.i. No viral-specific T cells are noted in the spleens of any of the groups examined at any Day p.i. (data not shown).

Allo BMT leads to CD8⁺ T cell upregulation of PD-1 that is enhanced by SeV infection

Since AlloSeV mice fail to develop a normal of CD8⁺ T cell antiviral response, we sought to determine whether there is a functional alteration in pulmonary CD8⁺ T cells that could further explain this phenomenon and contribute to impaired viral clearance. PD-1 is a marker of T cell exhaustion that has been associated with impaired viral clearance [34]. Therefore we measured the PD-1 cell surface expression on both CD4⁺ and CD8⁺ cells at baseline and after viral infection in the lungs of Allo and Syn mice. Within the CD4⁺ T cells population, PD-1 expressing cells are increased at Day 8 and return close to baseline by Day 15 in a manner that is similar between both AlloSeV and SynSeV groups (Figure 7B). In contrast, several important differences are noted in the CD8⁺ T cell population. First, after AlloBMT alone, CD8⁺ T cells in the lung have a higher expression of PD-1 as well as an increase in PD-1⁺ CD8⁺ T cell numbers when compared to Syn controls (Figure 7A and C). Consistent with this point, a significant increase in the number of CD8⁺ T cells, but not CD4⁺ T cells, expressing PD-1 is also noted in the spleen of Allo mice as compared to Syn mice (Supplemental Figure 2A and B). Second, SeV induces differential responses in CD8⁺PD-1⁺ population of cells among Syn vs. Allo mice. Specifically, in SynSeV mice the number of CD8⁺ PD-1⁺ expressing cells increases at Day 8 after infection and then returns close to baseline by Day 15 p.i. The AlloSeV mice, in contrast, have a significant elevation in CD8⁺PD-1⁺ expressing cells at Day 15 p.i. as compared to SynSeV (Figure 7A and C).

Adoptive transfer of CD8⁺ T cells from non-transplanted mice recovering from SeV restores viral clearance and improves virus-induced pathology in AlloSeV mice

Given the failure to develop virus-specific immunity after AlloBMT, it was of interest to examine whether or not an adoptive transfer of non-transplant CD8⁺ T cells could restore viral immunity in the lung. Therefore, CD8⁺ T cells were obtained from wild-type non-transplanted C57BL/6J mice infected with SeV and adoptively transferred into AlloSeV and SynSeV mice at Day 8 p.i., the timepoint of peak influx of CD8⁺ T cells after RVI (Figure 8A). After adoptive transfer, mice were necropsied at Day 15 p.i. to determine if viral immunity was restored. AlloSeV mice again have increased viral burden at Day 15 p.i. when compared to SynSeV mice (Figure 8B–D). Adoptive transfer did not alter viral titers in SynSeV mice (Figure 8B and E). However after adoptive transfer of CD8⁺ T cells, AlloSeV mice have significantly less viral load in the lungs with levels comparable to levels in SynSeV (Figure 8B and F). In addition to increased viral clearance in the lung after adoptive transfer, pulmonary pathology of AlloSeV mice is ameliorated with little to no peribronchiolar and perivascular lymphocytic inflammation (Supplemental Figure 3A–D). This improvement in pathology is also coupled with an increase in Scgb1a1 production by the airway epithelium, although not as abundant as SynSeV mice (Supplemental Figure 3E–H).

After CD8⁺ T cell adoptive transfer, CD8⁺ T cell numbers are also increased in lung tissue of AlloSeV mice when compared to AlloSeV mice alone (Figure 8G), along with decreased expression of PD-1 on CD8⁺ T cells (Figure 8H), indicating that these CD8⁺ T cells remained functional. Granzyme B levels in the BALF are decreased in AlloSeV mice after adoptive transfer, mirroring the viral clearance and the levels seen on Day 15 p.i. in SynSeV (Figure 8I).

5. DISCUSSION

In this present study we make the novel observation that respiratory infection with SeV after AlloBMT leads to decreased viral clearance, increased lung injury, and significantly increased mortality as compared to SynSeV or NTSeV. Furthermore, we demonstrate that infected AlloBMT mice have impaired CD8⁺ T cell responses with reduced viral-specific T cells and an increase in PD-1 expression that is reversed with an adoptive transfer of non-exhausted CD8⁺ T cells. These data suggest that pulmonary viral immunity is impaired after AlloBMT as a result of CD8⁺ T cell dysfunction.

Our results resemble those observed with impaired immunity to *Pseudomonas aeruginosa* or herpes simplex virus type-1 (HSV1) after SynBMT or AlloBMT [11–13, 35]. However, in those studies, increased susceptibility to bacterial or viral infection after BMT was related to decreased interferon and increased transforming growth factor β (TGF- β 1) production by CD4⁺ cells as well as impaired alveolar macrophage function but did not imply a mechanism of increased infection related to CD8⁺ T cell responses. In contrast to some of the previous studies, SynSeV mice in our model respond to infection in a manner similar to NTSeV mice and impaired immunity to RVI is observed only in the setting of AlloBMT. These differences likely reflect variations in the BMT protocols used in these experiments as well as differences in pathogen-specific immune responses after BMT [11–13, 35]. For

example, delayed clearance of HSV1 after SynBMT is a result of decreased IFN- γ production after transplant [36]. However, decreased IFN- γ levels were not seen in AlloSeV mice when compared to SynSeV controls. Additionally, previous studies on nontransplanted mice have determined that SeV virus clearance is not dependent on IFN- γ production [37] but is dependent on CD8⁺ T cell function [20]. Thus, in comparison to previous studies that focused on bacterial and non-SeV viral infections, our manuscript confirms that pulmonary immunity is impaired after BMT independent of additional immunosuppression and also provides new insight into CD8⁺ T cell impairment specifically in the context of RVI.

Previous studies of antiviral immunity during SeV infection have demonstrated that CD8⁺ T cells function to kill virus-infected cells while the CD4⁺ T cells are important in memory and antibody production [37–39]. In the study presented here, we make the novel observation that impaired immunity to SeV after AlloBMT occurs as a result of pulmonary CD8⁺ T cell dysfunction. Furthermore, our data suggest this T cell dysfunction occurs as a result of both reduced CD8⁺ T cell numbers as well as T cell exhaustion. In support of this idea, increased PD-1 expression was observed in AlloBMT CD8⁺ T cells in the setting of impaired viral clearance. Additionally, adoptive transfer of normal SeV-primed PD-1^{low} CD8⁺ T cells into AlloBMT mice infected with SeV restores immunity. These results are consistent with the growing body of literature that suggests CD8⁺ T cells expressing PD-1 lose effector function during chronic infection [23, 24, 34]. T cell exhaustion has been correlated with lack of viral clearance in many models of chronic infection such as HIV and LCMV [40], and more recently has been linked to impaired function in the setting of acute pulmonary viral infections in non-transplant models [23]. Interestingly, PD-1 expression was increased on CD8⁺ but not CD4⁺ T cells after AlloBMT, indicating that the exhaustion phenotype was restricted to CD8⁺ T cells. We observed minimal differences in the CD4⁺ T cell population, although the exact pattern of CD4⁺ T cell recruitment was different between AlloSeV and SynSeV.

We also found that AlloBMT increases PD-1 expression on CD8⁺ T cells, both in the lung and spleen, independent of SeV infection. This is consistent with studies showing increased PD-1 expression on T cells in peripheral blood samples of patients that have undergone AlloBMT (32, 33). Additionally, experimental studies have found that upregulation of PD-1 on CD8⁺ T cells is required for engraftment after a mixed chimera BMT [41, 42]. The increase in PD-1 expression after AlloBMT has also been shown to occur in response to alloantigen expression on non-hematopoietic cells and may contribute to reducing graft-versus-leukemia responses [43]. Thus, our findings of increased PD-1 expression after allotransplantation is likely a result of chronic alloantigen stimulation after BMT and predisposes these CD8⁺ T cells to further exhaustion when challenged with RVI as was observed in our study.

The CD8⁺ T cell impairment after allotransplantation could be due to several potential mechanisms besides T cell exhaustion. First, this could reflect failure to locally generate appropriate CD8⁺ SeV cells in the lung. We find this idea intriguing and consistent with recent publications that indicate that the lung can function as a tertiary lymphoid organ and that T cell priming can occur directly in pulmonary lymphoid aggregates after transplantation [44, 45]. It is possible that allotransplantation acceptance results in impaired

ability of the lung to support proper CD8⁺ T cell priming. Second the lack of CD8⁺ T cells in lung tissue after allotransplantation could indicate that there is a defect in recruitment of CD8⁺ T cells to the lung during SeV infection as defects in CD8⁺ T cell recruitment to the lung have previously been associated with delayed viral clearance [46]. Future studies will need to determine the mechanism by which CD8⁺ T cells are impaired after allotransplantation and unable to contain respiratory viral infections.

We also found that adoptive transfer of SeV-primed CD8⁺ T cells increases viral clearance and improves pulmonary pathology after AlloBMT. This is consistent with previous human BMT studies showing that a therapeutic transfer of cytomegalovirus (CMV) specific CD8⁺ T cells early after syngeneic BMT decreased viral disease [47–49]. Additionally, in this setting of experimental BMT and murine CMV infection, transfer of CD4⁺ T cells and other effector T cells did not compensate for CD8⁺ T cells in the control of viral replication and disease [50]. These studies as well as the data presented here provide evidence that the CD8⁺ T cell compartment is compromised after transplantation and there is a benefit from CD8⁺ T cell transfer with BMT-associated infections. Our study is the first to show that transfer of CD8⁺ T cells can be beneficial for RVIs that commonly plague human BMT patients, however the mechanism of this association is still unknown.

Although SeV infection is exacerbated in AlloBMT at later timepoints, there was evidence of decreased viral titers at an early timepoint (Day 4 p.i.) as compared to NTSeV and SynSeV despite viral persistence, impaired clearance, and increased mortality at Day 8 and 15 p.i. This might reflect reduced viral attachment early during infection potentially due to changes to the pulmonary epithelium after AlloBMT alone. Respiratory viruses, such as SeV, primarily infect the respiratory epithelium by attaching to apical cell surface carbohydrates, which may be altered after AlloBMT [31]. Consistent with our results, in a previous study of AlloBMT mice infected with HSV1, lung viral titers were also decreased early after BMT when compared to Syn controls [35].

Despite the novelty of our results, we also recognize certain inherent limitations to our study. First, our BMT model employs transplantation of a small number of alloreactive T cells transferred with no immunosuppression and minimal baseline GVHD, thus differing from the usual clinical scenario. Nevertheless, it provides a useful way to elucidate the mechanism by which alloreactivity itself impairs antiviral immunity while avoiding any confounding effects by immunosuppression or significant GVHD. Second, due to the severity of infection and increased mortality in AlloSeV mice, we were unable to study longer timepoints or memory responses in the context of viral infection. Perhaps use of a lower dose or a less pathogenic virus would allow adequate analysis of these endpoints in the context of alloimmunity. Third, the adoptive transfer of CD8⁺ T cells is likely a mixed population of viral-specific, regulatory, and memory CD8⁺ T cells, as has been described in the spleens of mice after viral infection [51]. Given the fact that adoptive transfer of CD8⁺ T cells increased viral clearance but not GVHD indicates that a regulatory CD8⁺ T cell population may have been transferred since previous reports have shown that a significant amount of regulatory cytokines are produced by CD8⁺ T cells after RVI [52]. To better understand which population of CD8⁺ T cells is compromised after transplant, future studies

will focus on transferring specific sub-populations. Finally, we acknowledge that SeV is not a human virus but has proven useful as a model to understand RVI in humans.

In conclusion, our findings indicate that allotransplantation decreases CD8⁺ T cell numbers in the lung and impairs pulmonary clearance of RVI. Additionally, restoring CD8⁺ T cell immunity in AlloSeV promotes viral clearance and restores host antiviral responses. This study adds to our growing knowledge of altered pulmonary immunity after allotransplantation. A better understanding of post-transplantation pulmonary immune defects will help better prevent and treat common and clinically important infections that cause severe and persistent complications after BMT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

BMT	bone marrow transplant
AlloBMT	allogeneic bone marrow transplant
SynBMT	syngeneic bone marrow transplant
RVI	respiratory viral infections
mPIV-1	mouse parainfluenza virus type 1
SeV	Sendai virus
p.i.	post-inoculation
RSV	respiratory syncytial virus
PIV	parainfluenza virus
IAV	influenza A virus
hMPV	human metapneumovirus
ITIM	immunoreceptor tyrosine-based inhibition motif
BALF	bronchoalveolar lavage fluid
H&E	hematoxylin and eosin
Scgb1a1	secretoglobin 1A1

AlloSeV	allogeneic bone marrow transplant mice that have been inoculated with SeV
SynSeV	syngeneic bone marrow transplant mice that have been inoculated with SeV
NT	nontransplanted
NTSeV	non-transplanted mice that have been inoculated with SeV
NP	SeV nucleocapsid protein
GVHD	graft-versus-host disease
HSV-1	herpes simplex virus type-1
CMV	cytomegalovirus

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HIGHLIGHTS

- Severity of respiratory viral infection is increased in a mouse model of allogeneic BMT.
- Allogeneic BMT mice have inadequate antiviral CD8⁺ T cell responses and consequent deficiencies in viral clearance and epithelial repair.
- The CD8⁺ T cell impairment correlates with increased markers of T cell exhaustion in CD8⁺ T cells.
- Adoptive transfer of non-exhausted CD8⁺ T cells improves antiviral immunity and viral clearance in allogeneic BMT mice.

Figure 1. Respiratory viral clearance is impaired after allogeneic BMT

Allogeneically-reconstituted BMT mice (Allo) or syngeneic controls (Syn) were infected with 1×10^5 PFU of Sendai Virus (SeV; AlloSeV or SynSeV respectively) and sacrificed at Day 1, 4, 8, and 15 post-inoculation (p.i.). B10.BR nontransplanted (NT) mice were used as controls (NTSeV). In NTSeV and SynSeV mice, viral burden peaked at Day 4 p.i. and cleared by Day 8 p.i. with no detectable viral load at Day 15 p.i. In AlloSeV mice, viral transcripts and immunohistochemical staining for SeV were significantly less at Day 4 p.i. but significantly higher at Day 8 and 15 p.i. when compared to NTSeV or SynSeV. (**A**)

Lung tissue was analyzed by real time PCR for SeV nucleocapsid protein RNA (N gene). Lung sections were stained for parainfluenza virus type 1 at various timepoints post-inoculation to further verify RNA analysis. **(B–G)** Representative immunohistochemistry staining for SeV (green=SeV, blue=DAPI; images are 200x) **(B)** SynSeV Day 4 p.i.; **(C)** AlloSeV Day 4 p.i.; **(D)** SynSeV Day 8 p.i.; **(E)** AlloSeV Day 8 p.i.; **(F)** SynSeV Day 15 p.i.; **(G)** AlloSeV Day 15 p.i.; (* = p-value less than 0.05 comparing AlloSeV to SynSeV and NTSeV) (n=3–5/group, data replicated in 3 independent experiments, graph represents data of 1 experiment).

Figure 2. Viral infection after allogeneic BMT leads to significant body weight loss and increased mortality

Allogeneically-reconstituted BMT mice (Allo) or syngeneic controls (Syn) were infected with 1×10^5 PFU of Sendai Virus (SeV; AlloSeV or SynSeV respectively) and weighed daily post-inoculation (p.i.) for 15 days. SeV infection causes a reduction in body weight by around Day 8 p.i. in SynSeV with recovery to starting body weight is seen by Day 10 p.i. AlloSeV mice had a significant loss in body weight by Day 4 p.i. that persisted by Day 8 and persisted to 15 p.i. This significant loss in body weight also correlated with a decrease in survival in AlloSeV mice when compared to SynSeV. **A)** Percent of starting body weights

after SeV infection. **B)** Survival analysis of experimental groups after SeV infection. (* = p-value less than 0.05 comparing SynBMT to AlloBMT or SynSeV to AlloSeV) (n=3–5/group, data replicated in 3 independent experiments).

Figure 3. Viral infection after allogeneic BMT leads to excessive peribronchiolar and perivascular pathology

Allo, Syn, and NT mice were infected with 10^5 PFU of SeV (AlloSeV, SynSeV, and NTSeV respectively) and sacrificed at Day 1, 4, 8, and 15 post-inoculation (p.i.). Lung pathology was evaluated at Day 4, 8, and 15 post-inoculation in NT, Syn and Allo mice. Saline controls were also analyzed for baseline pathology and displayed minimal peribronchiolar and perivascular infiltrates. At Day 4 p.i. after SeV infection, NTSeV, SynSeV, and AlloSeV mice have evidence of significant perivascular and peribronchiolar lymphocytic inflammation. AlloSeV mice had excessive peribronchiolar and perivascular pathology at

Day 8 and 15 p.i. when compared to NTSeV and SynSeV. Representative histology sections are shown for all groups (H&E stain, 200x). **(A)** Saline exposed NT; **(B)** Saline exposed Syn; **(C)** Saline exposed Allo; **(D)** NT SeV Day 4 p.i.; **(E)** SynSeV Day 4 p.i.; **(F)** AlloSeV Day 4 p.i.; **(G)** NT SeV Day 8 p.i.; **(H)** SynSeV Day 8 p.i.; **(I)** AlloSeV Day 8 p.i.; **(J)** NT SeV Day 15 p.i.; **(K)** SynSeV Day 15 p.i.; **(L)** AlloSeV Day 15 p.i. (n=3–5/group, data replicated in 3 independent experiments)

Figure 4. Impaired viral clearance after allogeneic BMT is not associated with decreased type I or II interferon production

BAL fluid was analyzed by ELISA for **A**) IFN- β levels or by multiplex ELISA for **B**) IFN- γ levels at Day 1, 4, 8 and 15 post-inoculation (p.i.) in Allo and Syn and uninfected controls. Allo or Syn BMT alone did not affect IFN- β or IFN- γ production, although production was increased with virus infection at Day 4 and 8 p.i. in both groups. At Day 15 p.i. AlloSeV mice had a significant increase in IFN- β in BAL fluid when compared to SynSeV and

uninfected controls (* = p-value less than 0.05 comparing SynBMT to AlloBMT or SynSeV to AlloSeV) (n=3–5/group, data replicated in 3 independent experiments).

Figure 5. Impaired viral clearance after allogeneic BMT is associated with decreased CD8⁺ T cell numbers and Granzyme B production

After BMT and infection with SeV, lung cells were analyzed by flow cytometry. SynSeV mice had a significant influx of CD8⁺ T cells into the lungs at Day 8 and 15 post-inoculation (p.i.), but AlloSeV mice never recruited CD8⁺ T cells at either timepoint. This lack of CD8⁺ T cells was reflected in impaired Granzyme B production in the BAL fluid at Day 4 and 8 p.i. **A)** CD3⁺CD4⁺ cells for each experimental group at Day 4, 8, and 15 post-inoculation. **B)** CD3⁺CD8⁺ cells for each experimental group at Day 4, 8, and 15 post-inoculation. **C)**

BAL fluid was analyzed by ELISA for Granzyme B levels at Day 4, 8 and 15 p.i. in Allo and Syn and uninfected controls (* = p-value less than 0.05 comparing SynBMT to AlloBMT or SynSeV to AlloSeV) (n=3–5/group, data replicated in 3 independent experiments).

Figure 6. Impaired viral clearance after allogeneic BMT is associated with decreased viral-specific CD8⁺ T cell numbers

CD8⁺ T cells specific for the nucleocapsid protein of SeV (NP⁺) were noted in the lungs of SynSeV at Day 8 and 15 post-inoculation (p.i.) but this population was not seen in the lungs of AlloSeV mice at any timepoints p.i. **A)** Representative flow cytometric plots show CD3⁺CD8⁺NP-tetramers⁺ (SeV specific T cells) populations in lung cells for each experimental group at Day 15 post-inoculation in Allo and Syn and uninfected controls. Percentage of SeV specific T cells in upper right corner. **B)** Total number of CD3⁺CD8⁺NP-tetramers⁺ (SeV specific T cells) population within small cells of the lung for each

experimental group at Day 4, 8, and 15 post-inoculation. (* = p-value less than 0.05 comparing SynSeV to AlloSeV) (n=3–5/group, data replicated in 2 independent experiments).

Figure 7. Allogeneic BMT is associated with increased PD-1 expression on CD8⁺ T cells
 After BMT and infection with SeV, lung cells were analyzed by flow cytometry for PD-1 expression on the CD3⁺CD8⁺ and CD3⁺CD4⁺ T cells for each experimental group at Day 4, 8, and 15 post-inoculation (p.i.). Allo mice have a significant increase in PD-1 expression on CD8⁺ T cells in the lung when compared to Syn mice and this upregulation of PD-1 was exaggerated when Allo mice were infected with SeV. **A)** Representative histograms of PD-1 expression on CD3⁺CD8⁺ T cells in lung cells for each experimental group at Day 15 post-inoculation in Allo and Syn and uninfected controls. Percentage of CD3⁺CD8⁺ T cells that

are PD-1⁺ are listed in histogram. Grey filled histogram is isotype control. **B)** Percentage of CD3⁺CD4⁺ T cells that are PD-1⁺ for each experimental group. **C)** Percentage of CD3⁺CD8⁺ T cells that are PD-1⁺ for each experimental group. (* = p-value less than 0.05 and ** = p-value less than 0.01 comparing SynBMT to AlloBMT or SynSeV to AlloSeV) (n=3–5/group, data replicated in 2 independent experiments).

Figure 8. Adoptive transfer of $CD8^+$ T cells restores impaired viral clearance, restores $CD8^+$ T cell numbers, and decreases PD-1 expression in the lung after allogeneic BMT

$CD8^+$ T cells were isolated on Day 15 post-inoculation (p.i.) from splenocytes of nontransplanted C57Bl/6 mice that were infected with 10^5 PFUs of SeV and adoptively transferred into AlloSeV or SynSeV on Day 8 post-inoculation. After adoptive transfer of $CD8^+$ T cells, AlloSeV mice had significantly less virus in the lungs with levels comparable to levels in SynSeV at Day 15 p.i. **A**) Schematic of adoptive transfer. **B**) Lung tissue was analyzed by real time PCR for SeV nucleocapsid protein RNA on Day 15 p.i. **C-F**) Lung

sections were stained for parainfluenza virus type 1 at Day 15 p.i. to further verify RNA analysis. Representative immunohistochemistry staining for SeV (green=SeV, blue=DAPI; images are 200x) **C)** SynSeV Day 15 p.i.; **D)** AlloSeV Day 15 p.i.; **E)** SynSeV + CD8⁺ T cells Day 15 p.i.; **F)** AlloSeV + CD8⁺ T cells Day 15 p.i.; **G)** CD3⁺CD8⁺ population within small cells for each experimental group on Day 15 post-inoculation. **H)** Percentage of CD3⁺CD8⁺ T cells that are PD-1⁺ for each experimental group. **I)** BAL fluid was analyzed by ELISA for Granzyme B levels at Day 15 post-inoculation in Allo and Syn with or without adoptive transfer of CD8⁺ T cells; (* = p-value less than 0.05 comparing SynSeV to AlloSeV, ns= not significant) (n=5/group, graph is representative of 1 experiment).