BLT1-mediated T cell trafficking is critical for rejection and obliterative bronchiolitis after lung transplantation

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Leukotriene B4 is a lipid mediator that recently has been shown to have potent chemotactic activity for effector T lymphocytes mediated through its receptor, BLT1. Here, we developed a novel murine model of acute lung rejection to demonstrate that BLT1 controls effector CD8+ T cell trafficking into the lung and that disruption of BLT1 signaling in CD8+ T cells reduces lung inflammation and mortality in the model. In addition, we used BLT1-deficient mice and a BLT1 antagonist in two tracheal transplant models of lung transplantation to demonstrate the importance of BLT1 for the recruitment of T cells into tracheal allografts. We also show that BLT1-mediated CD8+ T cell recruitment plays an important role in the development of airway fibroproliferation and obliteration. Finally, in human studies of lung transplant recipients, we found that BLT1 is up-regulated on T lymphocytes isolated from the airways of patients with obliterative bronchiolitis. These data demonstrate that BLT1-mediated CD8+ T lymphocyte recruitment plays an important role in the development of airway fibroproliferation and obliteration. Finally, in human studies of lung transplant recipients, we found that BLT1 is up-regulated on T lymphocytes isolated from the airways of patients with obliterative bronchiolitis. These data demonstrate that BLT1 contributes to the development of lung rejection and obliterative bronchiolitis by mediating effector T lymphocyte trafficking into the lung. This is the first report that describes a pathologic role for BLT1-mediated T lymphocyte recruitment in disease and identifies BLT1 as a potential therapeutic target after lung transplantation.

BLT1 is a G protein-coupled receptor (GPCR) that is activated by leukotriene B4 (LTB4), a lipid mediator derived from arachidonic acid that is potent in cardiovascular and respiratory systems. It is expressed on many cell types, including monocytes, granulocytes, eosinophils, mast cells, and T lymphocytes. The activation of BLT1 by LTB4 results in a series of signaling pathways that can lead to inflammation, hyper-responsiveness, and tissue injury. In this study, the authors investigated the role of BLT1 in the rejection of lung allografts after transplantation.

The authors developed a novel murine model of acute lung rejection to demonstrate that BLT1 controls effector CD8+ T cell trafficking into the lung and that disruption of BLT1 signaling in CD8+ T cells reduces lung inflammation and mortality in the model. They also used BLT1-deficient mice and a BLT1 antagonist in two tracheal transplant models of lung transplantation to demonstrate the importance of BLT1 for the recruitment of T cells into tracheal allografts. Additionally, they showed that BLT1-mediated CD8+ T cell recruitment plays an important role in the development of airway fibroproliferation and obliteration.

In human studies of lung transplant recipients, the authors found that BLT1 is up-regulated on T lymphocytes isolated from the airways of patients with obliterative bronchiolitis. These data demonstrate that BLT1 contributes to the development of lung rejection and obliterative bronchiolitis by mediating effector T lymphocyte trafficking into the lung. This is the first report that describes a pathologic role for BLT1-mediated T lymphocyte recruitment in disease and identifies BLT1 as a potential therapeutic target after lung transplantation.
Lung transplantation remains the only effective therapy for the large number of patients with end-stage lung disease (8, 9). However, despite advances in immunosuppressive therapies and surgical techniques, overall 5-yr survival remains only 50% (10). Mortality after lung transplantation results mainly from the development of chronic graft dysfunction from obliterative bronchiolitis (OB), which develops in >60% of lung transplant recipients (11–13). The histologic hallmark of OB is an inflammatory and fibroproliferative response in the small airways that can lead to significantly reduced lung function and death. Clinical studies have implicated acute rejection (AR) as the major causative factor for the development of OB (14–16). AR is orchestrated primarily by activated graft-specific T lymphocytes (17), which initiate a cascade of events that leads to an intense inflammatory reaction resulting in damage to the transplanted organ. It is thought that effector T lymphocytes specific for airway epithelial cells traffic into the airway and damage the airway epithelium; this damage then leads to an exuberant repair process and OB (18, 19). Thus, donor-specific T lymphocyte infiltration in AR is probably crucial for the subsequent development of OB (20–22). Support for this hypothesis has come from experiments that have demonstrated donor-specific T lymphocytes in bronchoalveolar lavage (BAL) fluid and lung tissue taken from transplant recipients with OB (20, 23). It follows that interventions that block lymphocyte recruitment into allografts should reduce the intensity of rejection and delay the development of OB.

In this study, we investigated the role of the LTB4/BLT1 pathway in effector T lymphocyte recruitment into the lung during rejection of the airways. We used BLT1-deficient mice (BLT1−/−) as well as mice treated with a specific small-molecule antagonist of BLT1 (24) in three murine models of airway rejection, including a novel murine model of antigen-specific T cell–mediated lung injury. These experiments demonstrated that disruption of BLT1 signaling not only reduced effector T cell trafficking into the lung but also diminished mortality and the pathologic changes induced by airway rejection. In addition, we demonstrated that BLT1 is up-regulated on human T lymphocytes infiltrating the grafts of lung transplant recipients with OB, which is the first demonstration of BLT1 expression on human lymphocytes. These data provide a definitive link between BLT1-mediated lymphocyte recruitment and the pathogenesis of an inflammatory disease. Furthermore, the findings suggest that BLT1 is a potential therapeutic target in lung transplant rejection and OB.

RESULTS

A novel murine model of effector CD8+ T cell–mediated lung rejection

To create a novel model of lung rejection that involved the entire intact lung, we generated transgenic mice that specifically expressed a membrane-bound exogenous antigen, chicken OVA, on airway-lining cells in the lung. Lung-specific injury then was induced by adoptive transfer of activated OVA-specific T cells. Lung-specific expression was achieved by ligating the OVA gene containing the epitope recognized by OT-I cells was ligated to the transmembrane (TM) domain of the human transferrin receptor and the CC10 promoter. (B) OVA RNA levels in the indicated organs as measured by QPCR analysis and expressed as copies per copy of GAPDH. WT, wild type. (C) Mortality of wild-type mice and CC10-OVA transgenic mice after adoptive transfer of 5 × 10^5 effector OT-I cells (n = 6 mice/group).
try of these cells demonstrated an effector phenotype: low CD62L expression, high CD25 expression, high perforin expression, and high IFNγ expression (Fig. S1 A, available at http://www.jem.org/cgi/content/full/jem.20042481/DC1, and unpublished data). Further analysis of these cells demonstrated BLT1 mRNA expression (Fig. S2 A, available at http://www.jem.org/cgi/content/full/jem.20042481/DC1) and efficient chemotaxis to LTB4 (Fig. S2 B). Chemotaxis to LTB4 was progressively inhibited by increasing doses of CP 105,696, a specific BLT1 antagonist (Fig. S2 B and reference 24). Adoptive transfer of 5 × 10⁶ effector OT-I cells into the CC10-OVA mouse led to death in 100% of mice by 6 d (Fig. 1 C). Injection of these cells into wild-type mice did not result in illness or mortality (Fig. 1 C). The OT-I mice...
were in a Thy1.1+ background allowing us to track the transferred cells in the CC10-OVA mouse, which is in a Thy1.2+ background. Analysis of BAL, lung tissue, spleen, and thoracic lymph nodes 3 and 5 d after transfer revealed accumulation of the OT-I cells in the BAL and lung and reduced numbers of the cells in the spleen and lymph node of CC10-OVA mice when compared with wild-type mice (Fig. 2 A).

At 5 d there was a 400-fold and threefold increase in the number of OT-I cells in the BAL and lung, respectively, of CC10-OVA mice compared with wild-type mice (Fig. 2 B). Histological analysis of the lung demonstrated perivascular and peribronchial inflammation 3 d after adoptive transfer (Fig. 2 C, iii and iv) that progressed to diffuse infiltration of the lung 5 d after adoptive transfer (Fig. 2 C, vii and viii). Adoptive transfer of OT-I effector cells into nontransgenic wild-type mice did not induce significant inflammation of the lung at 3, 5, and 14 d (Fig. 2 C, i, ii, v, and vi and data not depicted).

BLT1-deficient T cells induce reduced mortality and lung inflammation

To characterize further the precise role of BLT1 in lymphocyte recruitment in lung rejection, we crossed the OT-I strain with BLT1−/−/− mice to generate OT-I/BLT1−/−/− mice (3). CD8+ lymphocytes generated from these mice proliferated normally and had similar staining for activation markers, perforin, and IFNγ compared with OT-I/BLT1−/−/− cells (Fig. S1 B and not depicted). CC10-OVA mice that received OT-I/BLT1−/−/− effector cells had significantly reduced mortality (Fig. 3 A), longer median survival (7 d vs. 5 d, log-rank analysis, P < 0.0001), and had reduced inflammation of the lung as assessed by histological analysis (Fig. 3 B, ii and iv) compared with CC10-OVA mice that received OT-I/BLT1−/−/− cells (Fig. 3 B, i and iii).

Mice that received OT-I/BLT1−/− CD8+ effector cells had mild inflammation around the vasculature in the lung (black arrows) and almost no involvement of the airways (white arrows). The OT-I/BLT1−/− mice are in the same Thy1.2+ background as the CC10-OVA mice, so we used a
tetramer specific for the OT-I TCR to analyze recruitment of transferred OT-I/BLT1<sup>+/+</sup> cells or OT-I/BLT1<sup>−/−</sup> cells into the BAL of CC10-OVA mice 3 d after transfer. These experiments revealed a defect in recruitment of OT-I/BLT1<sup>−/−</sup> cells into the airway with a twofold reduction in the number of tetramer-positive cells in the BAL of mice that received OT-I/BLT1<sup>−/−</sup> cells compared with mice that received OT-I/BLT1<sup>+/+</sup> cells (Fig. 4 A).

In the surviving mice that received OT-I/BLT1<sup>−/−</sup> cells, the transferred cells were still present in the BAL 14 d after transfer; however, there was no histological evidence of acute inflammation (unpublished data).

In addition to trafficking defects, studies have suggested that LTB<sub>4</sub> can influence the cytotoxicity of T cells (29, 30). To assess this influence, we analyzed cytotoxicity using an in vitro caspase activation assay. These experiments revealed a small reduction (12%) in cytotoxicity with the OT-I/BLT1<sup>−/−</sup> cells compared with OT-I/BLT1<sup>+/+</sup> cells (Fig. 4 B). Similar data were obtained if OT-I cells were treated with the BLT1 inhibitor CP 105,696 (Fig. S2 C). This small difference in cytotoxicity did not seem adequate to explain the dramatic decrease in inflammation and mortality evident when OT-I/BLT1<sup>−/−</sup> cells were used in this model. We thus focused our subsequent experiments on the more significant defect we observed in the recruitment of OT-I/BLT1<sup>−/−</sup> effector T cells.

To evaluate further the ability of BLT1 to mediate effector T cell recruitment in this model, we performed a competitive homing assay by cotransferring Thy1.1 OT-I/BLT1<sup>+/+</sup> cells and Thy1.2 OT-I/BLT1<sup>−/−</sup> cells into CC10-OVA mice. In these experiments, an OT-I–specific tetramer was used to identify the transferred cells, and the Thy1 antigen was used to differentiate between transferred OT-I/BLT1<sup>−/−</sup> (Thy1.1) cells and OT-I/BLT1<sup>+/+</sup> (Thy1.2) cells in the same animals. This competitive homing assay provides a sensitive test of BLT1-mediated recruitment in the model.
Figure 6. Fibroproliferation and cellular recruitment in heterotopic tracheal transplants. (A) BLT1 RNA expression on murine CD3+/CD4+ and CD3+/CD8+ lymphocytes isolated from heterotopic tracheal transplants 1 or 2 wk after transplantation and compared with expression on naive CD3+/CD4+ and CD3+/CD8+ lymphocytes (cells pooled from 12 tracheas at each time point). (B) Hydroxyproline content in heterotopic tracheal transplants harvested 2 and 3 wk after transplantation (n = 4 sets of 2 tracheas in each group). (C) Lymphocyte recruitment into tracheas 7 d after transplant as assessed by flow cytometry (n = 4 sets of 2 tracheas in each group). (D) Activated lymphocyte recruitment (CD25+) into tracheas 7 d after transplant as assessed by flow cytometry (n = 4 sets of 2 tracheas in each group).
Analysis of BAL and lung tissue 4 d after cotransfer revealed a significant fourfold decrease in the recruitment of OT-I/BLT1−/− cells into the lung and BAL compared with OT-I/BLT1+/+ cells (Fig. 5, A and B).

Role of BLT1 on airway fibroproliferation and obliteration
The results in this novel model of antigen-specific T cell–induced lung injury suggests that BLT1-mediated lymphocyte recruitment is important for the development of acute lung rejection. However, because none of the mice that received wild-type effector OT–I cells survived past 6 d, we were unable to evaluate the effects of disruption of the LTβR/BLT1 pathway on the development of airway obliteration and fibroproliferation as seen in OB. To assess this important component of human lung transplant failure, we used two murine tracheal transplant models that mimic the changes of OB. In the first set of experiments, we used the heterotopic model of OB in which tracheas from two syngeneic or allogeneic donors are transplanted into the backs of wild-type or BLT1−/− mice. The tracheas can be removed at various time points for measurement of RNA expression, cellular recruitment, and fibroproliferation. In the second set of experiments, we used an orthotopic tracheal transplant model in which the allogeneic trachea is ligated to the native trachea of a wild-type or BLT1−/− mouse with two end-to-side anastomoses (31). After 2 wk, airway hyper-responsiveness can be measured, and the tracheas can then be removed for histopathology.

BLT1 expression on T lymphocytes isolated from tracheal transplants
The first analysis we performed was the expression of BLT1 on CD3+ T lymphocytes isolated from allogeneic heterotopic tracheal transplants into wild-type mice. For these experiments, tracheas from BALB/c mice were transplanted into C57BL/6 mice and harvested after 7 and 14 d. RNA was isolated from cell-sorted CD3+/CD4+ and CD3+/CD8+ lymphocytes from the transplanted tissue and then analyzed by real-time QPCR. BLT1 expression levels were compared with expression levels in naive CD4+ and CD8+ T cells isolated from the spleens of C57BL/6 mice. BLT1 expression was up-regulated 15-fold on CD8+ lymphocytes and ninefold on CD4+ lymphocytes isolated from tracheas 1 wk after transplantation. Two wk after transplantation, BLT1 expression was up-regulated 180-fold on CD8+ lymphocytes and 1.4-fold on CD4+ lymphocytes isolated from the transplanted tracheas (Fig. 6 A).

BLT1 deficiency in recipient animals reduced collagen deposition
Having demonstrated that BLT1 is highly induced on effector T cells recruited into tracheal allografts, we were interested in the role of BLT1 in the development of airway fibroproliferation. To assess this role, we performed heterotopic tracheal transplants into wild-type mice and BLT1−/− mice. We analyzed the collagen content in transplanted tracheas by measuring hydroxyproline levels in transplanted tracheas removed from recipients 2 and 3 wk after transplant. Allogeneic transplants into wild-type mice develop significantly more fibrosis than syngeneic transplants at 2 and 3 wk as evidenced by greater hydroxyproline content in the transplants (sixfold and twofold, respectively). Allogeneic transplants into BLT1−/− recipients had significantly less fibrosis than allogeneic transplants into wild-type mice with hydroxyproline values at 2 and 3 wk that were not statistically different from values in syngeneic transplants (Fig. 6 B).

BLT1 deficiency in recipient animals reduced recruitment of T lymphocytes
To determine if the difference in fibroproliferation seen in BLT1−/− recipient mice compared with wild-type recipient mice could be explained by differences in effector T cell trafficking, we used flow cytometry to analyze T lymphocyte recruitment into heterotopic tracheal transplants into wild-type and BLT1−/− mice after 2 and 7 d. 2 d after transplantation there was minimal recruitment of T lymphocytes and no difference between the groups (unpublished data). At 7 d, however, there was a dramatic increase in the recruitment of CD4+ and CD8+ lymphocytes into allogeneic transplants, with 15% of the CD4+ lymphocytes and 6% of the CD8+ lymphocytes also staining positive for the activation marker CD25. Transplants into BLT1−/− mice had a statistically significant twofold reduction in CD4+ and threefold reduction in CD8+ lymphocyte recruitment compared with transplants into wild-type mice (Fig. 6 C). Consistent with data in the BLT1−/− mice, there was a significant reduction in CD4+ and CD8+ lymphocyte recruitment into the allografts transplanted into wild-type mice treated with the BLT1–specific inhibitor CP 105,696 when compared with mice treated with vehicle alone (seven- and eightfold, respectively). Additionally, there was a significant fivefold and threefold reduction in activated CD4+ and CD8+ lymphocyte (defined by CD25+ staining) recruitment into allografts in BLT1−/− mice (Fig. 6 D). Similar reductions were seen with treatment of wild-type recipients with CP 105,696. Assessment of tissue macrophage and neutrophil numbers by myeloperoxidase levels and cell counts demonstrated only a 20% reduction in neutrophil recruitment 1 d after transplantation in BLT1−/− mice but no decrease at other time points (unpublished data).

BLT1 deficiency in recipient animals reduced obliteration of tracheal transplants
To assess further the role of BLT1 in airway obliteration, we performed orthotopic tracheal transplants into BLT1−/− mice or wild-type mice or, in some cases, wild-type mice treated with vehicle or CP 105,696. In this model, the transplanted trachea is ligated to the recipient animal’s native trachea with two end-to-side anastomoses, thus preserving the movement of air through the graft (31). This orthotopic model has several advantages over the standard heterotopic model, including the natural anatomic location of the graft, easier removal
for histologic analysis, and the ability to assess patency by an indirect measurement of respiratory distress (enhanced pause or Penh) (32). Allogeneic transplants into wild-type recipients developed prominent inflammation and fibroproliferation by 2 wk (Fig. 7 A, iii and iv), whereas syngeneic transplants seemed to be normal at 2 wks (Fig. 7 A, i and ii).

Allogeneic transplants into BLT1−/− recipients had minimal inflammation and fibroproliferation within the airway lumen and attenuated inflammation and fibrosis in the tissue surrounding the transplanted tracheas (Fig. 7 A, v and vi). Allogeneic transplants into wild-type mice treated with CP 105,696 (Fig. 7 A, ix and x) also had reduced fibroproliferation when compared with wild-type mice treated with vehicle (Fig. 7 A, vi and vii). The epithelial cell layer seemed to be normal in the syngeneic transplants (arrow in Fig. 7 A ii.), was completely absent in the allogeneic transplants (Fig. 7 A, iv and vii), and was present but seemed to be damaged in transplants into BLT1−/− mice or wild-type mice treated with CP 106,696 (arrow in Fig. 7 A, vi and x). These data demonstrate that BLT1 contributes to airway epithelial cell injury and the development of OB. To quantify better the degree of fibroproliferation, slides were evaluated by an investigator blinded to the genotype of each animal, and the degree of fibroproliferation was scored on a scale of 0 (no fibroproliferation) to 4 (complete obliteration). N = 4–6 animals/group. Airways hyper-responsiveness as measured by Penh 2 wk after orthotopic tracheal transplant (n = 8 mice/group; P = 0.038 by repeated measures ANOVA between allogeneic transplants into wild-type mice and allogeneic transplants into BLT1−/− mice).
methacholine similar to effects in mice with allergic airway inflammation (34). To test this obstruction of airflow and tracheal smooth muscle cell reactivity, we measured the Penh, a noninvasive measurement that uses airway pressure and respiratory time to quantify respiratory distress in response to methacholine administration (32). In this assay, the allogeneic transplants into wild-type mice developed a prominent increase in Penh consistent with increased airway resistance compared with syngeneic transplants (Fig. 7 C). This increase was abrogated completely when allogeneic transplantsations were performed into BLT1−/− recipient mice, suggesting reduced obliteration and inflammation of the transplanted tracheas (Fig. 7 C).

BLT1 expression on antigen-specific CD8+ lymphocytes contributes to airway obliteration

Experiments in the CC10-OVA mice demonstrated that BLT1 expression on CD8+ lymphocytes plays a central role in mediating antigen-specific acute lung injury. The tracheal transplant experiments demonstrated that BLT1 expression in the recipient contributes to airway obliteration. Analysis of cellular recruitment in this model of OB showed defects in CD4+ T cell, CD8+ T cell, and neutrophil recruitment into the allografts. Based on our findings in our model of acute rejection, we hypothesized that effector CD8+ lymphocytes were the major mediators of the lung injury that stimulates airway fibroproliferation. Thus, to link the results in these models better and to determine if airway obliteration can be directly mediated by BLT1-activated CD8+ T cells, we transplanted CC10-OVA tracheas into MHC-matched C57BL/6 recipient mice that had received effector OT-I cells. Because CC10-OVA tracheas express the OVA transgene (unpublished data), transfer of effector OT-I cells should induce rejection. Orthotopic tracheal transplants of CC10-OVA tracheas into C57BL/6 mice demonstrated minimal fibroproliferation (Fig. 8 A i).

If we injected 2 × 10⁶ activated OT-I/BLT1+/+ effector cells into the recipients 24 h before the transplantation, the tracheas developed moderate fibroproliferation after 2 wk (Fig. 8 A ii). However, if we injected 2 × 10⁶ activated OT-I/BLT1−/− effector cells into the recipients 24 h before transplantation, only minimal fibroproliferation was seen (Fig. 8 A iii). Histology slides were evaluated by an investigator blinded to the origin of the tissue, and the degree of fibroproliferation was scored on a scale of 0 to 4. Tracheas transplanted into recipients that received OT-I/BLT1+/+ cells had significantly higher fibroproliferation scores than tracheas transplanted into recipients that received OT-I/BLT1−/− cells (Fig. 8 B).

To quantitate further any differences in fibroproliferation, we analyzed hydroxyproline content in heterotopic tracheal transplants performed with CC10-OVA tracheas into MHC-matched C57BL/6 recipients that had received 2 × 10⁶ activated OT-I/BLT1+/+ effector cells or 2 × 10⁶ activated OT-I/BLT1−/− effector cells 24 h before transplantation. Tracheas were harvested 2 wk after transplantation. Tracheas transplanted into recipients that had received OT-I/BLT1+/+ effector cells had a significant threefold increase in hydroxyproline content compared with tracheas harvested from recipients that had received OT-I/BLT1−/− effector cells (Fig. 8 C).

Figure 8. Fibroproliferation in transplanted tracheas after adoptive transfer of OT-I/BLT1+/+ or OT-I/BLT1−/− cells. (A) Representative histology from orthotopic tracheal transplants of CC10-OVA mouse tracheas into C57BL/6 recipients after adoptive transfer of no cells (i), 2 × 10⁶ OT-I/BLT1+/+ cells (ii), or 2 × 10⁶ OT-I/BLT1−/− cells (iii) harvested 2 wk after transplantation. (B) Scoring of fibroproliferation in the lumens of the donor tracheas after orthotopic transplantation. The scoring was based on a scale of 0 (no fibroproliferation) to 4 (complete obliteration). N = 3 animals/group. (C) Hydroxyproline content in heterotopic tracheal transplants of CC10-OVA mouse tracheas into C57BL/6 recipients after adoptive transfer of 2 × 10⁶ OT-I/BLT1+/+ cells or 2 × 10⁶ OT-I/BLT1−/− cells harvested 2 wk after transplantation. N = 3 sets of two tracheas in each group.
To determine if effector T cells that mediate human lung rejection express BLT1, we analyzed BLT1 expression on T lymphocytes isolated from BAL fluid taken from surveillance bronchoscopies of 40 lung transplant recipients using an antibody to BLT1 and flow cytometry. These patients had undergone transplantation at least 45 d earlier and were not acutely ill. Eighteen patients were classified as clinically normal, 13 were diagnosed as having OB, and the rest were diagnosed as having either AR (n = 4) or infection (n = 2) (excluded from analysis). The diagnosis of OB was based on specific criteria published by the International Society of Heart and Lung Transplantation that uses changes in pulmonary function tests compared with baseline values (35, 36).

Characteristics of the patients in the two groups were not different except for lung function, which was significantly worse in the patients with OB (Table S1, available at http://www.jem.org/cgi/content/full/jem.20042481/DC1).

In addition, there were no significant differences between patients with and without OB in the time since transplantation, the amount of fluid recovered, the number of cells recovered, or total lymphocyte number recovered. Cells isolated from the BAL were stained with antibodies to CD3, CD4, CD8, and BLT1, and the percentage of CD4+ and CD8+ lymphocytes expressing BLT1 was determined by gating on either CD3+/CD4+ or CD3+/CD8+ lymphocytes. BLT1 was expressed on a significantly greater percentage of CD4+ and CD8+ lymphocytes isolated from patients with OB compared with normal patients (CD4+: 19 ± 5.5% vs. 6 ± 1.5%; CD8+: 26 ± 7.8% vs. 4.5 ± 1.6%; Fig. 9, A and B).

**DISCUSSION**

Previous research into the role of LTB4 and its receptor BLT1 in organ transplantation has been limited. In an animal model of kidney transplantation, LTB4 was shown to be up-regulated during acute allograft rejection (5), and inhibition of 5-lipoxygenase significantly reduced rejection of kidney allografts and prolonged graft function. In a heart transplant model and a liver transplant model, blockade of BLT1 with a chemical antagonist prolonged graft survival and reduced mononuclear cell recruitment (6, 7). However, these studies did not provide a clear mechanism for these findings and did not specifically examine the role of LTB4-mediated T lymphocyte recruitment in rejection. Based on our recent data demonstrating that BLT1 contributes to impaired T cell trafficking to the airways in a mouse model of asthma, we hypothesized that impaired T lymphocyte recruitment from inhibition of LTB4 activity may have been the major mechanism behind the prolonged allograft function seen in these studies.

In the experiments reported here, we have found that BLT1 is important for the development of CD8+ T cell–dependent airway rejection in a novel transgenic model of acute lung rejection. In this model, OVA-specific effector T cells transferred into CC10-OVA transgenic mice induced airway injury and inflammation. Similar approaches have been used to model heart and pancreatic islet rejection (28, 37). Using this model, we found that CC10-OVA transgenic mice that received BLT1−/− CD8+ T cells had a reduction in the number of T cells recruited into the airway, reduced airway inflammation, and a significant reduction in mortality compared with CC10-OVA mice that received wild-type OT-I effector T lymphocytes. OT-I/BLT1−/− cells had similar activation and cytokine production com-
pared with wild-type OT-I cells; however, there was a small defect in an in vitro assay of cytotoxicity consistent with findings in previous studies (29, 30). Although reduced cytotoxicity may contribute to the phenotype in the model, we believe that the primary mechanism for the reduced mortality was the defect in lymphocyte recruitment. These experiments demonstrate that BLT1-mediated CD8+ lymphocyte recruitment into the airways is important for the development of significant pulmonary inflammation and mortality in this model and suggest that BLT1 may play a role in the development of acute lung rejection.

One of the important advantages of our new transgenic model of acute lung rejection was that it allowed us to isolate the mechanism of rejection to effector antigen-specific CD8+ T cells and thus explore the role of BLT1 for the recruitment of the key cellular mediator of lung rejection. Although CD4+ T cells are clearly important for allore cognition and the development of the rejection response, CD8+ lymphocytes are recruited in greater numbers than CD4+ lymphocytes in acute lung rejection (38) and are considered critical cellular mediators of graft injury (4). This new model has the additional benefits of involving the entire lung with intact vascular and lymphatic systems and allowing a physiologic assessment of graft failure in that the transfer of OT-I effector cells into CC10-OVA mice induced lung rejection and death within 6 d. This model differs from a previously described transgenic mouse model of asthma in which alveolar epithelial cells were engineered to secrete OVA into the lung (39). In that model, foreign antigen was not found on native lung cells, whereas in our new model OVA is membrane bound on the airway epithelium, resulting in CD8+ T cell-mediated injury to the airway lining, which closely mimics the pathophysiology of acute rejection. Our new model also has an important difference from a transgenic model of viral pneumonia that used alveolar epithelial cell expression of a viral antigen (influenza hemagglutinin) to induce a pneumonitis after adoptive transfer of virus-specific T cells (40). In our model, the foreign antigen is expressed predominantly in the airways rather than in the alveoli, providing a model of acute bronchiolitis (the primary lesion in acute lung rejection) rather than alveolitis (the primary lesion in viral pneumonitis).

In human lung transplantation, clinical studies have shown that acute rejection is the primary risk factor for the development of chronic allograft dysfunction from OB, which is the major cause of morbidity and mortality after transplantation. To explore the role of BLT1 in the development of OB, we used two murine models of airway obliteration. In these experiments we have shown that BLT1 is expressed selectively on graft-infiltrating T lymphocytes and that disruption of BLT1 signaling, either by genetic manipulation or by a specific chemical antagonist, leads to reduced airway fibrosis and obliteration (as assessed by histology, airway collagen content) and to airway hyper-reactivity (as assessed by the Penh). Although there has been criticism of the Penh in recent years (41), we believe the differences seen in our experiments probably relate to the differences in tracheal obliteration. The fact that syngeneic and BLT1−/− recipients are breathing through two tracheas and the allogeneic recipients have only one fully patent trachea should lead to differences in airway resistance, because the cross-sectional area of the airways at the level of the trachea will be double in the syngeneic and BLT1−/− hosts. In addition, airway inflammation leads to smooth muscle cell reactivity, so rejection may make the tracheas more responsive to methacholine and lead to more prominent narrowing compared with transplants with little inflammation.

The most prominent defect noted in these models in BLT1−/− mice or wild-type mice treated with the BLT1 inhibitor was a significant reduction in effector CD4+ and CD8+ lymphocyte recruitment into the airways. There was also a small difference in early neutrophil recruitment in the model. Neutrophils are recruited into tracheal transplants in response to surgical manipulation, ischemic injury, and during acute rejection and may contribute to airway obliteration. Although our data demonstrate that LTB4 contributes to neutrophil recruitment after transplantation, the defect in effector lymphocyte recruitment in BLT1−/− mice was more profound, suggesting that the effect on lymphocytes was the major cause for the reduced airway fibrosis. To confirm this possibility, we performed experiments using CC10-OVA mouse tracheal transplants into MHC-matched C57BL/6 recipient mice that had received OVA-specific BLT1+/+ or BLT1−/− CD8+ effector cells. Consistent with findings by others (42), we found that CD8+ T cell-mediated injury was sufficient to induce fibroproliferation in transplanted airways, although the fibroproliferation was less than that seen with a complete MHC-mismatch transplant. We also demonstrated that the transfer of BLT1−/− antigen-specific CD8+ effectors induced significantly less fibroproliferation compared with wild-type effector cells. These experiments clearly demonstrate that the reduced fibroproliferation seen in tracheal transplants into BLT1−/− recipients result at least in part from effects on CD8+ lymphocyte recruitment separate from effects on neutrophil recruitment.

We have correlated our findings in these mouse models of OB to the disease state in humans by demonstrating the expression of BLT1 on graft-infiltrating lymphocytes isolated from the lungs of patients after lung transplantation. When compared with patients with normal lungs after lung transplantation, the percentage of T cells expressing BLT1 was significantly higher in the lymphocytes isolated from patients with OB. These data suggest that the LTB4/BLT1 pathway may play a role in the recruitment of pathogenic T cells into the lungs during the development of OB.

In previous experiments, we found that BLT1 contributed to early T cell recruitment into the airways in a murine model of asthma (3); however, this defect did not affect late T cell recruitment, the overall development of airway inflammation, or airway hyper-reactivity (unpublished data). In or-
gan transplantation, however, early epithelial cell injury mediated by graft-specific T cells is a primary factor driving acute rejection and the development of chronic organ dysfunction such as OB (43), and thus defects in early lymphocyte recruitment may have a greater impact on the overall pathology. Therefore, our findings suggest that disruption of BLT1 expression on CD8+ T cells will reduce their recruitment into the airway and possibly reduce their cytotoxicity, leading to reduced T lymphocyte–mediated epithelial cell injury. This reduced epithelial cell injury then leads to reduced airway obliteration. The data in human lung transplant recipients correlate with the findings in the animal models and suggest that the LTB4/BLT1 pathway may be involved in recruiting T cells into the airways of humans with OB.

In summary, we have shown that the LTB4/BLT1 pathway provides an important signal for the recruitment of T lymphocytes into the airway. Our findings suggest that disruption of LTB4/BLT1 signaling will decrease the intensity of rejection and inhibit the formation of OB. It has been suggested that measures that reduce the intensity of AR could have profound effects on long-term allogeneic recognition, allowing the development of tolerance to allografts (44). Thus, inhibition of BLT1 may have a therapeutic role by limiting the injury associated with rejection, delaying the progression to OB, and potentially allowing the induction of tolerance to lung transplants.

MATERIALS AND METHODS

Mice. Wild-type BALB/c and C57BL/6 mice were purchased from Charles River Laboratories Inc. The OT-I TCR transgenic mouse strain in the C57BL/6-Thy1.1 background was provided by P. Shrikant (Roswell Park Cancer Institute, Buffalo, NY), and OT-I TCR mice in the C57BL/6-Thy1.2 background were obtained from Jackson ImmunoResearch Laboratories. BLT1-deficient mice (BLT1−/−) were generated in our laboratory and backcrossed into C57BL/6 for nine generations (45). These mice were then crossed with OT-I mice to generate OT-I/BLT1−/− mice. Mice were used at 8–12 wk of age. All protocols were approved by the Massachusetts General Hospital Subcommittee on Research and Animal Care.

Generation of CC10-OVA mice. The transferrin receptor–OVA construct in the pCDNA3.1V5-His6-TOPO plasmid (Invitrogen) was made as previously described (28). This construct was then ligated to the lung-specific promoter for CC10 (provided by J. Whitsett, University of Cincinnati, Cincinnati, OH) (Fig 1 A). The construct was successfully injected into pronuclei of C57BL/6 eggs, implanted into C57BL/6 pseudopregnant females, and a founder mouse line was then expanded.

BAL analysis from patients after lung transplantation. The protocol was reviewed and approved by the Massachusetts General Hospital Human Studies Committee. BAL samples were taken from patients undergoing surveillance bronchoscopy after lung transplantation. Patients were classified by clinicians blinded to the analysis as normal after transplantation, AR, OB, or infected. AR patients were diagnosed based on transbronchial biopsies, infected patients by a positive culture or serology in the proper clinical setting, and OB patients were diagnosed based on specific published clinical criteria (35, 36). Only patients with OB stage 1 or higher were classified as OB. The cells were isolated from the fluid and incubated with an unconjugated anti-human BLT1 mAb (Serotec Ltd.). The samples were then stained with a secondary mAb conjugated to PE. The cells were also stained with mAb to CD3, CD4, and CD8 (BD Biosciences). Staining was analyzed using a FACSCalibur analytical flow cytometer (Becton Dickinson) and Flowjo software (Tree Star, Inc.).

OT-I cell preparation and adoptive transfer. Isolation and preparation of OT-I and OT-I/BLT1−/− CD8+ effector T cells were performed as described previously (28). Cells were resuspended in PBS, and 5 × 10^3 cells were injected intraperitoneally.

Orthotopic and heterotopic tracheal transplants. Orthotopic transplants were performed as described (31). Briefly, donor mice received a lethal ketamine injection (100 mg/kg) followed by sterile removal of the trachea. Recipient mice were anesthetized using ketamine (80 mg/kg)/xylazine (12 mg/kg). The trachea was exposed, and openings were made in the distal trachea and the proximal trachea. The donor trachea was anastomosed to the openings using 10–0 polypropylene suture. For the heterotopic transplantations, two donor tracheas were implanted subcutaneously in the back of the recipient mouse as described (46). Some mice were given CP 105,696, a small-molecule antagonist of BLT1 (provided by H. Showell of Pfizer Inc.) (24). 10 mg/kg of CP 105,696 was administered by gavage suspended in a vehicle solution of 10% ethanol/0.45% methylcellulose/0.1% Tween 80. Control mice were given vehicle alone. In certain experiments, 2 × 10^6 effector OT-I or OT-I/BLT1−/− cells were injected intraperitoneally into recipient animals 24 h before transplantation. Mice were killed at various time points for analysis.

Airway responsiveness. Airway hyperresponsiveness was measured noninvasively using a whole-body plethysmograph (Buxco Electronics) as previously described (34).

Chemotaxis. Chemotaxis was used for OT-I and OT-I/BLT1−/− cells to give the indicated concentration.

OT-I cell cytotoxicity. Cytotoxicity was measured using the commercially available kit according to the manufacturer’s protocol (Cytotoxicity Assay Kit; OncoImmun, Inc.). Briefly, EL4 target cells were suspended in PBS at 2 × 10^4 cells/ml in the presence of a fluorescent target cell marker and then incubated in a 37°C for 1 h with or without OVA peptide (2 μg/ml). The target cell suspension was then cultured with effector cells at the various effector cell: target cell ratios. The cells were then incubated in capase substrate for 1 h. The cells then analyzed by flow cytometry. In other experiments, both the target cells and the T cell effectors were treated with increasing concentrations of the BLT1 inhibitor, CP 105,696 in a 2:1 effector cell:target cell ratio.

CC10-OVA mouse tissue sampling and processing. Animals were killed with a lethal injection of ketamine (100 mg/kg). The lungs were harvested and processed for 0.5 ml aliquots of PBS containing 0.6 mM EDTA. The spleen and thoracic lymph nodes were removed. The lungs were flushed free of blood by slowly injecting 10 ml of PBS into the right ventricle before excision for all studies.

Histopathologic examination. Tissue was placed into 10% buffered formalin. Multiple paraffin-embedded 5-μm sections were prepared and stained with hematoxylin and eosin. The slides were evaluated by light microscopy, and the amount of fibroproliferation was assessed using a 0-to-4 scale given by an investigator blinded to the genotype of the animals.

Tissue digests and isolation of single cell suspensions. Lung, trachea, or spleen was excised and minced into small pieces with a scissors. The pieces were digested for 45 min in RPMI with 0.28 Wunsch U/ml Liberase Blendzyme (Roche) and DNase 30 U/ml (Sigma-Aldrich) for 45 min at 37°C. The digested tissues were then extruded through a mesh strainer.

Flow cytometry and cell sorting. Cells recovered from culture, BAL, or suspensions of trachea, lung, lymph node, spleen, or lung were stained using a FACSCalibur analytical flow cytometer (Becton Dickinson) and Flowjo software (Tree Star, Inc.).
and analyzed as previously described (34). The tetramer specific for OT-I cells was obtained from Beckman Coulter. The anti-perforin antibody was from eBioscience. All other antibodies were from BD Biosciences. Single-cell suspensions of 12 combined tracheas were stained and sorted using a FACS Vantage cell sorter.

Real-time PCR. Total RNA was isolated and analyzed by real-time PCR using MX-4000 software (Stratagene) as previously described (48). Specific primers used for sequence detection of message for the BLT1 gene are 5'-GCTCAGTCTCCCTTTTCCCT-3' and 5'-GCGGCAAC-CCTCCTCTCCTA-3' and for the GAPDH gene are 5'-GGCAAT-TCAACGGGCAAGT-3' and 5'-AGATGGTGAATGGGCCTCCC-3'.

Fibroproliferation. Hydroxyproline was assayed as described (49).

Myeloperoxidase. Myeloperoxidase was assayed as described (50).

Online supplemental material. Fig. S1 shows the expression of CD25, CD62L, and IFNγ on BLT1+ and BLT1− OT-I effector CD8+ lymphocytes. Fig. S2 includes additional data quantifying expression of OT-I effecter CD8+ lymphocytes from eBioscience. All other antibodies were from BD Biosciences. Single-cell suspensions of 12 combined tracheas were stained and sorted using a FACSVantage cell sorter.

This work was supported by grants from the National Institutes of Health K08 BLT1 by effector OT-I cells, measuring chemotaxis of these cells to LTB4, and for the average lung function. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20042481/DC1.

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