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Immune Complex-Dependent Remodeling of the Airway Vasculature in Response to a Chronic Bacterial Infection

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Chronic inflammation in the airways is associated with dramatic architectural changes in the walls of the airways and in the vasculature they contain. In this study, we show that the adaptive immune system is essential for airway remodeling that occurs in mice that are chronically infected with the respiratory pathogen Mycoplasma pulmonis. Angiogenesis, lymphangiogenesis, and epithelial remodeling were greatly reduced in mice that lacked B cells. Substantiating a role for Ab and airway immune complexes, we found that the transfer of immune serum to B cell-deficient mice could reconstitute pathogen-induced angiogenesis. Inflammatory cells recruited to the infected airways were activated by the humoral response, and this activation correlated with the induction of genes for remodeling factors such as vascular endothelial growth factor-D. The results reveal a novel pathway whereby T cell-dependent humoral immunity to a persistent airway infection can induce inflammation-dependent angiogenesis, lymphangiogenesis, and chronic airway pathology. The Journal of Immunology, 2005, 175: 6319–6326.

T
he entry of leukocytes into tissues during inflammation is essential for pathogen control and wound healing (1). Inflammation can, however, cause significant damage to tissues and organ systems especially when it develops into a chronic condition (2–6). Examples of this include the destruction of bone and cartilage in rheumatoid arthritis, and the dramatic thickening of the colonic mucosal epithelium in colitis (3, 7). In the airways, persistent inflammation is also associated with chronic changes that collectively can be termed “airway remodeling” (8–11). In addition to a thickening and reorganization of the epithelial barrier, airway remodeling events include the proliferation of submucosal glands, subepithelial fibrosis and collagen deposition, and substantial reorganization and growth of the local vasculature. As a consequence of these changes there is diminished airway function and life-threatening hyperresponsiveness to bronchoconstrictive stimuli.

Transgenic experiments involving airway-restricted expression of cytokines that feature prominently in various forms of chronic airway inflammation have helped to define some of the factors that have the potential to contribute to specific forms of airway remodeling. For instance, cytokines from Th2 cells, such as IL-4, IL-5, IL-9, and IL-13 can induce epithelial cell proliferation, fibrosis, and mucus secretion to differing extents (12–15). It is unclear, however, which of these cytokines are truly critical for remodeling in the context of airway infections or other types of airway disease.

Although they are prominent aspects of airway remodeling, there is little mechanistic understanding of what induces vascular growth and reorganization in chronically inflamed airways (8). These processes can, however, be readily visualized in mice infected with the respiratory pathogen Mycoplasma pulmonis, which is a bacterium that can establish a life-long airway infection in mice (16, 17). M. pulmonis bacteria attach themselves to the epithelial cells lining the alveolar spaces and airways where they replicate prolifically despite a vigorous host immune response against them (18–23). Over time after infection, the inflamed airways manifest the type of remodeling symptoms mentioned above accompanied by substantial growth of new blood vessels, the enlargement of pre-existing blood vessels, and considerable expansion of the local lymphatic network (24).

In this study, we show that airway remodeling in response to M. pulmonis infection does not occur in the absence of adaptive immunity. We describe a pathway that connects a T cell-dependent anti-bacterial Ab response to exacerbated inflammation and consequently vascular and epithelial remodeling. We show that a humoral response to the bacteria correlated with increased recruitment and activation of inflammatory leukocytes in the airways. Humoral immunity also promoted localized increases in the expression of leukocyte genes that have potential direct remodeling roles. Cumulatively, the data shed light on a mechanism by which adaptive immunity can potentially induce airway remodeling, and provide the novel perspective that immune complex-dependent inflammation is a possible cause of the type of structural changes observed in the airways of M. pulmonis-infected mice.

Materials and Methods

Animals

C57BL/6J, C57BL/6-Igh-6m1Cgn (IghMTMT) referred to in this article as IghMTMT, C57BL/6-Jocmtm1Mom (Jocmtm1−/−), and C57BL/6-Terbtm1Mom (Terbt−/−) mice were obtained from The Jackson Laboratory or the National Cancer Institute, and/or were bred in the Parnassus Heights Barrier Facility at the University of California San Francisco. All of the experimental mice were 6–12 wk-old unless otherwise specified. In the majority of experiments, control animals were age- and sex-matched.
C57BL/6 mice purchased from the same commercial source. Infected and pathogen-free (PF*) control mice were housed separately.

**M. pulmonis infection and serum transfer**

Cultures of *M. pulmonis* were derived from stocks of the UAB CT7 strain kindly provided by Dr. J. R. Lindsey (University of Alabama, Birmingham, Alabama). The cultures were grown in broth and frozen in 1 ml aliquots as described previously (25). Mice were anesthetized (87 mg/kg ketamine and 13 mg/kg xylazine i.m.) and given *M. pulmonis* intranasally. The dose of organisms varied from strain to strain due to differences in background susceptibility, but ranged from 10^6 to 10^8 CFU in a total volume of 50 μl/mouse.

Immune serum came from the blood of female C57BL/6 mice that had been infected with *M. pulmonis* for at least 2 wk. Sera from multiple bleeds were pooled and tested for *M. pulmonis* reactivity by ELISA before use. Serum injections were given i.p. (200–250 μl total volume/injection) beginning at day 3 postinfection and every third day thereafter until the mice were analyzed.

**Perfusion and whole-mount staining of airway vasculature**

Lectin staining followed by perfusion to visualize blood vessels in the trachea was performed as described previously (26). In brief, biotinylated *Lycopersicon esculentum* lectin (100 μg in 100 μl; Vector Laboratories), which binds to the luminal endothelial cell surface, was injected i.v. followed by perfusion with a 1% paraformaldehyde, 0.5% glutaraldehyde fixative. Tracheas were removed from the mice, permeabilized with PBS containing 0.1% Triton X-100, and stained with ABC (avidin-biotin complex; Vector Laboratories) followed by the peroxidase-diaminobenzidine substrate. The tracheas were then flattened and mounted with Permount (Fisher Scientific) before imaging with a Leica DMLB microscope and a CCD camera. The images were analyzed and cropped using Adobe Photoshop.

To generate whole-mount immunofluorescence images of both the lymphatic and vascular endothelium, infected or PF mice were first perfused with 1% paraformaldehyde. The tracheas were removed and fixed for an additional 1 h in 1% paraformaldehyde in PBS (pH 7.4). They were then stained overnight with goat anti-mouse vascular endothelial growth factor (VEGF) receptor (VEGFR)-3 (R&D Systems) and rat anti-mouse CD31 (BD Pharmingen). Washing was followed by staining with a Cy3-conjugated donkey anti-goat Ig secondary Ab (Jackson Immunoresearch Laboratories) and FITC anti-rat Ig (Jackson Immunoresearch Laboratories). Tissues were washed, fixed for 1 min in 4% paraformaldehyde, and then mounted using Vectashield mounting medium for immunofluorescence (Vector Laboratories). The whole mounts were imaged using a Zeiss LSM 510 confocal microscope.

**Morphometric analysis of epithelial remodeling**

Mouse tracheas were fixed in 1% paraformaldehyde and sent to Biopathology Medical Sciences Corporation (South San Francisco, CA) for paraffin embedding, sectioning, and staining with H&E and the periodic acid-Schiff (PAS) stain. The stained sections were subsequently examined using a Nikon Eclipse E600 microscope, and photographed with a Zeiss Axioscam camera using AxioVision 3.0 software. Epithelial remodeling in the trachea was measured by measuring the thickness of the epithelium from the base of the columnar epithelium to the outer limit of the adventitia. Ten different measurements were taken around the lumen of each section to calculate the average thickness of the epithelial layer in different types of mice. Measurements were taken from midtracheal sections from at least three different mice in each group.

**Serology and determination of bacterial burden in infected organs**

An alkaline phosphatase-based ELISA was used to quantify *M. pulmonis*-specific IgG2a, IgG1, and IgA Abs in sera collected 4 wk after infection. ELISA plates (Nunc) were coated overnight at 4°C with 50 μl of carbonate-bicarbonate buffer (0.05 M; pH 9.6) containing 10^7 CFU of lysed *M. pulmonis* bacteria. The plates were subsequently blocked with PBS containing 1% BSA for 2 h at room temperature before incubation with serially diluted sera for 4 h at room temperature. After four washes with PBS containing 0.05% Tween 20, the wells were incubated overnight at 4°C with biotinylated goat anti-mouse IgG2a, IgG1, or IgA (Southern Bio-technology Associates and Caltag Laboratories). The wells were washed another four times before applying alkaline phosphatase-conjugated streptavidin for 45 min at 37°C. After further washes, p-nitrophenyl phosphate was added to the plate wells. Finally, the absorbance at 405 nm was determined using a Molecular Devices plate reader and SoftMax software.

To quantify numbers of *M. pulmonis* in infected mice, tissues were removed and homogenized in 4 ml of Hayflick’s broth. Homogenates were centrifuged, and serial dilutions of supernatant fluids were plated on *Mycoplasma* culture plates. The plates were incubated at 37°C for 2 wk before counting colonies.

**BrdU labeling and immunofluorescence**

BrdU (Sigma-Aldrich) was administered to mice continuously in their drinking water (0.8 mg/ml) after infection with *M. pulmonis*. Tracheas were removed from the mice at 7–14 days after infection, embedded in OCT, and stored at −80°C. Sections (7-μm thick) were cut using a cryostat and fixed for 10 min in cold acetone before staining. Endothelial cells in the sections were identified by staining with rat anti-mouse CD31 (BD Biosciences) followed by Cy3-conjugated donkey anti-rat Ig (Jackson Immunoresearch Laboratories). The sections were permeabilized and stained with biotinylated anti-BrdU (Zymed) followed by Cy3-conjugated streptavidin (Jackson Immunoresearch Laboratories).

Sections were analyzed under UV illumination using a Nikon Eclipse E600 microscope equipped with a Zeiss Axioimacamera, and AxioVision software. To count BrdU* CD31* endothelial cells, a series of images was taken around the entire circumference of each tracheal section both the red and green filters. The red and green images were then merged using Adobe Photoshop software. Each independent, yellow (i.e., double-stained) event mapping to CD31* vessels of the trachea was scored as an endothelial cell that had proliferated and thus incorporated BrdU into its DNA. At least three sections in midtrachea from three or more mice of each genotype were analyzed for each experiment. The significance of differences between groups was assessed by ANOVA.

For IgG immune-complex detection, 7-μm thick sections were cut from tracheas embedded in OCT and stored at −80°C. Sections were fixed, dried in cold acetone for 10 min, and stained with Cy2-conjugated anti-mouse IgG (Jackson Immunoresearch Laboratories). The sections were then analyzed with UV illumination using a Nikon Eclipse E600 microscope equipped with a Zeiss Axioscam camera, and AxioVision software.

**Isolation of airway inflammatory cells and analysis of cell types by flow cytometry**

Single-cell suspensions from tracheas were generated by a combination of mechanical and enzymatic digestion methods. Tracheas were removed from mice and minced with a razor in 1.5 ml of PBS on ice. Collagenase type II (Worthington Biochemical) and DNase type I (Sigma-Aldrich) were added to a final concentration of 1.5 mg/ml and 10 μg/ml, respectively. The samples were incubated for 20–30 min at 37°C before adding 5 ml of PBS containing 0.1% BSA and 1 mM EDTA, filtering through 70-μm cell strainers (Falcon) and washing twice by centrifugation. Finally, the cells were counted using a hemacytometer and stained for flow cytometry using standard procedures and directly conjugated Abs specific for Gr-1, CD11b, CD62L, and I-Ab (from BD Biosciences, eBioscience, and Caltag Laboratories). Flow cytometry data were analyzed using FlowJo software (Tree Star).

**Bronchoalveolar lavage (BAL) and RNA preparation**

After anesthesia (87 mg/kg ketamine and 13 mg/kg xylazine i.m.), the tracheas of anesthetized mice were surgically exposed, cannulated using a 20-gauge catheter, and then the lungs were flushed five times with 0.8 ml of PBS. The cells were washed twice with PBS containing 0.1% BSA, counted, and either analyzed by flow cytometry as described above or flash-frozen in RLT buffer (Qiagen RNeasy; Qiagen) for extraction of RNA. The RNA was quantified using an Agilent Bioanalyzer 2100 and analyzed using a multiplexed real-time PCR assay as described previously (30). Sequence data for the oligonucleotide primers that were used may be viewed at (http://asthmagenomics.ucsf.edu).

**Results**

**Immunological control of *M. pulmonis*-induced airway remodeling**

Angiogenesis in the tracheas of *M. pulmonis*-infected mice is evident both as the formation of new blood vessels and the enlargement of existing ones (26). Vascular remodeling begins within
FIGURE 1. Involvement of the adaptive immune system in mucosal remodeling in *M. pulmonis*-infected mice. **A**, Angiogenesis induced by *M. pulmonis* infection in WT and mutant mice at 4 wk postinfection. The airway vasculature in infected C57BL/6, *Rag1*^-/-^, *Tcrα*^-/-^, *Tcrβδ*^-/-^, and *Igμ*MT mice and PF C57BL/6 mice was visualized in tracheal whole-mount preparations by perfusion of mice with biotinylated *L. esculentum* lectin followed by staining tracheas with HRP-conjugated streptavidin and the diaminobenzidine substrate. Scale bar represents 100 μm. The results are representative of those obtained in three independent experiments involving at least four mice per group. **B**, Remodeling of the airway epithelium induced by *M. pulmonis* infection in C57BL/6, *Igμ*MT, and *Tcrβδ*^-/-^ mice. Tracheas from mice that had been infected for 4 wk were fixed, sectioned, and stained with H&E and PAS. The scale bar represents 25 μm. The graph at right shows the mean thicknesses (and associated SEMs) of the epithelium in the different mice (10 measurements from each of three separate mice per group). With the exception of the comparison between the *Igμ*MT and *Tcrβδ*^-/-^ means, all of the means were different one from another (*p* < 0.05) by ANOVA and Bonferroni’s multiple comparison test. **C**, Lymphangiogenesis induced by *M. pulmonis* infection in WT but not *Rag1*^-/-^ mice at 4 wk postinfection. The lymphatics in infected and PF mice were visualized in tracheal whole-mount preparations by staining with anti-VEGFR-3 (red), whereas blood vessels were detected with anti-CD31 (green). The scale bar represents 200 μm. The results are representative of those obtained in three independent experiments.
days following infection, and persists chronically because the bacteria are typically never cleared from the airways of the infected mice (16, 18, 23). At late time points (i.e., 1 mo or more after infection), the tracheas of infected wild-type (WT) C57BL/6 background mice show complex growth and reorganization of the vascular beds with a spaghetti-like interlaced pattern of small vessels connecting to larger ones. This increased complexity contrasts with the simpler pattern of vessels that is uniformly present in uninfectect tracheas (compare PF vs B6 in Fig. 1A).

Lung pathology in M. pulmonis-infected SCID mice is much reduced compared with that in infected WT controls (19). To determine whether adaptive immunity might also influence the angiogenesis that accompanies infection, we initially infected mutant mice that lacked all lymphocytes because of homozygosity of a null mutation in the Rag1 gene. Four weeks after infection, vascular remodeling was dramatically impaired in Rag1–/– mice compared with infected WT controls (Fig. 1A). Similarly, mice lacking either B cells (IgκMT) or all T cells (Tcrβδ–/–) showed an absence of remodeling, even though (like the Rag1–/– mice) normal numbers of bacteria could be recovered from their lungs (Table I). Consistent with previous work showing that Abs prevent systemic dissemination of M. pulmonis (19), we found that bacteria were also present in the livers and kidneys of infected T cell-deficient or B cell-deficient mice, but not WT mice (Table I). Tcrαδ–/– mice that lacked αβ, but retained γδ T cells showed diminished remodeling in most cases, but had an intermediate remodeling phenotype in others (Fig. 1A).

Epithelial cell hyperplasia was evident in the tracheas of infected WT mice but not B cell-deficient mice (Fig. 1B). Interestingly, although PAS-positive mucin was present in the infected WT mice, there was considerably more of it in the tracheas from infected B cell-deficient mice. Moreover, despite the absence of hyperplasia, the epithelial layer was nonetheless abnormally elongated in B cell-deficient mice. In both mucus secretion and epithelial cell differentiation, the tracheas of T cell-deficient mice showed an intermediate phenotype resembling WT mice in some sections and B cell-deficient mice in others (Fig. 1B and data not shown).

Growth of airway lymphatic vessels constitutes one of the more dramatic remodeling events in M. pulmonis-infected mice (24). Vessel growth is dependent on VEGF-C and/or VEGF-D because treating mice with inhibitory doses of VEGFR-3 blocks it (24). To determine whether lymphatic remodeling might also be dependent on adaptive immunity, we infected mice with M. pulmonis and then examined their tracheas using a whole-mount procedure that simultaneously revealed the local organization of both the lymphatic and blood vasculature (24). Whereas PF mice had very few lymphatic vessels in the parts of their tracheas that were ringed by cartilage, M. pulmonis infection caused a striking invasion of these areas with VEGFR-3+ lymphatic vessels (Fig. 1C). Like the angiogenesis, this lymphatic remodeling was largely absent from the tracheas of infected Rag1–/– and B cell-deficient mice (Fig. 1C and data not shown) indicating that it too was dependent on lymphocytes.

The results just summarized indicate that infection-associated remodeling of the vasculature (blood and lymphatic), and the epithelial barrier of the trachea are all processes that hinge on the presence of an adaptive immune system in M. pulmonis-infected mice. Although the factors immediately responsible for remodeling of these diverse airway structures are likely to be different, the common dependence on lymphocytes (B cells in particular) suggested that a common general cellular mechanism might be responsible for orchestrating all forms of remodeling. With this in mind, we chose to focus primarily on determining the causes of infection-associated vascular remodeling in the studies that follow.

Abs are required for M. pulmonis-induced airway remodeling

Mice lacking T cells are deficient in both T cell responses and also T cell-dependent Ab responses (27). Defective airway remodeling in these mice could therefore have resulted from either a specific requirement for T cells in remodeling, or alternatively, the absence of a pathogen-specific T cell-dependent Ab response. Infected WT mice made a strong Ab response to M. pulmonis (Fig. 2A) that could be detected as soon as 1 wk after infection (data not shown). Not surprisingly, mice lacking T cells failed to produce IgG following infection, although they did have M. pulmonis-specific IgM in their sera. Consistent with the vigor of the Ab response and the chronic nature of the bacterial infection, immune complexes could be detected by staining for IgG (Fig. 2B) or C3 (not shown) in the tracheas of infected WT mice.

To test for the specific involvement of pathogen-reactive Abs in remodeling, we infected B cell-deficient mice with M. pulmonis and then attempted to rescue tracheal angiogenesis by providing them with serum pooled from infected WT or PF mice. Serum was given to the mice every third day starting at day 3 after infection. Strikingly, by 14 days after infection, immune, but not PF serum induced dramatic remodeling of both the blood and lymphatic vasculature in infected B cell-deficient mice (Fig. 3A, bottom panels) with the extent of remodeling resembling that which was evident...
in infected WT C57BL/6 mice (Fig. 3A, top panels). As an additional means to quantify angiogenesis in these experiments, we gave the mice BrdU continuously in their drinking water. We then detected endothelial cells that had proliferated during the labeling period by immunofluorescence microscopy using Abs specific for CD31 and BrdU-containing DNA. This procedure consistently informed on the remodeling that occurred in WT, but was much reduced in Rag1−/− or IgμMT mice (Fig. 3B and data not shown).

Serum from infected WT mice generated immune complexes in the tracheas of infected B cell-deficient mice (Fig. 2B), and strikingly, it also rescued endothelial cell proliferation (Fig. 3C). These data indicate that serum and the anti-M. pulmonis Abs it contained could substitute for B cells in causing remodeling in the infected airways. Moreover, the data suggested that an important contribution of T cells to remodeling (Fig. 1) would be to provide help for the Ab response.

Kinetics and form of the inflammatory response to M. pulmonis

To determine the impact of humoral immunity and M. pulmonis immune complexes on leukocyte recruitment, we infected Rag1−/− and WT mice and then examined their tracheas for the presence of inflammatory cells 2 wk or 1 mo later. Like WT mice, Rag1−/− mice had many neutrophils present in their tracheas 14 days following infection (Fig. 4A). These cells could be seen in whole-mount preparations as CD31+ cells that were not part of the vascular network (not shown), and they could also be enumerated by flow cytometry following enzymatic digestion of infected tracheal tissue (Fig. 4A). At the two time points, but especially at 1 mo postinfection, there were significantly more cells present in the tracheas of the infected WT mice than in the Rag1−/− mice (Fig. 4A).

To assess the activation state of the inflammatory cells present in Rag1−/− and WT tracheas, we analyzed them by flow cytometry using Abs specific for CD62L and MHC class II molecules. Whereas CD62L is shed from the surface of leukocytes in response to activation, MHC class II levels are up-regulated (28, 29). Neutrophils from infected WT mice showed much lower cell surface expression of CD62L than those from PF mice, suggesting that they had been activated (Fig. 4B). By contrast, the neutrophils from infected Rag1−/− mice resembled those of PF mice in terms of their CD62L expression levels. Similarly, we found that macrophages in the tracheas of WT but not Rag1−/− or PF mice had slightly elevated levels of MHC class II on their surfaces (Fig. 4B). Collectively, the data indicate that adaptive immunity to M. pulmonis enhanced both the recruitment and activation of inflammatory leukocytes.
Expression levels on neutrophils (Gr-1 macrophages, whereas samples from PF and infected WT mice comprised on average 40–50% neutrophils and 40–50% macrophages. At 1 mo following infection, BAL samples from infected mice were analyzed per group. CD62L and MHC class II staining were determined by flow cytometry using FlowJo software. Four to five mice were analyzed per group. The data are representative of those obtained in two separate experiments. A, The graph at left shows CD62L expression levels on neutrophils (Gr-1/ Mac-1/) from the tracheas of infected WT and Rag1−/− mice 31 days after infection with M. pulmonis. The graph at right shows MHC class II levels (I-Ab) on the Mac-1/ I-Aα population (80–90% macrophages) in tracheas from WT and Rag1−/− mice at 14 days after infection. The mean fluorescence intensities (MFI) of CD62L levels were reduced on infected WT but not Rag1−/− neutrophils compared with PF controls (p < 0.05, ANOVA). MHC class II levels were increased on WT but not Rag1−/− macrophages compared with PF controls (p < 0.01, ANOVA).

Airway inflammatory cells produce soluble factors that regulate angiogenesis and lymphangiogenesis

Given that neutrophils and macrophages were activated in the tracheas of WT but not Rag1−/− mice, we next tested whether their activation might be associated with the up-regulation of genes that could be important for remodeling. For this purpose, we turned to a sensitive multiplexed real-time PCR assay that allows for a determination of relative gene expression in cell populations (30). Initial attempts to obtain high quality mRNA from tracheal cell populations were unsuccessful in large part because of the small numbers of cells that could be recovered following flow sorting of enzyme-digested tissue samples. We therefore turned to BAL as a means to increase our recovery of inflammatory cells from infected mice. At 1 mo following infection, BAL samples from infected WT mice comprised on average 40–50% neutrophils and 40–50% macrophages, whereas samples from PF and infected Rag1−/− mice comprised an average of 80–90% macrophages (data not shown).

The expression of 15 genes was determined in BAL cells taken from infected Rag1−/− but not WT mice (MIP-2, matrix metalloproteinase (MMP)-2, VEGF-A, MMP-9, TNF-α, and VEGF-C); roughly equivalent expression in cells from all types of mice (TGF-β, VEGF-B, TWEAK, VGSQ, and ephrin B2); and finally, elevated expression in infected WT compared with Rag1−/− mice (fibroblast growth factor (FGF)-2, platelet-derived growth factor (PDGF)-α, Ephrin B4, and VEGF-D). The first class of genes includes those that are likely induced in macrophages and/or neutrophils as a consequence of dissemination of the bacteria throughout the bodies of the infected Rag1−/− mice. By contrast, the third class of genes probably includes some that lie downstream of signaling in response to immune complexes. These genes are either directly up-regulated because of immune complex signaling and/or the cells that express them are more abundant in the presence of immune complexes than in their absence.

Discussion

In this study, we describe the immunological underpinnings of the dramatic vascular and lymphatic remodeling that occurs in the airways of mice infected with M. pulmonis. Remodeling failed to occur in the absence of adaptive immunity, and the data suggest that for angiogenesis in particular, remodeling required a pathogen-specific T cell-dependent Ab response. Without B cells remodeling was largely absent, whereas the transfer of serum restored angiogenesis and lymphangiogenesis to normal levels. Adaptive immunity to the bacteria promoted the accumulation of inflammatory leukocytes in the infected airways and correlated both with their activation and localized increases in the expression of gene products (such as PDGF-α, FGF-2, and VEGF-D) that...
might account directly for remodeling outcomes. Cumulatively, the data reveal a novel mechanism by which adaptive immunity to a persistent pathogen can result in chronic changes in the architecture of the airways via the involvement of inflammatory leukocytes.

Immune complexes were present in the airways of the infected mice and appeared to represent the critical trigger for remodeling. This follows from the fact that B cells and Abs were necessary for infection-dependent changes in the airways, and from the finding that vascular remodeling is significantly impaired when leukocytes are defective in their capacity to respond to immune complexes (specifically, when they lack FcR-γ expression; our unpublished observations). Thus, M. pulmonis-induced chronic lymphatic and blood vessel remodeling appears to be a consequence of immune complex-dependent inflammation and is therefore mechanistically related to type III hypersensitivity responses such as the Arthus Reaction (31).

Humoral immunity to M. pulmonis could potentiate remodeling through two nonexclusive mechanisms. The first would depend simply on increasing the number of inflammatory cells present in the airways, whereas the second would require the activation of the cells before or after their arrival. In the first case, an accumulation of leukocytes would cause remodeling if the cells constitutively generate remodeling factors (e.g., those in the second and third classes represented in Fig. 5). In the second case, immune complex-dependent signaling would lead to up-regulated expression of genes that have critical remodeling roles (such as perhaps some of the genes in the third class represented in Fig. 5).

There is evidence that leukocytes have the potential to influence remodeling through both of the above pathways. With respect to the first one, proliferative changes in the blood and lymphatic vasculature can be detected in C3H/HeJ mice 3 days after infection (32), i.e., accompanying the early recruitment of neutrophils to the airways and before T cell-dependent humoral immunity would likely make a significant impact. Similarly, as shown in this study, vascular endothelial cell proliferation was diminished but not completely absent in B cell-deficient mice. Thus, changes in the vasculature can occur without humoral immunity, although clearly they are minor by comparison to those that occur in immunocompetent mice.

The possibility that immune complex-dependent signaling might induce leukocytes to up-regulate the production of remodeling factors follows from the observation that changes in leukocyte MHC class II and CD62L levels correlated with a humoral response to the pathogen. Furthermore, PDGF-α, FGF-2, Ephrin B4, and VEGF-D all appeared to be expressed more highly by BAL cells from infected WT mice than by BAL cells from PF or infected Rag1−/− mice. Although changes in the expression of some of these genes may simply be due to differences in the composition of the BAL in the different mice, it is also possible that some of the changes are representative of alterations in leukocyte gene expression that are a direct consequence of immune complex signaling. Immune complex-dependent up-regulation of VEGF-D in BAL cells is of particular interest because blockade with soluble VEGFR-3 (the receptor for VEGF-D and VEGF-C) greatly reduces M. pulmonis-induced lymphangiogenesis (24). Furthermore, neutrophils and macrophages in infected airways appear to be a prominent source of VEGF-D (24). Additional work will be required to determine the expression of these and other candidate remodeling genes in different types of inflammatory leukocytes in BAL. It should then be possible to use genetic and other approaches to test which of the leukocyte-expressed genes are essential for remodeling to occur.

In addition to their potential to secrete factors that would act directly on endothelial cells, leukocytes may also act indirectly to cause remodeling. As an example, angiogenesis in mouse models of skin or pancreatic tumors is dependent on MMP-9, and in the skin model, the relevant source of this is inflammatory cells (33–36). Observations made in the pancreatic model, in which islet-derived VEGF-A is of crucial importance (37), indicate that MMP-9 promotes angiogenesis by causing the release of VEGF-A from the extracellular matrix (33). Although MMP-9 does not appear to be essential for M. pulmonis-induced angiogenesis (38), there is obviously precedent for invoking an indirect effect of inflammatory leukocytes on remodeling.

T cells appear to be essential for M. pulmonis-induced airway remodeling because they provide help to B cells for the production of T cell-dependent Ab isotypes. It is unclear, however, whether there are other roles for T cells in M. pulmonis-induced remodeling. Thus, through the secretion of Th2 cytokines, in particular, T cells have the potential to influence airway remodeling, in a substantial fashion (10, 39, 40). Additional experiments will therefore be required to determine whether the recruitment of T cells to the mucosa has any influence on the kinetics, magnitude, or form of the remodeling response in M. pulmonis-infected mice. Interestingly, mice lacking only αβ T cells (Tcrβ−/− mice) showed evidence of some remodeling in occasional cases (Fig. 1A), more so than was observed in mice lacking all T cells (Tcrβ−/−Tcrδ−/− mice). Mice lacking only γδ T cells, however, showed apparently normal remodeling of their airways, indicating that there was no indispensable role for these cells (data not shown). Tcrδ−/− mice generated a much impaired but nonetheless detectable IgG response to the pathogen, raising the possibility that this residual Ab response accounted for their sporadic partial remodeling phenotype (data not shown). In other settings, γδ T cells have demonstrated the capacity to provide help for B cell responses (41–43), and this might also be the case for M. pulmonis.

In summary, we show in this study that Abs specific for M. pulmonis are critical for the changes in the airway vasculature, lymphatics, and epithelium that occur in chronically infected mice. The data suggest that the remodeling syndrome is a form of immune complex-dependent inflammation, and raise the possibility that humoral immunity to other chronic respiratory pathogens, such as Chlamydia, Pseudomonas, or Mycoplasma pneumoniae could lead to similar airway pathology in humans. Moreover, the data are consistent with an expanded role for inflammation in the induction of localized angiogenesis and lymphangiogenesis.

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Disclosures

The authors have no financial conflict of interest.

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VASCULAR REMODELING IN M. pulmonis-INFECTED MICE

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