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Simultaneous LFA-1 and CD40 Ligand Antagonism Prevents Airway Remodeling in Orthotopic Airway Transplantation: Implications for the Role of Respiratory Epithelium as a Modulator of Fibrosis

Tomohiro Murakawa,* Michelle M. Kerklo,† Martin R. Zamora,‡ Yi Wei,§ Ronald G. Gill,‡ Peter M. Henson,¶ Frederick L. Grover,* and Mark R. Nicolls²†

Airway remodeling is a prominent feature of certain immune-mediated lung diseases such as asthma and chronic lung transplant rejection. Under conditions of airway inflammation, the respiratory epithelium may serve an important role in this remodeling process. Given the proposed role of respiratory epithelium in nonspecific injury models, we investigated the respiratory epithelium in an immune-specific orthotopic airway transplant model. MHC-mismatched tracheal transplants in mice were used to generate alloimmune-mediated airway lesions. Attenuation of this immune injury and alteration of antidonor reactivity were achieved by the administration of combined anti-LFA-1/anti-CD40L mAbs. By contrast, without immunotherapy, transplanted airways remodeled with a flattening of respiratory epithelium and significant subepithelial fibrosis. Unopposed alloimmune injury for 10 days was associated with subsequent epithelial transformation and subepithelial fibrosis that could not be reversed with immunotherapy. The relining of donor airways with recipient-derived epithelium was delayed with immunotherapy resulting in partially chimeric airways by 28 days. Partial epithelial cell chimerism was sufficient to prevent luminal fibrosis. However, epithelial chimerism was also associated with airway remodeling. Therefore, there appears to be an intimate relationship between the morphology and level of chimerism of the respiratory epithelium and the degree of airway remodeling following alloimmune injury. The Journal of Immunology, 2005, 174: 3869–3879.

Traditionally, the respiratory epithelium has been considered a simple barrier between the external environment and the lung. It is now generally accepted that the epithelium is far more complex and can play a critical role in controlling many airway functions beyond its barrier function (1). The respiratory epithelium has diverse secretory functions including the liberation of a variety of cytokines, chemokines, growth factors, lipid mediators, adhesion molecules and integrins (2). It is a rich source of arachidonic acid metabolites and inducible NO synthase, which regulate airway contraction, mucus secretion, neurotransmitter release, and inflammatory responses. The epithelial membrane is capable of rapidly responding to injury to preserve the integrity of the mucosal barrier (3). However, in certain conditions, such as asthma or chronic lung transplant rejection, persistent inflammation can result in remodeling of the airways (4, 5). Airway remodeling is variably characterized by smooth muscle cell proliferation and the development of fibrosis. Many experimental airway models use nonspecific injury with inhaled charcoal, bleomycin, LPS, or mechanical trauma to induce remodeling (6–9). Although the Ag-specific model of experimental asthma, such as with OVA sensitization, induces a more immune-specific airway injury (10), the respiratory epithelium has not been well characterized in this model. In summary, a variety of animal models has been used to injure airways by both specific and nonspecific mechanisms, but the role of the respiratory epithelium in airway remodeling has not been well elucidated in immune-specific injury.

Allogeneic airway transplantation is another useful tool for studying the respiratory epithelium under Ag-specific immune attack. The contribution of respiratory epithelium to airway remodeling has been evaluated in heterotopically (ectopic) transplanted tracheas. In this model, the presence of an intact respiratory epithelium is required to prevent fibrous obliteration of the airway (11), and epithelial Ags can serve as a major target for immune responses (12). The respiratory epithelium is likely important as a source of alloantigen, because immediately following transplantation, it is shed (13), and epithelial alloantigens are presumably presented by dendritic cells in draining lymph nodes to naive T cells (14). Remarkably, the epithelium in a transplanted airway is replaced within several hours to days in a manner similar to epithelium that has been nonspecifically (e.g., mechanically) damaged (3, 13). However, in the absence of protective immunotherapy, allogeneic transplants undergo immune injury, followed by epithelial reshedding and luminal fibrosis (13).

The heterotopic tracheal transplantation (HTT)³ model has been used in lung transplantation research because rejecting airways

³ Abbreviations used in this paper: HTT, heterotopic tracheal transplantation; OTT, orthotopic tracheal transplantation; LTS, long-term surviving.
develop pathology similar to bronchiolitis obliterans, the pathology of chronic rejection in clinical lung transplantation (15). Recently, the orthotopic tracheal transplantation (OTT) model has been developed by some groups in which the donor trachea is directly anastomosed to recipient trachea (16, 17). The OTT model is distinguished from HTTs in several ways. Most notably, OTTs do not develop luminal fibrosis, and because they are contiguous with graft recipient airways, are re-epithelialized with recipient-type respiratory epithelium within 4 wk following transplantation (18, 19). Additionally, dendritic cell clearance of shed epithelial cell Ags to draining lymph nodes for Ag presentation to T cells is reproduced in its native position in the neck and thorax. Transplanting airways into their normal anatomic sites may preserve the regular dendritic cell and lymphocyte trafficking pathways critical for the study of normal airway immunity (20). Finally, OTTs are exposed to ambient air, and so the unique respiratory interface between host and environment is maintained. Thus, with OTTs, the lymphatic sensitization pathways and environmental exposures mirror those of native airways.

We developed an OTT model that used potent immunotherapy to selectively block alloimmune responses. Simultaneously targeting LFA-1 and CD40L pathways with mAb therapy has been found by our group to be highly effective in experimental pancreatic islet transplantation (21) and was a useful tool for the current airway transplantation studies. This therapy resulted in attenuated airway immune injury and decreased antidonor reactivity. By contrast, in the absence of immunotherapy, transplanted airways underwent a change in architecture characterized mainly by flattened epithelium and subepithelial fibrosis. Additionally, epithelial chimerism was strongly associated with airway remodeling. Cumulatively, the current study demonstrates a strong relationship between the state of the respiratory epithelium and airway fibrosis.

Materials and Methods

Tracheal transplantation

All animal studies were approved by the University of Colorado Health Sciences Center Institutional Animal Care and Utilization Committee. C57BL/6 (B6, H-2b) or C57BL/6 RAG-1 knockout mice (B6-rag1−/−, H-2b) were transplanted with tracheas from B6, and MHC-mismatched BALB/c ByJ (BALB/c, H-2a), C3H/HeJ (C3H, H-2b) mice obtained from The Jackson Laboratory. Seven ring tracheal segments were removed from C57BL/6 mice that were matched for recipient age and male sex. Recipient mice were anesthetized with Avertin, and a short incision was made in the midline neck region. Division of the strap muscles allowed visualization of the entire laryngotracheal complex. After the recipient’s trachea was transected, the donor trachea was sewn in with 10-0 nylon visualization of the entire laryngotracheal complex. After the recipient’s trachea was reanastomosed in an orthotopic position, B6 mice underwent OTT and received anti-LFA-1/anti-CD40L therapy. At day 75, these mice underwent HTT with B6, BALB/c, or C3H tracheas but received no immunotherapy. These grafts and the original OTTs were excised for histology 28 days following the second transplant.

Microarray study

To determine how the transcriptome of immune-injured airways compared with normal tracheas and with airways from anti-LFA-1/anti-CD40L-treated mice, a gene array study was performed. Total RNA was extracted from tracheas in TRIzol reagent and purified using RNeasy (Qiagen). RNA quality was evaluated with a 2100 Bioanalyzer system (Agilent Technologies). Amplified cRNA labeled with cyanine-3 CTP and cyanine-5 CTP (PerkinElmer/NEN Life Sciences) was produced from 1-μg total RNA. Hybridizations were performed using the human whole-genome microarray (Agilent Technologies). Fluorescent linear amplified cRNAs used in biological comparisons were hybridized to the oligo microarrays. RNA from tracheas were labeled with cyanine-3 and compared with a common reference RNA labeled with cyanine-5. Raw data were extracted from scanned array images using Feature Extraction 5.1.1 software (Agilent Technologies). Two tracheal samples per group were used and mean RNA expression for transplantations and non-transplantations were determined. OTTs and LTSs were calculated after subtracting the average of negative control signals (background) and normalization by nonlinear Lowess normalization.

Re-transplantation studies

To determine whether the state of the respiratory epithelium following immune injury correlated with the ability to prevent airway remodeling, B6 mice underwent BALB/c OTT. These OTTs were excised from the original recipient at either day 7, 10, 14, or 28. Tracheas were assessed for these time points with respect to the state of the respiratory epithelium and degree of lymphocyte infiltration. Tracheas not used for histology were re-transplanted orthotopically into immunologically naive B6 mice that received immunotherapy with anti-LFA-1/anti-CD40L. These grafts were re-excised 28 days following re-transplantation for histologic examination.

To determine the role for respiratory epithelium as a target of alloimmune attack (i.e., day 47), the number of spleen cells and 28-day time period were selected based on prior data suggesting that this was an adequate cell number and time period to reverse immunologic ignorance if this mechanism was at play (24, 25). As a final step to detect immune tolerance, a second transplant was performed into the originally transplanted mice. For this study, B6 mice underwent OTT and received anti-LFA-1/anti-CD40L therapy. At day 75, these mice underwent HTT with B6, BALB/c, or C3H tracheas but received no immunotherapy. These grafts and the original OTTs were excised for histology 28 days following the second transplant.

Histology

Tracheas were sagittally divided, with one-half being formalin fixed (H&E; Trichrome) and one-half frozen for immunohistochemistry. Paraffin-embedded tracheal segments were initially fixed in cold 10% neutral buffered formalin solution, and 5-μm sections were cut and stained for H&E and...
Masson’s Trichrome. For immunohistochemistry studies, 5- to 8-μm frozen sections were used. Lymphocyte infiltrates were characterized using rat anti-mouse CD4 (L3T4; BD Pharmingen), and rat anti-mouse CD8α (Ly-2; BD Pharmingen) Abs. For class I MHC staining, mouse anti-mouse H-2K\(^b\) (B6 class I MHC; AF6-88.5; BD Pharmingen), and mouse anti-mouse H-2K\(^d\) (BALB/c class I MHC; SFI-1.1; BD Pharmingen) were used.

**Histology assessment**

An Olympus BX51 microscope with Image-Pro Plus Image Analysis Software (Media Cybernetics) was used for histological and morphometric analysis. All grading was performed by scorers (M. M. Kerklo and M. R. Zamora) blinded to the identity of the samples, and slides were presented in random order for examination. Scores were based on a pathology scale described in Table I. We noted that, with rejection, epithelial height diminished and that lamina propria height generally increased. Therefore, to validate the subjective scores with objective measures, the ratio of the height of the epithelium to the height of the lamina propria was calculated. Three measurements were taken per Masson Trichrome-stained specimen and averaged. This number was correlated to the subjective scores obtained for each specimen from the blinded scorers.

**Chimerism assessment**

To determine relative chimerism of the respiratory epithelium, pathologic specimens stained for H-2K\(^b\) and H-2K\(^d\) were given a score of 0–3 as follows: 0, no (+) epithelial cells; 1, <50% (+) epithelial cells; 2, >50% (+) cells; and 3, all (+) epithelial cells. Thus, for a given sample, the net sum of the H-2K\(^b\) score and the H-2K\(^d\) score was equal to 3. Only BALB/c tracheas from OTT transplants in B6 mice were considered. Therefore, if H-2K\(^b\) staining was relatively greater than the H-2K\(^d\) staining, these specimens were considered “high percent chimerics,” whereas if H-2K\(^b\) staining was relatively less than the H-2K\(^d\) staining, these specimens were considered “low percent chimerics.” H-2K\(^b\) scores were subtracted from H-2K\(^d\) scores. A negative result was interpreted as relatively low chimerism and a positive result as relatively high chimerism.

**Statistics**

Intergroup statistical analysis was performed using the nonparametric Kruskal-Wallis test with Dunn’s post test for multiple group comparisons and the Mann-Whitney U test for individual group comparisons. Linear correlation with correlation coefficient (r) and coefficient of determination (r\(^2\)) was used to compare pathological scores with the ratio of epithelial height to lamina propria height. To compare rejecting from non-rejecting allografts for immune tolerance studies and to compare chimeric groups, the Fisher’s exact test was used.

**Results**

**OTT and HTT**

An in-circuit OTT model was developed to ensure that air passage through the graft occurred. In this system, airway allograft recipients were observed over several months without respiratory compromise, even in the control groups that demonstrated histological evidence of rejection. In the current study, inspiratory stridor was not audible in transplanted mice in contrast to a previously published report (17). Because of the lack of physical signs of rejection, mice were sacrificed at predetermined time points, and histology was evaluated. Serial histological evaluation of rejecting orthotopic tracheas led to the development of a pathological scoring system (Table I) that is similar to pathological scoring systems developed previously for the heterotopic tracheal model (26).

**Table I. Pathologic scoring system for OTTs and HTTs**

<table>
<thead>
<tr>
<th>Grade</th>
<th>Mononuclear Cell Infiltration</th>
<th>Respiratory Epithelium</th>
<th>Subepithelial Fibrosis</th>
<th>Luminal Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>Pseudostratified</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>Pseudostratified</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>Flattened/pseudostratified</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>Flattened/denuded</td>
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<td>0</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>Denuded</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Attenuation of airway immune injury and remodeling with combined anti-LFA-1/-anti-CD40L treatment**

To establish that combined anti-LFA-1/-anti-CD40L is efficacious in preventing immune injury to airways, mAbs were administered to mice receiving allogeneic orthotopic airway transplants. Use of anti-LFA-1/-anti-CD40L therapy strongly attenuated immune injury to allogeneic airways (Fig. 1). The only difference between grafts in syngeneic groups and anti-LFA-1/-anti-CD40L-treated groups was that LTS grafts were noted to occasionally have a limited accumulation of mononuclear cells. To confirm that this infiltrate did not represent impending rejection, additional groups were evaluated at day 105. No progression of pathology was noted in anti-LFA-1/-anti-CD40L-treated mice (Fig. 1c). For this reason, graft “acceptance” was defined as a pathological score of 0 or 1, although it remained a possibility that grade 1 pathology for any given graft could be transitioning to a higher grade at the time pathology was evaluated. Therapy with anti-LFA-1/-anti-CD40L resulted in reduced subepithelial fibrosis (Fig. 1b) and lowered pathologic scores (c). We noted that, in syngeneic and anti-LFA-1/-anti-CD40L groups, there was a preservation of the ratio of the epithelial height to the height of the lamina propria probably because of the maintenance of a well-differentiated columnar epithelium and the absence of significant mononuclear infiltrates and subepithelial fibrosis (Fig. 2a). This finding allowed an objective validation of the subjective pathological scores determined by our blinded observers (Fig. 2b). Taken together, results demonstrated that anti-LFA-1/-anti-CD40L therapy was highly effective in attenuating airway immune injury.

To better understand how airways undergoing immune injury contrasted at the level of the transcriptome compared with mice receiving immunotherapy, a microarray study comparing OTTs 21 days following transplantation was performed. Table II illustrates the most up-regulated mRNA in injured airways and the impact of immunotherapy on this expression (relative to control untransplanted tracheas). There was a significant reduction in transcripts for proteins implicated in allograft rejection (Table II). Notably, the monocyte attractants chemokine ligands 9 and 10, both strongly implicated in airway inflammation and fibrosis (28–30), were profoundly impacted with a >15-fold reduction in mRNA with immunotherapy. Thus, dampening of CXC chemokines responses, which are important components of the innate immune response (31), is a prominent feature of immunotherapy that successfully prevents airway injury and remodeling.

**Mice treated with combined anti-LFA-1/-anti-CD40L therapy develop altered donor reactivity to their transplanted airways**

We next sought to determine whether combined anti-LFA-1/-anti-CD40L therapy resulted in immunologic tolerance. Immune tolerance is distinguished from graft acceptance in that it implies a systemic change in the recipient’s immune responsiveness toward the donor. First, spleen cells from mice bearing LTS allografts were used to reconstitute immunodeficient B6 RAG1\(^-/-\) mice, which lack B and T cells that had been transplanted with either B6 (syngeneic), BALB/c (donor-type), or C3H (third-party) orthotopic tracheas (Fig. 3a). In contrast to the prior pancreatic islet transplant study that used the same strain combinations and dosing of anti-LFA-1 and anti-CD40L (21), immune tolerance was not
demonstrated in this system. Rejection (i.e., grade 2 or higher pathology) rates were significantly higher for LTS reconstituted mice compared with control mice \( (p < 0.005) \). To demonstrate that anti-LFA-1/anti-CD40L therapy did not work by simply inducing immunological ignorance of the allograft, treated B6 mice bearing BALB/c OTTs were immunized with BALB/c spleen cells 4 wk

![Architectural changes that occur with airway remodeling correlate with pathological scores.](image)

**FIGURE 2.** Architectural changes that occur with airway remodeling correlate with pathological scores. *a.* The height of the respiratory epithelium and the height of the lamina propria were measured, and the ratio was calculated. In syngeneic and anti-LFA-1/anti-CD40L-treated mice, there was generally a preservation of epithelial height without profound thickening (by fibrosis or by mononuclear cells) of the lamina propria. The opposite was noted in control groups. *b.* The epithelium/lamina propria ratio permitted an objective validation of the subjective pathologic scores. OTT pathological scores \((n = 172)\), with some points overlapping on graph, were significantly and inversely related to the ratio values.
before being harvested at day 75. Allografts were not rejected with donor cell immunization \((n = 7; \text{pathological score} = 0.6 \pm 0.2)\), suggesting that immune ignorance did not account for airway allograft prolongation with anti-LFA-1/-anti-CD40L therapy.

Because it did not appear that therapy was working by inducing immunological ignorance and because a prior study (21) had suggested that anti-LFA-1/-anti-CD40L therapy did not rely on the deletion of alloreactive cells, we questioned whether the original graft recipient exhibited a change in donor reactivity. Therefore, mice bearing LTS transplants were subsequently transplanted with a second transplant in the heterotopic position (Fig. 3b). In contrast to the adoptive transfer model, immune tolerance was observed in this setting. In summary, anti-LFA-1/-anti-CD40L administration resulted in a systemic alteration in antidonor reactivity in the original transplant recipient that persisted after the cessation of therapy and was reflected in a preservation of normal airway architecture.

### Table II. Differential mRNA expression in OTTs 3 wk following transplantation compared with untransplanted tracheas

<table>
<thead>
<tr>
<th>Gene Description</th>
<th>Fold Change without Therapy</th>
<th>Fold Change with Anti-LFA-1/-Anti-CD40L Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genes Most Up-Regulated with Rejection (Reference)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemokine (C-X-C motif) ligand 9(^a) (29)</td>
<td>94</td>
<td>4.2</td>
</tr>
<tr>
<td>Serum amyloid A (^a) (56, 57)</td>
<td>77</td>
<td>7.6</td>
</tr>
<tr>
<td>Chemokine (C-X-C motif) ligand 10(^a) (58)</td>
<td>73</td>
<td>4.4</td>
</tr>
<tr>
<td>Cystatin F (leukocystatin)(^a) (59)</td>
<td>52</td>
<td>3.9</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor(^a) (60)</td>
<td>45</td>
<td>15</td>
</tr>
<tr>
<td>Granzyme B(^a) (61)</td>
<td>40</td>
<td>1.5</td>
</tr>
<tr>
<td>CD8 Ag (\beta)-chain(^a) (62)</td>
<td>38</td>
<td>2.7</td>
</tr>
<tr>
<td>Serine (or cysteine) protease inhibitor, clade A, member 3G</td>
<td>32</td>
<td>5.3</td>
</tr>
<tr>
<td>Chemokine (C-C motif) ligand 5 (RANTES)(^a) (63)</td>
<td>32</td>
<td>3.2</td>
</tr>
<tr>
<td>Chemokine (C-C motif) ligand 8(^a) (64)</td>
<td>31</td>
<td>5.7</td>
</tr>
<tr>
<td>CD8 Ag (\alpha)-chain(^a) (62)</td>
<td>30</td>
<td>2.7</td>
</tr>
<tr>
<td>CD48 Ag(^a) (65)</td>
<td>30</td>
<td>13</td>
</tr>
<tr>
<td>Chemokine (C-C motif) ligand 2(^a) (32)</td>
<td>21</td>
<td>7.1</td>
</tr>
</tbody>
</table>

\(^a\) Previously reported in clinical or experimental lung transplant rejection.

\(^b\) Previously reported in any organ rejection.

![FIGURE 3.](http://www.jimmunol.org/) Immune tolerance assays. a. Immune tolerance to BALB/c OTTs was not detected in the adoptive transfer model. b. Secondary (2\(^{nd}\)) transplants into anti-LFA-1/-anti-CD40L-treated mice bearing BALB/c tracheas were performed 75 days after the original transplant. Control 2\(^{nd}\) B6 syngeneic secondary transplants were accepted, and control 2\(^{nd}\) third party C3H tracheas were rejected. The majority (four of six) of BALB/c 2\(^{nd}\) HTTs were accepted in mice that had BALB/c OTTs and anti-LFA-1/-anti-CD40L treatment. All control (1\(^{st}\)) BALB/c HTTs from untreated B6 mice were rejected at 28 days (BALB/c HTT 2\(^{nd}\) vs BALB/c 1\(^{st}\) control: \(p < 0.002\)).
Having characterized the effectiveness of anti-LFA-1/anti-CD40L therapy, we next used this as rescue therapy following defined periods of alloimmune injury.

**The duration of alloimmune injury inversely correlates with the ability for immunotherapy to prevent airway remodeling that follows immune injury.**

In this study, it was clear that the progression from normal to remodeled airways in rejecting allografts began with mononuclear infiltration, followed by a morphological change in respiratory epithelium and the appearance of subepithelial fibrosis. We investigated whether a period of alloimmune injury could transpire and then be rescued with anti-LFA-1/anti-CD40L therapy. The rationale of this approach was that by re-transplanting injured airways into immunologically naive animals that received highly protective immunotherapy, a period of alloimmune injury could be identified that indicates when irreversible fibroproliferation begins. To this end, BALB/c OTTs were exposed to immune injury for 7, 10, 14, or 28 days (Fig. 4). These tracheas were excised and re-transplanted into immunologically naive mice treated with anti-LFA-1/anti-CD40L. After 28 days, re-transplanted OTTs were excised and examined histologically. Seven days of injury to OTTs were characterized by a well-differentiated epithelium and no subepithelial fibrosis. After re-transplantation, these injured airways did not develop progressive pathology. However, if 10 days or more of alloimmune injury occurred, airways became remodeled after re-transplantation. Of note, 7 and 10 days of alloimmune injury were histologically indistinguishable. Therefore, in this study, a critical period for the initiation of fibroproliferation was identified.

**Epithelial chimerism prevents the development of luminal fibrosis in HTTs but is strongly associated with flattening of respiratory epithelium and subepithelial fibrosis in OTTs.**

In this study, airway fibrosis was initiated following mononuclear cell infiltration driven by a response to alloantigens. Given a recent study (12) implicating the donor respiratory epithelium as a primary antigenic target of alloimmunity in experimental airway transplantation, we investigated whether replacing donor epithelium with recipient epithelium before transplantation could attenuate immune injury. To accomplish this, donor tracheas were allowed to re-epithelialize with recipient-type epithelium in the first transplant recipient. In contrast to a previously published study...
demonstrating that cyclosporine promotes respiratory epithelial chimerism in OTTs (18), anti-LFA-1/anti-CD40L therapy only resulted in partial chimerism at 28 days (Fig. 5). As previously reported (12), if no therapy was administered during the period of initial transplantation, donor epithelium was more efficiently replaced by recipient epithelium. In the heterotopic position, B6 epithelium was found to protect against luminal fibrosis in B6 recipients and to promote luminal fibrosis in BALB/c recipients (data not shown), confirming the importance of epithelial Ags in the luminal fibrotic response. Immunotherapy with anti-LFA-1/anti-CD40L resulted in only partial re-epithelialization by B6 cells. However, this partial chimerism was also sufficient to prevent luminal fibrosis (Fig. 6). In contrast, partially chimeric epithelium was not sufficient to prevent the development of subepithelial fibrosis when transplanted in either the orthotopic or heterotopic position in B6 recipients (data not shown).

Finally, OTT groups used in this study were analyzed for relative expression of donor vs recipient respiratory epithelial class I MHC (Table III). In contrast to untreated OTT allografts, therapy resulted in greatly delayed epithelial chimerism. In fact, significant chimerism was not observed even at 105 days posttransplantation in anti-LFA-1/anti-CD40L-treated animals, and donor-derived epithelium remained the most abundant. By contrast, in actively remodeling airways, epithelial chimerism was first observed 14 days following transplantation. This time period coincides with the first appearance of subepithelial fibrosis as well as the initial appearance of flattened respiratory epithelium. When the groups were considered collectively, there was a strong correlation between the morphology of the respiratory epithelium, the presence of subepithelial fibrosis, and the degree of chimerism. There were nine groups in Table III that had normal respiratory epithelium, and these groups were all scored as low chimerics, whereas the remaining six groups with flat epithelium were highly chimeric (p = 0.0002; Fisher’s exact test) (note that one group was excluded because of the presence of mixed normal/flat pathology). Furthermore, nine groups in Table III had no subepithelial fibrosis, and these were scored as low chimerics, whereas the remaining seven groups with subepithelial fibrosis were highly chimeric (p < 0.0001). In summary, the epithelium is an important antigenic target in the alloimmune response, and the migration of recipient-derived epithelium following a period of injury coincides with an altered epithelial phenotype and the development of fibrosis.

**Discussion**

This study examined the evolution of immune-mediated injury in functional airway transplantation. Following the characterization of anti-LFA-1/anti-CD40L attenuation of airway immune injury, we used this immunotherapy as a late intervention to arrest the development of airway remodeling. Furthermore, use of anti-LFA-1/anti-CD40L was helpful in facilitating a study on the effects of

**FIGURE 5.** Respiratory epithelial chimerism in orthotopic airway grafts. Airway respiratory epithelium is shed soon after transplantation. In OTT where donor airways are contiguous with host airways, the shed respiratory epithelium is replaced by contiguous donor respiratory epithelium (H-2Kb\(^{+}\); class I MHC). OTT is from a day 28 BALB/c (H-2Kd\(^{+}\); class I MHC) transplanted in a B6 (H-2Kb\(^{+}\)) recipient. With anti-LFA-1/anti-CD40L therapy, only partial epithelial chimerism is noted by day 28 compared with significant chimerism in untreated allografts.

**FIGURE 6.** Partial epithelial chimerism prevents luminal fibrosis. Chimeric airways composed of BALB/c tracheas partially lined with recipient (B6) respiratory epithelium were excised from mice receiving anti-LFA-1/anti-CD40L 28 days after transplantation. Partially chimeric airways were retransplanted in a heterotopic position in B6 recipients. Luminal fibrosis was prevented in HTTs when lined with recipient-type respiratory epithelium (p = 0.005 compared with nonchimeric day 28 BALB---B6 HTTs).
immunotherapy on epithelial chimerism. In this study, combined anti-LFA-1/anti-CD40L therapy was found to be effective in preventing alloimmune injury, and in altering antitumor immunoreactivity to transplanted airways. A gene array study of the most up-regulated transcripts in airway immune injury revealed that among the most affected genes were chemokine ligands 2, 9, and 10, which are important in pulmonary innate immunity and fibrotic responses (28–30, 32). These transcripts are greatly attenuated with immunotherapy, and decreased levels of these chemokines may, in part, explain the decreased influx of mononuclear phagocytes and lymphocytes and the absence of fibrosis with therapy. Additionally, decreased expression of plasminogen activator inhibitor-1 and chemokine ligand 5 (RANTES) may be associated with the prevention of subsequent subepithelial airway fibrosis (33, 34). Strieter and Keane (35) have recently suggested that, in pulmonary fibrosis, polarization toward cell-mediated immunity and Th1 cytokines are protective against the development of fibrosis, whereas humoral immunity and Th2 cytokines predispose toward fibrosis. Whether this mechanism is in effect in airway fibrosis induced by alloimmune injury is unclear as cell-mediated immune injury would be expected to significantly contribute to subsequent fibrotic responses.

Unlike results obtained from conventional adoptive transfer studies (e.g., Ref. 21), we could not demonstrate tolerance in the adoptive transfer model but rather did so in a secondary transplant into the same animal that had received a transplant followed by a limited period of immunotherapy. Several possibilities for an inability to demonstrate tolerance in this adoptive transfer system include the following: 1) immune tolerance may not reside in spleen cells in OTT; 2) homeostatic expansion of nontolerant memory T cells in immunodeficient mice may override tolerant T cells (36); and 3) because immunodeficient mice never received anti-LFA-1/anti-CD40L therapy, donor respiratory epithelial Ags were presented by APCs not conditioned for tolerance (37). Such conditioned APCs may explain the donor-specific decreased immunoreactivity unique to treated OTT-transplanted mice that were subsequently transplanted with HTTs. Cumulatively, results demonstrated that anti-LFA-1/anti-CD40L therapy was potent and durable in this model of airway transplantation. Subsequently, this therapy was used to rescue tracheal allografts in which alloimmune injury had already commenced.

Initial studies in this project demonstrated that orthotopic airway remodeling consisted of two prominent features: flattening of the respiratory epithelium and subepithelial fibrosis. When allogeneic airways were allowed to undergo immune injury before re-transplantation into naive second recipients receiving protective immunotherapy, it was determined that factors leading to flattened epithelium and subepithelial fibrosis appear between 7 and 10 days of injury. These factors are not arrested with a potent therapy that primarily targets lymphocytes. King et al. (38) similarly noted that heterotopic allografts re-transplanted into syngeneic recipients would not develop luminal fibrosis if only 7 days of injury occurred but would develop luminal fibrosis following 14 days of alloimmune attack. These results are consistent with the clinical observation that antilymphocyte therapy is not effective in established fibroproliferative lung disease. Possible explanations for why alloimmune injury becomes irreversible between 7 and 10 days include the following: 1) a new cell population migrates into the allograft in this time period (e.g., fibrocytes; Ref. 39); 2) continued lymphocytic attack against allogeneic subepithelium results in activation of myofibroblasts; and/or 3) alloimmune injury to respiratory epithelium results in stressed epithelial cells that are known to secrete profibrotic factors (40).

The role of the respiratory epithelium in the development of airway fibrosis has been extensively studied in other model systems. In heterotopic airway transplantation, the respiratory epithelium has been assigned two seemingly conflicting roles in both the prevention and generation of airway lumen fibrosis. Morris and colleagues (11) demonstrated that denuding the respiratory epithelium before grafting syngeneic tracheas resulted in luminal fibrosis. Thus, this airway lesion occurred even in syngeneic transplants if respiratory epithelium was removed before transplantation. But as Fernandez et al. (12) recently observed, the respiratory epithelium may also be an important antigenic target for immune responses that lead to luminal fibrosis pathology.

### Table III. Tracheal chimerism

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>H-2Kb Score</th>
<th>H-2Kd Score</th>
<th>Subepithelial Fibrosis</th>
<th>Respiratory Epithelium</th>
<th>H-2Kb-H-2Kd</th>
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<tbody>
<tr>
<td>Anti-LFA-1 + anti-CD40L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Day 28</td>
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<td>2</td>
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<td>Normal</td>
<td>−1</td>
</tr>
<tr>
<td>Day 56</td>
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<td>1</td>
<td>2</td>
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</tr>
<tr>
<td>Day 75</td>
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<td>1</td>
<td>2</td>
<td>No</td>
<td>Normal</td>
<td>−1</td>
</tr>
<tr>
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<td>2</td>
<td>No</td>
<td>Normal</td>
<td>−1</td>
</tr>
<tr>
<td>No Rx</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Day 7</td>
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<td>−3</td>
</tr>
<tr>
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<td>−3</td>
</tr>
<tr>
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<td>2</td>
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<td>Normal/flat</td>
<td>1</td>
</tr>
<tr>
<td>Day 28</td>
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<td>2</td>
<td>1</td>
<td>Yes</td>
<td>Flat</td>
<td>1</td>
</tr>
<tr>
<td>Day 75</td>
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<td>3</td>
<td>0</td>
<td>Yes</td>
<td>Flat</td>
<td>3</td>
</tr>
<tr>
<td>Day 105</td>
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<td>3</td>
<td>0</td>
<td>Yes</td>
<td>Flat</td>
<td>3</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd Transplant: B6 OTT</td>
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<td>2</td>
<td>1</td>
<td>Yes</td>
<td>Flat</td>
<td>1</td>
</tr>
<tr>
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<td>6</td>
<td>1</td>
<td>2</td>
<td>No</td>
<td>Normal</td>
<td>−1</td>
</tr>
<tr>
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<td>2</td>
<td>1</td>
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<td>1</td>
<td>2</td>
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<td>Normal</td>
<td>−1</td>
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<td>2</td>
<td>1</td>
<td>Yes</td>
<td>Flat</td>
<td>1</td>
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*a* H-2Kb-H-2Kd scores were <0; then chimerism was considered relatively low. A result >0 was considered relatively high chimerism.

*b* First transplant received anti-LFA-1/anti-CD40L treatment (Rx), was extracted and retransplanted into second animal. This transplant was extracted after 28 more days without therapy.

*c* First transplant received no Rx and was harvested on day 7 or on day 28 and retransplanted into second animal. The second animal received anti-LFA-1/anti-CD40L Rx, and graft was excised 28 days after transplantation.
Consequently, we sought to clarify whether the respiratory epithelium was also an important target for the immune response against orthotopic transplants. We repeated and confirmed results from the Washington University study (12) that show in the absence of therapy, efficient relining with recipient epithelium occurs and is sufficient to prevent luminal fibrosis when re-transplanted into recipient strain mice. Conversely, recipient-derived epithelium promotes luminal fibrosis when re-transplanted into syngeneic mice. In contrast to results reported with cyclosporine immunotherapy (18), anti-LFA-1/anti-CD40L immunotherapy resulted in delayed chimerism. Why cyclosporine would promote epithelial chimerism and anti-LFA-1/anti-CD40L would inhibit chimerism is not immediately clear. Cyclosporine may simply not be as effective as anti-LFA-1/anti-CD40L therapy in preventing epithelial injury. Additionally, a recent provocative report demonstrated that cyclosporine directly influenced respiratory epithelial cells to secrete factors that enhance fibroblast proliferation (41). Although it is not known what factors were liberated that promoted fibroblast proliferation in this study, cyclosporine inhibition of PGE_2 release could culminate in airway fibrosis (41–45). Therefore, it has been postulated that the influence of cyclosporine on epithelium could contribute to the development of fibrotic lung disease (obliterative bronchiolitis) after lung transplantation (41). In summary, in keeping with previous studies, recipient-derived respiratory epithelium efficiently replaced injured airways, but, in contrast to a prior study that used cyclosporine, immunotherapy with anti-LFA-1/anti-CD40L inhibited the level of chimerism in donor airways. The current study confirmed that even partial repopulation of tracheal allografts with recipient-derived epithelium conferred a protective effect against luminal fibrosis following heterotopic retransplantation (12). However, partial chimerism did not otherwise prevent airway remodeling. Partially chimeric airways developed flattened respiratory epithelium and subepithelial fibrosis in all re-transplanted allogeneic recipients. The mechanism by which partial chimerism prevents luminal fibrosis is not known. It is intriguing that, in human lung transplantation, partial respiratory epithelial chimerism is also observed but that this is associated with chronic injury (46). When the current study’s experimental groups were examined for chimerism, it became clear that, similar to human respiratory epithelium in injured lung allografts, there was a correlation between the extent of chimerism and airway remodeling. It is possible that the strong antigenicity of donor epithelium contributes to driving the alloimmune response and that migration of recipient epithelium replaces shed or injured donor epithelium. Flattening of columnar respiratory epithelium to cover an epithelial defect can result in activation of underlying myofibroblasts (47). Thus, epithelial stress and migration may lead to collagen deposition and airway remodeling.

Another pathologic feature of interest in the OTT model was that lymphocytic infiltration was not always a precursor to airway remodeling. In the current study, airway lymphocytes were noted in several mice receiving anti-LFA-1/anti-CD40L therapy. To confirm that this pathology did not represent early rejection, treated mice were followed out beyond 100 days, and the pathological scores remained constant with no further progression to dedifferentiated respiratory epithelium and subepithelial fibrosis. The current study also showed the significant lymphocytic infiltration that occurs by day 7 in an untreated OTT allograft will not lead to airway remodeling if immunotherapy is initiated at this time. When lymphocytes infiltrate a transplant, it is often unclear...
whether rejection or benign colonization is occurring. T lymphocytes have been well demonstrated to be precursory to tissue damage, and yet it is quite common to note an accumulation of lymphocytes without subsequent tissue damage. An example of lymphocyte accumulations not known to progress to transplant damage is the Quilty lesion in heart transplantation (48). Colonizing T cells in transplants have been shown to contain members with regulatory function that can prevent nontolerant lymphocytes from rejecting transplanted allografts (49). In summary, the current study demonstrated that lymphocytic infiltrates chronologically preceded subepithelial fibrosis, but lymphocytic infiltration, in and of itself, did not always result in subepithelial fibrosis. Besides lymphocytic infiltration and fibrotic responses, the other major pathologic feature of airway immune injury was a change in the phenotype of the respiratory epithelium from columnar to flattened. Sequentially, this pathologic change in morphology occurred after lymphocytic infiltration and was always associated with subepithelial fibrosis. We questioned whether the impact of immune injury on the respiratory epithelium would correlate with subsequent airway remodeling.

In this study, airways that were allowed to reject for 10 or more days without intervention, were incapable of re-epithelialization with normal epithelium despite re-transplantation into an immunologically naive (i.e., nonprimed) host that received powerful immunotherapy. This finding speaks to several points: 1) that subepithelial fibrosis in this model is nonreversible; 2) that normal respiratory epithelium does not replace flattened undifferentiated epithelium overlying subepithelial fibrosis; and 3) that the cell phenotype of overlying epithelium may therefore be influenced by the underlying lamina propria. Fig. 7 is an integrated model of how immune injury and remodeling in airway transplantation could occur. Dendritic cell trafficking is modeled after the OVA sensitization model of asthma in which dendritic cell clearance of permeable Ag with mediastinal lymph node presentation is considered to be of central importance in the primary immune response (14). In summary, preserving epithelial integrity in allotransplantation may be important for preventing early fibrosis. This can be achieved either by limiting early immune injury or, ideally, by inducing immune tolerance.

If lymphocytic infiltration damages or signals a change in morphology to overlying respiratory epithelium, then it is possible to link immune injury to fibrosis, because the communication between epithelial cells and underlying myofibroblasts is significant. Following the mechanical scraping of healthy epithelium, the transformed flattened epithelial cells migrate over the damaged area just as myofibroblasts are transiently activated (47). This activation is inhibited by blocking TGF-β and thrombospondin. Other profibrogenic factors that are liberated by epithelial cells following mechanical damage or chemical damage (polyarginine) include insulin-like growth factor-1, platelet-derived growth factor-BB, fibroblast growth factor-2, and endothelin-1 (40). Activated myofibroblasts result in up-regulation of procollagen type I and type III mRNAs and increased collagen deposition. In contrast to wound healing models in which self-limited subepithelial fibrosis and increased smooth muscle cells are followed by re-epithelialization with normal pseudostratified columnar cells within 5 days (3, 50), the specific airway damage inflicted by alloimmune attack may have less capacity for such resolution. It is intriguing that TGF-β may be critically important in the development of immune tolerance (reviewed in Ref. 51), and yet it is also implicated in maladaptive fibrosis development (52-55). If quelling inflammation and generating regulatory T cells require factors such as TGF-β that are also profibrotic, then there may be particular difficulty in achieving immune tolerance in organs prone to fibrotic responses such as the lung. The current study demonstrates that, once fibrosis reaches a critical stage, blockade of alloimmunity no longer has benefit to the transplant.

In conclusion, we propose that the state of the respiratory epithelium is directly related to the presence of subepithelial fibrosis. The respiratory epithelium serves as an important alloantigen in airway transplantation. The replacement of donor epithelium with recipient-derived epithelium was correlated with a flattened phenotype and subepithelial fibrosis. Finally, at a time point between 7 and 10 days of allograft injury, fibroproliferative events are initiated that cannot be reversed with potent immunotherapy. The resulting pathology consists of a flattened epithelium and subepithelial fibrosis despite ongoing treatment with anti-LFA-1/anti-CD40L therapy. Preserving the integrity of the respiratory epithelium while effectively regulating the immune response to epithelial Ags may be of key importance in minimizing airway damage in some Ag-specific immune-mediated respiratory diseases such as transplantation rejection, or allergen-mediated asthma.

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Disclosures
The authors have no financial conflict of interest.

References
33. Oh, C. K., B. Ariue, R. F. Alban, B. Shaw, and S. H. Cho. 2002. PAI-1 promotes...
31. Magor, B. G., and K. E. Magor. 2001. Evolution of effectors and receptors of...
25. Coulombe, M., and R. G. Gill. 1996. T lymphocyte indifference to extrathymic...
22. Dorsch, S., and R. Roser. 1977. Recirculating, suppressor T cells in transplan-
1. Coulombe, M., and R. G. Gill. 1996. T lymphocyte indifference to extrathymic...

The Journal of Immunology 3879

15. Coulombe, M., and R. G. Gill. 1996. T lymphocyte indifference to extrathymic...
13. Coulombe, M., and R. G. Gill. 1996. T lymphocyte indifference to extrathymic...
11. Coulombe, M., and R. G. Gill. 1994. T lymphocyte indifference to extrathymic...
10. Coulombe, M., and R. G. Gill. 1996. T lymphocyte indifference to extrathymic...
7. Coulombe, M., and R. G. Gill. 1996. T lymphocyte indifference to extrathymic...
6. Coulombe, M., and R. G. Gill. 1996. T lymphocyte indifference to extrathymic...
5. Coulombe, M., and R. G. Gill. 1996. T lymphocyte indifference to extrathymic...

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