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The Role of Intrapulmonary De Novo Lymphoid Tissue in Obliterative Bronchiolitis after Lung Transplantation

Masaaki Sato,* Shin Hirayama,* David M. Hwang,* Humberto Lara-Guerra,* Dirk Wagner,* Thomas K. Waddell,* Mingyao Liu,* and Shaf Keshavjee2*

Chronic rejection after lung transplantation is manifested as obliterative bronchiolitis (OB). The development of de novo lymphoid tissue (lymphoid neogenesis) may contribute to local immune responses in small airways. Compared with normal lungs, the lung tissue of 13 lung transplant recipients who developed OB demonstrated a significantly larger number of small, airway-associated, peripheral node addressin-positive (PNAd⁺) high endothelial venules (HEVs) unique to lymphoid tissue (p < 0.001). HEVs were most abundant in lesions of lymphocytic bronchiolitis and “active” OB infiltrated by lymphocytes compared with those of “inactive” OB. T cells in lymphocytic bronchiolitis and active OB were predominantly of the CD45RO⁺CCR7⁺ effector memory phenotype. Similar lymphoid tissue was also observed in the rat lung after intrapulmonary transplantation of allograft trachea (Brown Norway (BN) to Lewis), but not after isograft transplantation. Subsequent orthotopic transplantation of the recipient Lewis lung containing a BN trachea into an F1 (Lewis × BN) rat demonstrated stable homing of Lewis-derived T cells in the lung and their Ag-specific effector function against the secondary intrapulmonary BN trachea. In conclusion, we found de novo lymphoid tissue in the lung composed of effector memory T cells and HEVs but lacking delineated T cell and B cell zones. This de novo lymphoid tissue may play a critical role in chronic local immune responses after lung transplantation. The Journal of Immunology, 2009, 182: 7307–7316.

However, formation of TLO after lung transplantation has not been reported and is controversial. Chronic rejection after lung transplantation is manifested by obliterative bronchiolitis (OB), a chronic inflammatory and fibroproliferative condition in small airways, and its clinical correlate, bronchiolitis obliterans syndrome (BOS) (10). In the lung, TLO is considered to be represented by an inducible form of bronchus-associated lymphoid tissue (BALT), namely iBALT (11). Formation of iBALT has been reported in various chronic inflammatory lung diseases such as emphysema (12), pulmonary fibrosis (13), and chronic hypersensitive pneumonitis (14). In contrast, Hasegawa et al. demonstrated a negative result regarding the relationship between OB/BOS and BALT, which should theoretically include iBALT (15). Although the study using transbronchial biopsies was limited in its sensitivity to detect OB lesions and (i)BALT, it is possible that de novo lymphoid tissue after lung transplantation does not form the conventional structure of TLO with HEVs, T and B cell zones, and germinal center formation.

Interestingly, an evolving concept proposed by van Panhuys et al. indicates the existence of “effector lymphoid tissue” (ELT) that exerts effector function by collecting effector and effector memory T cells but does not necessarily take the conventional anatomical form of TLO or iBALT (16). Increasing evidence suggests that the lung might be an important reservoir of effector and effector memory T cells (17, 18). In a s.c. tracheal transplant model of OB, preferential localization of effector memory CD4⁺ and CD8⁺ T cells to the lung parenchyma and airways has been demonstrated (19).

Thus, we hypothesized that lymphoid neogenesis plays an important role in chronic rejection after lung transplantation. To address the hypothesis, we conducted careful histological and immunohistochemical analyses of the lungs that were explanted at the time of retransplantation due to OB/BOS after the initial lung transplantation. Furthermore, we have devised an animal model that combines a rat intrapulmonary tracheal transplant model of
Committee of the Toronto General Research Institute. Use of Experimental Animals formulated by the Canadian Council on An-orthotopic lung transplantation were sacrificed 28 days after the concurrent tracheal graft using the same technique as regular intrapulmonary tracheal transplantation. After reperfusion of the left lung graft, a secondary tracheal graft from a previously described (21). The recipient animals of orthotopic lung transplantation conducted as described above at day 0. After 7 or 28 days, the left lung of intrapulmonary tracheal transplantation (i.e., day 35 or 56 after transplantation in combination with concurrent second intrapulmonary transplantation (BN to Lewis) was conducted as described above.

Table I. Demographics of lung transplant recipients who were diagnosed with OB/BOSa

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age at 2nd Tx (yr)</th>
<th>Time from 1st Tx (min)</th>
<th>Primary Diagnosis</th>
<th>Examined Lung Area (cm²)</th>
<th>No. of Lymphocytic Bronchiolitis (area in cm²)</th>
<th>No. of Active OB (area in cm²)</th>
<th>No. of Inactive OB (area in cm²)</th>
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<tr>
<td>Male</td>
<td>20</td>
<td>10.4</td>
<td>CF</td>
<td>4.30</td>
<td>3 (2.0)</td>
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<tr>
<td>Female</td>
<td>36</td>
<td>10.7</td>
<td>CF</td>
<td>5.91</td>
<td>3 (2.0)</td>
<td>6 (1.0)</td>
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<tr>
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<td>20</td>
<td>21.5</td>
<td>CF</td>
<td>1.15</td>
<td>0 (0)</td>
<td>1.0 (0.9)</td>
<td>0 (0)</td>
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<tr>
<td>Male</td>
<td>36</td>
<td>34.2</td>
<td>BE</td>
<td>1.93</td>
<td>2 (0.4)</td>
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<td>7 (3.6)</td>
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<tr>
<td>Female</td>
<td>26</td>
<td>35.0</td>
<td>IPF</td>
<td>8.62</td>
<td>7 (4.0)</td>
<td>7 (0.8)</td>
<td>12 (1.4)</td>
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<td>43</td>
<td>40.6</td>
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<td>4 (0.3)</td>
<td>12 (0.8)</td>
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<td>60</td>
<td>52.2</td>
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<td>12.08</td>
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<td>0 (0)</td>
<td>23 (1.9)</td>
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<tr>
<td>Male</td>
<td>23</td>
<td>69.3</td>
<td>OB</td>
<td>3.85</td>
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<td>3.90</td>
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<td>OB</td>
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<td>7 (2.2)</td>
<td>9 (1.4)</td>
<td>11 (1.7)</td>
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<tr>
<td>Female</td>
<td>39</td>
<td>16.7</td>
<td>OB</td>
<td>3.32</td>
<td>0 (0)</td>
<td>1 (0.2)</td>
<td>7 (2.1)</td>
</tr>
<tr>
<td>Average ± SEM</td>
<td>38.0 ± 3.8</td>
<td>52.9 ± 10.4</td>
<td>6.59 ± 1.4</td>
<td>3.31 ± 0.8 (1.89 ± 0.5)</td>
<td>3.31 ± 0.5 (0.77 ± 0.1)</td>
<td>5.85 ± 1.9 (1.00 ± 0.3)</td>
<td></td>
</tr>
</tbody>
</table>

*TX, Transplantation; BE, bronchiectasis; CF, cystic fibrosis; COPD, chronic obstructive pulmonary disorder; IPF, idiopathic pulmonary fibrosis; OB, obliterative bronchiolitis (idiopathic or secondary to hematopoietic stem cell transplantation).

Ob (20) and orthotopic lung transplantation to examine the effector function of intrapulmonary de novo lymphoid tissue after transplantation.

Materials and Methods

Tissue samples of BOS lungs and normal control lungs

Human tissue samples of OB/BOS lungs were obtained from patients during their follow-up at Toronto General Hospital (Toronto, Canada). Twelve lung specimens were obtained from patients with established diagnoses of BOS at the time of retransplantation and one lung specimen was obtained at the time of open lung biopsy, which confirmed the diagnosis of OB. Fifteen control lung tissue samples were obtained from patients with early stage lung cancer without any evidence of previous or current pulmonary disorders (age, 57.85 ± 4.0 (mean ± SEM); seven males and eight females). The human study was approved by the Research Ethics Board of University Health Network, University of Toronto (Toronto, Canada).

Animal models

Male Brown Norway (BN; RT1A) and Lewis rats (RT1A) were purchased from Charles River Laboratories, Inc. Rats were bred from male BN and female Lewis rats at the Toronto General Research Institute (Toronto, Canada). Intrapulmonary tracheal transplantation was conducted as previously described (20). Animals were sacrificed at postoperative days 7, 28, 60, and 120 for the following analyses (n = 5 for each time point and group).

Intrapulmonary tracheal transplantation followed by orthotopic lung transplantation (Fig. 5A). Rat intrapulmonary tracheal transplantation was conducted as described above at day 0. After 7 or 28 days, the left lung of the intrapulmonary tracheal transplant recipient was used as a donor lung for orthotopic left lung transplantation using surgical techniques described previously (21). The recipient animals of orthotopic lung transplantation were sacrificed 28 days after lung transplantation (i.e., days 35 or 56 after the initial intrapulmonary tracheal transplantation; n = 4 for each group).

Intrapulmonary tracheal transplantation followed by orthotopic lung transplantation in combination with concurrent second intrapulmonary and s.c. tracheal transplantation (Fig. 8A). Rat intrapulmonary allograft tracheal transplantation (BN to Lewis) was conducted as described above. After 28 days, the left lung of the initial intrapulmonary tracheal transplant recipient was used as a donor lung for orthotopic left lung transplantation. After reperfusion of the left lung graft, a secondary tracheal graft from a BN rat was implanted in the left lung adjacent to the first intrapulmonary tracheal graft using the same technique as regular intrapulmonary tracheal transplantation. Another tracheal graft from a BN rat was implanted in the dorsal s.c. tissue through the use of thoracotomy. The recipient animals of orthotopic lung transplantation were sacrificed 28 days after the concurrent transplantation (i.e., 56 days after the initial intrapulmonary tracheal transplantation; n = 4).

All animals received care in compliance with the Guide to the Care and Use of Experimental Animals formulated by the Canadian Council on Animal Care. The experimental protocol was approved by the Animal Care Committee of the Toronto General Research Institute.

Histology and immunofluorescence labeling

Formalin-fixed, paraffin-embedded tissues cut into 5-μm-thick sections were used for standard H&E stain, elastic trichrome stain, and all human immunofluorescence labeling studies. CD3 (rabbit anti-human), CD20 (mouse anti-human), Ki-67 (mouse anti-human), and anti-rat RT1Au (OX27; Serotec) for frozen sections were used with the appropriate isotype-specific Ig as negative control. For rat specimens, the following primary Abs reported to react with rat tissue were used: rabbit anti-human CD3 (DakoCytometry) and mouse anti-human CD79a (Abcam) for formalin-fixed paraffin-embedded or frozen sections; and mouse anti-rat mucosal addressin cell adhesion molecule (MAdCAM)-1 (BD Biosciences), rabbit anti-human von Willebrand factor (Milipore), and anti-rat RT1A* (OX27; Serotec) for frozen sections. Immunofluorescence labeling was conducted as described previously (22).

Evaluation and quantification of airway lesions and lymphocyte aggregates

In the human study, serial sections were cut from two to five blocks obtained from different areas of a single lung specimen and stained for OB/BOS, H&E, elastic trichrome, and CD3/CD20. H&E and trichrome staining was used to identify bronchioles, whereas in individual bronchioles H&E and CD3/CD20 staining was used to evaluate the extent of inflammation and lymphocyte infiltration. Trichrome staining was used to evaluate lumenal obliteration. Bronchioles with lymphocyte infiltration and without lumenal obliteration were classified as lymphocytic bronchiolitis; bronchioles with obliteration were classified as OB, among those with lymphocyte infiltration in or around the bronchiole were classified as “active OB” and those without such infiltration were classified as “inactive OB.” Each bronchiole was also evaluated for HEVs using PNAd staining.

In the animal study, the size of lymphocyte aggregates was morphometrically classified using ImageJ, version 1.30 (W. Rasband, National Institute of Mental Health, Bethesda, MD). The number of CD3+ T cells infiltrating into the tracheal graft was counted in 10 randomly selected high power fields confined to the obliterated area.

To standardize the number of HEV+ bronchioles (human study) and the size of lymphocyte aggregates (animal study), the whole lung area was scanned using SNAPSCAN e50 (Agfa) and the size was calculated using ImageJ software.

Flow cytometric analysis

The following anti-rat Abs and appropriate IgG isotype controls were purchased from BD Pharmingen: FITC-labeled CD3 (G4.18), PE-Cy5-labeled CD4 (G045.1), Alexa Fluor 647-labeled CD6 (OX9), PE-labeled CD45RC (OX22), and PE-labeled RT1A* (OX27). Cell surface staining was conducted as described previously (22).

Statistics

Data are expressed as means ± SEM. When comparing two groups, data were analyzed with t tests. When comparing three groups, one-way ANOVA was followed by post hoc Tukey tests. All statistical analyses
were performed using JMP 5.0 (SAS Institute). Values of $p \leq 0.05$ were considered to be significant.

**Results**

**Effector memory T cells in small airways of BOS lungs**

We examined 13 human lungs from patients that developed BOS after lung transplantation and compared them with 15 normal lung controls. Patient demographics are shown in Table I. Histologically, all of the posttransplant lungs contained multiple OB lesions and 11 of them also contained airway inflammation without obliteration (lymphocytic bronchiolitis). Among 164 OB lesions examined, 65 (39.6%) were classified as “active” lesions while the others were classified as “inactive” based on H&E, elastic trichrome, and CD3/CD20 staining as described in Materials and Methods.

In immunofluorescence labeling for T cells and B cells, we observed a number of T cells and a relatively small number of B cells in lymphocytic bronchiolitis and active OB lesions, whereas inactive OB lesions and normal lungs contained only a small number of T cells and B cells (Fig. 1A). In lymphocytic bronchiolitis and active OB lesions, the memory T cell marker CD45RO was largely colocalized to CD3$^+$ T cells, demonstrating that T cells in active inflammatory lesions in BOS lungs are mostly memory T cells (Fig. 1B). Furthermore, CCR7, a chemokine receptor expressed by naive and central memory T cells but not by effector and effector memory T cells (23), was not localized to CD45RO$^+$ cells, demonstrating that memory T cells in BOS lungs are of an effector memory phenotype (Fig. 1C).

**Development of high endothelial venules in small airways after lung transplantation**

The aggregates of effector memory T cells in BOS lungs do not completely meet the anatomical criteria of secondary or tertiary lymphoid tissue because they do not include segregated T cell and B cell zones (Fig. 1A) or B cell follicles positive for CD21$^+$

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**FIGURE 1.** Effector memory T cells in airways after lung transplantation. A, H&E staining of a normal airway and lesions of lymphocytic bronchiolitis, active OB, and inactive OB (top panels) and the corresponding immunofluorescence labeling for T cell (CD3) and B cell (CD20) staining (bottom panels). B, Immunofluorescence labeling for CD3 and CD45RO, a marker of memory T cells. Pictures were taken from the same active OB lesions as in A. C, Immunofluorescence labeling for CD45RO and CCR7, a marker for naive and central memory T cells. Scale bar, 50 μm. NC, Negative control.
follicular dendritic cells (data not shown). Conversely, immunofluorescence labeling for Ki-67 (a cell proliferation marker) demonstrated a relatively small number of proliferating B cells as well as T cells in the lymphoid tissue (data not shown). Representative pictures of a normal bronchiole (top) and lesions of lymphocytic bronchiolitis (second from top), active OB with lymphocyte infiltration in the lumen (third from top) active OB with luminal fibrosis and peribronchiolar lymphocyte aggregates (fourth from top), and two inactive OB lesions (fifth and sixth from top, only one of which shows positive PNAd staining). B, Higher magnification of a PNAd⁺ HEV in an active OB lesion. Scale bar, 100 μm. NC, Normal control.

**FIGURE 2.** Development of HEVs in small airways after lung transplantation. A, H&E staining, trichrome staining, and double immunofluorescence labeling for CD3 and PNAd, a marker of high endothelial venules. Representative pictures of a normal bronchiole (top) and lesions of lymphocytic bronchiolitis (second from top), active OB with lymphocyte infiltration in the lumen (third from top) active OB with luminal fibrosis and peribronchiolar lymphocyte aggregates (fourth from top), and two inactive OB lesions (fifth and sixth from top, only one of which shows positive PNAd staining). B, Higher magnification of a PNAd⁺ HEV in an active OB lesion. Scale bar, 100 μm. NC, Normal control.

HEVs are specialized endothelial lined vessels that exist uniquely in lymphoid tissue and play critical roles in lymphocyte trafficking (24). Interestingly, immunofluorescence labeling for PNAd, a marker specific for HEVs, demonstrated a large number of HEVs composed of characteristic cuboidal endothelial cells in the airways of BOS lungs, whereas bronchioles in normal lungs did not show HEVs (Fig. 2). Because HEVs positive for PNAd are considered to be unique to secondary and tertiary lymphoid organs, a similar finding in a nonlymphoid organ was surprising to us. Through quantitative evaluation we found that HEVs existed in almost all of the lesions of lymphocytic bronchiolitis and active OB lesions in the bronchiolar wall (Fig. 2A), whereas a portion of inactive OB lesions were also accompanied by a small number of
HEVs (Fig. 3A). In some active OB lesions, HEVs were also observed in the lumen of obliterated airways, suggesting that the induction of HEVs can occur through angiogenesis.

The percentages of lymphocytic bronchiolitis lesions, active OB lesions, and inactive OB lesions that are accompanied by HEVs is shown in Fig. 3A. The number of bronchioles with accompanying HEVs per unit lung area was significantly larger in BOS lungs than in normal lungs (Fig. 3B; p < 0.01). The small number of HEVs observed in normal lungs is considered to represent constitutive BALT.

**Alloantigen-dependent ELT formation after rat intrapulmonary tracheal transplantation**

A rat intrapulmonary tracheal transplant model of OB is an animal model of OB that reflects the influences of the pulmonary milieu (20, 22). This model enables examination of the pulmonary immune system’s reaction to allogenic stimuli. In the present study, we examined whether lymphoid tissue also develops in the lung in fully MHC-mismatched intrapulmonary tracheal transplantation (Lewis to Lewis). Thus, the intrapulmonary tracheal transplantation is likely to be a de novo lymphoid tissue, similar to those observed in transplanted human lungs.

*Early lymphocyte aggregates are insufficient for allograft airway rejection*

We subsequently addressed the question regarding whether or not the lymphocyte aggregates become a stable structure in association with the stable homing of memory T cells. Following initial intrapulmonary allograft tracheal transplantation (BN to Lewis), we conducted orthotopic transplantation of the Lewis lung containing a BN tracheal graft into an F1 (BN × Lewis) rat at day 7 or day 28 (Fig. 5A). Because F1 rats should accept both Lewis-derived and BN-derived grafts, the only components that could reject BN-derived grafts are Lewis-derived lymphocytes that may persist in the lung after orthotopic lung transplantation into an F1 rat.

In the first set of experiments wherein orthotopic lung transplantation was conducted at day 7, peribronchiolar lymphocyte aggregates in the Lewis lung disappeared and the BN graft showed recovery of the epithelium with little subepithelial fibrosis at day 35 (Fig. 5B). The “negative” control of Lewis-Lewis-F1 transplantation also demonstrated minimum lymphocyte aggregates and complete recovery of the tracheal graft in contrast to the “positive” control of BN-Lewis-Lewis transplantation in which graft rejection should continue, and indeed lymphocyte aggregates were still observed in the lung with obliterated BN trachea (Fig. 5B). Morphometric quantification demonstrated significantly larger lymphocyte aggregates in BN-Lewis-Lewis transplantation at day 35 compared with the other two groups (Fig. 5C). Thus, the intrapulmonary lymphocyte aggregates at day 7 are still immature and incapable of stably harboring memory T cells.

Conversely, the second set of experiments wherein orthotopic lung transplantation was conducted at day 28 demonstrated stable homing of memory T cells. In BN-Lewis-F1 transplantation, the lymphocyte aggregates in the peribronchiolar tissue were obviously enlarged compared with those in the other two groups and extended into the perigraft tissue at day 56 (i.e., 28 days after orthotopic lung transplantation) (Fig. 6A). The “negative” control of Lewis-Lewis-F1 transplantation showed the small size of lymphocyte aggregates, whereas those of the “positive” control of BN-Lewis-Lewis transplantation were similar to those of the untreated allograft transplantation control. Morphometric quantification confirmed the significantly larger size of lymphocyte aggregates in the group of BN-Lewis-F1 transplantation (p < 0.05; Fig. 6B).

Flow cytometric analyses demonstrated that a significant percentage of Lewis-derived RT1A<sup>−</sup>CD4<sup>−</sup> T cells and CD8<sup>−</sup> T cells exist in the left lung in BN-Lewis-F1 transplantation as compared with the other groups (Fig. 7A). Immunofluorescence labeling for RT1A<sup>+</sup> in the lymphocyte aggregates in the lung after BN-Lewis-F<sub>1</sub> transplantation demonstrated chimerism of F<sub>1</sub>-derived RT1A<sup>−</sup> cells and Lewis-derived RT1A<sup>+</sup> cells (Fig. 7B). Moreover, the majority of CD4<sup>+</sup> T cells in the lung of the BN-Lewis-F<sub>1</sub> transplantation group demonstrated a higher ratio of the
FIGURE 4. Development of effector lymphoid tissue in airways after intrapulmonary tracheal transplantation. A and B, H&E staining (top panels) and double immunofluorescence labeling for CD3 (T cell) and CD79a (B cell) (bottom panels). Representative pictures of recipient lungs of intrapulmonary isograft and allograft tracheal transplantation at days 7 and 28 are shown. Insets in B are corresponding tracheal grafts (original magnification, ×40). C, H&E staining of recipient lungs of intrapulmonary isograft and allograft tracheal transplantation at day 120. Arrows indicate peribronchiolar lymphocyte aggregates. D, Morphometric quantification of lymphocyte aggregates in the recipient lungs that received an isograft and an allograft. Allograft recipient lungs demonstrated a significantly larger number of lymphocyte aggregates than those of isograft recipient lungs at day 28 and after (p < 0.05). n = 4–5; mean ± SEM. E, Flow cytometric analysis of CD4 and CD45RC expression on CD3+ T cells in the spleen, mediastinal lymph nodes, and the left lung at day 28 after intrapulmonary allograft tracheal transplantation. Cells were first gated by CD3 positivity. F, Double immunofluorescence labeling for von Willebrand factor (vWF) and MadCAM-1 (top panel) and CD3 and CD79a (bottom panel) taken from a similar area of a well-developed lymphocyte aggregates at day 56 after intrapulmonary allograft tracheal transplantation. The arrow indicates a HEV-like vessel observed in the aggregate of T cells. Scale bar, 100 μm. NC, Negative control.
CD45RClow memory phenotype as compared with the other groups (Fig. 7C). This ratio was also higher than those of the opposite lung, mediastinal lymph nodes, and spleen (~60%), indicating that memory T cells dominantly exist in the orthotopically transplanted lungs in association with lymphocyte aggregates. This overexpansion of lymphocyte aggregates, which was even greater than that of the BN-Lewis-Lewis “positive” control transplantation, was somewhat surprising to us. We speculate that Lewis-derived RT1A(-) lymphocytes homing to the lung reacted to F1-derived RT1A(+) lymphocytes. The Lewis lung containing the BN trachea was orthotopically transplanted into an F1 host because an F1 rat should not reject either Lewis or BN-derived tissue; however, the opposite is not true and, if there were significant Lewis-derived lymphocytes they could theoretically be reactive to the F1 host-derived cells through a mechanism similar to graft-vs-host disease, which can lead to OB after hematopoietic stem cell transplantation (26).

Memory lymphocytes homing to the lung exert effector function

The previous experiment could not demonstrate the effector function of intrapulmonary lymphocytes against the airway graft because the allograft trachea was already obliterated when orthotopic lung transplantation was conducted at day 28. Thus, we added a second intrapulmonary tracheal transplantation at the time of orthotopic lung transplantation at day 28 (Fig. 8A). At day 56 after the initial intrapulmonary tracheal transplantation (i.e., 28 days after orthotopic lung transplantation), the first BN-derived tracheal grafts showed total loss of the epithelium and lumenal obliteration with fibrous tissue (Fig. 8Ba, top graft). The histological characteristics of the fibrous tissue of the first BN grafts were similar to those of untreated control allografts (Fig. 3A). In contrast, the second intrapulmonary BN-derived tracheal grafts showed a massive infiltration of inflammatory cells with total loss of the epithelium and luminal obliteration with fibrous tissue (Fig. 8Bb, top graft). The second intrapulmonary Lewis-derived trachea (i.e., isograft) implanted adjacent to the first BN-derived trachea showed minimum inflammatory infiltration (Fig. 8Bc). Conversely, the second intrapulmonary Lewis-derived trachea (i.e., isograft) implanted adjacent to the first BN-derived trachea showed minimum inflammatory infiltration (Fig. 8Bd).
with fibrous tissue similarly to that of the other group (data not shown). Semiquantification of CD3⁺ T cells demonstrated a significantly larger number of T cells infiltrating into the secondary BN-derived “allo” graft compared with that of the Lewis-derived “iso” graft (Fig. 8C).

Moreover, another BN graft implanted in the s.c. tissue at the time of orthotopic lung transplantation showed complete recovery of the epithelium with no evidence of T cell infiltration (Fig. 8D).

Thus, the effector function of the intrapulmonary de novo lymphoid tissue has a significant impact on the local immune response against allogenic airways.

**Discussion**

In the present study, we hypothesized that lymphoid neogenesis plays an important role in chronic rejection after lung transplantation. The human study for the first time demonstrated lymphoid neogenesis in the lung affected by chronic rejection. The animal study demonstrated that intrapulmonary lymphoid tissue has an effector function sufficient to reject allograft airways. These results support our hypothesized role of lymphoid neogenesis in chronic rejection after lung transplantation.
In the human study, we observed a number of lymphoid tissues composed of T cells, B cells, and HEVs (Fig. 1) in association with lesions of OB and lymphocytic bronchioles after lung transplantation (Fig. 2), suggesting lymphoid neogenesis in chronic rejection after lung transplantation. Although lymphoid neogenesis has been observed in chronically rejected human kidney, liver (7), and heart (27, 28), lymphoid neogenesis has not been reported in lung transplantation. It is possible that the presumed existence of “constitutive BALT” has made lymphoid neogenesis in the lung less obvious. However, BALT has been demonstrated not to exist in normal human lungs (29). We also observed a significantly larger number of lymphoid tissues containing HEVs in BOS lungs than in normal control lungs (Fig. 3), suggesting the process of lymphoid neogenesis in transplanted lungs.

Compared with reported lymphoid neogenesis (3–6), lymphoid tissue in transplanted lungs is unique in the lack of delineated T cell and B cell zones and follicular dendritic cells (Fig. 1). In the lung, similar, less organized lymphoid tissue that does not meet the conventional criteria of secondary and tertiary lymphoid tissue has been reported (13). However, the functional significance of such anatomical variations among intrapulmonary lymphoid tissue remains unclear. Indeed, the most important factor in lymphoid neogenesis may be the distribution of effector lymphocytes in peripheral organs, particularly at the sites of pathogen entry such as mucosal surfaces of the lung and gut (30). ELT has been proposed to describe a functionally unique subset of lymphoid tissue that collects effector and effector memory T cells regardless of anatomically defined structures (16). Our finding of predominant CD45RO/CCR7 effector memory T cells supports the formation of ELT after lung transplantation (Fig. 1). Furthermore, even in a s.c. tracheal transplant model of OB, the trafficking of effector T cells to the lung has been reported (31). This evidence suggests the important role of the lung as a “reservoir” of effector and effector memory T cells that may contribute to allograft airway rejection after lung transplantation (19).

Our human study was, however, limited in the functional assessment of lymphoid neogenesis. To overcome the challenge, we used an animal model of OB in which the allograft airway is exposed to the pulmonary milieu (22). In the model, we observed a larger number of lymphoid tissues in the lung after allograft transplantation compared with that of isografts (Fig. 4), demonstrating that allograft airway rejection is accompanied by significant intrapulmonary lymphoid neogenesis. In contrast, the lymphoid tissue did not result in obliteration of the airways around which lymphoid tissue is formed, even after 120 days (Fig. 4). This is likely explained by the nature of the intrapulmonary tracheal transplantation in which lymphoid tissue is formed in the native (recipient) lung instead of the donor lung as seen in human lung transplantation. Because the epithelium is considered to be the primary target of allograft airway rejection (32), the airway that is not allogenic may not be significantly affected.

To further assess the role of lymphoid neogenesis in allograft airway rejection, we orthotopically transplanted the lung containing de novo lymphoid tissue into an F1 rat, which dissected the immune response mediated by the de novo lymphoid tissue from that mediated by conventional secondary lymphoid tissue such as regional lymph node tissue (Fig. 5A). Interestingly, using this newly devised animal model of OB, we found that lymphocyte aggregates at day 7 after intrapulmonary allograft transplantation are still reversible (Fig. 5) while the lymphocyte aggregates become more stable after day 28 (Fig. 6 and 7), suggesting that there is a maturation process in lymphoid neogenesis. Recent studies in chronic inflammatory disorders such as rheumatoid arthritis suggest that the critical switch from acute, resolvable inflammation to persistent, poorly resolvable inflammation lies in phenotypical alterations of resident cells, including endothelial cells (33, 34). These cells begin to express adhesion molecules (e.g., PNA d) and ectopic lymphoid chemokines (e.g., CCL21) in response to inflammatory stimuli (35) such as TNF (36), and the altered tissue microenvironment allows for a large influx and survival of Ag-specific lymphocytes in peripheral tissue (35). Interestingly, we found that lymphocytic bronchiolitis, a presumed precursor of OB (37, 38) but still a reversible condition (39), accompanies abundant HEVs and lymphocytes (Fig. 2), indicating that lymphoid neogenesis is initiated before the development of OB. Moreover, considering alloantigen-independent inflammatory stimuli such as ischemia-reperfusion injury at the time of transplantation (40), bacterial colonization in airways (41, 42), and bile/acid aspiration (43, 44) that contribute to OB/BOS, these Ag-independent factors might be enough to activate resident cells such as endothelial cells in airways to initiate lymphoid neogenesis. An interesting question to be answered in the future is how soon such phenotypical changes in the resident cells (e.g., endothelial cells) leading to lymphoid neogenesis start after lung transplantation.

The initial set of retransplantation experiments (Figs. 5–7), however, did not clarify the role of the mature intrapulmonary lymphoid tissue in allograft airway rejection, because the first tracheal graft was already rejected and obliterated at day 28 when the orthotopic lung transplantation was conducted. Thus, we further implanted secondary tracheal graft at the time of orthotopic lung transplantation into an F1 rat (Fig. 8A). In this experiment, we observed massive infiltration of T cells into allografts but not isografts (Fig. 8), suggesting an allospecific effector function of the lymphoid tissue in the lung or in ELT in general (16). Moreover, because the s.c. allograft remained intact, the effector function of intrapulmonary lymphoid tissue was more important in local than in systemic immune response as indicated in another report on iBALT (11). Interestingly, we found that the secondary isografts implanted in the lung containing lymphoid tissue also show pathological changes such as epithelial loss and loose fibrosis formation, albeit free of T cell infiltration (Fig. 8). It is possible that the inflammatory microenvironment created by intrapulmonary lymphoid tissue affected the isograft in an alloantigen-independent manner and delayed normal epithelial regeneration.

To further clarify the role of memory T cells, Ab-mediated T cell depletion would be the ideal experiment. However, the efficacy of Ab-mediated T cell depletion was limited in rats in our own experience as well as in the literature (45). A mouse intrapulmonary tracheal transplant model that we have recently developed (46) may help to overcome the challenge.

We acknowledge that the animal model we used has multiple limitations, including the initial intrapulmonary tracheal transplantation (not transplantation of the entire lung) and the lack of immunosuppression. Differences among species are another important limitation of an animal experiment, particularly in the organization of intrapulmonary lymphoid tissues such as BALT (29, 47). Despite these limitations, we emphasize that the animal experiments in the present study complemented the limitations in the human study in many ways and provided important insights regarding the role of intrapulmonary de novo lymphoid tissue in OB after lung transplantation.

In conclusion, we found de novo lymphoid tissue in the lung affected by chronic rejection after human lung transplantation. The de novo lymphoid tissue likely plays a role in chronic local immune responses after lung transplantation. Further studies are obviously necessary to improve our understanding of this novel form of lymphoid tissue and its contribution to the development of OB after lung transplantation.
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Disclosures
The authors have no financial conflict of interest.

References