Association of Local Intrapulmonary Production of Antibodies Specific to Donor Major Histocompatibility Complex Class I With the Progression of Chronic Rejection of Lung Allografts

Ei Miyamoto, MD,[1] Hideki Motoyama, PhD,[1] Masaaki Sato, PhD,[2] Akihiro Aoyama, PhD,[1] Toshi Menju, PhD,[1] Kei Shikuma, MD,[1] Terumasa Sowa, MD,[1] Akihiko Yoshizawa, PhD,[3] Masao Saito, MD,[1] Akihiro Takahagi, MD,[1] Satona Tanaka, MD,[1] Mamoru Takahashi, MD,[1] Keiji Ohata, MD,[1] Takeshi Kondo, PhD,[1] Kyoko Hijiya, MD,[1] Toyofumi F. Chen-Yoshikawa, PhD,[1] and Hiroshi Date, PhD[1]

Background. Antibody-mediated rejection may lead to chronic lung allograft dysfunction, but antibody-mediated rejection may develop in the absence of detectable donor-specific antibody (DSA) in recipient serum. This study investigated whether humoral immune responses develop not only systemically but locally within rejected lung allografts, resulting in local production of DSA. **Methods.** Lewis rats received orthotopic left lung transplantation from Lewis (syngeneic control) or Brown-Norway (major histocompatibility complex-mismatched allogeneic) donor rats. Rats that underwent allogeneic lung transplantation were subsequently administered cyclosporine until day 14 (short immunosuppression) or day 35 (long immunosuppression). The lung grafts and spleens of recipient animals were tissue cultured for 4 days, and the titer of antibody against donor major histocompatibility complex molecules was assayed by flow cytometry. Explanted lung grafts were also evaluated pathologically. **Results.** By day 98, DSA titers in supernatants of lung graft ($P = 0.0074$) and spleen ($P = 0.0167$) cultures, but not serum, from the short immunosuppression group were significantly higher than titers in syngeneic controls. Cultures and sera from the long immunosuppression group showed no production of DSA. Microscopically, the lung grafts from the short immunosuppression group showed severe bronchiole obliteration and parenchymal fibrosis, along with lymphoid aggregates containing T and B cells, accompanying plasma cells. These findings suggestive of local humoral immune response were not observed by days 28 and 63. **Conclusions.** DSA can be locally produced in chronically rejected lung allografts, along with intragraft immunocompetent cells. Clinical testing of DSA in serum samples alone may underestimate lung allograft dysfunction.

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Antibody-mediated rejection (AMR) after lung transplantation (LTx) remains a major cause of graft failure, hampering long-term survival of recipients.[1-3] Diagnostic criteria for AMR include evidence of donor-specific antibody (DSA) in recipient serum, physiological deterioration, positive histology suggestive of AMR, and positive staining of lung grafts for complement component 4d (C4d).[4] Especially, serum DSAs were reported to have a significant impact on the development of AMR,[5,6] mixed rejection,[6] and even chronic lung allograft dysfunction.[7] Although the presence of DSAs and humoral rejection after LTx are considered as efficacy endpoints of immunosuppression (IS) drugs, their

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[1] Department of Thoracic Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan.

[2] Department of Thoracic Surgery, The University of Tokyo Hospital, Tokyo, Japan.

[3] Department of Diagnostic Pathology, Kyoto University Hospital, Kyoto, Japan.

E.M., H.M., and M.S. equally contributed to this study.

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Correspondence: Masaaki Sato, PhD, Department of Thoracic Surgery, The University of Tokyo Hospital, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. (SATOM-SUR@h.u-tokyo.ac.jp).

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predictive value for long-term outcome is still controversial.[8] The current animal model could be used to better demonstrate the existence of locally produced DSA and/or non-DSA, and the causal relationship between AMR and CLAD.allograft dysfunction may develop or progress in the absence of detectable serum DSA. These absences have been traditionally interpreted as being due to phasic release of DSA, limitations of the diagnostic test, or absorption of DSA into lung grafts.[4] Paradoxically, false-negative findings of serum DSA remain an important unresolved obstacle that may result in underdiagnosis of subclinical AMR. DSAs were recently identified in eluates from lung graft biopsies, as well as in other solid organ transplant recipients,[9,10] suggesting that analyzing only serum DSA may underestimate the occurrence of DSA bound to donor tissue.[11]

Over the last decade, it has become clear that tertiary lymphoid tissues (TLTs) are associated with allograft rejection after solid organ transplantation.[12,13] Intragraft TLT, also called de novo lymphoid tissue, contains both T cells and B cells. These cells may generate alloantibodies, suggesting that intragraft lymphoid tissue may be a source of alloantibodies.[14]

Intrapulmonary lymphoid neogenesis has been observed in the lung allografts of transplant recipients who developed obliterative bronchiolitis (OB).[15] Moreover, stable homing of recipient-derived T cells and their donor-specific effector function have been demonstrated in orthotopically transplanted lung allografts of major histocompatibility complex (MHC) mismatched rats. Intrapulmonary de novo lymphoid tissue was shown to have the ability to reject allograft airways, even in the absence of secondary lymphoid organs.[16] To date, however, it remains unclear whether humoral alloimmune responses could be elicited locally in lung allografts and could contribute to allograft dysfunction.

We hypothesized that humoral immune responses could be activated not only systemically but also locally within rejected lung allografts. Because localized antigen-antibody reactions may be independent of the systemic circulation,[17] this hypothesis may explain the progressive deterioration of lung grafts in the absence of circulating DSA. To test this hypothesis, we used an MHC-mismatched rat orthotopic LTx model to investigate whether antibodies directed against alloantigens are produced locally in lung allografts undergoing rejection, along with intragraft lymphoid neogenesis.

MATERIALS AND METHODS

Animal Models

Specific pathogen-free inbred male rats weighing 250 to 300 g were obtained from Japan SLC (Hamamatsu, Japan). All animals received adequate care according to the animal protocols approved by the Kyoto University Institutional Animal Care and Use Committee (MedKyo15525). Lewis rats were used as recipients and as syngeneic donors, and Brown-Norway rats were used as MHC fully mismatched donors. Orthotopic left LTx was performed as previously described.[18] Briefly, the donor rat was mechanically ventilated with room air during the procedure. After intravenous administration of 500 IU heparin, the lungs of each rat were flushed with 20 mL of ET-Kyoto solution (Otsuka, Tokushima, Japan). The heart-lung bloc was removed, and the left lung graft was prepared using plastic cuffs. Recipient rats were ventilated using the same settings as for the donors. After intraperitoneal injection of methylprednisolone sodium (10 mg/rat), the graft was orthotopically transplanted using the cuff technique.[19] The graft was reperfused, the chest cavity was closed, and the recipient rat allowed to recover from general anesthesia.

IS Protocols

Allogeneic LTx recipients were treated with cyclosporine A (CsA), administered subcutaneously at a dose of 25 mg/kg per day, on posttransplant days 0, 1, 2, and 4 (induction period) and twice weekly thereafter (maintenance period) until day 14 (short IS protocol) or day 35 (long IS protocol), after which immunosuppressive therapy was discontinued (free period) (Figure 1). Syngeneic recipients were not administered CsA. Each group consisted of 5 animals.

Allogeneically transplanted rats administered the short IS protocol were sacrificed on posttransplant days 28, 63, or 98, and those administered the long IS protocol were sacrificed on day 98. Syngeneically transplanted control rats were sacrificed on day 28 or day 98. Briefly, blood samples were obtained from each rat, followed by intravenous administration of 500 IU heparin. After flushing with 10-mL cold saline, the lungs and spleen were removed. The explanted lung graft and the spleen were dissected for tissue culture, and the remaining lung was inflated by injecting 10% formalin into the trachea at a pressure of 30 cm H_2O for fixation.

Tissue Culture

Each explanted lung graft and spleen was placed in 5 mL of cold sterile X-VIVO 15 serum-free medium (Lonza; Chiba, Japan) containing 100 units/mL of penicillin/streptomycin (Thermo Fisher Scientific K.K.; Yokohama, Japan) and 25 μg/mL of amphotericin B (Thermo Fisher Scientific K.K.), as described.[14] The tissue samples were washed 3 times with culture medium, with these washes collected as control samples, and 100 mg of each tissue sample were microdissected with a sterile razor blade in 3 mL of X-VIVO 15 culture

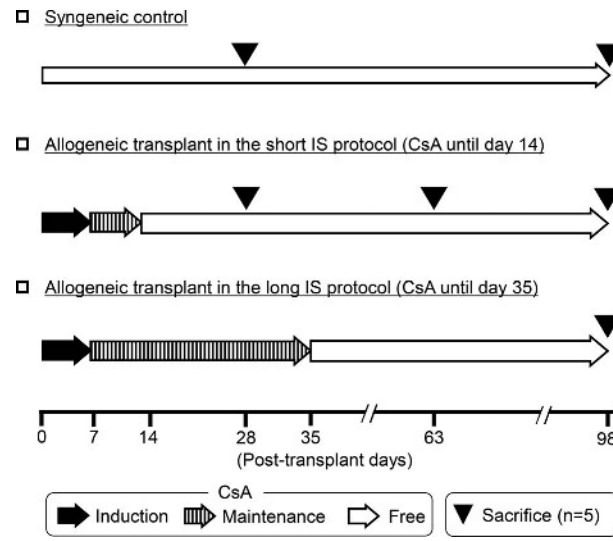

FIGURE 1. Schedule of cyclosporine administration for each IS protocol. Syngeneic control rats did not receive CsA. The IS protocols for allogeneic transplantation consisted of an induction period (CsA administration on days 0, 1, 2, 4, and 7), a maintenance period (CsA twice weekly), and a treatment-free period. Syngeneic recipient rats were sacrificed on day 28 or 63; allogeneic recipient rats that received the short IS protocol were sacrificed on day 28, 63, or 98; and allogeneic recipient rats that received the long IS protocol were sacrificed on day 98. Each group consisted of 5 animals.

medium and cultured in 6-well plates at 37°C under normoxic conditions (20% O_2/0.5% CO_2). After 2 or 4 days, the culture supernatants were recovered.

Histological Examinations

Formalin-fixed paraffin-embedded lungs cut into 5-μm-thick sections were stained with hematoxylin-eosin (HE) and Elastica-Masson (EM) trichrome stains. Standard HE staining was used to determine whether perivascular mononuclear cell infiltrates (A grade) were present, based on International Society for Heart and Lung Transplantation criteria.[20] Capillaritis was defined as capillary neutrophilic infiltration with karyorrhexis.[21] The positivity rate for each histological characteristic was defined as the percentage of specimens with that characteristic. Graft vascular sclerosis attributable to fibrointimal thickening of pulmonary vessels was defined as described.[22] The numbers of obliterated and nonobliterated bronchioles were counted in 10 randomly selected high-power fields, and the percentage of obliterated bronchioles in each lung specimen was calculated. The percentage of parenchymal fibrosis was quantified in 5 random EM-stained samples at 200× magnification using ImageJ version 1.49 (National Institutes of Health, Bethesda, MD). All pathological grading and semiquantification were performed by a single pathologist (A.Y.) in a blinded manner.

Immunohistochemical Staining and Immunofluorescence Labeling

Formalin-fixed 5-μm-thick paraffin sections were used in all immunohistochemical stainings. After deparaffinization and antigen retrieval in boiling 10 mM citrate buffer (Target Retrieval Solution; DAKO; Tokyo, Japan) for 20 minutes, the sections were blocked and incubated overnight at 4°C with a primary antibody. The following anti-rat primary antibodies were used: anti-CD3 (polyclonal rabbit; 1:200; Abcam, Tokyo, Japan), anti-CD8a (monoclonal mouse; 1:200, Abcam), anti-CD79a (monoclonal mouse; 1:300; Abcam), anti-CD20 (monoclonal mouse; 1:300, DAKO), anti-Ki-67 (polyclonal rabbit; 1:800; Abcam), anti-rat IgG (polyclonal rabbit; 1:1000, Vector Laboratories, Burlingame, CA), anti-C4d (polyclonal rabbit; 1:100; Hycult Biotech, Uden, The Netherlands), anti-CD138 (polyclonal rabbit; 1:100; Proteintech, Chicago, IL), and anti-RT1-B (His 19: monoclonal mouse; 1:300; Santa Cruz Biotechnology, Dallas, TX). The sections were washed and incubated with VECTASTAIN Elite ABC Kits (Vector Laboratories), according to the manufacturer's instructions, followed by counterstaining with hematoxylin.

DSA Assays

The reactivity of antibodies against Brown-Norway rat MHC class I molecules (RT1-A1^n) was analyzed by flow cytometry using mouse fibroblasts (L cells; negative control) and L cells transfected with RT1-A1^n-expressing retroviral vectors (L cells plus A1n), prepared as described.[14] These cells were cultured in Dulbecco modified Eagle medium (Sigma-Aldrich, St. Louis, MO) containing 10% fetal calf serum and 0.5 mg/mL G418 (Nacalai Tesque, Kyoto, Japan). For flow cytometry, 100-μL aliquots of each serum sample or tissue-culture supernatant were incubated with 200 000 L cells plus A1^n or control L cells for 30 minutes at 4°C. After washing with phosphate-buffered saline, the cells were incubated with anti-rat IgG heavy chain and light chain APC-conjugated antibodies (R&D Systems, Minneapolis, MN), followed by flow cytometry using a LSRFortessa flow cytometer (BD Biosciences, San Jose, CA). Mean fluorescence intensity (MFI) was assessed using FlowJo software (TOMY Digital Biology, Tokyo, Japan). Positive and negative control cells, consisting of cultured L plus A1^n cells and L cells, respectively, were gated by flow cytometry using a fluorescein isothiocyanate (FITC)-conjugated OX27 monoclonal antibody (mouse anti-RT1-A1^n; eBioscience, San Diego, CA). The DSA titer was calculated as the MFI of anti-rat IgG binding to RT1-A1^n-positive L cells divided by the MFI of anti-rat IgG binding to RT1-A1^n-negative L cells. To validate that RT1-A1^n–positive L cells have similar surface expression of donor-MHC molecules, the reactivity of antibodies was also analyzed using Brown-Norway rat T cells as the positive control and Lewis rat T cells as the negative control. Splenocytes isolated from rat spleens using BD Falcon Cell Strainers (BD Biosciences) were incubated with a FITC-conjugated mouse antirat CD3 antibody (eBioscience) for T cell gating by flow cytometry.

Statistical Analysis

All data are presented as means ± standard error of the mean. Student *t* tests were used to determine differences between 2 groups and 1-way analysis of variance followed by post hoc Tukey tests for differences among 3 or more groups. All statistical analyses were performed used JMP version 11.0.0 (SAS Institute Inc., Cary, NC) statistical software, with *P* values less than 0.05 considered statistically significant.

RESULTS

Pathological Features of Lung Grafts

Figure 2 shows a representative explanted lung graft obtained after each transplantation and IS protocol. All syngeneic grafts were well preserved, with slight inflammation, on days 28 and 98. Lung grafts of allogeneically transplanted animals that received the short IS protocol and were sacrificed on days 28 and 63 could be air inflated to a certain degree, with marked inflammatory cell infiltration observed at the circumferences of vessels and airways. Bronchiole obliteration was often observed in day 63 lung samples. Air inflation was difficult in allogeneic lung grafts of animals that received the short IS protocol and were sacrificed on day 98. These lungs showed intensive spread of fibrotic tissue to the subpleural area, bronchioles and vessels. In contrast, the allogeneic lung grafts in animals that received the long IS protocol and were sacrificed on day 98 were well preserved. Microscopically, perivascular inflammatory cell infiltration was common in these grafts, but fibrotic changes were not apparent.

Figure 3 shows the histological semiquantification of these lung grafts. Grafts of allogeneically transplanted rats that underwent the short IS protocol and were sacrificed on day 28 showed significantly higher A grade than syngeneic controls. Vascular sclerosis scores were significantly higher in the short IS than in the syngeneic control group. There were no significant differences in the percentages of obliterated bronchioles and parenchymal fibrosis. Grafts of allogeneically transplanted rats that underwent the short IS protocol and were sacrificed on day 98 did not differ significantly in International Society for Heart and Lung Transplantation A grade from syngeneic rats. Vascular sclerosis scores and the percentage of parenchymal fibrosis were significantly higher in the short IS than in the long IS and syngeneic control groups, and the percentage of

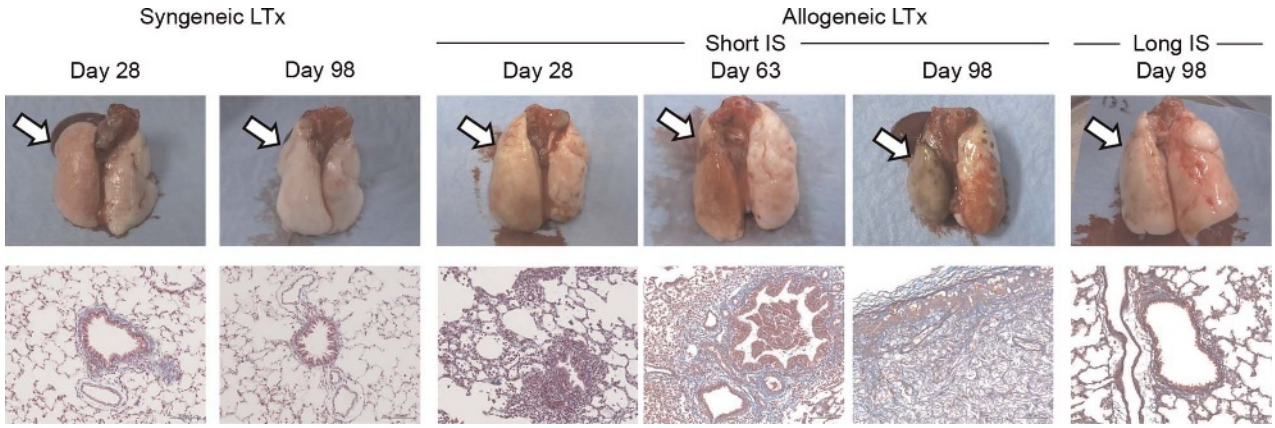

FIGURE 2. Macroscopic and microscopic findings of lung grafts. Macroscopic (upper photographs: arrowheads indicate lung grafts) and microscopic (lower micrographs: EM trichrome staining, scale bars = 50 μm) findings of the lung grafts. The syngeneic lung grafts explanted on days 28 and 98 were well preserved, with infiltration of few inflammatory cells and no obliterated bronchioles. The allogeneic lung grafts of rats that received the short IS protocol and were sacrificed on days 28 and 63 could be air inflated to some degree, with moderate inflammatory cell infiltration around vessels and airways. Some bronchioles were obliterated in grafts obtained on day 63, but not on day 28. The lung grafts of rats that received the short IS protocol and were sacrificed on day 98 were difficult to inflate, with intense collagen fibrosis spreading not only to the subpleural area but throughout the entire lung graft involving the bronchioles and lung parenchyma. The lung grafts of rats that received the long IS protocol were relatively well ventilated, even on day 98. Inflammatory cell infiltration was observed around the vessels and airways, but most of the lung grafts were free of bronchiole obliteration.

obliterated bronchioles was significantly higher in the short IS group than in the syngeneic control group.

Diffuse alveolar damage, alveolar hemorrhage, and neutrophilic capillaritis were observed more frequently in the lung grafts of the short IS group than in grafts of the other 2 groups (Figure 4A). All the explanted lung grafts of the short IS group sacrificed at day 98 showed patchy deposition of C4d in the capillaries (Figure 4A). Alveolar hemorrhage

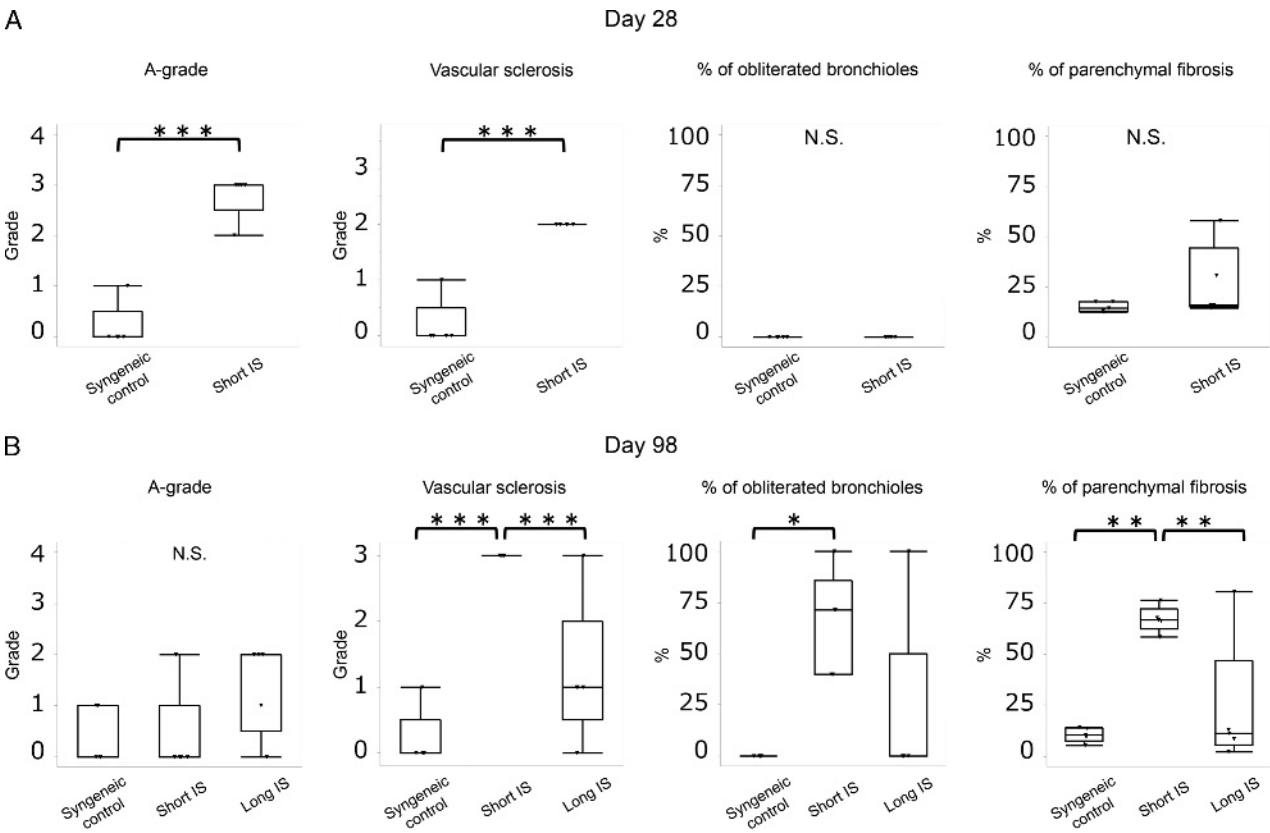

FIGURE 3. Histological grading and quantification of lung grafts. A, Pathological features of the lung grafts explanted on day 28. A grade and vascular sclerosis were significantly higher in the allogeneic short IS group than in the syngeneic control group. There were no significant differences in the percentages of obliterated bronchioles and parenchymal fibrosis. B, Pathological features of the lung grafts explanted on day 98. Vascular sclerosis grade and the percentage of parenchymal fibrosis were significantly higher in the short IS than in the syngeneic control and long IS groups. The percentage of obliterated bronchioles was significantly higher in the short IS than in the syngeneic control group. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. N.S., not significant.

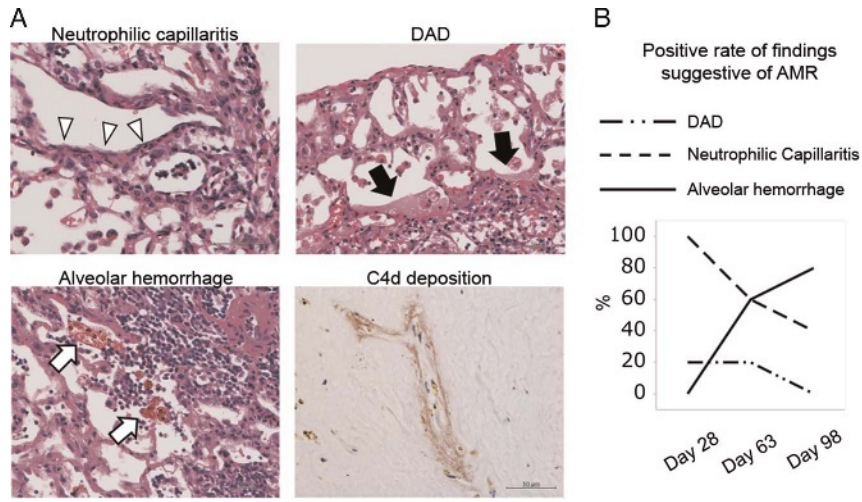

FIGURE 4. Histological findings suggestive of AMR and C4d deposition in the lung grafts of rats receiving the short IS protocol. A, HE-stained samples, showing neutrophils migrating into the capillaries (white triangles) and fibrin exudates in alveolar spaces (black arrowheads) in rats sacrificed on day 63; and alveolar hemorrhage (white arrowheads) in rats sacrificed on day 98. Samples stained with the anti-C4d antibody showed strong linear deposition of C4d in capillaries of rats sacrificed on day 98. B, Longitudinal presentations of histological findings suggestive of AMR. Alveolar hemorrhage was observed predominantly in rats sacrificed on days 63 and 98. Scale bars = 50 μm. DAD, diffuse alveolar damage.

increased over time in the short IS group (Figure 4B; P = 0.0236, analysis of variance). Neutrophilic capillaritis was observed in all lung grafts of the short IS group sacrificed at day 28, but in only 2 of the 5 lung grafts of the short IS group sacrificed at day 98, with the degree of neutrophilic capillalitis lower in the latter animals.

Detection of DSA in Supernatants of Lung Graft Culture and Serum

Figure 5 shows the representative result of DSA analysis using rat T cells. The MFI of APC against Brown-Norway rat T cells was approximately threefold higher than that against Lewis rat T cells.

To confirm the production of DSA against donor MHC class I, we further used mouse fibroblasts expressing RT1-A1^n. Figure 6 shows the high reactivity of DSA in the supernatant of a cultured lung graft of an allogeneically transplanted rat treated with the short IS protocol and sacrificed on day 98. The MFI of APC against RT1-A1^n–expressing mouse fibroblasts was more than fivefold higher than that against RT1-A1^n–negative mouse fibroblasts.

Figure 7A shows the titers of antibodies specifically reacting with donor-MHC class I antigens in rat sera and tissue culture supernatants. Of the allogeneically transplanted rats treated with the short IS protocol and sacrificed on day 28, only spleen-culture supernatants showed a significant higher titer than the syngeneic control. Supernatants of lung graft and spleen cultures of allogeneically transplanted rats in the short IS group sacrificed on day 98 had higher titers than supernatants of the other groups and higher titers than rats in the short IS group sacrificed on day 28 (Figure 7B). Serum titers, however, were not significantly different among groups. The titers of antibodies specifically reacting with donor-MHC class I antigens in the supernatants of lung graft cultures of the short IS group sacrificed on day 98 increased significantly with time in culture (Figure 7C).

Intragraft Lymphoid Aggregates

Figure 8 shows component analyses of de novo lymphoid tissue in the chronically rejected lung grafts, along with their high reactivity with antibodies against donor-MHC class I molecules. Lymphocyte aggregates were observed in peribronchiolar

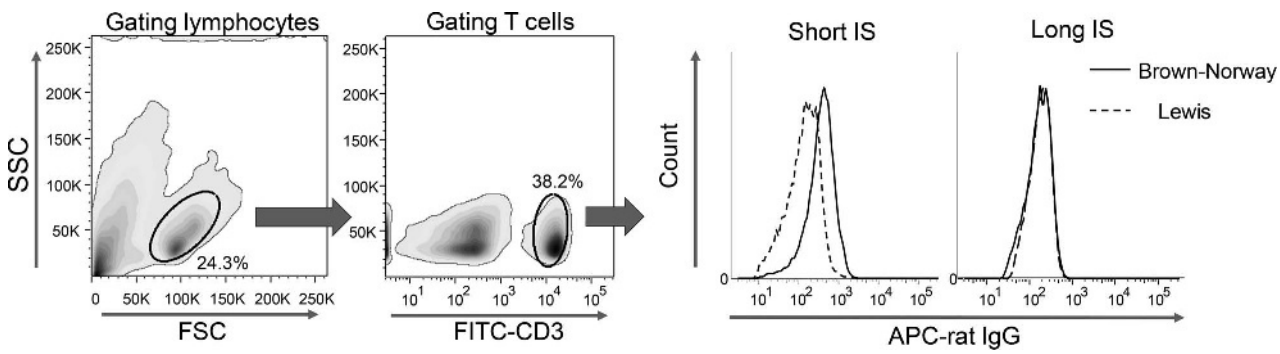

FIGURE 5. Representative DSA assay data using rat T cells. Rat T cells were gated from isolated splenocytes by flow cytometry using the FITC-conjugated anti-CD3 antibody. The MFI of APC-rat IgG was approximately threefold higher in Brown-Norway rat T cells than in Lewis rat T cells incubated with supernatants of lung graft cultures of a rat treated with the short IS protocol and sacrificed on day 98. The MFI of APC-rat IgG was similar against both Brown-Norway and Lewis rat T cells incubated with supernatants refined from lung graft cultures of a rat treated with the long IS protocol and sacrificed on day 98.

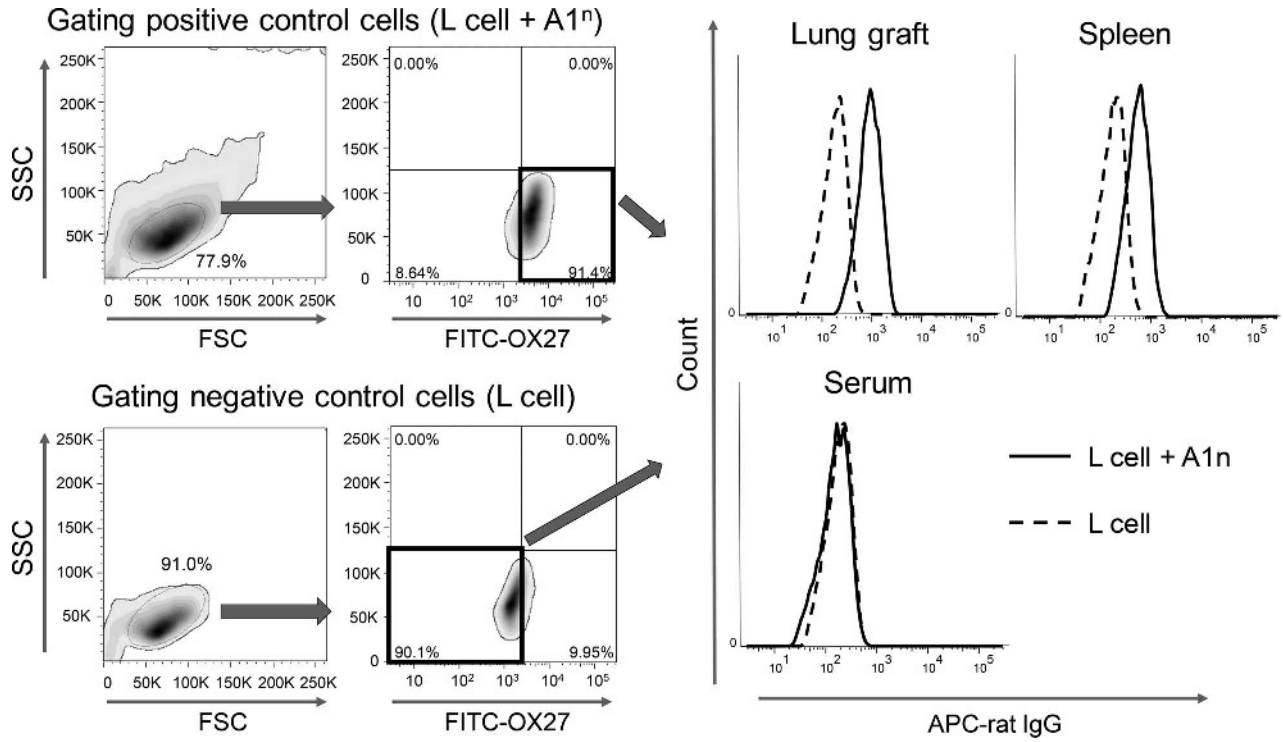

FIGURE 6. Representative data showing high reactivity of DSA in the supernatant of a lung graft culture against RT1-A1^n. RT1-A1^n–positive and RT1-A1^n–negative cultured L cells plus A1^n and L cells, respectively, were gated by flow cytometry using FITC-OX27. The MFI of APC-rat IgG was more than fivefold higher in supernatants of RT1-A1^n–positive than RT1-A1^n–negative L cells after lung graft culture of a rat treated with the short IS protocol and sacrificed on day 98. The MFI of APC-rat IgG against RT1-A1^n–positive L cells was threefold higher in supernatants of spleen culture of the same rat than that against than RT-1-A1^n–negative L cells. The MFI of APC-rat IgG was similar against RT-1-A1^n–positive and RT-1-A1^n–negative L cells.

and perivascular areas, which also contained large areas of collagen fibrosis. These de novo lymphoid tissues contained T cells (CD3), B cells (CD79a), plasma cells (CD138), and recipient-derived MHC class II-positive cells (His 19).

Figure 9 shows T cell and B cell aggregates in de novo lymphoid tissue of chronically rejected lung grafts. Immunofluorescence double staining showed the juxtaposition of B cell clusters and T cell aggregation within these de novo lymphoid tissues (Figure 9A). CD8-positive effector T cells were also found in the lung grafts (Figure 9B). Almost all of the B cells in the B cell clusters were positive for rat IgG, suggesting that these cells were producing DSA (Figure 9C). B cells were also positive for the proliferation marker Ki-67 (Figure 9D). A few B cells were sparsely distributed in the stroma apart from intragraft lymphoid aggregation and were not always positive for rat IgG (data not shown).

DISCUSSION

This study showed that antibodies against donor MHC class I molecules were locally produced within chronically rejected lung allografts, despite serum DSA being undetectable, suggesting that localized humoral immunity is an aspect of AMR after LTx. Although locally produced alloantibodies and resulting humoral immunity have been associated with solid organ graft rejection,[13-15] to our knowledge, neither intrapulmonary production of DSA nor local humoral immune response after LTx has been demonstrated to date. Generally, a diagnosis of AMR after LTx is based on allograft dysfunction, the presence of circulating DSA, and suggestive histology such as positive C4d staining and capillaritis in the absence of infectious pneumonia.[4] These indicators, along with the possibility that locally produced antibodies react with lung allografts in situ independent of the systemic circulation,[17] may explain findings of progressive AMR in the absence of detectable serum DSA.[4] Tissue elution assays have shown the significance of intragraft DSA as a risk factor for graft loss in lung[11] and kidney[9] transplant recipients. Moreover, ectopic lymphoid structure have been shown important in grafts with intragraft DSAs, indicating locally activated humoral immunity.[9-11,23] The tissue culture assay results reported in this study showed that DSAs could be produced locally in chronically rejected lung allografts.

The finding of parallel longitudinal increases in DSA titers in lung allografts and the spleen suggests that local humoral alloimmune responses in the lung did not develop independent of systemic responses during the progression of chronic lung allograft rejection. Rather, local responses developed in addition or as a complement to systemic responses including those in the spleen. Although primary systemic adaptive immune responses are initiated in secondary lymphoid organs, such as the lymph nodes and spleen,[24] local respiratory immune responses, such as those against viral infection, can be initiated in intrapulmonary lymphoid aggregates, which can be a site of response to antigens.[25] Our results indicate that these intrapulmonary lymphoid aggregates can also contribute to local humoral immunity against alloantigens. Indeed, DSAs were detected only in the lung graft culture assay while not in serum nor even in spleen culture assay in some rats treated with short IS protocol at day 98. Further

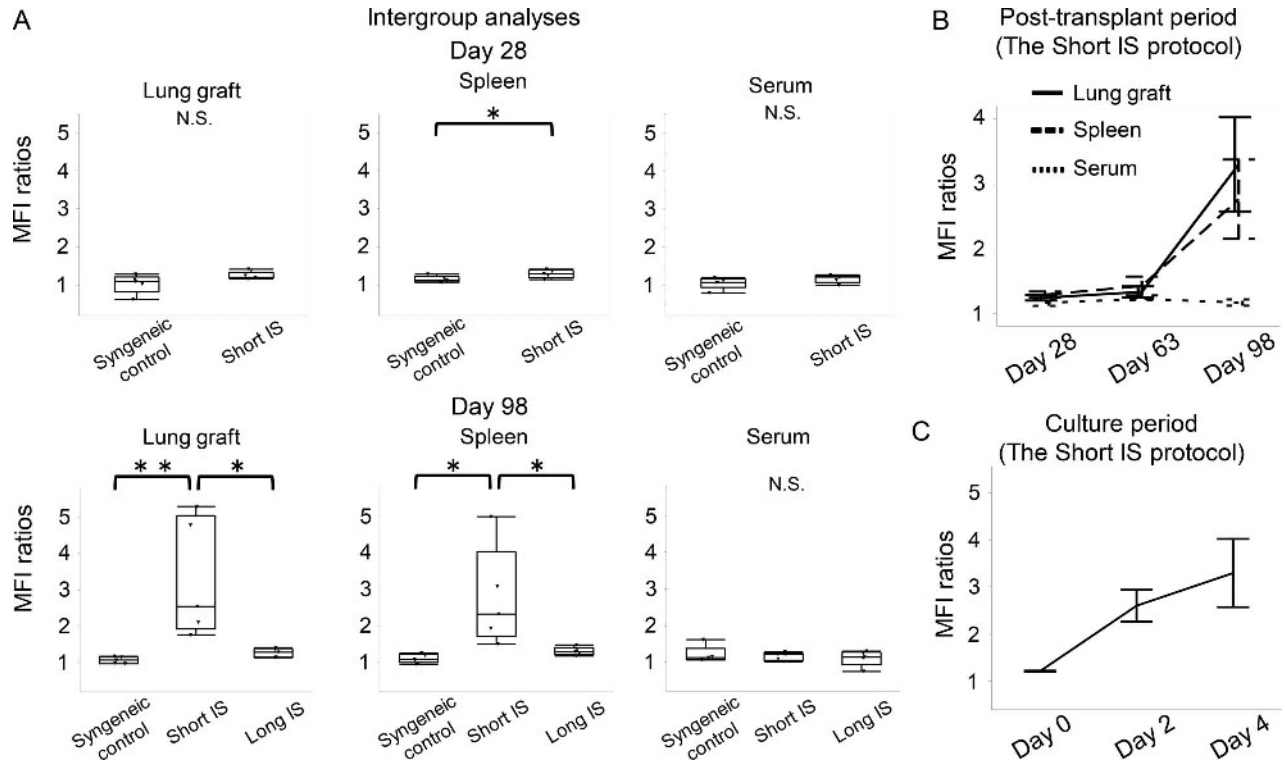

FIGURE 7. Intergroup and longitudinal analyses of DSA titers in tissue-culture supernatants and serum. A, Inter-group comparisons of DSA titers in tissue culture supernatants and sera. DSA titers were significantly higher in supernatants of spleen cultures of rats treated with the short IS protocol and sacrificed on day 28 than in spleen cultures of syngeneic control rats. However, there were no significant differences between lung graft cultures and serum titers. DSA titers in the supernatants of both lung graft and spleen cultures of rats treated with the short IS protocol and sacrificed on day 98 were significantly higher than in syngeneic control rats, although there were no differences in the serum titers. B, Longitudinal analysis of posttransplant period, showing that DSA titers increased significantly over time in the supernatants of both lung graft ($P = 0.0075$) and spleen ($P = 0.0263$). C, The MFI ratio in the supernatants of lung graft ($P = 0.0224$) increased as culture period increased. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

studies should be needed to speculate the determining influencer of the development of locally and systemically activated humoral immune mechanisms.

Stromal lymphoid aggregates that produce DSA locally were observed in chronically rejected lung allografts. These intragraft de novo lymphoid tissues were mainly composed of T cells and B cells, along with plasma cells and recipient-derived MHC class II-positive cells, which act as antigen-presenting cells. B cell clusters positive for anti-rat IgG and proliferation markers were thought to play a role in the local production of DSA. Furthermore, the presence of recipient-derived MHC class II-expressing cells, which are considered essential for interactions with helper T cells, suggest local intralung activation of a recipient's humoral immune system. B cell clusters within kidney grafts during rejection were reported associated with reduced graft survival and resistance to steroid therapy, independent of C4d deposition or the presence of serum alloantibody.[26] In systemic humoral immunity, B cell responses lead to the generation of long-lived plasma cells, which migrate into the bone marrow and continue to produce antibodies indefinitely.[27-30] The presence of de novo lymphoid tissue also suggests that the lung itself may become a niche for long-lived plasma cells, in addition to being a site for T cell–B cell interactions.[28,31] Intrapulmonary TLT after LTx may act as a locus of activated local humoral immune responses and may therefore be a potential therapeutic target. In addition, humoral responses within intragraft de novo lymphoid tissue may target a more diverse antigenic repertoire, including pathogens, auto-antigens, and molecules involved in allograft accommodation.[32-34] The results of this study do not exclude the possibility that, in addition to providing optimal protective immunity against respiratory pathogens at local tissue sites,[35,36] de novo lymphoid tissue after LTx may play a regulatory role in lung graft tolerance.

Pathological features observed in human lung grafts undergoing AMR were also observed in chronically rejected rat lung allografts. High-grade acute cellular rejection and neutrophilic capillaritis have been reported to be histopathologic indicators of AMR,[37] with DSA found to be associated with OB in lung transplant recipients.[38-41] The evidence of graft damage by inflammatory cells, such as A grade and DAD/neutrophilic capillaritis, may have become less pronounced because hypocellular collagen fibrosis had partially or extensively replaced the perivascular zone, into which inflammatory cells had infiltrated during early phases. OB has been observed in an animal model of intratracheal administration of antibodies against donor-MHC class I molecules.[42] DSA can also affect lung parenchymal cells, perhaps explaining the epithelial damage observed in lung transplants.[43] Antibodies directed against donor MHC class I molecules are particularly important in the pathogenesis of chronic vascular rejection.[17,44,45] Despite the evidence in the present study suggesting that locally produced alloantibodies contribute to allograft rejection, it remains unclear whether these locally produced antibodies

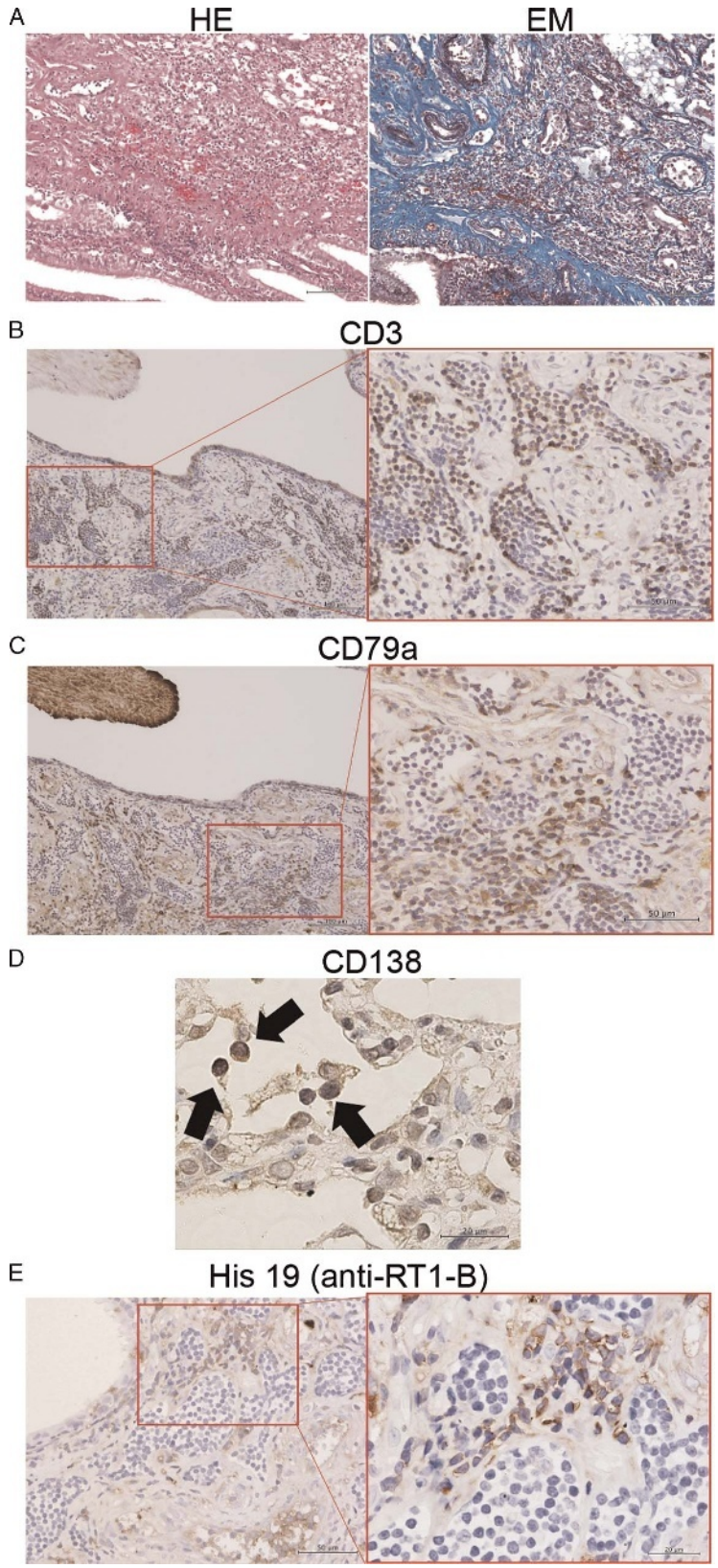

FIGURE 8. De novo lymphoid tissues in chronically rejected lung grafts of rats sacrificed on day 98. Representative lesion in a rat receiving the short IS protocol, showing lymphoid aggregation in a chronically rejected lung graft as well as the ability to produce DSA locally. A, Lymphocytic infiltration into collapsed lung parenchyma (dashed line), surrounded by stromal fibrosis spreading between the blood vessels (#) and airways (+) (HE, scale bars in the left and right panels, 100 μm and 50 μm, respectively). Immunohistochemical analyses showed that the de novo lymphoid tissue was mainly composed of T cells (B, anti-CD3, scale bars in the left and right panels, 100 μm and 50 μm, respectively) and B cells (C, anti-CD79a, scale bars in the left and right panels, 100 μm and 50 μm, respectively). Plasma cells (D, anti-CD138, scale bar = 20 μm) and recipient-derived MHC class II positive cells (E, His 19, scale bars in the left and right panels, 100 μm and 50 μm, respectively) were observed around the de novo lymphoid tissue.

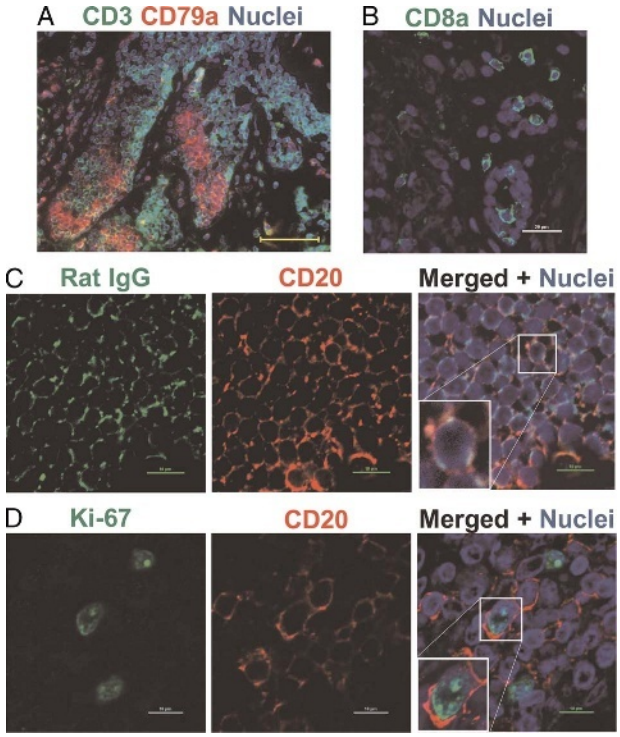

FIGURE 9. T cell and B cell components in de novo lymphoid tissue of chronically rejected lung grafts of rats sacrificed on day 98. A, Immunofluorescence staining showing B cell clusters (anti-CD79a, red) surrounded by T cells (anti-CD3, green) in the island structures (scale bar = 50 μm). B, CD8 positive effector T cells were also found (scale bar = 20 μm). C, Immunofluorescence staining (another pan-B cell marker and rat IgG) showing CD20-positive B cells stained positive for rat IgG (scale bar = 10 μm). D, Immunofluorescence staining (pan-B cell marker and cell proliferation marker) showing local proliferation of B cells in de novo lymphoid tissue (scale bar = 10 μm).

are essential in the context of cellular rejection and AMR caused by undetectable levels of circulating alloantibodies.

We acknowledge that the animal model used in this study has several limitations. First, the immune systems of rats and humans differ. The relative dose of immunosuppressants leading to complete acceptance of allografts is much lower in rats than in humans.[46] Thus, the optimal IS regimen required to reproduce conditions similar to human chronic lung allograft dysfunction in this study differed from that used in clinical settings. In addition to general limitations resulting from use of this lung transplant model, this study had specific limitations related to AMR. The full-MHC mismatched model is too simple to apply to highly heterogeneous human LTx. For example, this model cannot be used to assess DSA against the DQ-locus, the importance of which has recently been suggested.[7] Moreover, AMR in humans is likely modified in a complex manner with multiple IS, including by MMF, which has more potent effects on B cell proliferation than other agents.

Second, the tests used to detect DSA in the present study may not be directly applicable to human patients. Locally produced antibodies in this study were detected only after tissue culture. Because local DSA production in human lung allografts is similar to that in rats, the ability to detect these antibodies in, for example, bronchoalveolar lavage fluid, with sufficient sensitivity remains unclear. Third, the present study did not clarify the degree of contribution of locally produced DSA to allograft rejection. The local production of alloantibodies may only partially explain the entire process of AMR after LTx. The results of this study did not exclude the possibility that cellular immune responses could also cause these lung graft injuries.[47,48] A larger number of animals, sampling at additional time points, and further mechanistic analyses are required to determine a causal relationship between locally produced DSA and the progression of chronic lung allograft rejection. Furthermore, this model also can be applied for future animal study as to non-DSA and CLAD. Fourth, although this study focused on local DSA production, the details of intrapulmonary production of antibodies in association with lymphoid neogenesis remain to be explored further. For example, it is unclear whether nonspecific antibodies are produced, as suggested by a recent report in cardiac transplantation.[10] Moreover, local immunoglobulin class switch[49] and mutations in the variable domain may occur, suggesting the local evolution of antigen-specific immune responses. Notably, however, this study was designed as a proof-of-concept study, intending to demonstrate the local production of DSA in allograft lungs along with the progression of the rejection process. The finding that humoral alloimmune responses occur locally in lung allografts will lead to efforts to demonstrate a similar phenomenon in human lung allografts, to determine its possible clinical significance, and to develop novel strategies to detect local DSA or local AMR.

In conclusion, despite serum antibody levels being undetectable, locally produced antibodies against donor-MHC class I molecules were found in rejected lung allografts. Multiple pathological features suggestive of AMR were observed in intragraft de novo lymphoid tissue. Locally produced DSA, even in the absence of serum DSA, may play an important role in the progression of chronic lung allograft rejection after LTx.

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