A New Murine Model for Bronchiolitis Obliterans Post-Bone Marrow Transplant

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Rationale: Bronchiolitis obliterans (BO) is a major problem in lung transplantation and is also part of the spectrum of late-onset pulmonary complications that can occur after hematopoietic stem cell transplant. Better mouse models are needed to study the onset of this disease so that therapeutic interventions can be developed.

Objectives: Our goal was to develop a BO mouse model.

Methods: Recipients were lethally conditioned and given a rescue dose of T-cell-depleted, allogeneic bone marrow (BM) supplemented with a sublethal dose of allogeneic T cells.

Measurements and Main Results: At 2 months post-BM transplant, the lungs had extensive perivascular and peribronchiolar inflammation consisting of CD4⁺ T cells, CD8⁺ T cells, B cells, macrophages, neutrophils, and fibroblasts. In contrast to the acute model, histology showed airway obstruction consistent with BO. Epithelial cells of airways in the early stages of occlusion exhibited changes in expression of cytokeratins. Although the lung had severe allogeneic BM transplant-mediated disease, there was only mild to moderate graft-versus-host disease in liver, colon, skin, and spleen. High wet/dry weight ratios and elevated hydroxyproline were seen, consistent with pulmonary edema and fibrosis. Mice with BO exhibited high airway resistance and low compliance. Increases in many inflammatory mediators in the lungs of mice that develop BO were seen early post-transplant and not later at the time of BO.

Conclusions: This new mouse model will be useful for the study of BO associated with late post-hematopoietic stem cell transplant onset and chronic graft-versus-host disease, which also leads to poor outcome in the lung transplant setting.

Keywords: bronchiolitis obliterans; mouse models; transplantation

Bronchiolitis obliterans (BO) is a major obstacle that has limited the success of lung transplantation (1, 2). It is also part of the spectrum of late-onset pulmonary complications that can occur after hematopoietic stem cell transplant (HSCT) (3). The signature histopathologic finding in BO is the obliteration of the airway and airway epithelium by a fibroproliferative response (4–7). The formation of this lesion is generally believed to have two major components: airway epithelial injury, due to a directed alloimmune response, followed by fibroproliferation. This results in irreversible structural changes and impaired lung function. An increase in both CD8- and CD4-positive cells in BO lesions supports that this injury is immunologically mediated (8). The up-regulation of major histocompatibility complex (MHC) class II antigens in lesions together with bronchoalveolar lavage lymphocytes demonstrating reactivity to donor-specific class I HLA antigens suggest that alloreactivity directed against the epithelial

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The formation of bronchiolitis obliterans lesions is generally believed to have two major components, airway epithelial injury due to a directed alloimmune response followed by fibroproliferation. The cause of the heterogeneity seen in this disease is unknown.

What This Study Adds to the Field

We describe a new mouse model of bronchiolitis obliterans amenable to intervention strategies.

MHC antigens may be the inciting event (8). Within an affected lung, normal, inflamed, and fibrosed airways are histologically evident. This suggests a temporal and spatial continuum between injury and the fibroproliferative response.

We previously established a mouse model of the early-onset, acute form of this lung injury, caused by the influx of host monocytes and donor T cells into the lungs early post-allogeneic bone marrow transplant (BMT) of lethally irradiated mice (9). This model represents a noninfectious lung injury known as idiopathic pneumonia syndrome (IPS), which is a significant cause of death after BMT or HSCT and accounts for the majority of complications involving the lung in the early post-transplant period (10). Risk factors for developing IPS are related to the intensity of the conditioning regimen used and the degree of alloreactivity of the donor graft (11), but these same risk factors are beneficial in preventing relapse and promoting bone marrow (BM) engraftment. Intensifying the pre-BMT conditioning with cyclophosphamide potentiates the development of alloreactive T-cell-dependent lung injury. However, this early-onset model does not permit assessment of longer term consequences of interventional therapies. Furthermore, many patients develop late-onset lung disease with BO occurring several months post-BMT, typically as part of the sequelae of chronic graft-versus-host disease (GVHD). Therefore, we modified our acute murine model by reducing the donor T-cell content of the infusion to determine whether a chronic lung disease model could be established. Lowering allogeneic T-cell dose in chemoradiotherapy-conditioned mice given allogeneic BM resulted in lung injury with many characteristics distinct from the early-onset, acute form of IPS that results from high T-cell doses. Specifically, we found the presence of obstructive disease with fibrosis, thereby demonstrating the potential usefulness of this murine model for the study of BO in a transplant setting. Some of the results of these studies have been previously reported in the form of an abstract (12).

METHODS

Mice

C57BL/6 (H2^b) mice were purchased from the National Institutes of Health (Bethesda, MD). B10.BR (H2^k), BALB/c (H2^d), and FVB/N

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(H2^a) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in microisolator cages in the specific pathogen free (SPF) facility of the University of Minnesota and cared for according to the Research Animal Resources guidelines of our institution. Experiments were approved by the Institutional Animal Care and Use Committee of the University of Minnesota. Sentinel mice were found to be negative for infectious microorganisms known to cause pulmonary pathology, such as pneumonia virus, K virus, Sendai virus, mycoplasma, mouse adenoviruses 1 and 2, murine cytomegalovirus, and murine hepatitis virus. For BMT, donors were 8 to 12 weeks of age and recipients were 8 to 10 weeks of age.

BMT

Our BMT protocol has been described previously (13). Donor BM was T-cell depleted with anti-Thy 1.2 monoclonal antibody (mAb) (clone 30-H-12, rat IgG2_k; kindly provided by Dr. David Sachs, Charlestown, MA) plus complement (Neffenegger Co., Woodland, CA). Recipient mice received phosphate-buffered saline (PBS) or cyclophosphamide (Cytosan; Bristol Myers Squibb, Seattle, WA), 120 mg/kg per day intraperitoneally, as a conditioning regimen pre-BMT on Days -3 and -2. All mice were lethally irradiated on the day before BMT (7.5 Gy total body irradiation) by X-ray at a dose rate of 0.39 Gy/minute as described (13). Recipient mice were transplanted via caudal vein with 15×10^6 T-cell-depleted allogeneic marrow with or without 1×10^6 spleen cells as a source of allogeneic T cells.

Lung Weights

Mice were killed with sodium pentobarbital and the thoracic cavity partially dissected. Lungs were exsanguinated by perfusion with 1.0 ml saline via the right ventricle of the heart. To minimize the number of mice needed for the study without compromising the data, the right lung (bilobed) was used for weight determinations, whereas the left lung was processed for histopathology (*see below*). For each mouse, the wet weight was taken immediately after right lung was removed from the thorax. Lungs were dried overnight to a constant weight at 80°C followed by determination of dry weights. The wet/dry weight ratio was calculated and taken as a measure of the severity of lung injury (14). No correction for extravascular blood content was used in the calculations.

Hydroxyproline

Hydroxyproline (OH-proline) levels were determined by oxidation of 4-OH-L-proline to pyrrole and reaction with *p*-dimethylaminobenzaldehyde (absorbance read at 560 nm).

Pulmonary Function Tests

Lung function was assessed by whole body plethysmography using the Flexivent system (Scireq, Montreal, PQ, Canada) and Flexivent software version 5 was used. The Flexivent is calibrated for open- and closed-tube systems for each pulmonary test performed. Each mouse was anesthetized and weighed, and the weight was entered into the computer, resulting in parameters for the perturbations that were specific to that weight. The mice were allowed a brief period to acclimate to the ventilator. The maximum pressure was set at 30 cm H₂O for pressure/volume (PV) analysis and determination of total lung capacity (TLC). The positive end-expiratory pressure remained constant at approximately 2.5 cm H₂O. Five perturbations were performed on each mouse: snapshot-150, prime-8, prime-3, PV, and TLC.

Serum Collection

At the time of killing, blood was collected by cardiac puncture and placed immediately at 4°C; the serum was separated at 4°C and stored at -80°C.

Lung Protein Extracts

At the time of killing, post-exsanguination, the left lung was homogenized in 1 ml PBS containing protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN) and centrifuged at 3,000 rpm for 10 minutes. The supernatant was filtered through a 1.2- μ m syringe filter and stored at -80°C.

Chemokine/Cytokine Level Determination

Lung protein extract and serum levels of CCL2 (MCP-1/JE), CXCL1 (KC), CXCL2 (MIP-2), IFN- γ , tumor necrosis factor (TNF)- α , vascular endothelial growth factor (VEGF), IL-10, IL-5, IL-6, IL-13, IL-12p70, and IL-17 were determined by multiplex assay using the Luminex system (Austin, TX) and mouse-specific bead sets (R&D Systems, Minneapolis, MN; sensitivity, 1.5–3 pg/ml). PDGF-AB, CXCL10 (IP-10), and transforming growth factor (TGF)- β 1 were determined by ELISA (sensitivity, 2, 2.2, and 7 pg/ml, respectively; R&D Systems).

Frozen Tissue Preparation

A mixture of 0.5 ml Optimal Cutting Temperature compound (Miles, Inc., Elkhart, IN) and PBS (3:1) was infused via the trachea into lungs. Lung and conventional GVHD target organ tissues (liver, colon, skin, and spleen) were embedded in Optimal Cutting Temperature compound, frozen in liquid nitrogen, and stored at -80°C. Cryosections (6 μ m) were acetone-fixed (5 min at room temperature) and stained by hematoxylin and eosin for pathology and Masson's trichrome stain for detection of collagen deposition (Sigma, St. Louis, MO). Histopathologic features of GVHD were assessed as described (15).

Immunohistochemistry

Cryosections (6 μ m) were fixed in acetone and immunoperoxidase-stained using biotinylated mAbs as described (16) with avidin-biotin blocking reagents, avidin-biotin complex (ABC) peroxidase conjugate, and diaminobenzidine (DAB) chromogen purchased from Vector Laboratories (Burlingame, CA). The biotinylated mAbs used were specific for the following markers: I-A^k, I-A^b, CD4, CD8, CD11b, CD19, and Gr-1, all purchased from BD Pharmingen (San Diego, CA). Ki67 and vimentin antibody were purchased from Abcam (Cambridge, MA) and used with biotinylated donkey-anti-rabbit secondary Ab (Jackson ImmunoResearch, West Grove, PA). Anti-CC10 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA) and used with biotinylated donkey-anti-goat secondary Ab from Jackson ImmunoResearch. Anti-CK5 Ab was purchased from Covance Research (Denver, PA) and used with biotinylated donkey-anti-rabbit secondary Ab (Jackson ImmunoResearch). Biotinylated antibody for CK18 was from Progen Biotechnic (Heidelberg, Germany). Stained lung sections were counterstained with methyl green and examined under $\times 200$ and $\times 400$ magnification in coded fashion and photographed using an RT-Spot camera mounted on an Olympus BX51 microscope (Olympus, Hamburg, Germany). Lung tissue from de-identified lung transplant patients with BO was embedded in paraffin, and sections deparaffinized in xylene/alcohol series before epitope retrieval with antigen-unmasking solution (Vector Laboratories) in a water bath at 98°C. After washing, they were blocked with 2% normal rabbit serum/1% bovine serum albumin/0.1% Triton-X 100/0.05% Tween 20/PBS for 30 minutes, and subsequently stained with anti-CC10 antibody (Santa Cruz Biotechnology), as described for the mouse tissues above, and counterstained with hematoxylin.

In Situ Hybridization

Cryosections (5 μ m) were hybridized with digoxigenin-labeled antisense RNA probe corresponding to nucleotides 239–775 for granzyme B as described previously (17). Immunologic detection of digoxigenin-labeled RNA duplexes was accomplished with anti-digoxigenin antibody (alkaline-phosphatase conjugated; Boehringer Mannheim). After color development, sections were mounted in Crystalmount (Biomedica Corp., Foster City, CA).

Statistical Analysis

Data were analyzed by analysis of variance or Student *t* test. *P* values less than or equal to 0.05 were considered statistically significant.

RESULTS

BO after Murine BMT

C57BL/6 mice were conditioned with cyclophosphamide and total body irradiation before transplantation with B10.BR BM

Figure 1. Co-infusion of low-dose splenocytes with bone marrow transplant (BMT) results in lung disease with manifestations of bronchiolitis obliterans (BO). (A–C) Hematoxylin-and-eosin stains of 6- μ m lung cryosections of a representative C57BL/6 mouse that received allogeneic bone marrow (BM) only (A) and of C57BL/6 mice given allogeneic BM and 1 million splenocytes (B and C). At 60 days post-BMT, the lungs of mice receiving allogeneic BM and low-dose splenocytes exhibited extensive parenchymal inflammation as well as signs of BO (black arrows). Mice that received T-cell-depleted BM only did not exhibit lung injury. (A and B, original magnification $\times 100$; C, original magnification $\times 40$). (D–F) Masson's trichrome stains illustrate collagen deposition, shown in blue, surrounding a bronchiole in a representative C57BL/6 mouse that received allogeneic BM only (D) and in C57BL/6 mice given allogeneic BM and 1 million splenocytes (E and F) at 60 days post-BMT and showing increasing degrees of occlusion. E shows subepithelial collagen

deposition encroaching into the airway (white arrows), whereas F (depicting a serial section of an area shown in C) shows a completely occluded airway with central deposits of collagen (outlined in black). G shows another serial section of the same area shown in C, stained for vimentin, illustrating the colocalization of fibroblasts in areas of collagen deposition (D and E, original magnification $\times 1,000$ under oil immersion; F and G, original magnification $\times 400$). (H–J) Lung tissue sections from patients with bronchiolitis obliterans (OB) show illustrative examples of airways with varying degrees of occlusion similar to our murine model. I and J are from the same patient (original magnification $\times 200$).

with or without 1×10^6 splenocytes. All mice were engrafted by Day 60 post-BMT with engraftment levels greater than 90% of donor type as determined by flow cytometry for H2^k (donor) and H2^d (host) (data not shown). Histologic examination of the lungs procured on Day 60 post-BMT showed perivascular and peribronchiolar cuffing as well as signs of BO (Figures 1B and 1C, arrows) in recipients of allogeneic BM and low-dose splenocytes

that contain allogeneic T cells that cause lung injury (9). Masson's trichrome stains (Figures 1D–1F) showed that bronchioles undergoing occlusion in mice receiving allogeneic BM and low-dose splenocytes had more intense collagen deposition (blue stain) in the subepithelium, which extended through the epithelial barrier, and ultimately composed part of the lumen of completely occluded airways consistent with fibroobliterative

Figure 2. Mild to moderate graft-versus-host disease (GVHD) in classical target organs of mice with obliterative bronchiolitis, after allogeneic bone marrow transplant, given low-dose splenocytes. Hematoxylin-and-eosin stains of 6- μ m cryosections of GVHD target organs from a representative C57BL/6 mouse that received allogeneic bone marrow and 1 million splenocytes. Classical GVHD target organs taken 60 days post-transplant are indicated. Black arrows indicate mild epidermal hyperplasia. r = red pulp; w = white pulp. (Colon, liver, and spleen: original magnification $\times 100$; skin: original magnification $\times 200$).

TABLE 1. FREQUENCY OF BRONCHIOLITIS OBLITERANS AT 60 DAYS POST-TRANSPLANT IN MURINE RECIPIENTS GIVEN ALLOGENEIC BONE MARROW AND 1–2 MILLION SPLENCYTES

Donor to Recipient (n)*	Mice with BO (%) / BO Airways (%)†	Mice with Bronchiolitis (%) / Airways with Bronchiolitis (%)‡
B10.BR to C57BL/6 (39)	15.5/8.5	85.5/44
C57BL/6 to B10.BR (16)	50.0/4.0	75/25
C57BL/6 to FVB/N (36)	25.6/1.9	50/23
C57BL/6 to BALB/C (10)	40.0/7.7	100/36.7

Definition of abbreviation: BO = bronchiolitis obliterans.

* n = total number of recipient mice pooled from two to four experiments per strain combination.

† Denotes percentage of airways that are occluded in the mice with BO.

‡ Denotes percentage of airways with intense peribronchiolar cuffing (4- to 5-cell thickness) in the mice with such airways (i.e., mice with bronchiolitis).

bronchiolitis. Immunohistochemical staining for the fibroblast marker vimentin showed that fibroblasts were colocalized with collagen-positive occluded airways (Figure 1G). Figures 1H–1J show illustrative examples of airways with varying degrees of occlusion from de-identified patients with BO similar to our murine model. Figure 2 shows that the GVHD target organs (colon, liver, skin, and spleen) exhibited mild to moderate pathology in contrast to the severe lesions that are typically seen in murine recipients of high-dose allogeneic T cells that have early-onset lung injury (see Reference 18 for examples).

To determine how universal this murine BMT model was, we evaluated the development of BO using four different mouse strain combinations as outlined in Table 1. BO was seen in all fully MHC-mismatched strain combinations occurring at a frequency of 15 to 50% of recipient mice. Therefore, the pathology seen is not mouse strain specific. The frequency of occluded airways in affected mice ranged from 2 to 8% as determined semiquantitatively (not morphometrically) from microscopic examination of hematoxylin-and-eosin-stained sections taken midway through the larger left lung lobe. The occurrence of bronchiolitis, defined as intense peribronchiolar cuffing, was seen in much higher frequency, ranging from 50 to 100% of mice and 23 to 44% of airways affected. These frequencies correlated proportionally

with the frequencies of obliterated airways, implying that peribronchiolar cuffing is a prelude to airway occlusion.

In Mice That Develop BO after BMT, Cytotoxic T Lymphocytes Migrate to the Peribronchiolar Area before the Massive Cuffing by Inflammatory Cells in a Proliferative Phase

We have previously shown that the presence of the cells expressing the inducible cytolytic granzyme B is associated with increased frequency of injured alveolar type II cells and subsequent development of lung injury in our established acute model (17). To determine whether this association was also true in the current BO model, we stained cryosections of lungs taken on Day 14 post-transplant by *in situ* hybridization for granzyme B. In Figure 3, it can be seen that numerous granzyme B-positive cells (Figure 3B, arrows) surround a bronchiole of a representative mouse from a group given allogeneic BM and 1 million splenocytes (the BO group), whereas such cells were not seen in a representative mouse given allogeneic BM only (the non-BO group). The bronchiolar epithelium also demonstrated weak expression of granzymes consistent with a recent report of patients with chronic obstructive pulmonary disease and fibrosis (19). In a cohort of mice studies at 30 days after transplant, staining with the proliferative marker Ki67 antibody demonstrated the presence of numerous proliferating cells in the peribronchiolar area of a representative mouse given allogeneic BM and 1 million splenocytes (Figure 3D), whereas such cells were rarely seen in a mouse given allogeneic BM only (Figure 3C). Lungs were examined by immunohistochemistry to determine the nature of the inflammatory cells that had accumulated at sites of obliterated airways at 60 days post-transplant. Figure 4 shows that airways undergoing occlusion were associated with peribronchiolar accumulations of CD4⁺ T cells, few CD8⁺ T cells, donor MHC class II cells (CD11b⁺ macrophages, not shown), and small numbers of CD19⁺ B cells and neutrophils (Gr-1⁺, not shown). The accumulation of neutrophils was higher in areas where airways had been obliterated (not shown here, but seen morphologically in Figure 1F). Interestingly, the presence of donor MHC class II (IA^k) cells in the inflammatory cell infiltrate (and shed into the extracellular matrix) was much more abundant than recipient MHC class II (IA^b). This is the opposite of our findings in the early-onset lung injury model (9). It is also clear from Figure 4

Figure 3. In mice that develop bronchiolitis obliterans (BO) after bone marrow (BM) transplant, cytotoxic lymphocytes migrate to the peribronchiolar area before the massive cuffing by inflammatory cells in the proliferative phase. Cryosections of lungs taken on Day 14 post-transplant were stained by *in situ* hybridization for the inducible cytolytic protein granzyme B. A shows lack of staining in a representative mouse given allogeneic BM only, whereas B shows numerous granzyme B-positive cells (arrows) surrounding a bronchiole of a representative mouse from a group given allogeneic BM and 1 million splenocytes (referred to as BMS). Staining with Ki67 antibody illustrates proliferating cells in the peribronchiolar area of a representative mouse taken 30 days after transplant of allogeneic BM and 1 million splenocytes (D), whereas such cells are rarely seen in a mouse given allogeneic BM only (C). E is the secondary antibody control of a serial section of the lung shown in D.

Figure 4. Inflammatory cell phenotype in an airway with early-stage bronchiolitis obliterans (BO) after allogeneic bone marrow (BM) transplant with low-dose splenocytes. Cryosections (6 μ m) of lung from a representative C57BL/6 mouse taken at Day 60 post-transplant of allogeneic BM and 1 million splenocytes were immunoperoxidase stained for the indicated markers. Positive cells are *brown*. Cells positive for I-A^b are of recipient origin and are predominantly bronchiolar epithelial cells. The peribronchiolar area stains positively for I-A^k (donor major histocompatibility complex [MHC]), which is present on macrophages and B cells (shown as CD19 positive). Donor MHC also appears to have been shed and deposited into the extracellular matrix. Arrows indicate examples of the few CD8 T cells that are in the epithelial layer and the subepithelium, whereas CD4 cells are the predominant T cells in the formation of the BO lesion. BM control sections show few donor MHC class II cells that are predominantly macrophages of donor origin (not shown) and very little staining for host MHC class II on bronchiolar epithelium. (Methyl green counterstain, original magnification $\times 400$; BM control, original magnification $\times 200$). a = airway; H&E = hematoxylin and eosin.

that the peribronchiolar cells are predominantly of donor type and have begun to invade the bronchiolar epithelium, which is expressing host MHC class II (IA^b). In contrast, bronchiolar epithelium of BM control sections did not exhibit intense host MHC class II staining (Figure 4, *bottom panels*). The above results, taken in context, are consistent with the characteristics of fibroproliferative disease subsequent to an injurious insult.

Changes in Epithelial Marker Expression in Bronchioles Surrounded by Inflammatory Cells Indicate a Differentiation-patterned Response to Injury

Because Clara cells that express CC10 or CCSP are found in terminal bronchioles and are considered to be the precursors to the epithelial cells of the distal airways, we wanted to determine whether these cells were affected in the airways of mice that are developing BO after BMT. Figure 5 shows that, in mice that de-

Figure 5. Changes in epithelial marker expression in bronchioles surrounded by inflammatory cells. Cryosections (6 μ m) of lungs from a representative non-bone marrow (BM) transplant control C57BL/6 mouse, a C57BL/6 mouse that received allogeneic BM only, and a C57BL/6 mouse given allogeneic BM and 1 million splenocytes (BM + spleen). Lungs were taken at Day 60 post-transplant and were immunoperoxidase stained for the indicated epithelial markers. Positive cells are *brown*. Staining for CC10 is seen in the airways of control mice and mice given allogeneic BM only but not in periinflamed airways of mice given allo-BM and splenocytes. Airway epithelial staining for CK5 increases with inflammation and CK18 appears to stain a subset of airway epithelial cells in areas that are CK5 negative (original magnification $\times 200$, methyl green counterstain).

velop BO, epithelial cells of airways with intense peribroncholar cuffing lacked CC10-positive cells 60 days post-transplant. In contrast, staining for CC10 is seen in the airways of mice that received the allogeneic BM only. This indicates that the pre-transplant conditioning regimen alone does not lead to lack of CC10 expression in the BO group (i.e., absence of CC10 is associated with the presence of inflammatory cells). To further characterize the effects on epithelial cells in the mice that develop BO, staining for cytokeratins associated with differentiation responses was also evaluated. CK5 is a basal cytokeratin that is associated with mesothelium and squamous differentiation, whereas CK18 is a luminal cytokeratin and is expressed by simple epithelium. Staining for these cytokeratins in the lungs of mice developing BO (depicted in Figure 5, as indicated) showed that bronchiolar airway epithelial cell staining for CK5 increases with inflammation and CK18 appears to stain a subset of airway epithelial cells in areas that are CK5 negative.

Lung Dysfunction in Allogeneic Recipients of Low-Dose T Cells with BO

Chemoradiotherapy-conditioned C57BL/6 recipients of allogeneic B10.BR BM and low-dose splenocytes demonstrated elevated airway resistance on Day 60 post-BMT (Figure 6A). This would be consistent with the findings of BO and airway obstruction. Table 2 shows that such mice also had elevated levels of OH-proline, a measure of collagen accumulation consistent with fibrosis. However, the lungs of mice with BO had increased wet/dry weight ratios indicative of pulmonary edema (Table 1). The increase in pulmonary edema may account for the decrease in TLC (Figure 6B) as well as the decrease in pulmonary compliance (Figures 6C and 6D) in this model. Increased pulmonary edema, decreased compliance and decreased TLC are parameters that we have previously established to be correlated with the severity of lung injury in mice with IPS (9).

Differences in Inflammatory Mediator Expression in the Lungs of Mice That Develop BO Compared with Non-BO mice Are Seen Early Post-BMT

Because we and others have shown that many cytokines and chemokines are induced in the lungs and systemically in acute,

early-onset lung injury BMT murine models, we evaluated whether there were similar increases in such mediators in the lungs and systemic circulation of mice with post-BMT BO. This was done in a kinetic fashion (Days 7, 14, 30, and 60 post-BMT), and via side-by-side direct comparison of sera and lung extracts of mice given allogeneic BM alone or allogeneic BM with either high-dose splenic T cells (which causes mice to die early with acute GVHD and IPS) or low-dose splenic T cells (which causes mice to develop BO late post-BMT). Because the mice receiving high-dose T cells die before Day 14 post-BMT, levels of inflammatory mediators for this cohort could only be obtained for the Day 7 time point. In the analyses for a substantial, albeit not exhaustive, list of mediators (listed in METHODS), we found significant differences between the two T-cell dose groups for only five mediators (CXCL10, CXCL8, CCL2, IL-5, IL-6). Figure 7A shows that these five mediators were expressed at higher levels at the early Day 7 time point in the lungs of mice given the lower dose of T cells that go on to develop BO (compared with the mice given high-dose T cells). Similar results were found in the sera of these same mice (Figure 7B). The only mediator that we found to increase with time, especially in the group of mice that developed BO post-BMT, was CXCL8 (KC in the mouse), and this was seen only in the lung extracts (Figure 7A). The other four mediators declined within the first 30 days post-BMT in this group and did not differ markedly from the mice receiving allogeneic BM alone. The levels of CXCL10 and IL-6 in the lungs of all surviving mice returned to baseline (B6 control level) at later post-BMT time points, whereas the levels of CCL2 and IL-5, although decreased, still remained elevated above baseline in the lungs of the majority of surviving mice, including those receiving BM alone. In contrast, IL-5, IL-6, and CCL2 levels in the systemic circulation returned to baseline levels in the mice receiving BM alone or in mice receiving BM with low-dose T cells (i.e., those that develop BO) as early as Day 14 post-BMT (Figure 7B). The serum levels of CXCL10 and CXCL8 did not differ between these same two groups of mice at the later post-BMT time points, although the above-baseline elevated levels were maintained long-term post-BMT. Therefore, although the types of mediators produced in the cohorts receiving low-dose versus high-dose T cells did not differ, the time course and level of expression varied with higher levels of expression of these mediators early post-BMT in mice that later developed BO.

Figure 6. Mice with bronchiolitis obliterans (BO) (black bars) exhibit elevated airway resistance and decreased total lung capacity and compliance post–bone marrow (BM) transplant (Day 60). (A) Resistance; (B) total lung capacity; (C) specific compliance; (D) dynamic compliance. Mean values ± SE are indicated for eight mice per group pooled from two experiments. *P* values are versus C57BL/6 control mouse.

DISCUSSION

In this article, we present a new mouse model for the study of BO caused by low-dose allogeneic T-cell infusion in a BMT setting. Lowering the allogeneic T-cell dose in lethally conditioned mice given allogeneic BM results in histology consistent with BO, with many characteristics distinct from the early-onset, acute form that results from high T-cell doses. These characteristics are compared and contrasted in Table 3.

Classically, BO is believed to manifest as airflow obstruction with air trapping; however, evidence demonstrates this to be

a heterogeneous disease that can manifest with either obstructive or restrictive physiology (20). The cause of this heterogeneity is unknown, and in humans, no correlation has been found between the degree of subepithelial collagen deposition and airflow obstruction (20, 21). In our murine BMT model, the histologic lesion is bronchiolar with some alveolar involvement, yet the physiology is restrictive, demonstrated by an increase in resistance and decreased compliance. A murine model of BO is complicated by an increased percentage of airways with a concomitant fewer number of alveolar units per airway as compared with humans. Subsequently, fewer alveolar units would be subjected to air trapping from small airway occlusion and the murine physiology may not mirror the classical airflow obstruction seen in humans. In our initial description of early-onset, acute mouse IPS post-BMT (13), we demonstrated that lung dysfunction presents as reduced specific compliance, decreased TLC, and increased wet/dry lung weight ratios. An increase in lung water, as demonstrated by an increase in lung wet/dry ratios, was also seen in this BO model and may contribute to the restrictive physiology.

Typically, rodent models of BO are developed using either heterotopic (22) or orthotopic (23) tracheal transplants. The pathology of the airway rejection in these models is similar to that of BO involving T cells and macrophages (24, 25). However, heterotopic tracheal transplants develop luminal fibrosis, whereas orthotopic tracheal transplants develop subepithelial fibrosis. Orthotopically anastomosed tracheae are contiguous

TABLE 2. MICE WITH BRONCHIOLITIS OBLITERANS EXHIBITED ELEVATED OH-PROLINE LEVELS AND INCREASED WET/DRY LUNG WEIGHT RATIOS, CONSISTENT WITH FIBROSIS AND PULMONARY EDEMA (DAY 60 POST-BONE MARROW TRANSPLANT)

Group	OH-Proline ((μg)/Dry Lung (mg))	Wet/Dry Lung Weight Ratio
C57BL/6 control	5.1 ± 0.4	4.6 ± 0.3
Allogeneic BM only (no spleen)	5.2 ± 0.3	4.2 ± 0.4
Allogeneic BM + spleen	6.0 ± 0.3*	5.1 ± 0.4*

Definition of abbreviation: OH-proline = hydroxyproline.
Mean values ± SD are indicated for eight mice per group pooled from two experiments.
* *P* < 0.05 versus C57BL/6 controls and bone marrow transplant group.

Figure 7. Differential inflammatory mediator expression in the lungs of mice that develop bronchiolitis obliterans occurs early post-bone marrow transplant (BMT). Cytokine and chemokine levels were determined by Luminex methodology on lung protein extracts (A) and corresponding sera (B) at the post-BMT time points indicated. Data shown are mean $\pm$ SD for six mice/group pooled from two experiments.

with recipient airways and dendritic cell and lymphocyte trafficking appears to mimic that of the host tissue (26). The presence of both obstructive disease and subepithelial fibrosis in our current model demonstrates the potential usefulness of this murine model for the study of BO in a transplant setting (albeit, in a reverse scenario in which the lung is host-derived but the immune cells are donor-derived). The presence of an intact, vascularized whole lung in the presence of an allogeneic immune system in a BMT model is a good approximation of the lung transplant setting. The leading hypothesis is that airway epithelium is the primary target of allograft rejection (27). Epithelial cells and antigens are shed immediately after tracheal transplant (28) and dendritic cells carry these antigens to the draining lymph nodes where antigen-specific T cells become stimulated (29). Similarly, we have found that the pre-BMT conditioning regimen used in our mouse model causes epithelial cell injury and that this is most severe in the presence of allogeneic T cells (9, 17). In lung transplant models, there is much evidence that the early damage is initiated by the ischemia-reperfusion that causes oxidative stress (30). The conditioning

regimen used in our BMT model, that includes cyclophosphamide which depletes glutathione stores, also causes oxidative stress in the peri-BMT period (31). Therefore, the common denominators in comparing lung transplantation with our BMT model are as follows: (1) the initial insult of oxidative stress, leading to (2) pulmonary cell injury in the face of (3) foreign-tissue immune challenge.

Although a kinetic analysis of the progression to BO in the lungs of the mice shown in this study was not performed, a comparison can be made among bronchioles with varying degrees of occlusion within the same lung. We observed that nonoccluded bronchioles were surrounded by inflammatory cells similar in number and phenotype to those that surrounded partially occluded airways (shown in Figure 3) (i.e., $CD4^+$ T cells, $CD8^+$ T cells, with a large macrophage component and few numbers of B cells and neutrophils). In many ways, this resembles the early-onset murine IPS model, with the exception of the presence of B cells, in which there is peribronchiolar cuffing. However, BO was never observed in the early-onset, acute IPS model. It has also not been reported in other mouse models of IPS

Figure 7. (continued).

(32–34), including one model in which total body irradiation–only conditioning and low allogeneic T-cell doses led to a delayed onset of IPS (32). Another difference in our model and the aforementioned ones (32–34) is that our BO model is induced in a setting in which the donor is MHC class I and II disparate with the recipient. This may underscore the role of MHC in the pathogenesis of BO similar to clinical findings (1). It should be noted that our findings were reproducible in the reverse strain combination as well—that is, C57BL/6 mice as donors and B10.BR mice as recipients. We have also successfully replicated this model using BALB/c and FVB/N mice as recipients of B6 donor cells. Therefore, the pathology seen is not mouse strain specific and should lend itself well to the study of BO using various deletional mutant mice on a C57BL/6 genetic background as either donors or recipients.

Histologically, acute murine IPS was associated with injured alveolar type II cells and increased frequencies of cytotoxic T cells (9, 17). In our previous studies, bronchoalveolar lavage fluid and sera of mice with acute IPS contained elevated levels of inflammatory cytokines. A comparative kinetic analysis of the inflammatory mediators in early IPS injury versus late-onset

disease with BO showed that the systemic and lung-localized increase in cytokines that is induced in the peritransplant period (using this same strain combination) and that is associated with acute, early-onset IPS (9) has subsided by the time the mice developed BO in the current model. We found significant differences between the two T-cell–dose groups for five mediators (CXCL10, CXCL8, CCL2, IL-5, IL-6). Paradoxically, these mediators were expressed at higher levels at the early Day 7 post-BMT time point in mice given the lower dose of T cells and that go on to develop BO (compared with the mice given high-dose T cells). This is contrary to what one might expect because these mediators are usually considered as inflammatory and one would predict that they would be found at higher levels in the mice given high-dose T cells that die early post-BMT. CXCL8 (KC in the mouse) was the only mediator that we found to increase with time in the post-BMT lungs. CXCL8 may play a role in neutrophil recruitment and/or vascular remodeling in airway fibroblast (35). Consistent with what has been found in human lung transplant recipients with BO (36), we did not find increased levels of TNF- α or TGF- β in the lungs of mice with BO (data not shown). This does not mean that

TABLE 3. DIFFERENCES BETWEEN THE EARLY IDIOPATHIC PNEUMONIA SYNDROME AND LATER OCCURRING BRONCHIOLITIS OBLITERANS IN ALLOGENEIC MURINE BONE MARROW TRANSPLANT

Characteristic Findings	High-Dose T Cells with IPS*	Low-Dose T Cells with BO
Time of onset	1–2 wk	~2 mo
Lung compliance	Decreased	Decreased
Airway resistance	No change	Increased
Lung wet/dry ratio	No ratio change (both wet & dry are up)	Increased
Infiltrating cells	Donor T cells (CD4 and CD8) Host APCs No B cells Very few neutrophils	Donor T cells (CD4 and CD8) Donor APCs Donor B cells More neutrophils
Fibrosis	No	Yes
Bronchiolitis obliterans	No	Yes
GVHD in other target organs	Usually severe	Mild–moderate

Definition of abbreviations: APC = antigen-presenting cell; BO = bronchiolitis obliterans; GVHD = graft-versus-host disease; IPS = idiopathic pneumonia syndrome.

* Data from References 9 and 17.

inflammatory mediators play no role in BO, but rather, once established, BO may not be dependent on elevated levels of cytokines, at least not those that we were able to analyze. Furthermore, the temporal and spatial relationship of these cytokines may be important in the genesis of BO. Therefore, the roles of chemokines, lipid mediators, and their receptors that have been demonstrated to play roles in the pathogenesis of BO (24, 35, 37, 38), and the migration of circulating fibrocytes that mediate fibrosis (39) in tracheal transplant models, warrant investigation in the current mouse model.

The striking loss of the Clara cell protein in the epithelium supports epithelial cells being a target for injury in our model. In addition to the phenotypic change of the epithelium, this loss of Clara cell protein likely contributes to disease progression. Clara cell protein is an inhibitor of phospholipase A2 and is believed to have both antiinflammatory and antiproliferative properties (40). Therefore, the loss of this protein could contribute to further epithelial cell injury and cellular proliferation. In our model, proliferating cells are mostly seen in the peribronchiolar region, not in the epithelium. This increase in basal cell proliferation is consistent with the appearance of CK5 staining at the epithelial surface in the lungs with BO. CK5 is a cytokeratin associated with basal cells that lie beneath the epithelium (41). Few CK18-positive cells were seen in the airways and may represent the remaining epithelial cells attempting to repopulate the airways or could be indicative of epithelial–mesenchymal transitional changes (42, 43). Further injury results in the loss of Clara cell protein and proliferation of the basal cells that subsequently repopulate the epithelial layer with eventual obliteration of the airway by fibroblasts.

In conclusion, we present a new mouse model of BO with the hallmarks of obstructive airway disease and fibrosis that will be helpful for the study of late post-HSCT pulmonary complications and lung transplant rejection.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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