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Immunopathological airway remodeling in response to chronic infection with Mycoplasma pulmonis

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Immunopathological airway remodeling in response to chronic infection with *Mycoplasma pulmonis*

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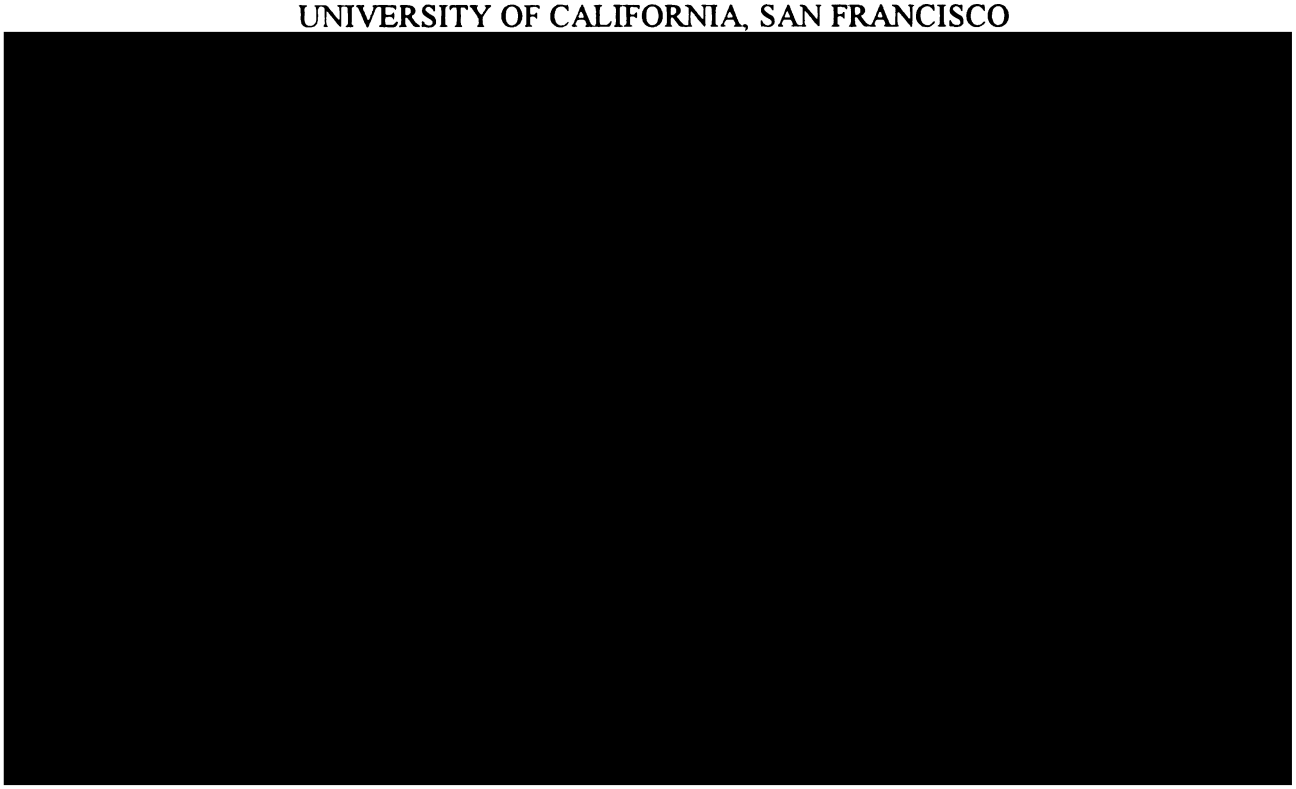

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Dong Ji Zhang contributed to much of the work presented here and in particular to Chapters 2 and 4. The following authors also contributed to the work presented in Chapter 2: Peter Baluk, Sukhvinder S. Sidhu, and Gregory M. Dolganov. The data presented in Chapter 3 was generated through collaboration between several members of the Killeen Laboratory including Nigel Killeen, Dong Ji Zhang, Sean Bell, and Joong Kyu Kim.

Immunopathological airway remodeling in response to chronic infection with *Mycoplasma pulmonis*

Arin B. Aurora

Abstract

Infection by bacteria or viruses, allergy, and/or a loss of tolerance to self-antigen can prompt an unbridled immune response that ultimately results in chronic disease. One common feature in many such immune responses is the development of chronic inflammation that causes both tissue destruction and changes or remodeling of the tissue. In many of these chronic diseases in the airway, remodeling of the airway mucosa, smooth muscle, and vasculature results in impaired airway function. While some of the soluble mediators responsible for these changes have been identified, the mechanism behind the development of the pathologic immune response remains undefined. The murine model of respiratory mycoplasmosis provides a chance to examine, step by step, the pathway starting from a required immune response to an infection and leading to tissue remodeling and disease. A genetic approach in which various strains of mice, deficient in specific components of the immune system, was used to determine which pathways contributed to tissue remodeling, specifically angiogenesis and lymphangiogenesis. By using this model, we have discovered that the humoral immune response to *Mycoplasma pulmonis* in mice is one inciting event that promotes remodeling.

The *M. pulmonis* specific antibody forms immune complexes in the airway, setting off a vicious cycle of recruitment and activation of the inflammatory cells that produce some of the key soluble mediators for remodeling. Furthermore, the recruitment and/or stimulation of the neutrophils and macrophages that make up the majority of the inflammatory cells in the airway depends on Fc receptors and is enhanced by the ability of complement to stabilize immune complexes. Finally, we begin to explore the development of T cell responses to chronic antigen in the airway by creating strains of *M. pulmonis* that express the LACK antigen. The specific T cell response to these recombinant bacteria in infected mice can successfully be detected by flow cytometry using a fluorescent peptide/MHC class II multimer.

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Chapter 1: Introduction

A. Infection and immunopathology

Chronic airway diseases like chronic obstructive pulmonary disease and asthma are caused, for the most part, by an overzealous immune response. The unrestrained response typically takes the form of chronic inflammation that is correlated with structural changes or remodeling of the airway tissue. The remodeling includes thickening and hyperplasia of airway epithelium, goblet cell hyperplasia and mucus secretion, collagen deposition and fibrosis of the smooth muscle, and vascular changes or angiogenesis. Often irreversible, these structural changes impair airway function, leading to severe illness and death. The chronic airway disease COPD (chronic obstructive pulmonary disease) describes a syndrome that can include various types of airway conditions like emphysema, bronchitis, and chronic airway obstruction. These conditions are the fourth most common cause of death in the United States after heart disease, cancer, and stroke. In addition, COPD is part of a group of five chronic syndromes that together are responsible for 75% of the health care costs in the United States. It is of the utmost interest, then, to describe the mechanisms that first instigate and then maintain the state of chronic inflammation in the airway. Understanding the ways in which inflammation contributes to tissue changes and tissue changes contribute to disease will be crucial for the development of treatments targeting the appropriate process.

Architectural changes that characterize airway remodeling are mediated by a network of cytokines

Inflammation contributes to tissue remodeling by the production of soluble factors that either directly or indirectly stimulate tissue injury or aberrant growth. Some of the major mediators that typically feature in chronic inflammatory diseases include TNFα, TGFβ, MMPs (i.e. MMP2, MMP9), IL-1, VEGF and IL-8. By overexpressing many of these cytokines in the lungs of mice, investigators have demonstrated that these mediators can have separable roles in tissue remodeling. For example, elevated levels of IL-11 are found in the bronchial epithelium of asthmatic airways [1]. This cytokine can be produced by a number of cells in the lung including epithelial cells, fibroblasts, airway myocytes, and eosinophils. Transgenic expression of IL-11 driven by the CC10 promoter in the lung leads to fibrosis of the airway wall and smooth muscle hyperplasia that causes airway obstruction and hyperresponsiveness [2, 3] IL-6 is another cytokine that induces fibrosis when expressed from a transgene controlled by the CC10 promoter in the mouse airway [4]. In contrast to IL-11, however, IL-6 does not cause mucus metaplasia, hyperresponsiveness, or infiltration of eosinophils.

Th2 cytokines also control remodeling. Activated T cells recruit leukocytes to the airway and then stimulate them to release inflammatory mediators. The production of IL-4 and IL-13 from T_H2 cells induces the production of IgE, which in turn causes mast cell degranulation and the release of inflammatory mediators. T cell-derived IL-5 also recruits and activates eosinophils and all of these recruited and activated cells in the airway continue to perpetuate the inflammation and subsequent tissue damage [5]. Transgenic experiments in which IL-5 or IL-13 were expressed in the airways from the CC10 promoter have shown that these cytokines can promote mucus secretion, fibrosis, and AHR [6] [7]. These effects occur by Th2 activation of a number of pathways. One

example is the ability of IL-13 to cause production of a number of other remodeling factors including TGFβ, VEGF, and IL-11 (see figure 1).

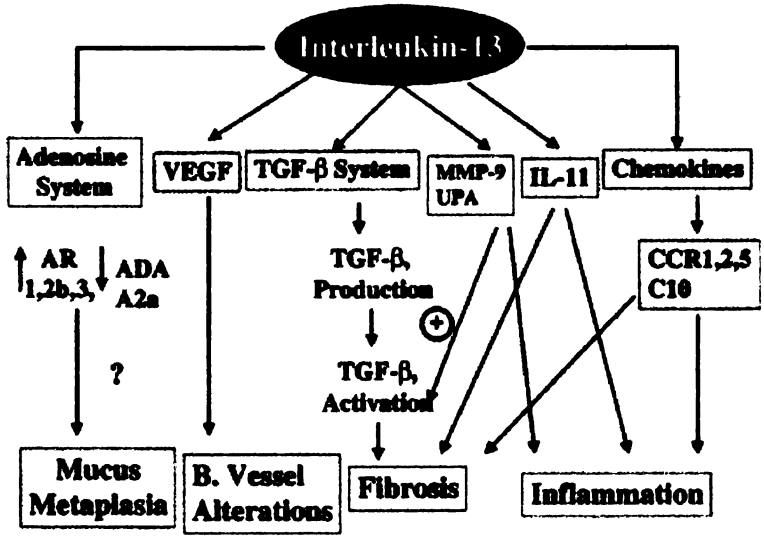

Figure 1: IL-13 contributes to tissue remodeling by initiating a number of pathways that cause specific phenotypes highlighted here.

(adapted from Elias and Zhu et al., Chest 2003 Mar;123:339S-45S)[8]

While many of the cytokines involved in tissue changes and even the cells that secrete them have been identified, the mechanisms that stimulate the initial production of Th2 cytokines in asthma and the mechanisms regulating the production of remodeling factors in other airway disease like COPD and pneumonia remain largely unknown.

Inflammation induced angiogenesis and lymphangiogenesis

Angiogenesis and lymphangiogenesis (*i.e.*, the growth of lymphatic vessels) feature prominently in asthma and airway diseases [9]. They are also, of course, key processes in tumorogenesis, wound healing, and chronic inflammatory diseases such as rheumatoid arthiritis. In all of these cases, inflammatory cells and the soluble factors they secrete contribute and, in most cases, are essential for vascular remodeling to occur.

The need for oxygen and nutrients drives tissues, reparative or pathological, to induce vessel growth to within a 100-200uM distance. Angiogenesis and lymphangiogenesis are regulated by a balance of factors that drive and inhibit vessel growth. The list for both pro-angiogenic factors and inhibitors continues to explode. Some of the mediators and their general function are summarized here by table 1. Angigogenesis can take many forms and occur by multiple mechanisms. New blood vessels can sprout and grow by proliferation of endothelial cells from existing vessels or can be formed by recruitment of endothelial precursors from the bone marrow (ref). Alternatively, existing vessels can be remodeled, physically expand, and become more tortuous. In addition to the growth of new endothelium, angiogenesis must be completed by the maturation and stabilization of blood vessels. Each of the steps in the process of vessel growth and development is regulated by different types of pro-angiogenic factors . (see table below).

Table 1: Activators of Angiogenesis (*adapted from Carmeliet and Jain, 2000)*[10]

Activators	Function
	Stimulate angiogenesis, vasculogenesis, permeability and leukocyte adhesion
Ang 1	Stabilize vessels, inhibit permeability
PDGF-BB	Recruit smooth muscle
TGFβ1, endoglin	Stimulate extracellular matrix production
FGF, HGF, MCP-1	Stimulate angio/arteriogenesis
Integrins αvβ3, αvβ5	Receptors for matrix macromolecules and proteinases
Ephrins	Regulate arterial/venous separation
MMPs, plasminogen activators	Remodel matrix, release and activate growth factors
Chemokines	Pleitropic role in angiogenesis
NOS; COX-2	Stimulate angiogenesis and vasodilation
Id1/Id3	Determine endothelial cell plasticity
AC133	Regulate angiogblast differentiation

The VEGF family of angiogenic and lymphangiogenic factors are by far the most studied, and perhaps the most prominent angiogenic factors in many settings. This family of five soluble factors signals through a family of receptor tyrosine kinases, expressed on the endothelium and some hematopoetic cells (see figure 2). VEGF-A, in particular, has emerged as a central regulator of angiogenesis in both physiological and pathological situations [10]. Expression of VEGF-A, regulated by hypoxia and cytokines, induces the initial sprouting of new vessels and increases vessel permeability. By activating several proangiogenic pathways, it induces a pleiotropic response that allows endothelial cells to proliferate, migrate, and assemble into tubes to form an interconnected network.

VEGF-B is expressed in the heart, skeletal muscle, and walls of pulmonary arteries in the adult [11]. In contrast to the haploinsufficiency of VEGF-A, VEGF-B knockout mice survive quite well but exhibit small hearts and some defects in vascular function [12]. In contrast to being regulated by hypoxia, VEGF-B can form heterodimers with VEGF-A, a mechanism thought to regulate its receptor binding and biological function [11, 13]. The function of VEGF-B has been difficult to elucidate but it has been implicated in collagen-induced arthiritis and chronic hypoxic pulmonary hypertension [14].

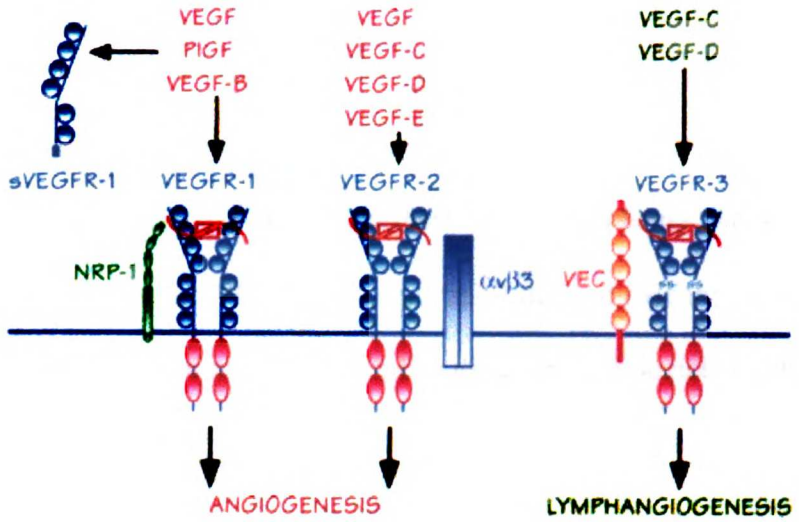

Figure 2: The VEGF family and their receptors.VEGFR-1 and VEGR-2 mediate angiogenesis while VEGFR-3 mediates lymphangiogenesis. αvβ3 integrin and VE-cadherin can complex with activated VEGFR-2.

-- *from Veikkola et al., Cancer Research, 2000*[15]

VEGF-C and VEGF-D are the only members that signal through VEGFR-3, a receptor specifically expressed on lymphatic endothelium. VEGF-C expression is not regulated by hypoxia but does have increased expression in response to pro-inflammatory cytokines. In addition to this, reports that activated macrophages can express VEGF-C and VEGFR-3 implicate this factor in inflammation. [16] [17] [18]. VEGF-D has angiogenic and lymphangiogenic properties *in vitro* and *in vivo* though its expression pattern, regulation, and physiological function is unknown [19].

Inflammatory cells are capable of secreting many pro-angiogenic factors. Activated neutrophils, macrophages and T cells can secrete VEGF-A and FGF2 [20], [21] [22] [23] Macrophages contribute to wound repair by inducing angiogenesis through the release of VEGF, FGF, and TGFβ. Furthermore, macrophages can be stimulated to make and release products like VEGF-A by neutrophil-derived oxidants at the site of injury [24]. Also, the extent of macrophage infiltration can be associated with the depth of invasion by primary melanomas. This association can be attributed to the ability of the macrophages to mediate angiogenesis [25]. VEGF expression and secretion by activated leukocytes is increased through interaction with synovial fibroblasts via integrins in the inflamed joint in rheumatoid arthritis patients [26].

In addition to their secretion of angiogenic cytokines, leukocytes also influence angiogenesis by the release of ELR+ chemokines [27]. One group has shown that MIP-2 is necessary to recruit and activate neutrophils to secrete VEGF in the *in vivo* matrigel sponge assay in a manner that depended on signaling through the Src family kinases Hck and Fgr. Furthermore, synovial macrophage derived IL-8 and TNF-a induce angiogensis in the RA joint [28]. Activated leukocytes can also mediate angiogenesis indirectly by making VEGF available from other sources such as the extracellular matrix. For example, in a mouse model of epithelial carcinogenesis, mast cell are responsible for the release of VEGF-A from the extra-cellular matrix in the skin stroma by releasing proteases such as MMP9 and activating dermal fibroblasts to do the same. The release of MMP9 and other proteases facilitates the release of VEGF-A from the extra-cellular matrix. By releasing sequestered angiogenic factors, carcinogenesis can progress from the early, wound-healing like stage to the second stage of carcinogenesis in which the

tumor can sustain its own angiogenesis [29]. The secretion of lymphangiogenic factors, VEGF-C and VEGF-D, has not been well studied to date though there is evidence that macrophages and neutrophils can produce these substances (Baluk et al., in preparation).

Correlation between infection and chronic disease

If inflammation instigates tissue remodeling such as angiogenesis, then what initiates the inflammation? Whether it is a response against a known pathogen or an autoimmune response, infections with viruses and bacteria have always been correlated with the development and exacerbation of chronic airway diseases. For example, the occurrence of a viral lower respiratory tract infection is an early risk factor for the development of childhood asthma, perhaps because the response to the virus causes an imbalance in Th1/Th2 cytokines that leads to the development of asthma. [30]. In addition, *Chlamydia pneumoniae* can contribute to the pathogenesis of severe asthma and COPD [31] [32] [33]. Also, respiratory viruses are frequently detected in asthmatics and COPD patients [34] [35]. While the correlations are numerous, the mechanisms by which an infection can instigate a chronic disease due to tissue remodeling remain a mystery.

For chronic airway disease, the murine model of respiratory mycoplasmosis offers a unique opportunity to study the process of how the immune response to an infection deviates into an immunopathological response that damages airway function through the remodeling of the airway tissue.

B. *Mycoplasma pulmonis*

General Characteristics

Mycoplasmas, the smallest self-replicating organisms, are bacteria grouped together in the class Mollicutes because of their minute size and lack of any cell wall. The genome of mycoplasmas is confined to a single chromosome and can be less than 600 kb in length. As a point of contrast, this is less than three times the size of some herpes virus genomes and about one eighth of the size of the *E. coli* genome. Because of this minimal genome, mycoplasmas can not synthesize purines, they lack the TCA cycle, and they lack the cytochrome-mediated electron transport chain[36]. Metabolic deficiencies such as these force mycoplasmas to live as parasites, gaining nutrients and biosynthetic precursors from the host cells.

In many mammals, parasitic mycoplasmas can be found living in joints and attached to the epithelial linings of the mucosal surfaces of the respiratory tract, urogenital tract, eyes, and the alimentary canal [37].

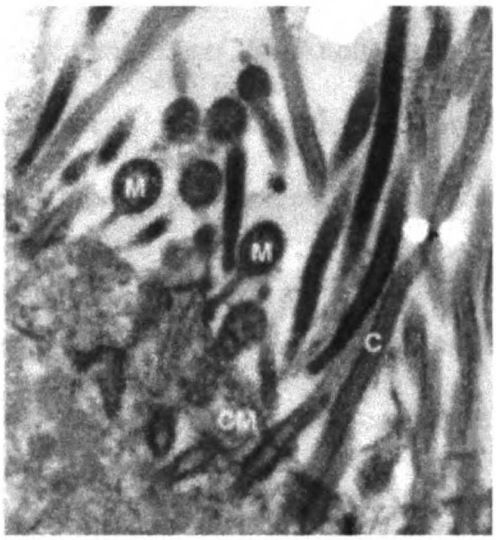

Figure 3: Electron micrograph of *M. pulmonis* attached to the epithelium in the airway.

Some species of mycoplasmas have a specialized organ or bleb structure that is used for attachment to the host cells and also for motility, but even without identifiable structures of this sort, mycoplasmas can still demonstrate adhesion. In the laboratory, mycoplasmas can be found inside cells grown in culture. It remains unclear, however, whether mycoplasma can enter epithelial or other cells in vivo. It is possible that in some cases mycoplasmas may be able to enter cells through a site-directed receptor mediated event involving clathrin-coated pits or by the specialized bleb structure they use for attachment [37].

Mycoplasma genome and genetic manipulation

The *M. pulmonis* genome, which was recently sequenced [38], is A-T rich and minimal in size at only 950kb. In spite of the small size of the genome, it contains several repetitive DNA elements. One of these repetitive elements is IS 1136, an

insertion sequence-like (IS) element, found in *M. pulmonis* that is able to transpose using an IS 1136-specific transposase[36]. Another, relatively well-studied, repetitive element in *M. pulmonis* is the *vsa* locus, a complex site-specific DNA inversion system, which consists of a series of sequences that code for the V-1 lipoprotein surface antigens. Only one of the seven genes present in the *vsa* locus can be expressed at a time and the expression is regulated by the site-specific DNA inversions that occur between a 34 base-pair sequence, called the vis box, which is conserved in each copy of the vsa gene. The ability to continually vary these surface proteins may contribute to disease by allowing mycoplasma to evade the host immune system. DNA inversions can be detected in 1-10% of *M. pulmonis* colonies grown *in vitro* suggesting that these inversion systems are very active. Because of this, individual mycoplasma colonies demonstrate considerable genetic heterogeneity, and it is usually not possible to maintain or propogate a homogenous mycoplasma culture[36, 39].

No plasmids are known to replicate in *M. pulmonis*. Transposons were therefore used to develop systems with which to engineer and modify gene expression in mycoplasmas. Tn4001 is a 4.7kb gram + transposon that can transpose into the genome of many mycoplasmas including *M. pulmonis*. In a recent paper by Dybvig et al. [40], the authors successfully expressed a lacz fusion protein in both *M. pulmonis* and *M. arthriditis* using Tn4001T, a derivitive of Tn4001 which confers a high level of tetracycline resistence.

In the body of work presented here, we have taken advantage of this system designed to engineer *M. pulmonis* to express the *Leishmania major* antigen LACK. (see Chapter 4)

Mechanisms of pathogenicity and relevance to disease

Mycoplasmas are known to be the etiologic agents that cause several human and animal diseases. In humans, respiratory infection with *M. pneumonia* is responsible for causing 20-30% of the cases of pneumonia [41] *M. pneumonia* infection has also been shown to be responsible for cases of pericarditis, peri-myocarditis, and hypertrophic cardiomyopathy [42-44].

Perhaps even more interesting, there are indications that mycoplasmas may contribute to many other human diseases. For example, *M. pneumonaie* has been found in the skin of patients with Stevens-Johnson syndrome, in the synovium of arthritic joints, and in the cerebrospinal fluids in patients with meningoencephilitis. There have also been correlations between the incidence of *M. pneumonia* infection and the incidence of juvenile arthritis. Furthermore, in a recent study, adults hospitalized for acute exacerbation of bronchial asthma were significantly enriched for simultaneous acute *Mycoplasma pneumoniae* infection [45].

M. pulmonis infection in mice and rats causes rhinitis, otitis, and bronchopneumonia. The ability of mycoplasmas to adhere to host cells is one virulence factor that determines how well the bacteria colonize the host and disrupt host cell function [37]. The pathogenicity of mycoplasma may also be attributed to the generation of hydrogen peroxide and superoxide radicals that can induce oxidative stress and host cell membrane damage. Because they are attached to the host cell, mycoplasmas can also secrete mycoplasmal enzymes such as proteases, phospholipases, and nucleases that disrupt the tissue and even contribute to chromosomal aberrations [46].

Perhaps the most pathogenic aspect of mycoplasma infection is the stimulation of the host immune response. Both the innate and adaptive immune responses are often ineffective at clearing these pathogens, leading to a state of chronic infection and chronic stimulation of the immune system. The result is an immunopathological response that causes extensive tissue remodeling at the site of infection and subsequent disease. The immune response to mycoplasma and how it causes tissue remodeling remains largely undescribed.

C. Innate and adaptive immune response to *M. pulmonis*

Immune response to *M. pulmonis*

The innate immune response plays an important role in controlling the initial infection of *M. pulmonis*, though it is not able to completely clear the bacteria. The numbers of mycoplasmas found in the lungs of experimentally infected mice depends on background strain [47] and is not dependent on the adaptive immune system [48]. During the first 72 hours of infection, the numbers of mycoplasmas decrease by greater than 83% in C57BL/6 lungs but increase by 18% in C3H mice. The maximum mycoplasmocidal activity occurs within 8 hours post-infection while appreciable numbers of macrophages, neutrophils, and lymphocytes don't accumulate until 72 hours[47]. This suggests that resident alveolar macrophages (AM) may be crucial for early mycoplasma killing and therefore that strain dependent susceptibility may be attributable to the varying ability of alveolar macrophages from different strains to clear *M. pulmonis*. In the C57BL resistant strain, alveolar macrophages were found to have increased activity compared to alveolar macrophages from more susceptible strains such as C3H[49]. In fact, AM depletion

before infection using liposome-encapsulated dichloromethylene bisphosphonate, resulted in a decrease in the numbers of mycoplasmas cleared in the C57BL lung to the level of clearance found in non-AM depleted C3H mice. Depletion in C3H mice had no effect on numbers of mycoplasma in the airway[49]. *M. pulmonis* infection leads to increased expression of MHC class II molecules on macrophages and B cells. Infection also induces the production of multiple cytokines in the lung including IL-1α, IL-1β, IL-6, TNF-α, IFN-γ, MCP-1, and MIP-1α and MIP-1β[50].

The adaptive immune response to *M. pulmonis* is also ineffective at clearing the infection as illustrated by the fact that similar numbers of bacteria can be found in the lungs of wild-type and SCID infected mice [48]. Nonetheless, lymphocytes are recruited to the infected airways where they are implicated in promoting tissue remodeling and airway disease. In C3H mice, lymphocyte numbers first increase in the draining lymph nodes and spleens of infected mice and T cell numbers peak in this tissue at day 7 post-infection. T cells (mostly CD4+) migrate to the lungs with the peak of their recruitment occurring 14 days after infection. Both IFN-γ and IL-4 production could be detected in the lungs of infected mice, suggesting that a mix of Th1 and Th2 responses was taking place [51]. Furthermore, examination of airway disease in IL-4 deficient and IFN-γ deficient BALB/c mice suggested that in the lower respiratory tract, IL-4 had no effect on disease progression while IFN-γ was important for disease severity by controlling numbers of mycoplasmas living in the airways [52]. While studies such as these just referenced provide information about the types of lymphocytes present in the airway and some of the cytokines they produce, they leave open the question of the importance of the T cell response for control of bacterial replication and remodeling.

Immune response to *M. pulmonis* infection and remodeling

Intranasal infection with the murine-tropic mycoplasma *M. pulmonis* induces a chronic airway inflammatory response and remodeling of the airway tissue that has many features in common with human asthma and chronic bronchitis. The idea that it is the host's immune response to mycoplasma infection and not the bacteria that causes disease was first put forward with the observation that infected mice lacking the combinatorial immune system (i.e. SCID mice) lacked airway disease. This observation was recently re-addressed by Cartner et al. [48] in a paper that showed that SCID mice infected intranasally with *M. pulmonis* have less severe lung disease than immune-competent controls, though both groups harbor the same titers of bacteria in their lungs. In this case, disease was assessed by scoring of severity of lesions viewed on H&E stained sections from lung. While it is clear that the adaptive immune response is necessary to promote airway disease, lymphocytes are important for preventing systemic disease. For example, infected SCID mice, which lack the pathology normally seen in the airway tissues, develop arthritis due to a dissemination of mycoplasma throughout the body and the accumulation of the bacteria in the joints where they bring induce an innate inflammatory response.

Vascular remodeling in murine mycoplasmosis features prominently and is evident in asthma and other airway diseases [53-56]. The significance of the adaptive immune response in causing airway angiogenesis in *M. pulmonis*-infected mice is readily apparent when one visually compares the vasculature from wild-type mice with that of immune deficient mice infected chronically with *M. pulmonis*. (Figure 4)[9]. Infection

with the bacteria leads to both the proliferation of new blood vessels and the remodeling of existing ones in C57BL/6 mice.

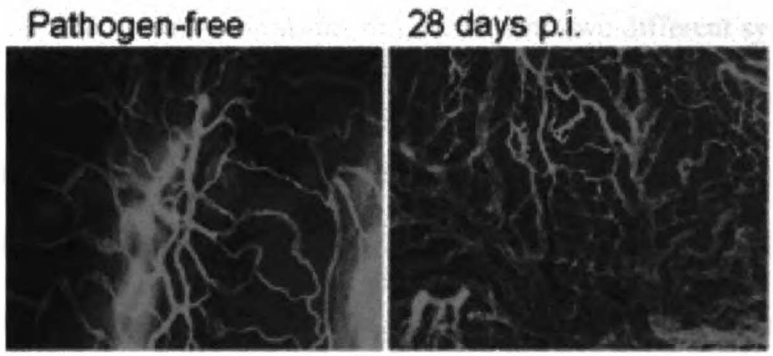

Figure 4: Images of whole-mount tracheas, flattened and stained with anti-mouse CD31 to visualize the blood vessels. The vessels in the pathogen-free airway are relatively sparse and ordered in the tissue interspersed with the cartilage rings of the trachea. After 4 weeks of *M. pulmonis* infection, the blood vessels have proliferated extensively and taken a more complex, spaghetti-like form.

The striking nature of the vascular changes observed, the ability to visualize such changes in three dimensions using tracheal whole mounts, and indications that lymphocytes are important for promoting remodeling make *M. pulmonis* infection a useful model for the study of immune regulation of angiogenesis. The initiation of remodeling by an infectious agent is of potential relevance to other human diseases such as asthma and rheumatoid arthritis where chronic encounter with a pathogen may be important for triggering or sustaining pathology.

D. Immune-complex induced inflammation

Antibodies have the ability to interact with two different systems that are involved in the inflammatory responses, the complement proteins and Fc receptors. It is these interactions that give antibodies their ability to carry out effector functions such as antibody-mediated endocytosis, opsonization, and complement activation. Immune complexes can interact with effector cells directly through Fc receptors or indirectly by binding to and activating complement. Both the isotype of the antibodies and the form in which they persist in the body dictates the type of effector functions they can mediate. The purpose of this section will be to review the pathways by which antibodies, specifically in the form of IgG or IgM immune complexes, and complement induce and regulate inflammation.

Complement and IgM mediated responses

Two major functions of complement are host protection and inflammation, and the regulation of B cell adaptive immunity. Complement is essential in the innate response to bacterial pathogens and participates in clearing infections by direct lysis via the membrane attack complex (C5b-C9), opsonization by C3b and iC3b, and activation and chemotaxis of neutrophils by C3a and C5a. However, by these same mechanisms, complement can also induce tissue damage.

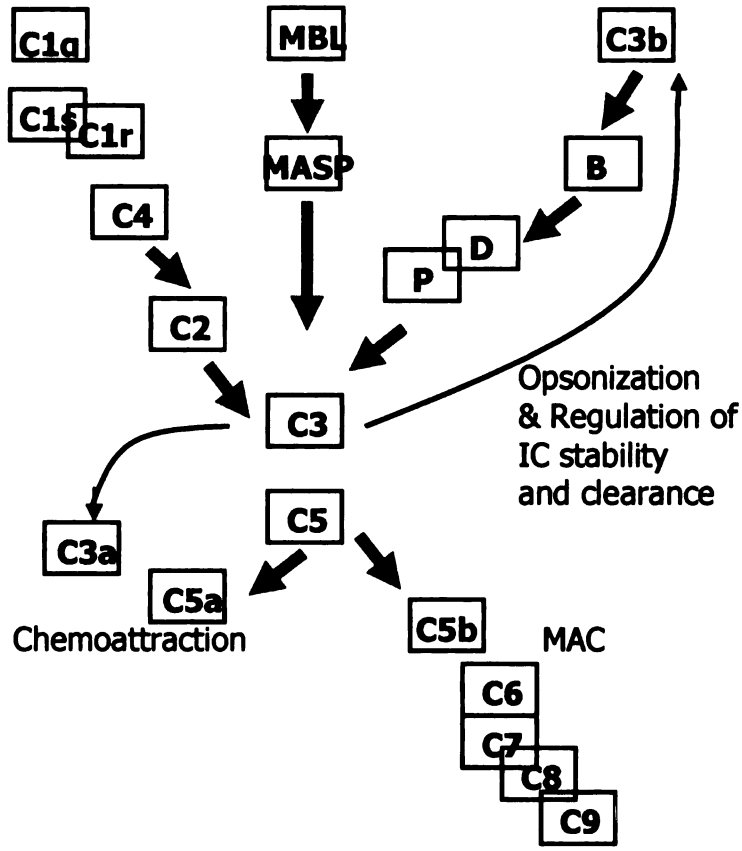

Figure 5: An overview of the complement pathway and the relative order of events occurring from initiation of the pathway to the effector functions.

The acute response to a bacterial infection depends on C3, C4, and natural antibody or secreted IgM. The requirement for the classical complement pathway, C3 and C4, was shown by the increased sensitivity of C3 and C4 knockout mice to streptococcal infection, LPS, and enterococci exposure in a model of cecal ligation and puncture (CLP) [57-59]. The CLP model was further used in mice expressing surface but not secreted IgM to show that bacterial immunity was also dependent on secreted natural antibody[60]. This secreted IgM is necessary for specific recognition of the pathogen and subsequent binding and activation of complement. The C3 coated bacteria then directly

activate mast cells to release TNF-α from their granules. TNF-α upregulates expression of E-selectin and P-selectin on endothelium. Neutrophils then infiltrate the tissue site followed by bacterial clearance. A similar pathway can also lead to inflammation induced injury. Reperfusion of ischemic vessels, which occurs in trauma, myocardial infarction, and stroke, leads to a revealing of new antigens that can be recognized by IgM. IgM then activates complement to form the MAC on the endothelium, leading to injury.

Complement can also regulate the adaptive immune response by affecting the localization of antigens to dendritic cells and by enhancing antibody production by B cells. The classical pathway of complement activation can enhance antibody production by signaling through the CR2 receptor complex (includes CR1 and CR2) on B cells. This process is dependent on IgM to activate complement [61, 62]. Additionally, dendritic cell expression of CR1 and CR2 is necessary for optimal maintenance of both antibody-secreting plasma cells and memory B cells [63].

IgG-mediated responses

Two general categories of IgG mediated inflammation exist. In type II inflammatory responses, fixed, cytotoxic antibodies can activate the cell types that cause cytotoxicity by engaging Fc receptors and the C3b receptor. These cells, which include NK cells, neutrophils, eosinophils, and monocytes/macrophages, then have an increased capacity to carry out ADCC, phagocytosis, and cytokine production. Cytotoxic IgG can also directly activate both early and late components of the complement pathway, leading to the formation of the membrane attack complex and direct lysis of the target cell.

The second class of IgG mediated inflammation, known as type III, involves the formation and deposition of soluble IgG complexes followed by distinct cellular responses exemplified in the Arthus reaction, a classic model of cutaneous IgG complex induced injury. Until the advent of gene-targeted knockout mice, it was generally thought that the Arthus cellular responses of edema, hemorrhage, and neutrophil influx were induced through the binding and activation of the components of the classical complement pathway. Activation of complement lead to the formation and release of chemotactic peptides leading to neutrophil influx, degranulation and finally tissue damage.

The above model was challenged in 1994, when showed that mice deficient in the common γ subunit of the Fc receptor were unable to initiate the cutaneous Arthus reaction[64]. While this showed a necessity for Fc receptors in the response, it was not until 1996 that the authors ruled out both a synergistic role and proximal role for complement in the Arthus reaction, showing that Fc receptors alone were sufficient to elicit the inflammatory response to soluble immune complexes [65]. Importantly, mice deficient in specific components of the complement system, either C3 or C4 were examined for their ability to mount a normal type II and type III inflammatory response. The results indicated that the complement-deficient mice developed thrombocytopenia, autoimmune hemolytic anemia (type II reactions), and a reverse passive Arthus reaction (type III response) that was indistinguishable from the response in wild-type mice. In contrast, FcR-γ deficient mice were resistant in the formation of both types of IgG-mediated inflammation.

Now that a clear role for Fc receptors had been defined, the same authors went on to define the cell type and specific Fc receptor responsible for mediating the Arthus reaction. The previous observation that mast cell deficient mice, *W/W^v*, exhibited an attenuated Arthus reaction lead Sylvestre and Ravetch to focus on the FcγRIII on mast cells. They found that reconstitution of *W/W^v* mice with wild-type mast cells but not $\gamma^{-/-}$ mast cells allowed for initiation of the cutaneous Arthus reaction. Interestingly, the partial Arthus reaction observed in *W/W^v* mice compared to the complete absence of the response in FcR-$\gamma^{-/-}$ mice suggested that an additional FcγR bearing cell is involved.

Fc receptors structure and function

Fc receptors are part of the immunoglobulin supergene family and can be divided into two main categories, activating and inhibitory receptors. The activating receptors, FcγRI, FcγRIII, and FcεRI are composed of a ligand-specific alpha chain and an associated gamma chain which contains the ITAM signaling domain. The inhibitory receptors are single chain molecules with extracellular domains that are homologous to their activating counterparts. The cytoplasmic tails contain a LxYxxL inhibitory sequence that functions only when co-ligated with an ITAM-containing receptor by recruitment of the phosphotase SHIP. FcgRIIB is the most widely expressed FcR and can be found on all hematopoetic linages except for RBCs and NK cells.

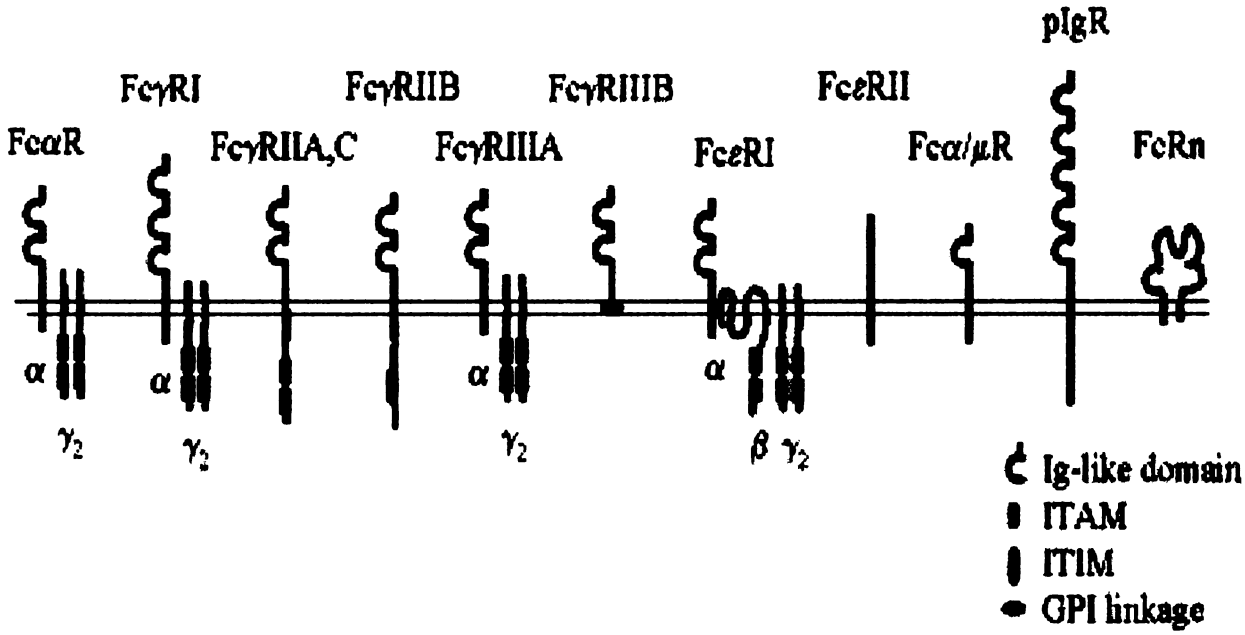

Figure 6: Schematic depiction of Fc receptors. Most Fc receptors (FcRs) contain immunoglobulin-like (Ig-like) domains in the extracellular portion of their αchain. Thus, they belong to the Ig-superfamily and signal to the inside of the cell trough an immunoreceptor tyrosine-based activation motif (ITAM). FcγRIIB contains an immunoreceptor tyrosine-based inhibitory motif (ITIM). FcγRIIIB is expressed only on neutrophils and is anchored to the cell membrane by a glycosidylphosphatidylinositol (GPI) linkage.

--from Ortiz-Stern and Rosales, Immunology Letters (2003)[66]

FcgRI is the high affinity activating receptor and binds monomeric IgG while FcgRII and FcgRIII the low affinity inhibitory and activating receptors respectively bind immune complexes. These three receptors share structurally related ligand binding

domains but have different transmembrane and intracellular domains. The differences in the intracellular domains dictate the different signaling pathways that are activated upon ligation. Both the activating and inhibitory receptors are co-expressed on the same cells, and it is the balance of expression of this pair of receptors that ultimately determines the cellular response. Regulation, then, of the relative expression levels of the activating and inhibitory receptor is an important means to modulate the immune response.

Triggering of activating Fc receptors, found on monocytes, macrophages, neutrophils, mast cells, and NK cells, can lead to a variety of cellular responses such as degranulation, phagocytosis, ADCC, and transcriptional activation and release of cytokines. The inhibitory receptor is the only FcR expressed on B cells but is also found on the same cell types that express the activating FcR. Signalling through the ITIM motifs in this receptor lead to a block of calcium mobilization and all cellular processes dependent on it. The inhibitory receptor also induces apoptosis in B cells through homo-typic interactions and aggregation on the surface, a mechanism for maintaining B cell tolerance in the periphery[66].

Resurfaced importance of complement in Arthus reaction

Though clearly distinct and independent roles were demonstrated for complement in response to bacterial infection and Fc receptors in the induction of the cutaneous Arthus reaction, others have been able to show evidence for a more complex situation in which both Fc receptors and complement can be involved in type III responses. Ravetch first showed that DBA mice, deficient in C5, were able to mount a normal cuntaeous Arthus reaction, suggesting no role for this complement protein in IC induced inflammation [65].

Others, however, had shown a dependence on C5 for inflammation in the lung and peritoneum [67, 68]. Hopken and Gerard went on to show that C5aR KO mice on the B6 background had a significantly impaired Arthus reaction in the skin, lung, and peritoneum[69]. These types of observations led to the suggestion that complement could also be involved in type III responses, that there were tissue-specific requirements for FcRs and complement, and that genetic background could influence the contribution of these two systems.

In 2000, Baumann et al. was able to demonstrate that both FcRs and C5aR were necessary for the Arthus reaction in both the skin and lungs but that there were tissue specific differences in the requirement for inducing specific aspects of the inflammatory response. For example, plasma exudation was dependent on both C5aR and FcgR in the skin and only on C5aR in the lung[69]. The same group went on to show that in the skin, C5a/C5aR activation leading to an Arthus reaction could occur in the absence of C3 while in the lung C3 is necessary to promote TNFa production and neutrophil chemotaxis [70]. The dependence of C5 in the Arthus reaction is not suprising when one considers that C5aR activation on cells like neutrophils, monocytes/macrophages, eosinophils, or activated B cells can lead to increased vascular permeability, chemotaxis and influx of neutrophils, and production of proinflammatory cytokines such as TNFα, IL-8, IL-6, and MIP-2 [71]. Also, C5a (and C3a) can be generated locally in tissue directly by proteases released by activated macrophages, neutrophils, and epithelial cells [72]. This may explain why deficiency of C3, the central regulator of the complement cascade, does not lead to impairment of the Arthus reaction. Furthermore, local production of C5a in a model system of immune-complex induced lung disease was shown to directly modulate

the expression levels of the FcgrIII and FcgrII activating and inhibitory receptor pair on macrophages[73] providing a satisfying mechanism by which the contribiution of complement to immune-complex-dependent inflammation could be routed through Fc receptors.

The above examples highlight some of the ways in which Fc receptors and complement, particularily C5a/C5aR, are linked together in generating type III inflammation. That these pathways coordinate to cause inflammatory disease is apparent in several model systems. In some cases, complement and Fc receptors may play redundant and alternative roles. For example, in an antibody-dependent model of autoimmune vitiligo, hypopigmentation could occur in $C3^{-/-}$ mice or FcgR $\gamma^{/-}$ mice but not in FcgR $\gamma^{/-}$ $C3^{-/-}$ double deficient mice[74]. In one model of autoimmune induced rheumatoid arthritis, the KBN TCR transgenic mouse, the onset of disease depends on both signaling through Fc receptors on mast cells and on the alternative pathway of complement [75, 76]. Complement had an additional role in C3b stabilization of immune complexes and C5a/C5aR recruitment and activation of neutrophils [75].

Concluding Remarks:

The murine model of respiratory mycoplasmosis offers a chance to study both the immune response to a chronic infectious agent and a mechanism of immunopathology in chronic airway disease. The complexity of the system reflects many of the complexities occurring in human disease such as the interaction between the innate and adaptive immune system, chronic inflammation, and a progression of tissue changes that result in disfunction of the organ and disease. As a result of these complexities, the work presented here will address how a chronic bacterial infection in the airway leads to tissue remodeling, and specifically vascular remodeling, dependent on lymphocytes.

Chapter 2:

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

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A. Abstract

Chronic inflammation in the airways is associated with dramatic architectural changes in the walls of the airways and in the vasculature they contain. Here 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 antibody 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 VEGF-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.

B. Introduction

The entry of leukocytes into tissues during inflammation is essential for pathogen control and wound healing. Inflammation can, however, cause significant damage to tissues and organ systems especially when it develops into a chronic condition. For example, chronic inflammation lies at the heart of diseases such as rheumatoid arthritis, ulcerative colitis, and asthma.

Chronic exposure to leukocytes and the mediators they release can lead to major structural changes in tissues. These can include the destruction of bone and cartilage in

rheumatoid arthritis and a significant thickening of the colonic mucosal epithelium in colitis. Chronic inflammation in the airways is also associated with many such changes that collectively can be termed 'airway remodeling'. In addition to a thickening and reorganization of the epithelial barrier, remodeling events include proliferation of submucosal glands, hyper-secretion of mucus, subepithelial fibrosis, and substantial growth of both blood and lymphatic vessels [9, 77-79]. As a consequence of these changes there is diminished airway function and life-threatening hyper-responsiveness 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 contribute to specific forms of airway remodeling[8, 80]. For instance, cytokines from T_H2 cells, such as IL-4, IL-5, IL-9 and IL-13 can induce epithelial cell proliferation, fibrosis, and mucus secretion to differing extents. It is unclear, however, whether all of these cytokines exert critical remodeling effects in the context of chronic airway infections or other airways disases.

Although they are prominent aspects of airway remodeling, there is little mechanistic understanding of what induces angiogenesis and lymphangiogenesis in chronically inflamed airways. These processes can be readily visualized in mice infected with the respiratory pathogen *Mycoplasma pulmonis*, which is a bacterium that establishes a chronic airway infection in mice. *M. pulmonis* bacteria attach themselves to the epithelial cells lining the alveolar spaces and airways and they replicate prolifically there despite a vigorous host inflammatory and adaptive immune response against them[48, 51, 81-83]. Over time after infection, the inflamed airways manifest the type of

remodeling symptoms mentioned above including substantial growth of new blood vessels, the enlargement of pre-existing blood vessels and considerable expansion of the local lymphatic network.

In this paper 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 antibody response to exacerbated inflammation and consequently vascular and epithelial remodeling. We show that inflammatory leukocytes in the airways are activated by humoral immunity to the bacteria and that this activation correlates with the expression of genes relevant to remodeling. Thus the data reveal a novel pathway that leads from immunologic recognition of an airway pathogen to inflammation-associated airway remodeling featuring localized induction of blood and lymphatic vessel growth.

C. Methods and Materials

Animals

C57BL/6J, C57BL/6-*Igh-6 tm1Cgn* (referred to here as $Ig\mu^{MT/MT}$), C57BL/6J-*Rag1 tm1Mom* ($Rag1^{-/-}$), C57BL/6-*Tcrb tm1Mom Tcrd tm1Mom* ($Tcr\beta^{-/-}$ $Tcr\delta^{-/-}$) mice were obtained from 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 experimental mice were 6-12-weeks-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 control mice were housed separately.

Mycoplasma 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). The cultures were grown in broth and frozen in 1mL aliquots as previously described [84]. Mice were anesthetized (87 mg/kg ketamine and 13 mg/kg xylazine intramuscularly) and given *M. pulmonis* intranasally. The dose of organisms varied from strain to strain due to differences in background susceptibility, but ranged from 10^4 -10^6 cfu in a total volume of 50µL/ mouse.

Immune serum came from the blood of female C57BL/6 mice that had been infected with *M. pulmonis* for at least two weeks. Sera from multiple bleeds was pooled and tested for its *M. pulmonis* reactivity by ELISA before use. Serum injections were given intraperitoneally (200-250uL total volume/injection) beginning at day 3 post-infection and every third day thereafter until the mice were analyzed.

Perfusion and whole mount staining of airway vasculature

Lectin staining followed by perfusion in order to visualize blood vessels in the trachea was performed as previously described[85]. In brief, biotinylated *Lycopersicon esculentum* lectin (100µg in 100µL, Vector Laboratories), which binds to the luminal endothelial cell surface, was injected intravenously 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-DAB substrate. The

tracheas were then flattened and mounted with Permount (Fisher) before imaging the stained vasculature with a Leica DMLB microscope (5x objective) using 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 pathogen-free mice were first perfused with 1% paraformaldehyde. The tracheas were removed and fixed for an additional 1 hour in 1% paraformaldehyde in PBS pH 7.4. They were then stained overnight with hamster anti-mouse VEGFR-3 (Chemicon) and rat anti-mouse CD31 (Pharmingen). Washing was followed by staining with a Cy3-conjugated goat anti-hamster Ig secondary antibody (Jackson ImmunoResearch) and FITC anti-rat Cy2 (Jackson ImmunoResearch). Tissues were washed, fixed for 1 minute in 4% PFA and then mounted and coverslipped using Vectashield mounting media for immunofluorescence (Vector). 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 Corp. (South San Francisco, CA) for paraffin embedding, sectioning, and costaining with hematoxylin-eosin and the periodic acid-Schiff stain. The stained sections were subsequently examined using a Nikon Eclipse E600 microscope, and photographed with a Zeiss Axioxcam camera using AxioVision 3.0 software. Epithelial remodeling in the tracheas was quantified 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 in order to calculate

the mean thickness of the epithelial layer in different types of mice. Measurements were taken from at least 3 different mid-tracheal 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 antibodies in sera collected 4 weeks after infection. ELISA plates (Nunc) were coated overnight at 4°C with 50 µl/well of carbonate-bicarbonate buffer (0.05M, pH 9.6) containing 10^5 CFU of lysed *M. pulmonis* bacteria. The plates were subsequently blocked with PBS containing 1% BSA for 2 hours at room temperature before incubation with serially diluted sera for 4 hours 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 Biotechnology Associates Inc., Birmingham, Ala. and CalTag Laboratories, Burlingame, CA). The wells were washed another four times before applying alkaline phosphatase-conjugated streptavidin for 45 min at 37°C. After further washes, ρ-nitrophenyl phosphate was added to the plate wells. Finally, the absorbance at 405nm 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 two weeks before counting colonies.

BrdU labeling and immunofluorescence

Bromodeoxyuridine (Sigma) 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. 7μm thick sections were cut using a cryostat and fixed for 10 minutes in cold acetone before staining. Endothelial cells in the sections were identified by staining with rat anti-mouse CD31 (BD-Biosciences) followed by Cy2-conjugated donkey anti-rat Ig (Jackson Immunoresearch). The sections were permeabilized and stained with biotinylated anti-BrdU (Zymed) followed by Cy3-conjugated strepavidin (Jackson Immunoresearch). Sections were analyzed under UV illumination using a Nikon Eclipse E600 microscope equipped with a Zeiss Axiocam camera, and AxioVision software. In order to count $BrdU^+CD31^+$ endothelial cells, a series of images were taken around the entire circumference of each tracheal section using 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 mid-trachea from three or more mice of each genotype were analyzed for each experiment. The significance of differences between groups was assessed using the Student's T-test.

For IgG immune-complex detection, 7μm thick sections were cut from tracheas embedded in OCT and stored at -80°C. Sections were dried, fixed in cold acetone for 10 minutes and stained with Cy2-conjugated anti-mouse IgG (Jackson ImmunoResearch).

The sections were then analyzed with UV illumination using a Nikon Eclipse E600 microscope equipped with a Zeiss Axiocam camera, and AxioVision software.

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

Single cell suspensions from trachea 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 Biochemicals) and DNAase type I (Sigma) were added to a final concentration of 1.5mg/mL and 10μg/mLrespectively. The samples were incubated for 20-30 minutes at 37°C before adding 5mL of PBS containing 0.1% BSA and 1mM EDTA, filtering through 70μm 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 antibodies specific for Gr-1, CD11b, CD62L and I-A^b (from BD-Biosciences, eBiosciences and CalTag Laboratories). Flow cytometry data were analyzed using FlowJo software (Tree Star Inc.).

Bronchoalveolar lavage (BAL) and RNA preparation

After anesthesia (87 mg/kg ketamine and 13 mg/kg xylazine intramuscularly), The tracheas of anesthetized mice were surgically exposed, cannulated using a 20 gauge catheter and then the lungs were flushed five times with 0.8ml 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) for

extraction of RNA. The RNA was quantified using an Agilent Bioanalyzer 2100 and analyzed using a multiplexed real-time PCR assay as previously [86].

D. Results

Immunological control of Mycoplasma pulmonis-induced airway remodeling

Three of the major features of *M. pulmonis*-induced airway remodeling in mice are angiogenesis, lymphangiogenesis and a general thickening and reorganization of the mucosal epithelial barrier. Mouse tracheas can be readily examined by staining 10μm-thick cross-sections or by cutting them longitudinally before staining them flattened in 'whole-mounts'. Whereas the former are useful for visualizing infection-dependent changes in the epithelial layer and its substrata, the latter are particularly appropriate for detecting localized angiogenesis and lymphangiogenesis. Here, we have employed two types of whole-mount staining approaches to detect blood and lymphatic vessels. The first depends on perfusion with a biotinylated lectin (*Lycopersicon esculentum*) that stains blood vessels but not lymphatic vessels[85] (Figure 1a). The second involves staining with monoclonal antibodies specific for vascular (anti-CD31; Figure 6) and/or lymphatic endothelium (LYVE-1; Figure 6 or anti-VEGFR3).

Tracheal angiogenesis in infected mice is evident both as the formation of new blood vessels and the enlargement of existing ones (Figure 1a). This vascular remodeling begins within days following infection, and persists chronically because the bacteria are typically not cleared from the airways [81, 87, 88]. At late time points (*i.e.*, one month or more after infection) the tracheas of infected wild-type C57BL/6 background mice show complex growth and reorganization of the vascular beds with a spaghetti-like interlaced

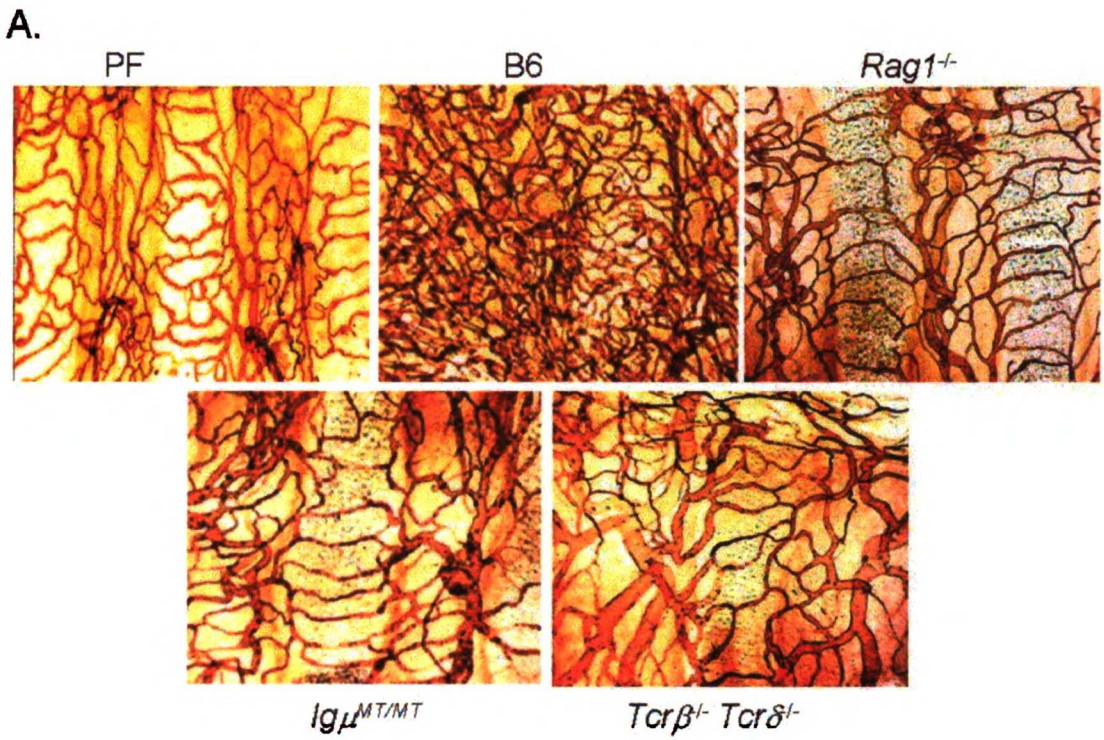

Figure 1 Involvement of the adaptive immune system in mucosal remodeling in *Mycoplasma pulmonis*-infected mice.

A. Angiogenesis induced by Mycoplasma pulmonis infection in wild-type and mutant mice at 4 weeks post-infection. The airway vasculature in infected C57Bl/6, *Rag1*$^{-/-}$, *Tcrβ*$^{-/-}$*Tcrδ*$^{-/-}$, and *Igμ*$^{MT/MT}$ mice and pathogen-free C57BL/6 mice was visualized in tracheal whole-mount preparations by perfusion of mice with biotinylated Lycopersicon esculentum lectin followed by staining tracheas with horse radish-peroxidase conjugated streptavidin and the DAB substrate. Images were taken using a 5x objective.

pattern of small vessels connecting larger ones. This increased complexity contrasts with the simpler pattern of vessels that is uniformly present in uninfected tracheas.

Changes in the tracheal lymphatic network are even more striking than those in the blood vessels. Whereas lymphatic vessels are normally absent from the cartilaginous rings in the trachea, infection with *M. pulmonis* causes substantial invasion of new lymphatic vessels into the rings (Figure 6 and data not shown). The vascular and lymphatic remodeling are both accompanied by inflammation and a characteristic thickening and differentiation of the mucosal epithelium (Figure 1b), which in turn would be expected to lead to impaired airflow.

All forms of tracheal remodeling brought on by *M. pulmonis* infection are absent or greatly reduced in *Rag1*$^{-/-}$ mice (Figure 1a and data not shown reinforcing prior indications [48, 89] that the adaptive immune system is important for the induced airway pathology. To gain insight into how the immune system regulates blood vessel remodeling in particular, we initially infected mice that lacked either T cells [90]or B cells [91]. Mice lacking all T cells due to homozygosity of null mutations in the genes encoding both TCRβ and TCRδ showed an absence of vascular remodeling indicating that T cells are required for this to occur (Figure 1a). Similarly, we found that mice lacking B cells were defective in angiogenesis and their tracheal vasculature reproducibly resembled that of pathogen-free or infected *Rag-1*$^{-/-}$ mice (Figure 1a and data not shown). Despite the absence of vascular remodeling, both types of mice were clearly infected with the organism because bacteria could be readily recovered from their lungs (Table 1). Consistent with previous work showing that antibodies prevent systemic dissemination of

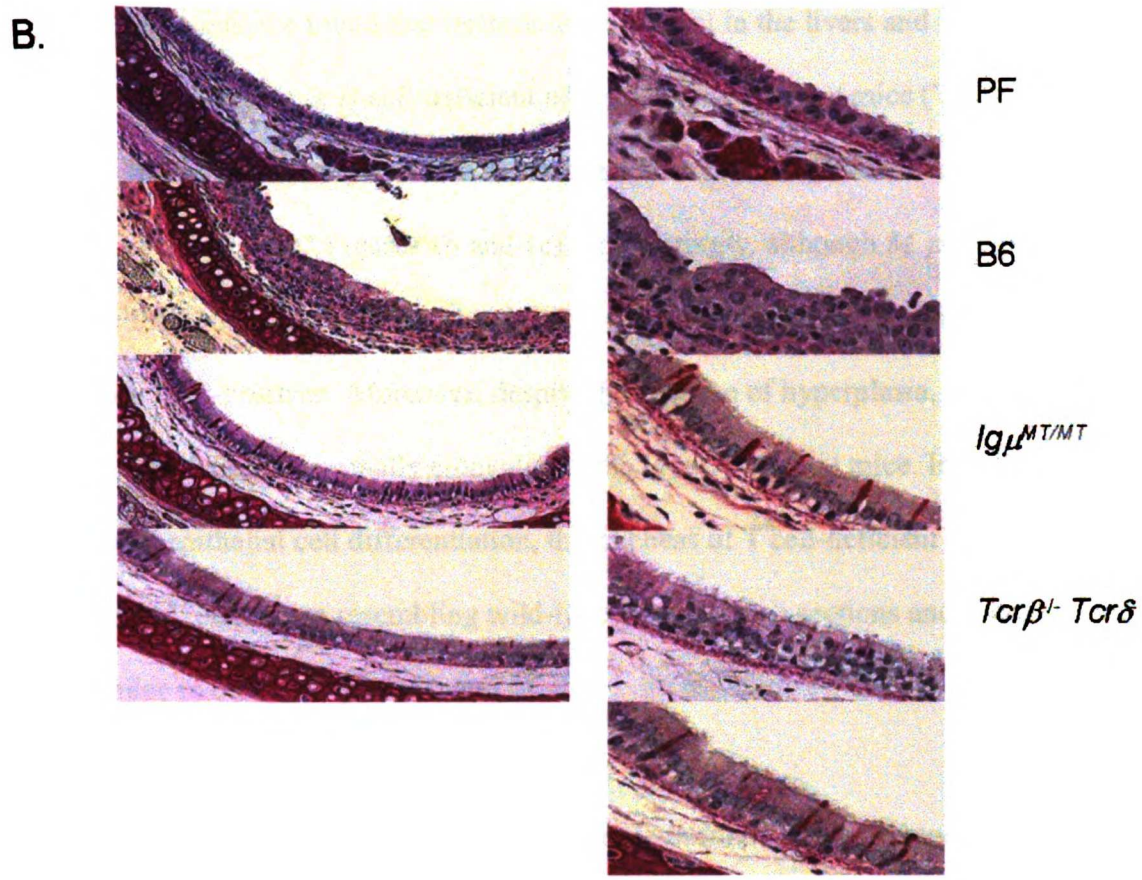

Figure 1 Involvement of the adaptive immune system in mucosal remodeling in *Mycoplasma pulmonis*-infected mice.

B. Remodeling of the airway epithelium induced by *Mycoplasma pulmonis* infection in C57Bl/6, $Ig\mu^{MT/MT}$ and $Tcr\beta^{-/-}Tcr\delta^{-/-}$ mice. Tracheas from mice that had been infected for 4 weeks were fixed, sectioned and stained with H&E and PAS. Images were photographed using 20x and 40x objectives.

M. pulmonis, we found that bacteria were present in the livers and kidneys of infected T cell-deficient or B cell-deficient mice, but not wild-type mice (Table 1).

Epithelial cell hyperplasia was evident in the tracheas of infected wild-type but not B cell-deficient mice (Figures 1b and 1c). Interestingly, although *M. pulmonis* did not induce PAS positive mucin in the wild-type mice, infected B cell-deficient tracheas were clearly PAS-positive. Moreover, despite the absence of hyperplasia, the epithelial layer was nonetheless abnormally elongated in the B cell-deficient mice. In both mucus secretion and epithelial cell differentiation, the tracheas of T cell-deficient mice showed an intermediate phenotype resembling wild-type mice in some sections and B cell-deficient mice in others (Figures 1b and c).

Antibodies are required for Mycoplasma pulmonis-induced airway remodeling

Mice lacking T cells are deficient in both T cell responses and also T cell-dependent antibody responses [92]. Thus, the defective remodeling in these mice could have resulted from either a specific requirement for T cells in remodeling, or alternatively, the absence of a pathogen-specific T cell-dependent antibody response. Infected wild-type mice make a strong antibody response to *M. pulmonis* that can be detected in the first week of infection (Figure 2). Mice lacking T cells failed to produce IgG in response to the pathogen, although they did have *M. pulmonis*-specific IgM in their sera. These data are consistent with a T cell-dependent antibody response being required for the airway changes.

To test for the specific involvement of pathogen-reactive antibodies in remodeling, we infected B cell-deficient mice with *M. pulmonis* and then attempted to

C.

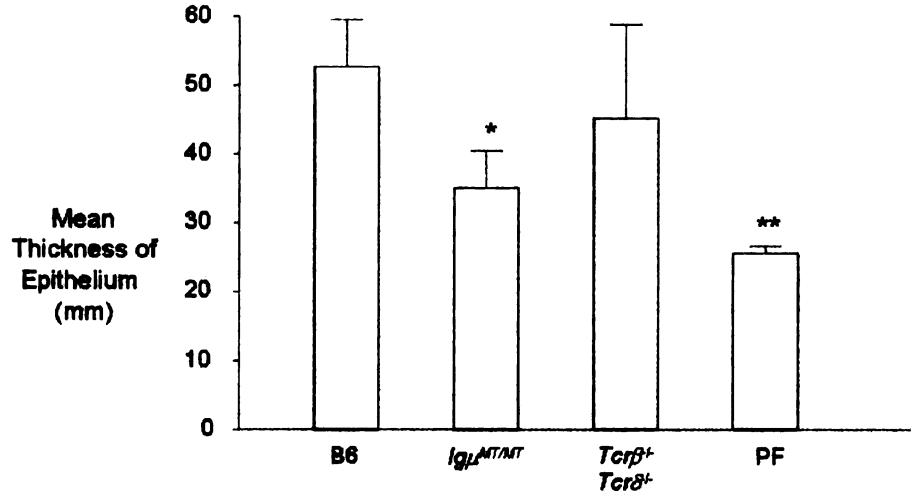

Figure 1 Involvement of the adaptive immune system in mucosal remodeling in *Mycoplasma pulmonis*-infected mice

C. Epithelial remodeling after 4 weeks of infection was quantified by measuring the thickness of the epithelium in tracheal sections. Four mice were analyzed for each group. Statistical significance was determined by the Student's T-test (* $p<0.02$, ** $p<0.002$ compared to B6).

Table 1 Recovery of Mycoplasma pulmonis from Organs of Infected Mice

	Mycoplasma pulmonis (Log_{10}CFU)		
Strain	Lung	Liver	Kidney
C57BL/6	7.1 ± 0.1	-	-
Igμ$^{MT/MT}$	8.0 ± 0.2	6.2 ± 0.2	6.2 ± 0.1
Tcrb$^{-/-}$*Tcrd*$^{-/-}$	7.5 ± 0.1	4.4 ± 0.2	4.1 ± 2.0
Pathogen-free	-	-	-

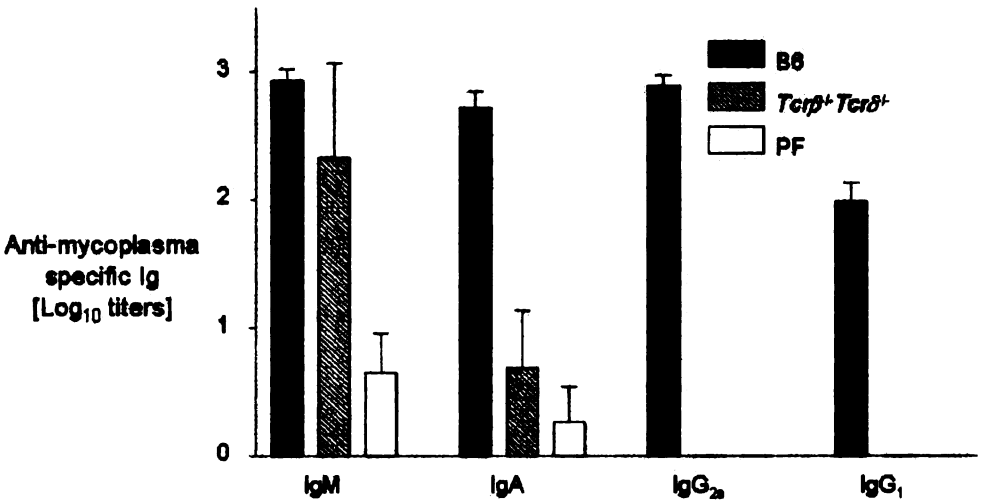

Figure 2 ***Mycoplasma pulmonis*-specific antibody responses in infected wild-type and mutant mice.**

Titers of *Mycoplasma pulmonis*-specific IgM, IgA, IgG2a, and IgG1 were measured by ELISA using blood from infected mice of the indicated genotypes at 4 weeks after infection. Four mice were analyzed for each group. (Black bars: C57Bl/6; gray bars: $Tcr\beta^{-/-}Tcr\delta^{-/-}$; clear bars: pathogen-free)

rescue tracheal angiogenesis by providing them with serum pooled from infected wild-type mice. Serum was given to the mice every third day starting at day 3 after infection. To monitor angiogenesis in a quantitative fashion, we gave the mice bromodeoxyuridine (BrdU) continuously in their drinking water. We then detected endothelial cells that had undergone proliferation during the labeling period by immunofluorescence microscopy using antibodies specific for CD31 and BrdU-containing DNA. This procedure consistently informed on the remodeling that occurred in wild-type, but not *Rag1*$^{-/-}$ or *Igμ*$^{MT/MT}$ mice (Figure 3a and data not shown).

As shown in Figure 3b, immune serum could effectively rescue tracheal angiogenesis in the infected B cell-deficient mice. Such data show that antibodies can substitute for B cells in *M. pulmonis*-induced remodeling and suggest that the identified contribution of T cells to remodeling (Figure 1) may be primarily to provide help for the antibody response.

Kinetics and Form of the Inflammatory Response to M. pulmonis

The persistent replication of *M. pulmonis* organisms and the vigor of the humoral response against them predicted that immune complexes would be detectable in the airways of infected mice. Consistent with this, there was deposition of antibody (Figure 4) and C3 (data not shown) in the tracheas of infected wild-type but not pathogen-free mice. Similarly, antibody deposition could be detected in the tracheas of infected B cell-deficient mice but only if they had previously been infused with serum from infected wild-type mice.

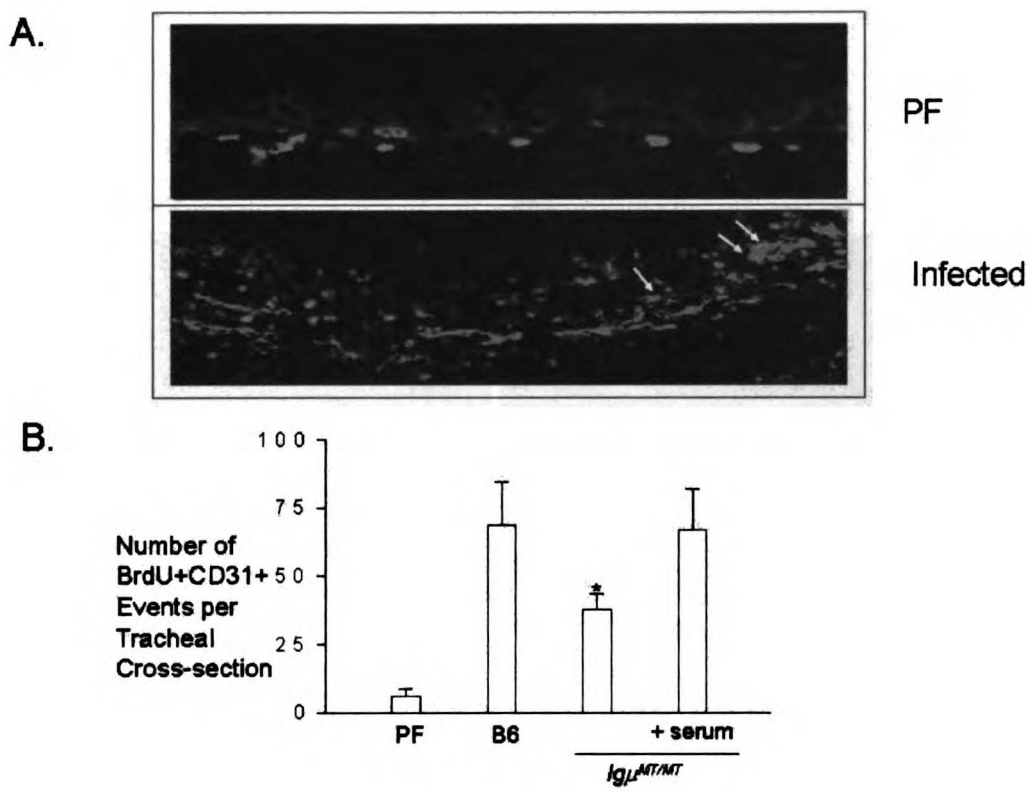

Figure 3 Passive transfer of serum from Mycoplasma pulmonis-infected wild-type mice restores remodeling in infected B cell-deficient mice.

A. Angiogenesis detected as the incorporation of BrdU into $CD31^+$ endothelial cells. CD31 and BrdU staining are shown in green and red respectively, with yellow representing double-labeled cells. The arrows identify representative $CD31^+BrdU^+$ cells. Inflammatory cells could be excluded from the analysis because of their location in the sections, and because they express CD31 at much reduced levels relative to endothelium. Images were photographed using a 20x objective.

B. Angiogeneis induced by the passive transfer of *Mycoplasma pulmonis*-immune serum into B cell-deficient mice. The graph shows the number of $BrdU^+CD31^+$ cells detectable eleven days after *Mycoplasma pulmonis* infection in wild-type, B cell-deficient and serum-infused B cell-deficient mice. Three or more sections from mice (three to four mice per group) of each genotype were analyzed (* $p < 0.03$ compared to $Ig\mu^{MT/MT}$ + serum).

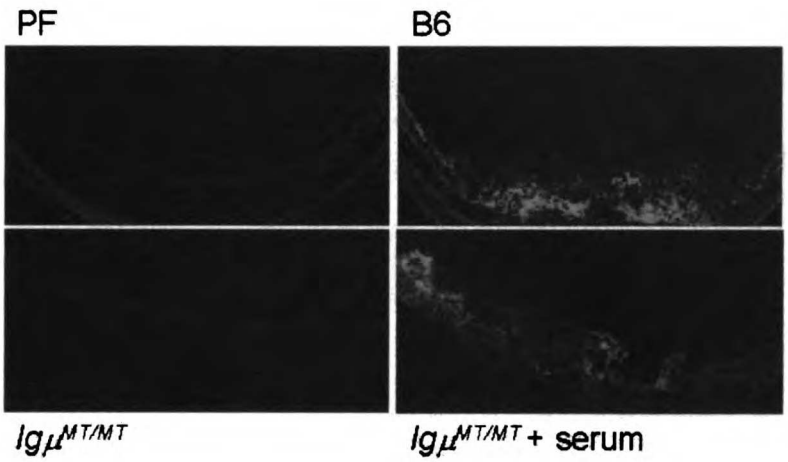

Figure 4 Immune complexes are present in remodeled airways

Immune complexes in the tracheas of *Mycoplasma pulmonis*-infected wild-type and $Ig\mu^{MT/MT}$ mice given *Mycoplasma pulmonis*-immune serum. Tracheal sections (10-14 days post-infection) were stained with Cy2-conjugated anti-mouse IgG and visualized by fluorescence microscopy.

To determine the impact of humoral immunity and the immune complexes it generated on leukocyte recruitment, we infected *Rag1*$^{-/-}$ and wild-type mice with *M. pulmonis*, and then examined their tracheas for the presence of inflammatory cells at various times later. Like wild-type mice, *Rag1*$^{-/-}$ mice had many neutrophils present in their tracheas 14 days following infection (Figure 5A). These cells could be seen on whole-mount preparations as CD31^{+} cells that were not part of the vascular network (Figure 6), and they could also be discriminated by flow cytometry following enzymatic digestion of infected tracheal tissue. One month after infection, the *Rag1*$^{-/-}$ mice had many fewer cells in their tracheas by both types of assays. Strikingly, at the two time-points, but especially at one month, there were significantly more cells present in the tracheas of the infected wild-type mice than in the *Rag1*$^{-/-}$ mice (Figure 5A).

To assess the activation state of the inflammatory cells present in *Rag1*$^{-/-}$ and wild-type tracheas, we analyzed them by flow cytometry using antibodies 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 upregulated. Neutrophils from infected wild-type mice showed much lower cell surface expression of CD62L than those from pathogen-free mice suggesting that they had been activated (Figure 5b,5c). By contrast, the neutrophils from infected *Rag1*$^{-/-}$ mice resembled those of pathogen-free mice in terms of their CD62L expression levels. Similarly, we found that macrophages in the tracheas of wild-type but not *Rag1*$^{-/-}$ or pathogen-free mice had elevated levels of MHC class II on their surfaces (Figure 5d). Cumulatively, therefore, these data show that adaptive immunity to *M. pulmonis* substantially enhances the recruitment and activation of inflammatory leukocytes.

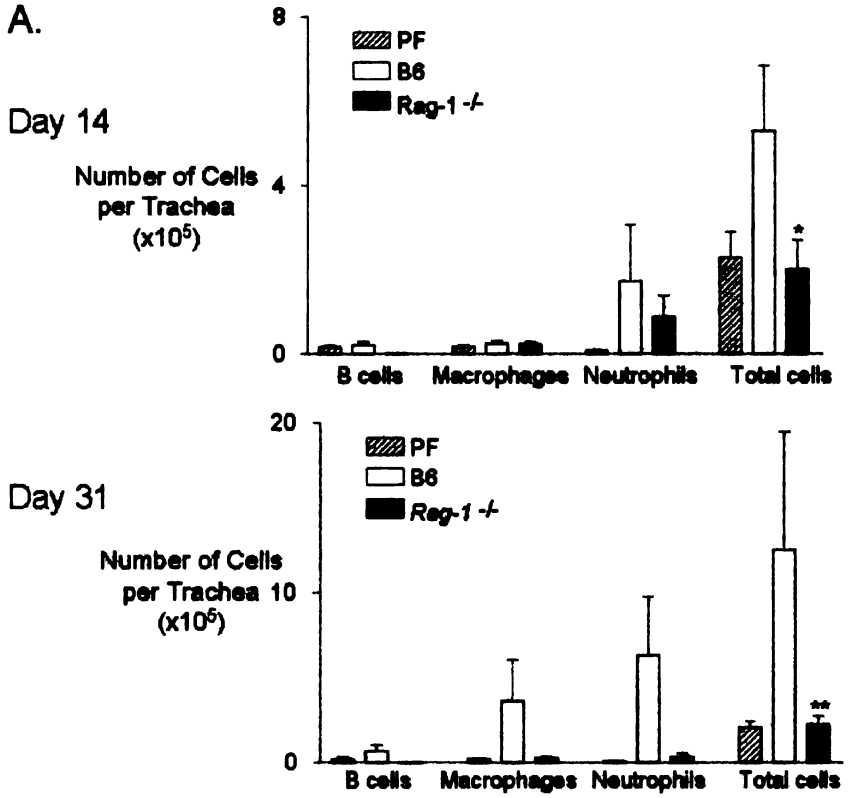

Figure 5 Inflammation from Rag-1$^{-/-}$ and μMT infected airways is quantitatively and qualitatively different from the inflammation in C57BL/6 mice.

A. Total cell numbers and types of inflammatory cells in the tracheas of *Rag-1*$^{-/-}$ and C57/BL6 mice infected with *M. pulmonis* 14 and 31 days after infection. Tracheas from four to five mice per group were minced and digested with collagenase to generate a single cell suspension. Cells were stained with antibodies to mouse Gr-1, Mac-1, and CD19 to identify neutrophil, macrophages, and B cells and analyzed on a FACScan. (*$p<0.05$, **$p<0.04$ compared to B6))

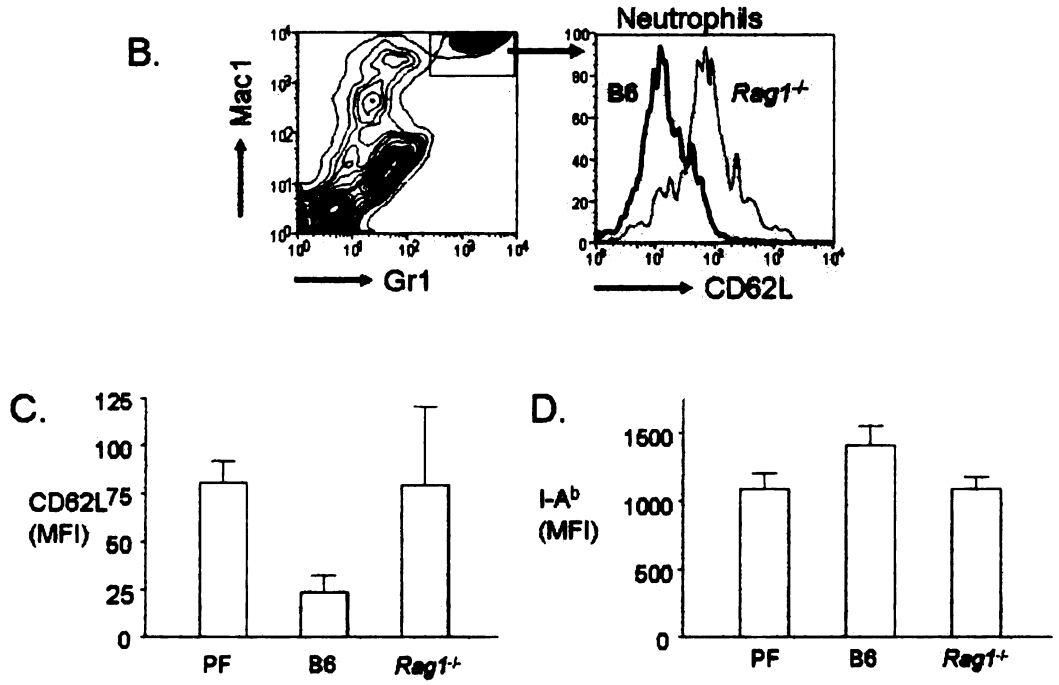

Figure 5 Inflammation from *Rag1*$^{-/-}$ infected airways is quantitatively and qualitatively different from the inflammation in C57BL/6 mice.

B. CD62L expression levels on neutrophils from infected WT mice compared to neutrophils from infected *Rag1*$^{-/-}$ mice 28 days post-infection. Neutrophils, identified as the Gr1+Mac1+ population , within a single cell suspension made from the trachea as previously described were stained with anti-mouse CD62L (L-selectin)

C. Mean fluorescence intensity (MFI) of CD62L was calculated using FlowJo software for each sample. The mean MFI for each group (4-5 mice per group)was compared between B6 and *Rag1*$^{-/-}$ using the Student's T test. (*$p<0.02$)

D. MHC class II levels (I-A^b) on the Mac-1$^+$I-A^{b+} population (80-90% macrophages) in tracheas from WT mice compared to *Rag1*$^{-/-}$ mice 14 days post-infection. Single cell suspensions from the trachea were stained with anti-mouse I-Ab, Gr-1, Mac-1 and CD19 and samples run on a FACScalibur. The mean fluorescence intensity of I-Ab on the Mac1+I-Ab+ population was calculated using FlowJo software. (*$p=0.002$ between B6 and *Rag1*$^{-/-}$.

Airway inflammatory cells produce soluble factors that regulate angiogenesis and lymhangiogenesis

We considered the possibility that remodeling of the airway vasculature might arise either as a consequence of the increased numbers of inflammatory cells present in the tracheas, or because activation of the recruited cells induced them to synthesize proteins that directly influence remodeling. At early times after infection, when cell numbers were increased in *Rag1*$^{-/-}$ mice (Figure 5a), we noted that some animals showed signs of vascular remodeling (Figure 6). In these cases, remodeling was typically not as extensive as at later time points, and strikingly, it was focal in nature being localized to areas where there were concentrations of inflammatory cells. Although these concentrations and the associated remodeling could be detected in some experiments at two weeks after infection, they were invariably not present at four weeks. Remodeling of lymphatic vessels in these mice was even more dramatic than the blood vessel remodeling, because it featured clearly identifiable buds and obvious new vessel growth in the immediate vicinity of clusters of inflammatory cells (Figure 6). Data such as these allow a link to be made between accumulations of inflammatory cells and remodeling, and they suggest that antibody-dependent activation of the innate immune system is not essential for the transient form of remodeling observed in *Rag1*$^{-/-}$ mice. Consistent with this, a previous study has shown *M. pulmonis*-induced tracheal endothelial cell proliferation as early as three days after infection in C3H/HeJ mice, correlating with an initial influx of neutrophils in the airways, and preceding the onset of the T cell-dependent humoral response against the organism.

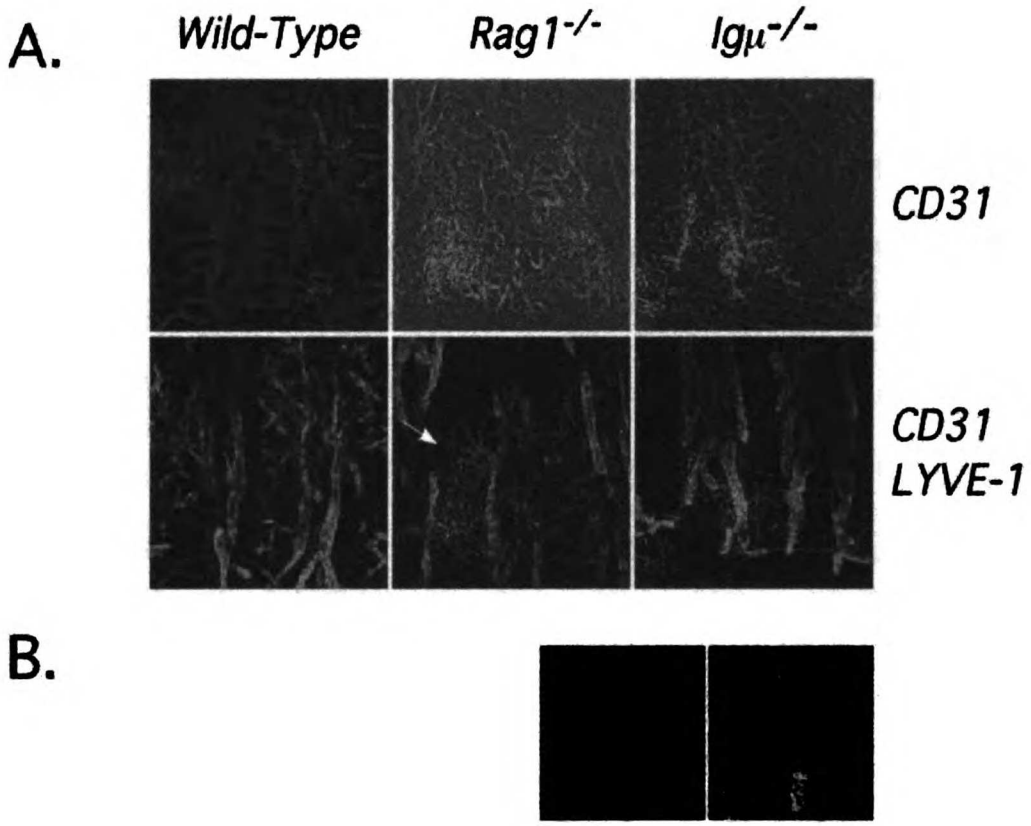

Figure 6 Transient remodeling in the Vicinity of Inflammation in Mycoplasma pulmonis-Infected Tracheas.

Blood vessels (stained with anti-mouse CD31, *green*) and lymphatics (stained with anti-mouse VEGFR-3, *red*) visualized in whole mount preparations of tracheas from wild-type mice, *Rag1*$^{-/-}$, and *Igμ*$^{MT/MT}$ mice at 2 weeks after infection with *M. pulmonis*. Focal angiogenesis and lymphangiogenesis can be seen in *Rag-1*$^{-/-}$ infected tracheas in the immediate vicinity of infiltrating leukocytes (stained green with anti-mouse CD31 and red with anti-mouse VEGFR-3 respectively) at 2 weeks post-infection (merge). The inflammation has resolved and remodeling is absent by 4 weeks post-infection in *Rag-1*$^{-/-}$. (data not shown)

Given that neutrophils and macrophages were activated in the tracheas of wild-type but not *Rag1*$^{-/-}$ mice, we next tested whether their activation was associated with the upregulation of genes that might be important for remodeling. For this purpose we turned to a sensitive multiplexed real-time PCR assay that allows for a determination of absolute gene expression activity in cell populations. 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 bronchoalveolar lavage (BAL) as a means to increase our recovery of inflammatory cells from infected mice. At 28 days following infection, BAL samples from infected wild-type mice were on average comprised of 40-50% neutrophils and 40-50% macrophages while samples from pathogen-free and *Rag1*$^{-/-}$ mice were comprised of an average of 80-90% macrophages (data not shown).

The expression of twenty genes was determined in BAL cells taken from pathogen-free, and wild-type, *Rag1*$^{-/-}$ or B cell-deficient mice 28 days after infection with *M. pulmonis*. (see Table 2) Of the genes analyzed, several have the capacity to induce angiogenesis (*e.g.*, VEGF-A, FGF2, PDGF) or lymphangiogenesis (VEGF-C and VEGF-D) in other settings. As shown in Figure 7, BAL cells generated abundant transcripts for many of these genes. For instance, VEGF-A levels were high in all samples, but they were especially high in the BAL from infected *Rag1*$^{-/-}$ (Figure 7) or B cell-deficient mice (data not shown).

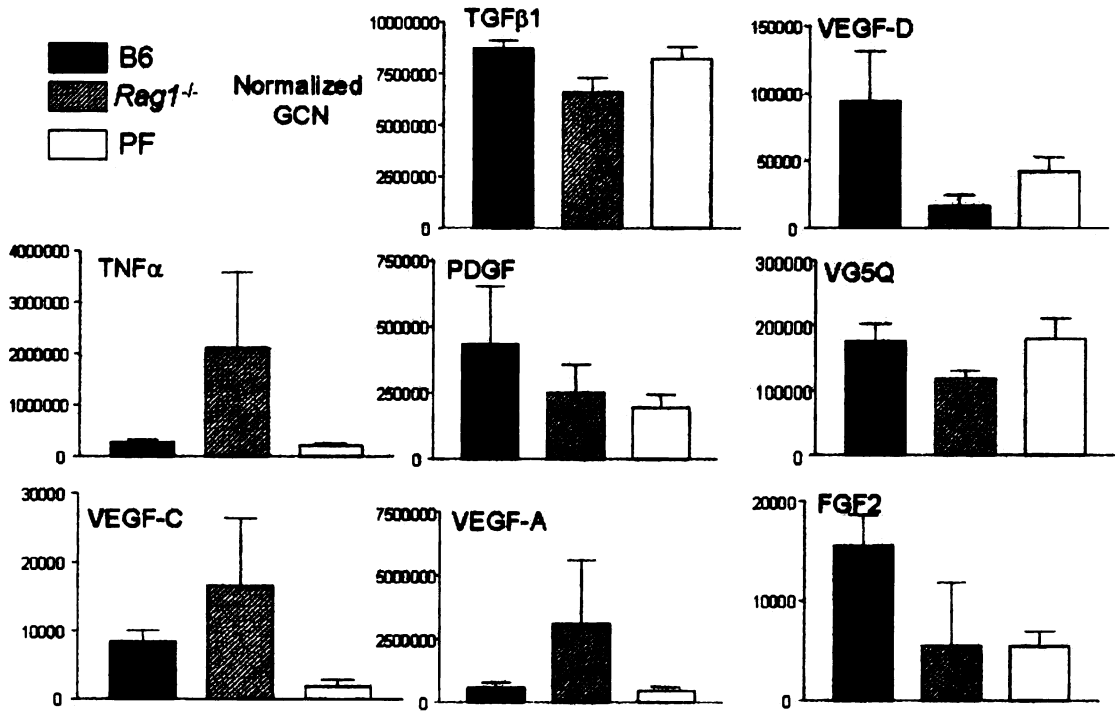

Figure 7 BAL neutrophils and macrophages from wild-type infected mice produce soluble factors that directly contribute to remodeling

Leucocytes from the mouse airway of three to four mice per group were isolated by bronchoalveolar lavage (BAL). Total RNA extracted from these cells was used for real-time PCR to quantify the expression levels of various soluble factors by these cells.

Table 2: Normalized Gene Copy Number (GCN) for real-time quantitative PCR

	PF		B6		*Reg1*$^{-/-}$	
	Mean	STDEV	Mean	STDEV	Mean	STDEV
TGFβ1	8234982	950058	4592520	5151219	4732194	2987148
IL-8	221075	54297	137686	117930	132747	68800
VEGF-A	459507	148726	304116	219755	283026	133703
PDGFa	192797	50327	121562	100742	116357	59088
MMP9	57558	71421	64489	9803	50818	27923
TWEAK	683409	151144	417276	376369	407050	218257
TNFα	214649	72314	143432	100575	132717	61895
VG5Q	178355	32434	105395	103182	104842	59582
VEGF-D	41988	10568	26278	22217	25263	12987
ephrin B4	27233	9829	18531	12307	16975	7757
MMP11	48038	4434	26236	30833	27385	17949
Ephrin B2	16589	9995	13292	4662	11135	5086
FGF-2	3612	3304	3458	218	2648	1625
MMP3	477	538	507	43	391	234
MMP2	458	511	485	37	373	225
VEGF-C	1838	1676	1757	115	1346	824
MMP7	797	1363	1080	400	910	411
VEGF-B	522	655	588	94	465	253
ANGP1	990	865	927	88	718	423
ANGP2	1	1	1	0	1	0

Similarly, VG5Q, TGFβ, and TNFα were also expressed in all samples. Expression data such as these therefore indicate that inflammatory cells could be a significant direct source of factors that are important for remodeling. Strikingly, VEGF-D, FGF2, and PDGF all stood out because it was substantially more highly expressed in BAL cells from wild-type mice than in BAL cells from infected *Rag1*$^{-/-}$ mice. Thus, there were 10-fold more VEGF-D transcripts in the wild-type BAL cells than in the mutant BAL cells. Whereas some of the other genes examined appeared to be unaffected by the activation status of the BAL cells, VEGF-D showed a pattern of expression that was highly suggestive of it being a direct target of the adaptive immune response. Consistent with this, VEGF-D can be detected within neutrophils in the airways of infected wild-type mice by immunostaining of tracheal sections. Moreover, infusion of a soluble form of the receptor for VEGF-D (and VEGF-C) dramatically reduces the extent of lymphatic remodeling in infected mice indicating that VEGFD, and perhaps VEGF-C may be critical for the process.

E. Discussion

In this report we describe the immunological basis of the dramatic vascular and lymphatic remodeling that occurs in the airways of mice infected with *M. pulmonis*. The full chronic form of this remodeling occurs only in mice that generate a T cell-dependent antibody response to the pathogen. Immune complexes formed as a consequence of this response then substantially exacerbate airway inflammation beyond the levels that occur solely because of innate immunity. Activation of the recruited inflammatory cells by immune complexes correlates with the production of mediators (*e.g.*, the

lymphangiogenic factor VEGF-D) that have the potential to orchestrate tissue remodeling directly. The data therefore reveal a novel mechanism by which adaptive immunity to a persistent pathogen can result in chronic changes in blood and lymphatic vasculature via inflammatory leukocytes.

Two types of observations implicate T cell-dependent antibodies in the vascular remodeling response to *M. pulmonis*. First, mutant mice lacking T cells or B cells showed markedly defective vascular and epithelial remodeling. Second, remodeling of the vasculature could be induced by the transfer of serum from *M. pulmonis*-infected wild-type mice to infected B cell-deficient mice. Immune complexes were present in the airways of the infected mice, and in other work we have also found that remodeling is impaired in mice that lack expression of either FcR-γ or C3 (Aurora *et al.*, in preperation). Such data show that *M. pulmonis*-induced chronic lymphatic and blood vessel remodeling is a consequence of immune-complex-dependent inflammation and therefore is mechanistically related to type III hypersensitivity responses such as the Arthus Reaction.

Humoral immunity to *M. pulmonis* is likely to potentiate remodeling through two nonexclusive mechanisms. The first would depend simply on increasing the number of inflammatory cells present in the airways. These cells would then express molecules that would directly or indirectly induce changes in the blood or lymphatic vasculature. In some cases, the expression of such molecules could be constitutive in the inflammatory leukocytes, while others could be induced in response to bacterial products (*e.g.*, those that engage Toll receptor family members) or the resident cytokine milieu. The second

mechanism would depend on the capacity of immune complexes to induce signaling in the recruited cells that results in the expression of remodeling genes.

There is evidence that leukocytes have the potential to influence remodeling through both of the above pathways. With respect to the first one, we have shown that even in *Rag1*$^{-/-}$ mice there can be sporadic indications of remodeling at two weeks after infection, and that such remodeling is both focal in nature and localized in the vicinity of accumulations of inflammatory cells. Similarly, endothelial cell proliferation can be detected in C3H/HeJ mice three days after infection – accompanying the early recruitment of neutrophils to the airways before the onset of T cell-dependent humoral immunity. We have also found that even in the absence of both FcR-γ and C3 expression, there are significant residual indications of remodeling. Cumulatively, therefore, such data suggest that some measure of blood and lymphatic vessel remodeling can occur without the involvement of signaling in response to immune complexes.
In support of the idea that immune complexes contribute to remodeling by inducing a signaling response in recruited leukocytes, we found that neutrophils and macrophages in the infected airways display an activated phenotype when the mice have an intact humoral response. More importantly, we found that the expression of genes such as VEGF-D, FGF-2, and PDGF was increased in BAL cells from such mice compared to BAL cells from mice that lack both T and B cells, or just B cells (Figure 7 and data not shown). Thus, in addition to provoking the continual accumulation of leukocytes in the airways, immune complexes also appear to contribute directly to the expression of genes that can induce remodeling effects.

Immune-complex-dependent upregulation of VEGF-D in BAL cells is of particular interest because this is one of just two factors that are clearly important for lymphangiogenesis in the airways. Blockade with soluble VEGFR-3 (the receptor for VEGF-D and VEGF-C) greatly reduces *M. pulmonis*-induced lymphangiogenesis, and consistent with the data presented here, neutrophils in infected airways appear to be a prominent source of VEGF-D (Baluk *et al.*, submitted). The analysis of mice lacking lymphocytes thus provides the novel perspective that upregulation of VEGF-D expression is a consequence of immune complex recognition and therefore provides a direct link between humoral immunity and remodeling of the airway lymphatics. Many mediators secreted by inflammatory cells have angiogenic properties that could allow for related connections to be drawn between chronic inflammation and remodeling of blood vessels. Most notably, activated neutrophils, macrophages and lymphocytes secrete the endothelial cell growth factors VEGF-A and FGF-2 [20-23, 93]; they also produce ELR^+ chemokines and sphingosine-1 phosphate that have mitogenic and/or chemotactic effects on endothelial cells [27, 94-96]. The gene expression data shown in Figure 7 show that BAL cells from *M. pulmonis*-infected *Rag1*$^{-/-}$ mice express high levels of VEGF-A, yet these mice do not support blood vessel remodeling. Consistent with this, other studies have failed to reveal a convincing role for VEGF-A in *M. pulmonis*-induced angiogenesis (Baluk *et al.*, unpublished). Whether expression of any of the other angiogenic factors shown in Figure 7 (such as VG5Q, FGF2, and PDGF) is likely to be important for *M. pulmonis*-induced airway remodeling requires further investigation.

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 matrix metalloproteinase-9, and in the skin model, the relevant source of this is inflammatory cells. Observations made in the pancreatic model, in which islet-derived VEGF-A is of crucial importance, indicate that MMP-9 promotes angiogenesis by causing the release of VEGF-A from the extracellular matrix. While this example, and others, provide a precedent for invoking a causative link between inflammation and angiogenesis, studies on MMP-9-deficient mice indicate that this particular protease is not essential for *M. pulmonis*-induced angiogenesis[97].

The requirement for T cells for *M. pulmonis*-induced airway remodeling is clear from the data presented in this paper. Nonetheless, whether this requirement reflects only the necessary contributions of these cells to the production of T cell-dependent antibody isotypes, or whether there are other roles for T cells in *M. pulmonis*-induced remodeling requires further work. Through the secretion of T_H2 cytokines, in particular, T cells have the potential to influence airway remodeling, in a substantial fashion [78, 80]. 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.

In summary, we show here that antibodies 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 *M. 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.

Acknowledgements

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Chapter 3:

Immune Complex-Dependent Angiogenesis and Lymphangiogenesis in the Airways of *Mycoplasma pulmonis*-Infected Mice

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A. Abstract

Angiogenesis and lymphangiogenesis are striking aspects of a remodeling syndrome that accompanies chronic infection of the airways with *M. pulmonis*. Mice infected with this pathogen develop a strong humoral response to it that results in the deposition of immune complexes in their tracheas. We show here that blood and lymphatic vessel remodeling in these mice is a consequence of a form of immune complex-dependent inflammation. Mice lacking expression of the FcR-γ chain show impaired tracheal angiogenesis and lymphangiogenesis consistent with the importance of this chain in inflammation driven by immune complexes. We found that complement influenced the kinetics of remodeling and that loss of C3 correlated with impaired stability of tracheal immune complexes. The results clarify an important mechanism by which exposure to a persistent airway infection can result in immunopathologic tissue remodeling.

B. Introduction

Prolonged inflammation in the respiratory tract due to chronic infections or asthma correlates with the induction of major structural changes in the airways. These include a general thickening of the airway walls due to the proliferation of epithelial cells, subepithelial fibrosis, hyperplasia of submucosal glands and smooth muscle, and the production of mucus. The term 'airway remodeling' is used to refer collectively to changes such as these.

Many remodeling events can be induced by ectopic airway-restricted expression of cytokines in transgenic mice. Whereas some of these cytokines derive from resident airway stromal cells, others are the product of leukocytes that are recruited to the mucosal

area by inflammatory stimuli. In the case of asthma, cytokines from allergen-specific T_H2 cells, such as IL-4, 5, 9 and 13 can contribute to airway inflammation and may also directly cause specific remodeling outcomes such as mucus secretion and fibrosis. Understanding the pathways that regulate airway remodeling is of critical importance for the development of therapies that would separate the beneficial contributions of innate and adaptive immune responses from those that cause acute or chronic pathology.

A prominent but poorly understood aspect of airway remodeling is the inflammation-dependent changes that occur in the number and form of blood and lymphatic vessels in the mucosa. These changes can be readily visualized in whole-mount preparations of rodent tracheas, particularly in the context of chronic airway infections. As an example, mice infected with the rodent respiratory pathogen *M. pulmonis* show dramatic tracheal angiogenesis, which depending on the mouse strain examined, results in the growth of many new vessels and/or the enlargement of existing vessels. Similarly, this infection also induces an even more striking growth of new lymphatic vessels. Both the angiogenesis and lymphangiogenesis are dependent on adaptive immunity and inflammation, but the mechanisms that connect lymphocytes to changes in the vasculature are unclear.

In this paper, we describe studies that provide new insight into the regulation of *M. pulmonis*-induced vascular remodeling. The work reported here is based on prior studies revealing a critical contribution of antibodies to remodeling. Here we have explored the possibility that remodeling is a consequence of a form of immune complex-dependent inflammation in which inflammatory cells respond to localized complexes of bacteria and the immunoglobulin specific for them. In diverse settings, inflammation in

response to such immune complexes shows primary dependence on Fc receptor signaling with variable contributions coming from complement fixation. We show that remodeling of blood and lymphatic vessels is markedly reduced in severity in mice that lack expression of the FcR-γ chain, but is only transiently impaired by absence of C3. Complement was required for early stabilization of immune complexes in the airways, and no role for C3 or C5 was clearly apparent by four weeks after infection commenced. Cumulatively, the data lend support to the view that airway remodeling in response to chronic *M. pulmonis* infection is a consequence of inflammation driven by immune complexes. The results raise the possibility that other forms of airway remodeling could likewise depend on inflammation brought on by localized complexes of antibodies and antigens derived from respiratory pathogens.

C. Methods and Materials

Animals

C57BL/6J, C57BL/6J-*C3* ^{tm1Crr} (*C3* $^{-/-}$), B10.D2-*Hc*^{0} *H2*^{d} *H2-T18*^{c}/oSnJ (C5-deficient) and B10.D2-*Hc1 H2d H2-T18c*/nSnJ (C5-sufficient) mice were obtained from Jackson Laboratories or the National Cancer Institute, and/or were bred in the Parnassus Heights Barrier Facility at the University of California San Francisco. B6.129P2-*Fcerg1*^{tm1} (*FcR-γ* $^{-/-}$) were obtained from Taconic. All experimental mice were 6-12-week-old females unless otherwise specified. In the majority of experiments, control animals were age- and sex-matched C57BL/6 mice purchased from the same commercial source. In some experiments (*e.g.*, the experiments involving *FcR-γ*$^{-/-}$ and *C3* $^{-/-}$ mice) the control animals

were littermates derived from crosses of mice harboring both mutations. Infected and pathogen-free control mice were housed separately.

Mycoplasma pulmonis infection and serum transfer

M. pulmonis stocks used in this study were derived from the UAB CT7 strain kindly provided by Dr. J.R. Lindsey (University of Alabama). Mice were given *M. pulmonis* intranasally under anesthesia (10^4 -10^6 cfu, depending on the strain of mouse, in a total volume of 50µl/ mouse).

Immune serum was pooled from the blood of female C57BL/6 mice that had been infected with *M. pulmonis* for at least two weeks. Serum samples were checked for *M. pulmonis* reactivity by ELISA and injected intraperitoneally (200-250uL total volume/injection) beginning at day 3 post-infection and every third day thereafter until the mice were analyzed.

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 in 10% CO_2 for two weeks before counting colonies.

BrdU labeling and immunofluorescence

Mice were infected with *M. pulmonis* and then fed bromodeoxyuridine (0.8 mg/ml; Sigma) continuously in their drinking water. Tracheas from the mice were then removed 7-14 days after infection, embedded in OCT, and stored at -80°C. 7µm thick sections were cut using a cryostat and fixed for 10 minutes in cold acetone before staining with rat

anti-mouse CD31 (BD-Biosciences) followed by Cy2-conjugated donkey anti-rat Ig (Jackson Immunoresearch). The sections were permeabilized and stained with biotinylated anti-BrdU (Zymed) followed by Cy3-conjugated strepavidin (Jackson Immunoresearch). Imaging was performed under UV illumination using a Nikon Eclipse E600 microscope equipped with a Zeiss Axiocam camera, and AxioVision software. In order to count $BrdU^+CD31^+$ endothelial cells, a series of images were taken around the entire circumference of each tracheal section using both the red and green filters. The two 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 mid-trachea from three or more mice of each genotype were analyzed for each experiment. The significance of differences between groups was assessed using the Student's T-test.

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

Whole mount immunofluorescene of airway lymphatics and blood vessels

The whole mount procedure used to visualize both the lymphatic vessels and blood vessels in the mouse trachea were done as previously described (Baluk et al. and Aurora et al., in preparation). In brief, infected or pathogen-free mice were first perfused with

1% paraformaldehyde. Tracheas were removed from the mice and fixed for an additional 1 hour in 1% paraformaldehyde in PBS pH 7.4 before washing with PBS containing 0.1% Triton X-100 for two hours followed by blocking for one hour in PBS containing 20% donkey serum. The samples were then stained overnight with hamster anti-mouse VEGFR-3 (Chemicon) and rat anti-mouse CD31 (BD-Biosciences) followed by a Cy3-conjugated goat anti-hamster Ig secondary antibody (Jackson ImmunoResearch) and Cy2-conjugated anti-rat Ig (Jackson ImmunoResearch). After washing and fixation in 4% PFA for 1 minute, the tracheas were mounted and coverslipped using Vectashield mounting medium for immunofluorescence. Finally, the whole-mounts were imaged using a Zeiss LSM 510 confocal microscope.

Area density quantification of blood vessels and lymphatics

In order to measure angiogenesis and lymphangiogenesis, the area density of both blood and lymphatic vessels was measured with a Zeiss Axiophot microscope equipped with digitizing software. Area density was defined as the number of points within a grid of 126 points that intersected with vessels. Vessel density measurements were made in ten different fields per trachea within the cartilage rings of the tissue (an area normally devoid of lymphatics and sparsely covered with blood vessels). Four to six tracheas (each from different mice) were included for each group. The significance of the area density measurements was determined using the unpaired Student's T test.

C3 and IgG immune-complex immunofluorescence

For C3 staining and IgG immune-complex detection, 7μm thick sections were cut from tracheas embedded in OCT and stored at -80°C. The sections were dried for at least 30 minutes, fixed for 5 minutes in cold acetone, and blocked for 15 minutes with either donkey serum (for anti-IgG) or goat serum (for anti-C3) (5% serum in PBS containing 0.1% Triton-X 100). The sections were then incubated with either FITC-conjugated goat anti-mouse C3 (ICN Pharmaceuticals-Cappel) or Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch) for 1.5 hours at room temperature. The slides were washed 4 times (5 minutes/wash) with PBS containing 0.1% Triton, mounted with *SlowFade*® Light Antifade Kit with DAPI - Molecular Probes Inc.) and imaged under UV illumination using a Nikon Eclipse E600 microscope equipped with a Zeiss Axiocam camera and Axiovision software.

D. Results

M. pulmonis bacteria attach themselves to the lumenal surfaces of the epithelial barrier in infected mice [81], where they normally survive and replicate despite a highly active immune response against them [48, 51, 82, 83]. Because of the persistent nature of the infection in the airways and the substantial amount of *M. pulmonis*-specific IgG the bacteria induce, immune complexes of antibody and bacteria can be detected in the airways of the infected mice (Aurora *et al.*, in preperation and see also Figure 3). These immune complexes are likely to represent an important trigger both for recruiting leukocytes to the infected airways and inducing their activation once they arrive there.

Given the demonstrated significance of Fc receptors and complement in immune complex-dependent inflammation, we sought to test whether airway remodeling might be defective in mice that lacked expression of C3 or the FcR-γ chain. C3 is the central component of the complement cascade, without which the inflammatory and membrane attack functions of the cascade are disabled. Similarly, FcR-γ is a crucial signaling component of the FcγRI, FcγRIII, FcεRI and FcαRI receptors for IgG, IgE and IgA.

Complement can kill some strains of M. pulmonis, so we first determined whether there were differences in the numbers of bacteria present in the lungs of the mutant mice compared to the lungs of control mice. We found that the absence of C3 had no impact on replication of the bacteria because both the mutant and wild-type mice had roughly equivalent numbers of organisms in their lungs (B6 5.8+/- 0.7, $C3^{-/-}$ 5.3+/- 0.6 $Log_{10}CFU$)

Vascular remodeling in the mutant mice was monitored using three different approaches. Initially, we used a procedure in which the mice were perfused with biotinylated *Lysopersicon esculentum*, which is a lectin from tomato that binds to blood vessels. We then removed the tracheas and visualized the lectin-labeled vessels in them by cutting them longitudinally and staining them in whole-mounts with streptavidin-conjugated horseradish peroxidase. A useful variation on this procedure involved preparing whole-mounts from mice that were not perfused with the lectin and then staining them for blood vessels with anti-CD31 and for lymphatic vessels with anti-VEGFRIII or LYVE-1-specific antisera. Finally, we also monitored vascular endothelial cell proliferation directly by giving mice BrdU in their drinking water for ten-to-fourteen days following infection and then staining 7μm cross-sections of their tracheas for $CD31^{+}$

cells that had incorporated BrdU into their nuclei. In all of the experiments, all of the groups of mice received repeated doses of immune serum from wild-type mice that had been infected with *M. pulmonis*. This serum was provided to eliminate any complicating variability in the T cell-dependent antibody response that might arise because of the C3 or FcR-γ deficiencies.

Tracheas from mice that had been infected for four weeks showed little evidence of impaired remodeling in the absence of C3 (Figure 1b,1c; Figure 2a,2b). The absence of an effect of C3 deficiency was apparent by visual inspection of tracheal whole mounts that had been stained with anti-CD31, and could be quantified by measuring the density of blood vessels in the cartilaginous zones of the tracheas. From this latter quantification, it was clear that the range of vessel densities exhibited by the mutant and wild-type mice were the same. We obtained a different perspective, however, when we looked earlier in the course of infection (ten days after infection) at the extent of endothelial cell proliferation in tracheal blood vessels. Specifically, the absence of C3 significantly reduced the amount of angiogenesis induced by the bacteria because there were reproducibly fewer $BrdU^{+}CD31^{+}$ cells in the tracheas of the C3-deficient mice compared to control tracheas (Figure 1a).

The structure of the trachea is maintained in part by the presence of repeating cartilaginous rings that surround the airway mucosa. These rings are separated from one another by an inter-cartilaginous space in which the larger veins, arteries and lymphatic vessels are located. In pathogen-free mice arterioles and collecting venules can be found spanning the area that lies on the cartilage rings, but this area is normally free of lymphatic vessels. *M. pulmonis* infection, however, leads to a dramatic invasion of the cartilaginous zones with lymphatic vessels that can be readily detected with anti-VEGFR-3 or LYVE-1-specific antisera.

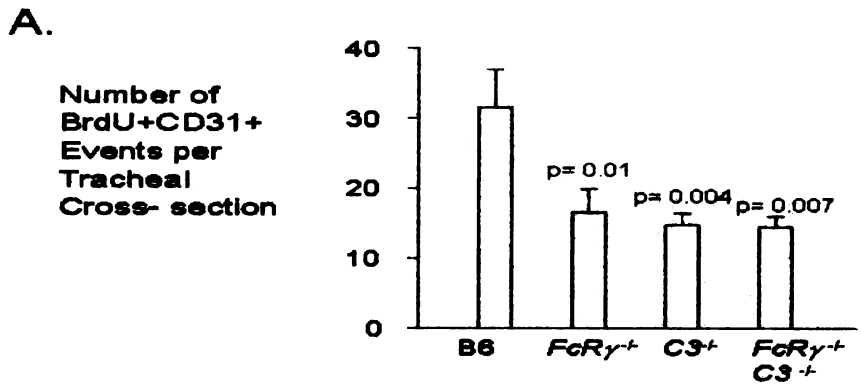
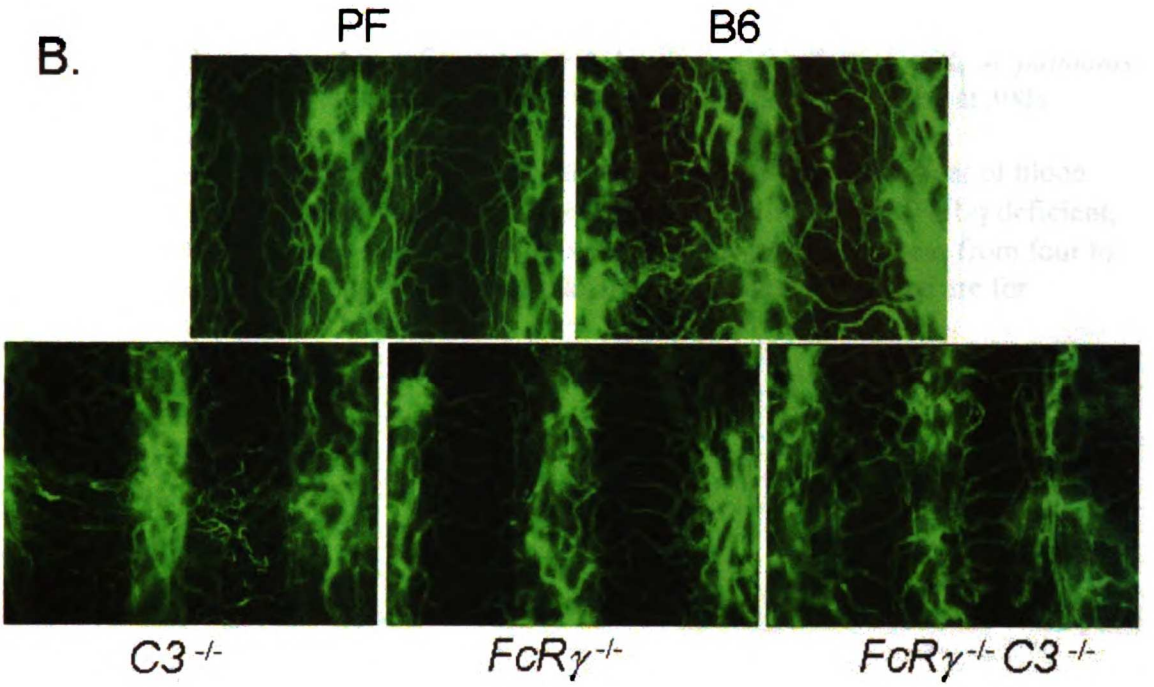


Figure 1 **Defective angiogenesis in mice lacking C3 or the FcR-γ chain.**

A. The graph shows the frequency of BrdU + CD31 + cells in tracheal sections from wild-type, FcR-γ deficient, C3-deficient mice or C3and FcR-γ - deficient mice ten days after infection with *Mycoplasma pulmonis*. All of the mice were given *Mycoplasma pulmonis*-immune serum on day 3, 6 and 9. At least four different mice per group were analyzed. Statistical significance was determined using the Student's t-test.


B.
PF
B6
C3 -/-
FcRγ -/-
FcRγ -/- C3 -/-

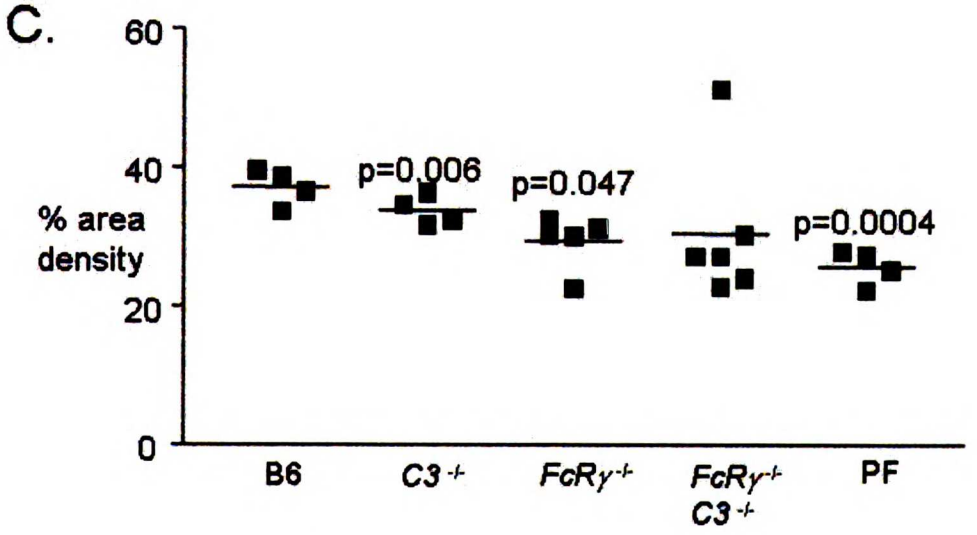

C.
60
40
20
0
% area density
p=0.006
p=0.047
p=0.0004
B6
C3 -/-
FcRγ -/-
FcRγ -/- C3 -/-
PF

Figure 1 **Defective angiogenesis in mice lacking C3 or the FcR-γ chain.**

B. Blood vessels (stained with anti-mouse CD31, *green*) visualized in whole mount preparations of tracheas from pathogen-free or wild-type, FcR-γ deficient or C3-deficient mice at 4 weeks after infection with *M. pulmonis*. Pictures were taken with with a Zeiss Axiophot microscope at 300x magnification.

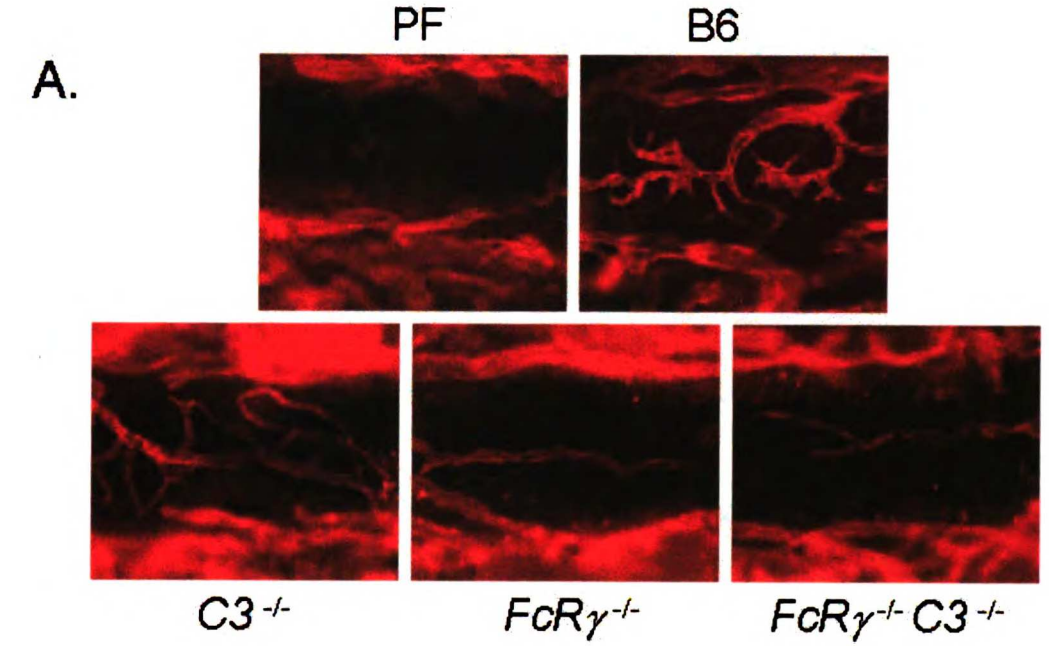
C. Angiogenesis was quantified using area density measurement of blood vessels in each trachea from pathogen-free or wild-type, FcR-γ deficient, C3-deficient, or FcR-γ C3 double knockout mice. Tracheas from four to six mice for each group were analyzed. (p values indicated are for comparison to B6)

To quantify the extent of lymphatic remodeling, we therefore measured the density of lymphatic vessels in the cartilaginous zones. At the four-week time point, we found that like blood vessel remodeling, lymphatic remodeling in *C3*$^{-/-}$ mice was indistinguishable from that in wild-type mice, with the tracheas from the mutant mice showing extensive invasion of the cartilaginous rings with lymphatic vessels (Figure 2).

Cumulatively, the data suggest that complement influences remodeling at early time points after infection, but this effect can not be detected as the disease enters its chronic phase.

From our analysis of mice lacking expression of FcR-γ we found clear evidence of a role for Fc receptors in the remodeling process. At the four-week time point, visual inspection of anti-CD31 and anti-VEGFRIII-stained tracheal whole-mounts showed diminished blood and lymphatic vessel growth in the mutant mice compared to controls. This conclusion was readily substantiated by quantifying the density of blood and lymphatic vessels in the cartilaginous areas of the infected tracheas (Figure 1b,1c and 2a,2b). At ten days after infection, we also found that the absence of FcR-γ impaired endothelial cell proliferation as detected by the incorporation of BrdU into CD31^{+} cells (Figure 1a). These data therefore show that FcR-γ signaling is crucial for efficient long-term remodeling of lymphatic and blood vessel networks in response to *M. pulmonis* infection.

Fc receptors make a dominant contribution to several, if not all, immune complex-dependent inflammatory responses. Thus, the involvement of FcR-γ in the remodeling of


A.
PF
B6
C3 -/-
FcRγ -/-
FcRγ -/- C3 -/-

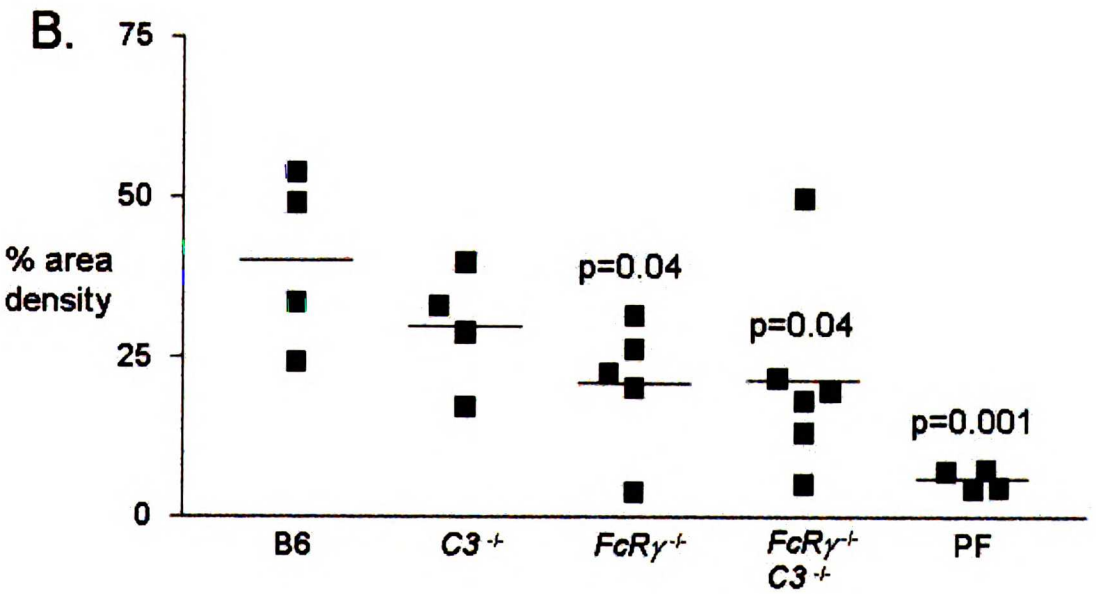

B.
75
50
25
0
% area density
p=0.04
p=0.04
p=0.001
B6
C3 -/-
FcRγ -/-
FcRγ -/- C3 -/-
PF

Figure 2 Defective lymphangiogenesis in mice lacking C3 or the FcR-γ chain.

A. Lymphatics (stained with anti-mouse Vegfr3, *red*) visualized in whole mount preparations of tracheas from pathogen-free or wild-type, FcR-γ deficient or FcR-γ C3-double deficient mice at 4 weeks after infection with *M. pulmonis*.

B. Lymphangiogenesis was quantified using area density measurement of lymphatic vessels in each trachea from pathogen-free or wild-type, FcR-γ deficient, C3-deficient, or FcR-γ C3 double knockout mice. Tracheas from four to six mice for each group were analyzed. P values are compared to B6.

tracheal vessels in *M. pulmonis*-infected mice is consistent with prior data showing that antibodies are critical for remodeling. Furthermore, the data support a model in which immune complexes provide a trigger that recruits and activates leukocytes in such a way that they provide the stimulus for the dramatic reorganization of the lymphatic and blood vessels.

We took two additional steps to explore the nature of the contribution of complement to remodeling. First, we generated mice that lacked both C3 and FcR-γ expression and examined lymphatic and blood vessel remodeling in these following *M. pulmonis* infection. These experiments revealed no clear additive effect of the two mutations on the extent of remodeling suggesting that the two systems make their contributions in a linear pathway rather than in two parallel or redundant pathways (Figures 1 and 2). Second, we stained for immune complexes in the tracheas of mice lacking either C3 or FcR-γ that had been infected for nine, fourteen or twenty-one days with *M. pulmonis*. This experiment was performed because of recent data supporting a role for C3 in the stabilization of immune complexes in a mouse model of immune complex-dependent arthritis [75]. Whereas immune complexes could be detected readily in the tracheas of wild-type or FcR-γ-deficient mice, they were much less prevalent in C3-deficient tracheas (Figure 3). This difference was apparent even though all of the groups of mice had been treated with immune serum from infected wild-type mice and therefore contained abundant *M. pulmonis*-specific Ig. These data therefore support a proximal role for complement in stabilizing immune complexes in the airways of infected mice.

Finally, as a further test of the involvement of complement in remodeling, we infected C5-deficient mice with *M. pulmonis* and analyzed them for vascular and lymphatic remodeling at four weeks after infection. C5 lies downstream of C3 in the complement cascade and is cleaved to generate C5a, a potent neutrophil chemoattractant, and C5b, the nucleating component of the membrane-attack complex. Like C3-deficient mice, the C5-deficient animals showed normal remodeling responses at the four week time point showing that neither the chemotactic contribution of C5a nor the membrane attack complex are essential for remodeling (Figure 4).

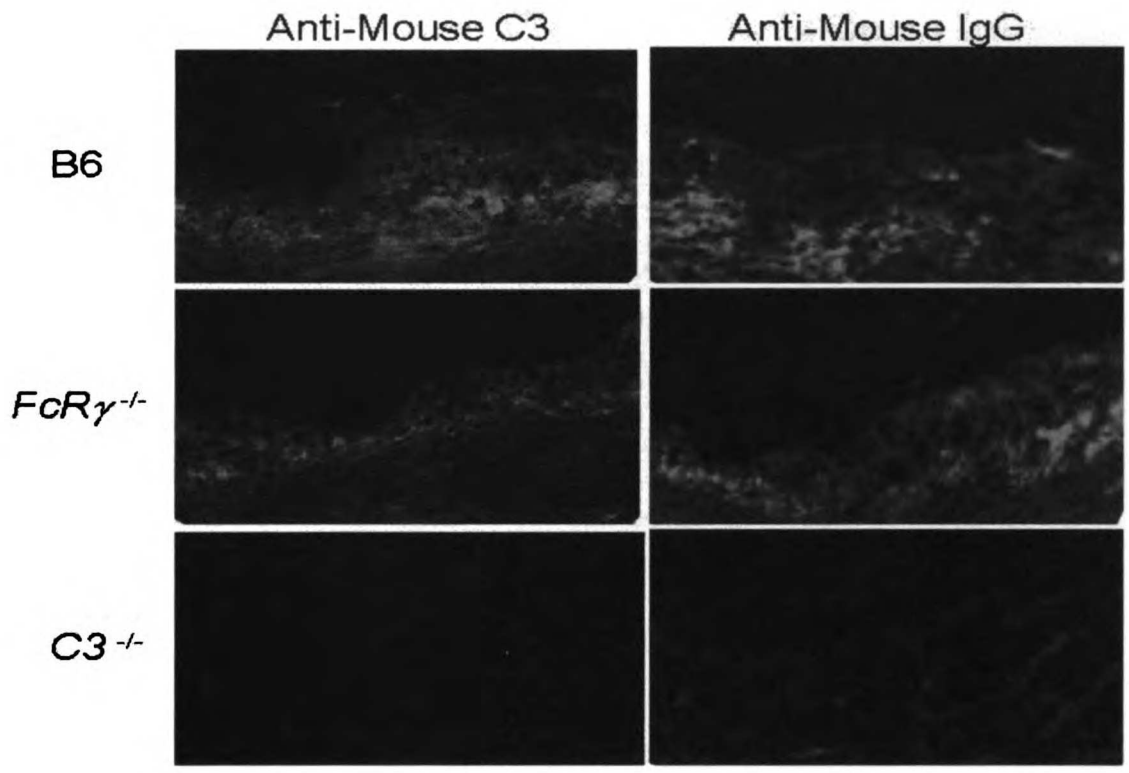


Figure 3 Immune complexes are diminished in *C3*$^{-/-}$ mice

Immune complexes and complement deposition in the tracheas of infected serum-treated mice at 9 days following infection with *M. pulmonis*. Tracheal sections from mice of the indicated genotypes were stained at left with an antibody specific for mouse C3, and at right with an antibody specific for mouse IgG. Complement and immune complexes could be detected in the tracheas of all mice. Twelve sections from each of 3-4 mice per group were imaged by imunofluorescence using a 20x objective.

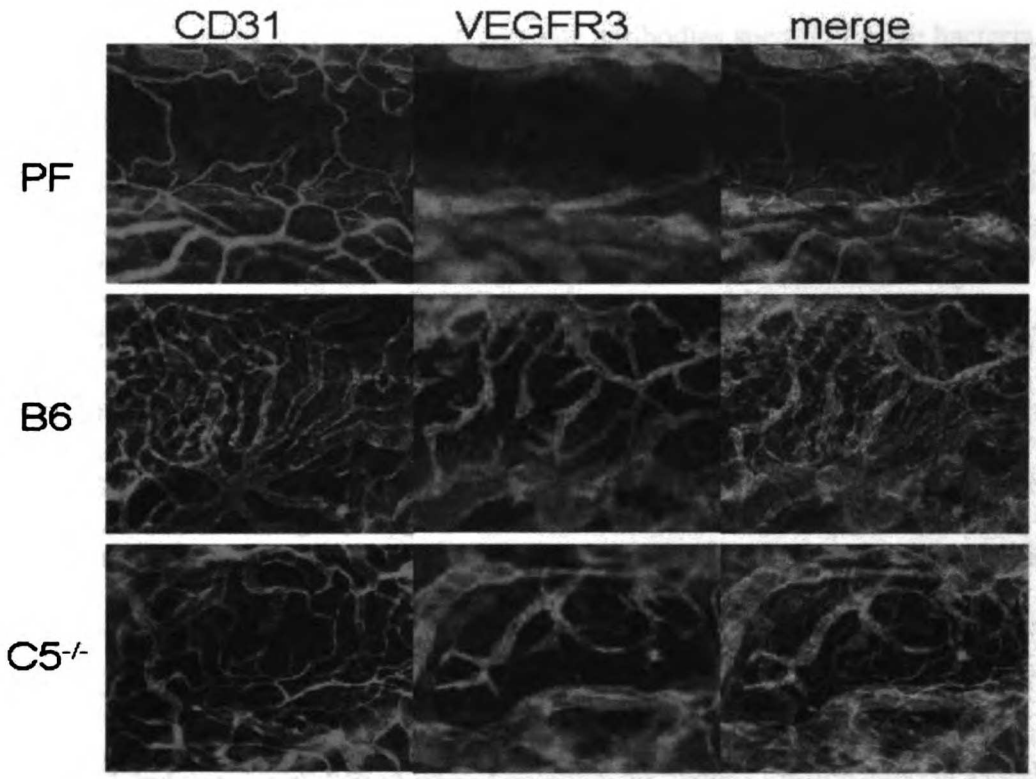


Figure 4 C5 does not play a major role in angiogenesis or lymphangiogenesis

Lymphangiogenesis and angiogenesis induced by *Mycoplasma pulmonis* infection in wild-type and *C5*$^{-/-}$ mice at 4 weeks post-infection. The lymphatics in infected C57BL/6, *C5*$^{-/-}$ and pathogen-free C57BL/6 mice (four mice per group) were visualized in tracheal whole-mount preparations by staining with VEGFR-3 (red); blood vessels were visualized by staining with CD31 (CD31). Images were taken using a 10x objective.

E. Discussion

Extensive remodeling of the tracheal blood and lymphatic vessels in *M. pulmonis*-infected mice occurs only if the mice generate antibodies specific for the bacteria. In the absence of an adaptive immune system or B cells, the infection fails to elicit lasting large-scale changes in the vasculature of the airways even though the mice ultimately become massively septic. Using mice deficient in expression of C3, C5 or FcR-γ, we now show that efficient vascular remodeling in *M. pulmonis*-infected mice is promoted by a stabilizing effect of complement on immune complex deposition, and by Fc receptor signaling. The data thus support a model in which a T cell-dependent antibody response leads to the formation of immune complexes that are important for the recruitment and/or activation of leukocytes. Airway remodeling is then a direct consequence of the accumulation of activated leukocytes in the airway tissues.

M. pulmonis infection in immunocompetent mice is localized to the airways and does not spread to other parts of the body if the mice make a robust humoral response. Antibodies are therefore critical for host defense against the organism because they prevent dissemination and thus stop complications such as the arthritis that occurs in infected SCID or *Rag1*$^{-/-}$ mice. As shown here and in other work from our laboratories, antibodies are also critical for airway remodeling, which is a process that is of unclear benefit to the host and may in fact be largely, if not entirely, harmful. This perspective follows from the finding that the numbers of bacteria present in the lungs of infected wild-type, *Rag1*$^{-/-}$, B cell-deficient, or T cell-deficient mice are not greatly different.

Thus, adaptive immunity and the inflammation-dependent airway remodeling it induces does not significantly influence bacterial replication in the airways.

The importance of complement in immune complex-directed inflammation has been the subject of considerable investigation and some controversy because of divergent and seemingly contradictory findings obtained from different model systems. The reverse Arthus reaction in the skin occurs normally in the absence of C3, but is blocked by loss of FcR-γ or FcγRIII pointing to a dominant role for Fc receptors in this type of antibody-dependent inflammation. By contrast, complement is essential for arthritis induced by passive transfer of immunoglobulin from KRN transgenic mice, although here disease is independent of C4 and relies on activation of the alternative pathway. In addition to roles for complement in regulating the stability of immune complexes in tissues, and in promoting leukocyte recruitment (through C5a, C3a and C4a), complement can also enhance the efficiency of FcR signaling by modulating the ratio of activating to inhibitory Fc receptors. Thus, C5a promotes upregulation of the activating receptor FcγRIII and down regulation of the inhibitory receptor FcγRIIb. Of these diverse roles, inflammation-dependent remodeling induced by *M. pulmonis* appears to require C3 for immune complex stability, but shows no requirement for C5, at least at the late time point examined here.

In contrast to the limited dependency on complement, *M. pulmonis*-induced remodeling clearly required expression of FcRγ. This reliance on FcR function was apparent at the early and late time points and was manifest in both an impaired angiogenic and lymphangiogenic response to the bacteria. In this respect, therefore, the remodeling conforms to the expectations of an immune complex-dependent inflammatory

process. Moreover, the involvement of Fc receptors in remodeling is entirely supportive of the view that leukocytes are essential participants in the process that brings about changes in the blood and lymphatic vasculature.

The factors released by inflammatory cells that control remodeling remain to be clearly identified. Efforts to determine the important angiogenic mediators are made difficult by the fact that multiple factors have the capacity to regulate angiogenesis. Leukocytes are a potential source of many of these factors, so a systematic approach employing inhibitors and/or conditional null alleles will be required to identify the ones that are critical for *M. pulmonis*-induced angiogenesis. At present, only VEGF-C and VEGF-D are candidates for regulators of the lymphangiogenesis that occurs in the infected tracheas. A soluble form of VEGFR-3 dramatically inhibits the growth of lymphatic vessels in response to the infection, and neutrophils are a prominent source of VEGF-D in the infected airways. Thus, resolving the cause of *M. pulmonis*-induced lymphatic remodeling may prove to be more straightforward than the cause of the angiogenesis.

Although remodeling was significantly impaired in mice that lacked FcR-γ, it was not completely absent. Residual remodeling of approximately the same magnitude was also observed in mice that lacked both FcR-γ and C3. At least two possible explanations could account for this residual FcR-γ-independent remodeling. One is that some FcR function, perhaps involving FcγRI, may persist despite the loss of FcR-γ. Another is that some remodeling may depend solely on the accumulation of leukocytes in the airways in response to triggers for innate signaling pathways such as those from Toll receptor family members. Leukocyte accumulation occurs rapidly before the onset of an adaptive

immune response, but is exacerbated and prolonged by the deposition of immune complexes in the airways that normally begins several days after infection. Consistent with FcR-independent accumulations of leukocytes having the potential to induce remodeling processes, we have observed sporadic and focal vascular remodeling in areas of the tracheas of some infected *Rag1*$^{-/-}$ mice where there were obvious accumulations of leukocytes. Whereas such accumulations are transient and infrequent in the absence of adaptive immunity, they would be massively exacerbated and prolonged by the presence of immune complexes.

Although it is possible that the major contribution of immune complexes could be simply to increase leukocyte numbers in the infected airways, it seems likely that leukocyte activation via FcR signaling could induce the expression of genes that would directly or indirectly cause angiogenesis and lymphangiogenesis. In support of this, we have found evidence of leukocyte activation in response to immune complexes and we have seen that this activation correlates with the induction of genes such as VEGF-D that may be critical for remodeling of lymphatic vessels.

In conclusion, the data presented here support the importance of humoral immunity and immune complexes in the remodeling events that accompany airway infection with *M. pulmonis*. Understanding the mechanisms that allow for leukocytes to regulate lymphatic and blood vessel growth is critical for the development of improved strategies for controlling unwanted inflammation-dependent vascular remodeling, and potentially for rescuing lymphatic growth in disease settings where it is defective.

Chapter 4:

Detection of Pathogen-Activated T Cells Following Infection of Mice with an Engineered Form of Mycoplasma pulmonis.

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A. Abstract

Mycoplasma pulmonis infects mice and rats and is able to establish a chronic presence in their airways. Here we have generated a recombinant form of the bacteria that expresses the LACK antigen derived from *Leishmania major*. These recombinant bacteria elicit a LACK-reactive T cell response in infected mice that can be detected by flow cytometry using a fluorescent peptide/MHC class II multimer. We found variability in virulence among the isolates of LACK-expressing bacteria that were generated in that some showed short-term persistence and others appeared to have the capacity for chronic infection. The results demonstrate the viability of expressing model T cell epitopes in *M. pulmonis* and suggest the promise of exploiting variation in virulence to study the consequences of chronic versus acute encounter to such antigens.

B. Introduction

Mycoplasmas fall within the Mollicutes class of bacteria and are distinguished by the complete absence of peptidoglycan-containing cell walls. These organisms have especially small genomes and because of the absence of genes for critical metabolic processes, they are obligate parasites[37]. Mycoplasmas are found in intimate contact with mucosal surfaces and are associated with respiratory and urogenital complications. For instance, *Mycoplasma pneumoniae* is the leading cause of pneumonia in school-age children and young adults, and is responsible for an estimated two million cases of pneumonia in the United States each year.

Mycoplasma pulmonis is a natural pathogen of mice and rats that like *M. pneumoniae* colonizes the respiratory tract and causes pneumonia. Susceptibility to some of the more severe consequences of *M. pulmonis* infection varies in different mouse (and rat) strains, but the bacteria can establish a chronic foothold even in relatively resistant backgrounds such as C57BL/6 [98].

The immune response to *M. pulmonis* infection in mice involves a robust humoral component that is critical for preventing systemic dissemination of the bacteria, but is largely ineffective in preventing persistent airway infection. In the absence of an antibody response, mice become septic and *M. pulmonis* organisms can be recovered from multiple organs throughout the body. In addition to their beneficial effect in preventing sepsis, however, *M. pulmonis*-specific antibodies are also responsible for significant airway pathology because they allow for chronic immune-complex-dependent inflammation.

M. pulmonis is attractive on many levels as an experimental infectious model system. First, as mentioned above it is representative of a class of organisms that cause significant human health problems, though *M. pulmonis* itself is incapable of crossing the murine-human species barrier. Second, it is normally restricted in its growth largely to the respiratory tract, which lends itself to morphological analysis and the facile recovery of large numbers of inflammatory cells and infectious organisms. Third, it is immunogenic and the immune response against it induces readily quantifiable forms of localized tissue remodeling. Fourth, there are genetic systems that allow for changes to be engineered into the *M. pulmonis* genome. Fifth, and perhaps most important, it establishes chronic residency in the airways allowing one the opportunity to study specific aspects of the immune response that derive from chronic exposure to a pathogen.

To begin to capitalize on some of the advantages just described, we have generated an engineered form of *M. pulmonis* that expresses a well-characterized model antigen that is highly immunogenic for CD4+ T cells. Here we report the initial description of this new model system and demonstrate its appeal as a means for studying the immunological consequences of chronic infection with a respiratory pathogen.

C. Materials and Methods

Animals

C57BL/6J were obtained from Jackson Laboratory or the National Cancer Institute, and/or were bred in the Parnassus Heights Barrier Facility at the University of California San Francisco. B10.D2.WT15β TCR transgenic (W15β) and B10.D2.WT15αβ TCR transgenic (W15αβ) were generated previously in the lab [99].

Mycoplasma pulmonis infection

$1x10^6$ cfus of wild-type *M. pulmonis* (strain UAB CT7 originally provided by Dr. J.R. Lindsey, University of Alabama) or $1x10^6$ cfus LACK-expressing derivatives were given intranasally to anesthetized mice.

To quantify numbers of *M. pulmonis* in infected mice, tissues were removed and homogenized in 4 mL of Hayflick's broth. The homogenates were centrifuged at 9000 rpm for 10 minutes and serial dilutions of the supernatant fluids were then plated on *Mycoplasma* culture plates. The plates were incubated at 37 °C for two weeks before counting colonies.

***In vitro* T cell proliferation and cytokine assays**

Mediastinal and peri-aortal lymph node cells recovered from *M. pulmonis*-infected wild type (C57BL/6) and mutant mice were washed and suspended in RPMI-1640 containing 10% fetal calf serum, 2 mM L-Glutamine, 5×10^{-5}M 2-mercaptoethanol and penicillin/streptomycin. The cells ($5x10^5$ /well) were mixed in triplicate in wells of 96-well flat-bottomed plates with variable doses ($1.25\text{-}5 \times 10^6$ CFU/ml) of heat-killed *M. pulmonis*. The cells were then incubated in 5% CO_2 at 37°C for 48 hours. ^{3}H-thymidine was added (0.5 µCi/well) for the final 6 hours of the culture and cell-associated radioactivity was then quantified using a Tomtek 96-well harvester and a BetaPlate scintillation counter (Wallac, Finland).

For cytokine analysis, the same cells ($1x10^7$/well) were incubated in 24-well flat bottomed plates with heat killed mycoplasma ($5x10^6$cfu/ml). After 48 and 72 hours in culture at 5% CO_2 at 37°C supernatants were collected and tested for IFN-γ by ELISA.

Th1 /Th2 intracellular cytokine assay

Lungs from either pathogen-free mice or mice infected for 14 days with *M. pulmonis* were dissociated by mashing through a 70μM cell strainer and put into culture with PMA/ionomycin (50ng/mL/500ng/mL). After incubating for 2 hours at 37 degrees, brefeldin A (5ug/mL) was added and the cultures incubated for another 2 hours at 37. Cells were washed twice with PBS and then resuspended in 2% paraformeldehyde for 15 minutes at room temperature. Fixed samples were washed twice with PBS and permeabilized by suspention in PBS+0.5% saponin+1%FCS. Fixed and permeabilized cells were then stained with antibodies to mouse CD4, IL-4 or IFN-g and samples run on a FACScalibur flow cytometry. Analysis of CD4+ cytokine+ cells was performed using FlowJo software.

Construction of LACK plasmids and PEG-mediated transformation of M. pulmonis

The LACK open reading frame was extracted from a bacterial expression vector ([100]; kindly provided by Dr. R.M. Locksley) by PCR using the following primers: 5'-atccatgaattcataactacgagggtcacctgaagggtcac-3' and 5'-attgacgcggccgcaggaaattactcggcgtcggagatggaccac-3'. These primers placed an *Eco*R I site at the 5' end of the LACK coding region and a *Not* I site at its 3' end. A putative transcriptional terminator from a gene upstream of the *arcA* gene in the *M. arthritidis*

genome was extracted by PCR from pZero2.1-arcA [40] using the following primers: 5'-attgacgcggccgctgataatgataaattaaa-3' and 5'- atcatggatcctgttaaagtccggtgttac-3'. These primers placed *Not* I and *Bam*H I sites at the 5' and 3' ends of the terminator sequence respectively. The two PCR products were digested with the enzymes that cut at the engineered flanking sites and then ligated to pZero2.1-arcA that had been digested with *Eco*R I and *Bam*H I. An *arcA-LACK*-terminator fragment was subsequently extracted by PCR using the following primers that hybridize in the pzero2.1 plasmid: 5'-tatcagtaagatctacttcgaatagggcgaattg-3' and 5'-ggctcgtatgttgtgtggaa-3'. The PCR product was subsequently digested with *Bgl* II and *Bam*H I and cloned into the Tn4001T transposon vector pIVT [40] that had been digested with *Bam*H I. pIVT plasmids containing the insert in both directions were isolated and ultimately shown to express the LACK protein in *M. pulmonis* transformants when they were tested in infected mice.

M. pulmonis bacteria were transformed using the polyethylene glycol procedure and tetracycline selection as previously described[40] [99]. Briefly, *M. pulmonis* cultures were grown to log phase in liquid broth (about $1x10^7$cfu/mL) and 5 mL was centrifuged for transformation. Cells were resuspended in PBS with 10ug of vector DNA, added to 2mL 40% PEG in PBS, and vortexed immediately. After adding 10mL of PBS and vortexing, the cells were pelleted by centrifuging for 12 minutes at 10,000xg. The supernatants were removed and cells resuspended in 500uL borth A. Cultures were incubated for at least 4 hours to allow recovery and then plated on broth A agar plates with tetracycline (3ug/mL). After two weeks, colonies were picked, inoculated into 1mL broth A cultures and flash frozen for future expansion and analysis.

Expression of the LACK open reading frame in the bacteria was confirmed using a flow cytometric assay that tested for upregulation of CD69 following stimulation of LACK-specific T cells (from W15αβ TCR transgenic mice) with heat-killed preparations of the bacteria. W15αβ cells T cells were stimulated *in vitro* for 48 hours with lysed LACK-transformed *M. pulmonis* and stained with CD4 and CD69. Samples were run on the FACScan and the percentage of CD69+ CD4+ T cells was calculated using FlowJo software.

Flow Cytometry

LACK-specific T cells from the draining lymph nodes and lung tissue of infected mice were detected using a fluorescent LACK/I-A^d multimer as previously described [99, 101]. In these experiments the fluorescent multimer was generated using LACK/I-A^d molecules purified from SF9 cells, biotin-conjugated protein-A (Pierce) and APC-labeled streptavidin (eBiosciences). The T cells were simultaneously stained with directly conjugated anti-CD4 and anti-CD69 antibodies.

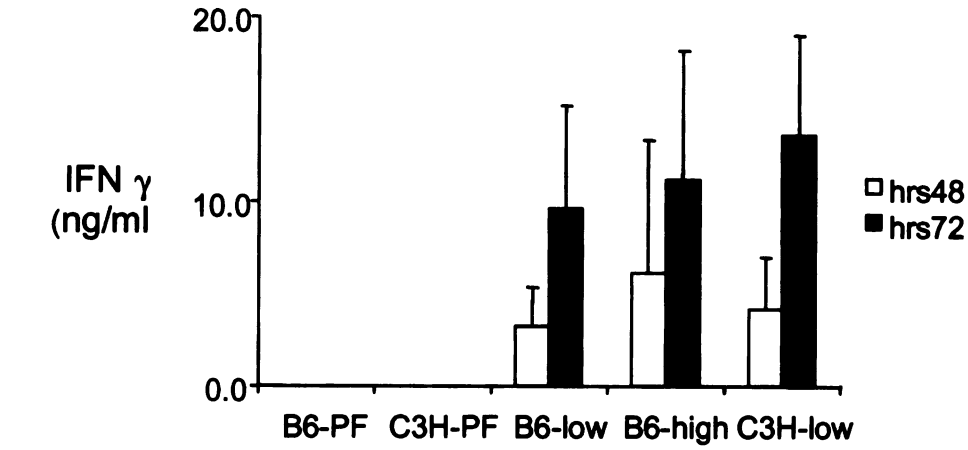
D. Results

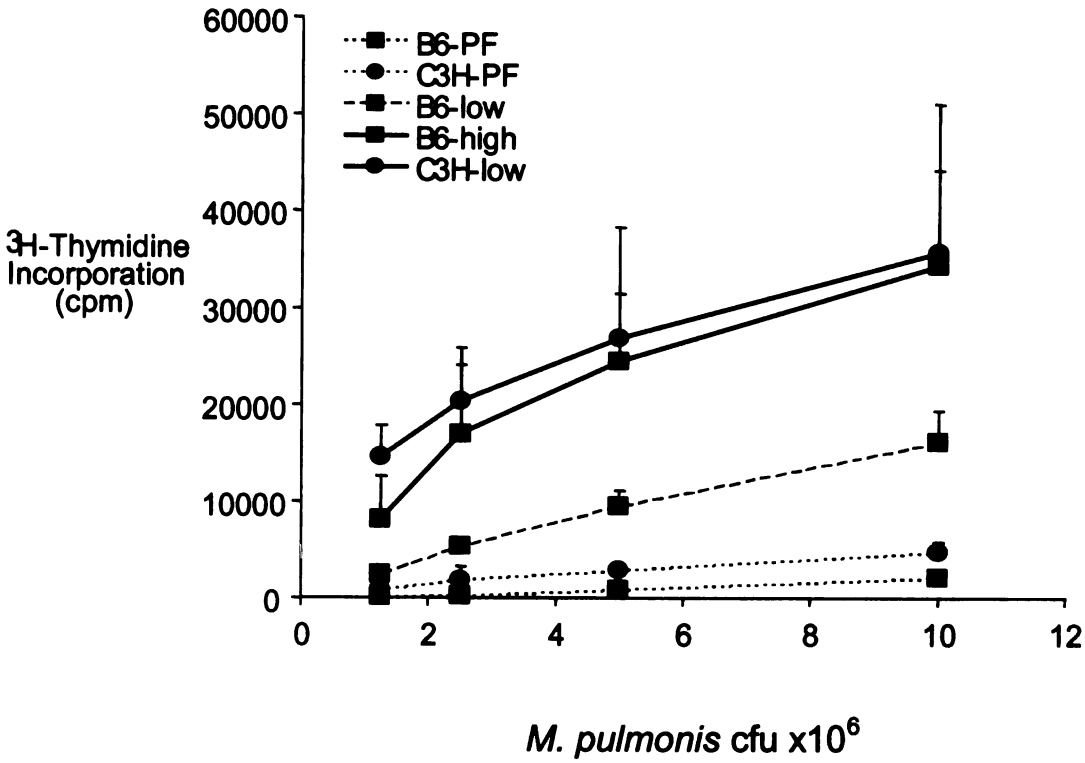
Mice infected with *M. pulmonis* show enlargement of the lymph nodes draining the airways. Accompanying this enlargement, there is a strong T cell response in both C3H and C57BL/6 mice (see Figure 1a) to the pathogen that involves both $CD4^+$ and $CD8^+$ T cells, and which is dominated by T_H1 cells in C57BL/6 background mice (Figure 1b). Although the T cell response provides help for the antibody response that prevents dissemination of the bacteria (Aurora et al., in preparation), it appears to be of little

overall significance for the control of bacterial replication because equivalent numbers of bacteria can be recovered from chronically infected T cell-deficient and T cell-replete mice.

Reagents for detecting and following T cells specific for *M. pulmonis* antigens have not yet been developed. For this reason, and because abundant tools are already in place for studying other infections, we decided to generate a model strain of *M. pulmonis* carrying a surrogate antigen from a different pathogen. Our choice for this antigen was the LACK protein from *Leishmania major*, which is the dominant target of the MHC class II-restricted T cell response in *L. major* infected H-2^d mice. This antigen was attractive because there are well-characterized fluorescent multimeric peptide/MHC class II reagents that allow for LACK-specific T cells to be detected using flow cytometry [99, 101, 102]. Furthermore, there are αβ and β TCR transgenic mice expressing heterodimers or β chains from heterodimers that recognize I-A^d presenting a minimal LACK antigenic epitope [99, 101, 103]. Finally, there is a useful precedent for the transfer of the LACK antigen into a heterologous pathogen, namely *Listeria monocytogenes*[104].

A.


20.0
10.0
0.0
IFN γ
(ng/ml
hrs48
hrs72
B6-PF
C3H-PF
B6-low
B6-high
C3H-low


60000
50000
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3H-Thymidine
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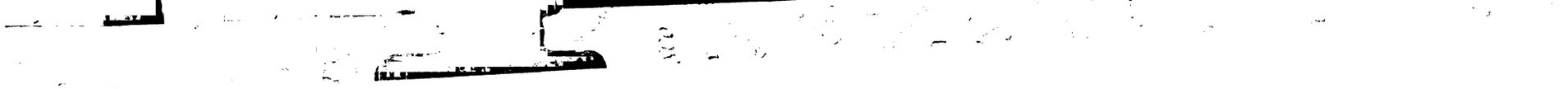
(cpm)
B6-PF
C3H-PF
B6-low
B6-high
C3H-low
0
2
4
6
8
10
12
M. pulmonis cfu x10^6

Figure 1 The T cell response to *M. pulmonis* is B cell dependent, predominately Th1 and does not effect bacterial clearance

A. Proliferation and cytokine production by cells from the draining lymph nodes of three C57BL/6 and three C3H mice infected for two weeks with *M. pulmonis*. Cells were re-stimulated *in vitro* with heat killed *M. pulmonis* and examined at 24, 48 or 72 hours for their proliferative and IFN-γ response respectively.



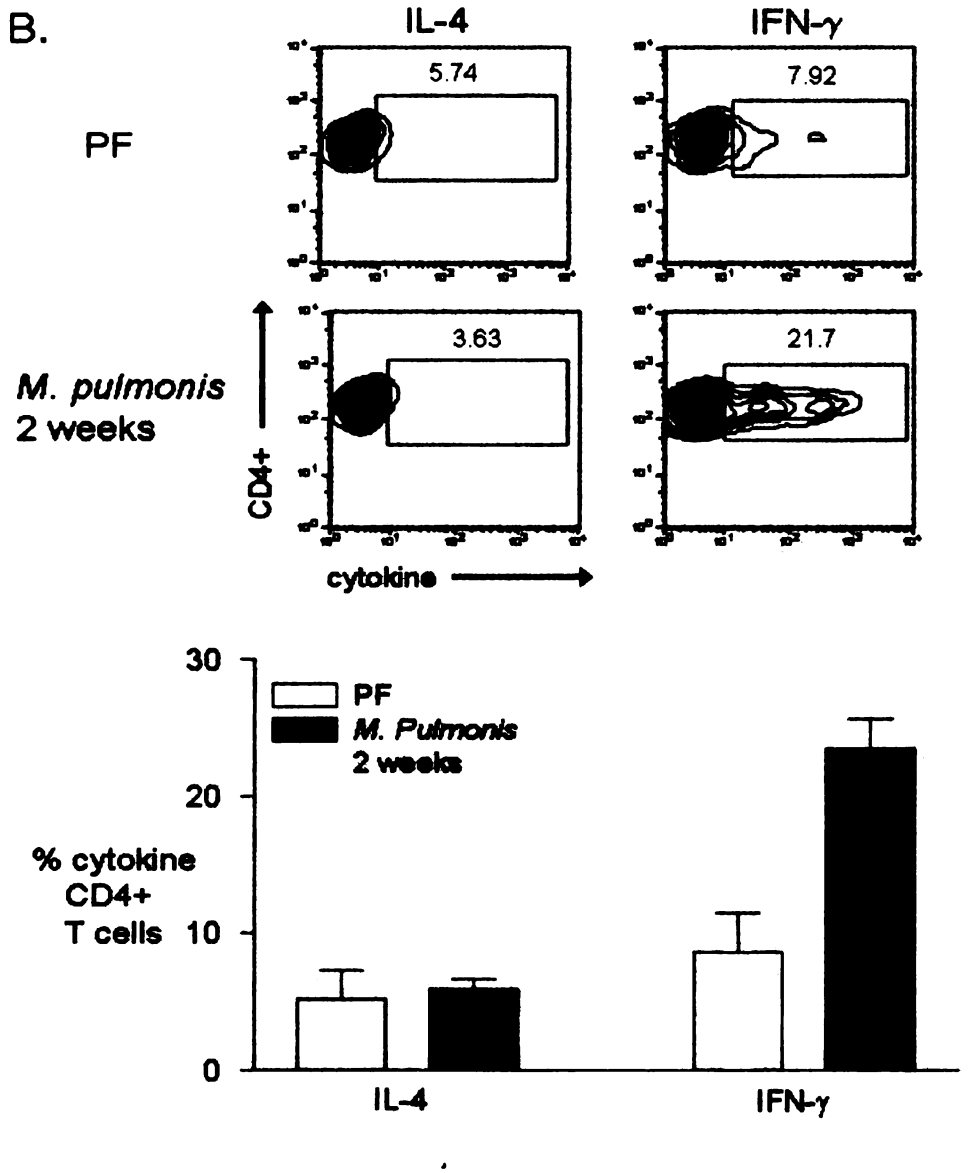


Figure 1 The T cell response to *M. pulmonis* is B cell dependent, predominately Th1 and does not effect bacterial clearance

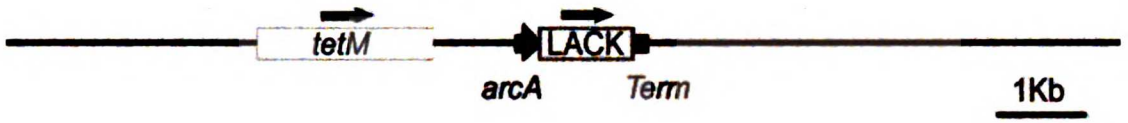
The Th1 versus Th2 response by T cells from the lungs of either pathogen-free (3 mice) C57BL/6 mice or mice infected for two weeks (6 mice) with *M. pulmonis*. Cells isolated from dissociated lungs were cultured with PMA/ionomycin and brefeldin A. Staining for intracellular cytokines, IL-4 or IFN-γ, was done followed by flow cytometric analysis of the percentage of cytokine positive CD4+ T cells.

To express LACK in *M. pulmonis*, we generated a transposon vector with the structure shown in Figure 2a. This vector was based on a design that had been successfully applied previously for the expression of *lacZ* in *M. pulmonis*[40]. An open reading frame encoding the *Leishmania major* LACK antigen was placed immediately downstream of the *M. arthritidis arcA* promoter and upstream of a transcriptional terminator from a gene that lies adjacent to *arcA* in the *M. arthritidis* genome. The vector was transformed into *M. pulmonis* and colonies were selected on tetracycline-containing plates. Twenty nine of these were then grown up further in broth and five were selected for further analysis.

As an initial test of whether the *M. pulmonis* bacteria expressed the LACK antigen, we stimulated LACK-specific αβ TCR transgenic T cells with heat-killed preparations of the bacteria and then monitored their activation by flow cytometry for CD69 expression. This assay is sufficiently sensitive to detect ≤1 ng/ml of recombinant LACK antigen in the culture medium. Preparations of *M. pulmonis* bacteria carrying the LACK expression vector stimulated an obvious response in the assay whereas *M. pulmonis* transformed with the empty vector carrying no LACK sequences failed to give a response. These data confirmed that the LACK-transformed *M. pulmonis* expressed the LACK protein in a form that could be presented to T cells *in vitro*.

To test whether the LACK-transformed *M. pulmonis* could induce a LACK-specific T cell response *in vivo* we initially infected cohorts of mice with one of the transformants that scored positively in the *in vitro* CD69 assay. We then monitored

A.



B.

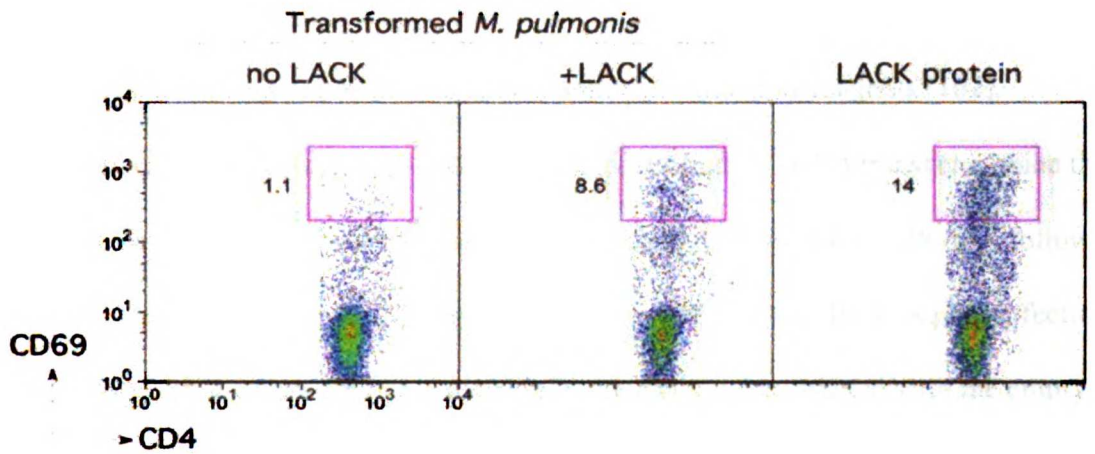


Figure 2 **Gene transfer and expression of LACK in *M. pulmonis***

A. Design of pIVT-LACK. The LACK sequence was inserted into The Tn4001T transposon vector under the control of the *arcA* promoter.

B. Transformed *M. pulmonis* expresses LACK. Transformed *M. pulmonis* was used to stimulate LACK-reactive T cells, isolated from W15αβ mice, *in vitro*. Activation was measured by CD69 expression of the CD4+ T cells population by flow cytometry.

clonal expansion of LACK-specific T cells in the draining lymph nodes of the infected mice using a well-characterized LACK/I-A^d fluorescent multimer. To increase the sensitivity of the experiment, the mice we used for this experiment expressed a transgenic TCRβ chain from a LACK-specific T cell hybridoma. These TCRβ transgenic mice have a diverse TCR repertoire with a frequency of multimer-positive cells (*i.e.*, LACK-reactive T cells) that is at or beneath the level of detection before exposure to LACK. The presence of the TCRβ chain, however, ensures that the mice make a large and rapid response to LACK immunization or *Leishmania major* infection. By contrast, the LACK response in nontransgenic mice infected with *Leishmania major* is difficult to detect without making use of dual-labeled fluorescent multimer reagents [102].

Multimer-positive cells could be readily identified in TCRβ transgenic mice that were infected with LACK-transformed *M. pulmonis* at 4, 7, 10, 14 and 28 days following infection, with the peak of the response occurring between 4 and 10 days post-infection. Multimer-positive cells were not detectable in mice that were infected with the empty vector, nor could they be reliably detected in mice that lacked the TCRβ chain. These data therefore confirmed that the transformed *M. pulmonis* expressed LACK and could elicit a LACK-specific T cell response in infected mice (Figure 3a).

M. pulmonis bacteria could be recovered from the lungs of mice infected with the LACK transformant, but only at 4 and 7 days after infection. Thereafter, the mice appeared to have cleared the organism. Consistent with this, the lungs of the mice lacked macroscopic signs of pneumonia and *M. pulmonis*-induced pathology. There was also no evidence of tracheal vascular remodeling at 28 days after infection, such as is commonly observed in mice that are infected with the parental strain of the bacteria (Figure 3b).

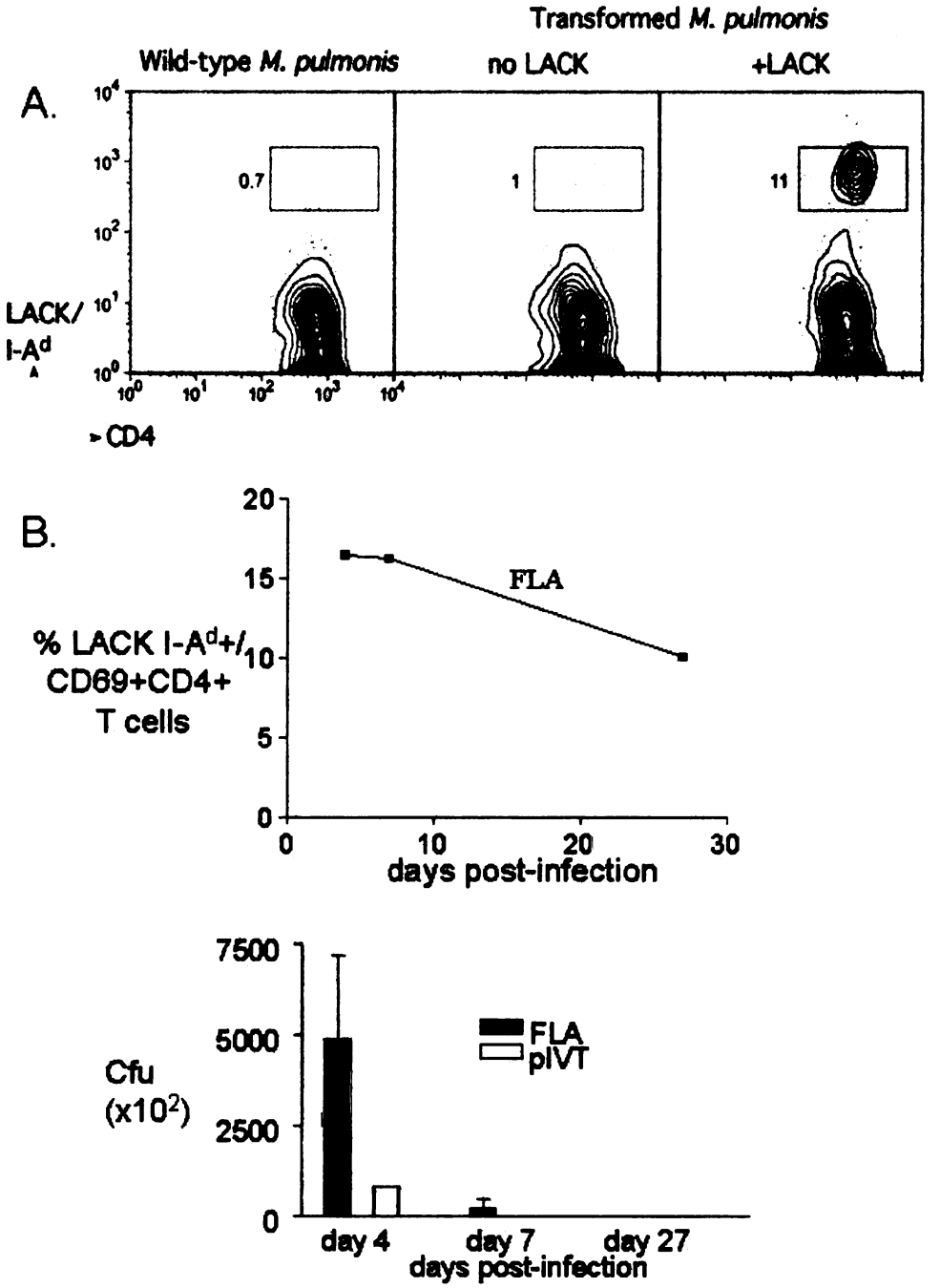

Transformed M. pulmonis
Wild-type M. pulmonis
no LACK
+LACK
A.
0.7
1
11
LACK/
I-Ad
A
CD4
B.
% LACK I-Ad+/
CD69+CD4+
T cells
FLA
days post-infection
Cfu
(x10²)
FLA
plVT
day 4
day 7
day 27
days post-infection

Figure 3 **Kinetics of the LACK-specific T cell response to LACK-transformed *M. pulmonis* infection**

A. Draining lymph nodes from W15β+ mice infected with LACK-transformed *M. pulmonis* for 14 days were isolated and stained with CD4, CD69 and the LACK I-A^d tetramer. Plots shown represent tetramer staining of the CD69+CD4+ T cell population.

B. Kinetics of the CD4+ CD69+T cell response to LACK-mycoplasma were measured at day 4, day 7, and day 27 post-infection in W15β+ mice (3 mice per group). The persistence of the bacteria in the airway was assessed during the same time course by plating supernatant from lung homogenates on Broth A agar plates. The numbers of cfu's were counted after two weeks.

Colonies of *M. pulmonis* demonstrate variability in virulence in part probably because of recombinase-induced changes in the expression of variable surface antigens. So, while it seemed possible that clearance of the transformed *M. pulmonis* could be a consequence of the strong LACK-specific T cell response, it was also possible that the LACK transformant was less virulent than its parent because of underlying genomic variation. To address this issue, we infected a cohort of mice with four additional LACK-expressing variants and tested them at 12 days after infection for the presence of bacteria and evidence of a LACK-specific T cell response against them by flow cytometry. For two of the new isolates tested and also for the original one, there were no *M. pulmonis* bacteria detectable in the lungs of the infected mice at the 12 day time point. For two others, however, this was not the case, and numbers of bacteria in the one case comparable with the parental UAB CT7 strain could be recovered from the infected lungs (data not shown). Lesions on the lungs were apparent for both of these isolates. For all of the isolates tested, there had been obvious clonal expansion of LACK-reactive T cells in the infected mice, as detected with the LACK/I-A^d fluorescent multimer, again confirming that the transformed bacteria expressed LACK. No such LACK expression was evident in mice infected with the control bacteria. These results show that LACK-expressing engineered *M. pulmonis* can persist at least to twelve days following infection and suggest that the clearance of the original isolate was likely to be a consequence of the expected underlying variation in virulence exhibited by colonies of *M. pulmonis*.

E. Discussion

The T cell response to *M. pulmonis* is of questionable significance with respect to control of replication of the bacteria in the respiratory system. This statement follows from the observation that SCID, *Rag1*$^{-/-}$ or T cell-deficient mice harbor little by way of obvious differences in bacterial numbers in their lungs as the infection enters its chronic phase. Given the propensity for variation in *M. pulmonis*, it is possible, however, that the T cell response imposes selection for variation and thus there may be more flux in the *M. pulmonis* antigenic profile in the presence of a vigorous T cell response than in its absence. Even if this is true, however, it seems clear that the T cell response is largely ineffectual in terms of clearing the organism from the body.

We sought to capitalize on the chronic nature of *M. pulmonis* infection and the absence of sterilizing T cell immunity by engineering the organism so that it would be highly visible to a sensitized (*i.e.*, TCRβ transgenic) T cell system. As shown in this paper, the introduction of the LACK expression vector into *M. pulmonis* accomplished this and allowed for readily detectable clonal expansion of LACK-specific T cells in the infected mice. The results therefore validate the experimental model and demonstrate some of its potential for studying airway infections with *M. pulmonis*, and in uncovering the impact of chronic antigen exposure on the form of the T cell response.

Variation in *M. pulmonis* due to genomic recombination is at least one explanation for differences in virulence exhibited by independently generated isolates of the organism. Here we initially found that one of the transformants we generated showed poor virulence and was cleared from the infected lungs by 12 days after infection. In contrast, other transformants showed equivalent LACK immunogenicity but a much-

enhanced capacity to persist to at least 12 days following infection. Whereas diminished virulence would be a problem if it completely interfered with persistence in all transformants, the results show that it is possible to derive isolates of *M. pulmonis* that differ in the length of time they persist *in vivo*. This is attractive experimentally because it should allow for a comparison to be made between the immune response elicited by acute versus chronic encounter with an airway pathogen. Although we have revealed the potential in the system with the data presented in this paper, additional experiments are now required to assess the fate of the transformants and their capacity to remain positive for LACK expression at much later time points. Moreover, it remains to be established which, if any of the isolates allows for the normal spectrum of remodeling events in the infected airways. With additional characterization of this sort as the foundation, future studies will focus on exploiting the system to provide new insight into the consequences of chronic antigen exposure in the airways.

Chapter 5:

Discussion:

Significance

An estimated 30.8 million Americans have been diagnosed with asthma within their lifetime; 11.2 million adults in the U.S. have COPD, and close to 342,000 Americans die of lung disease every year. Whether it is asthma, chronic bronchitis, emphysema, or pneumonia, a common feature of these diseases is impaired airway function resulting from changes in tissue structure. While we have an understanding of how some of these changes can be induced, most of what we know concerns the potential remodeling properties of specific mediators such as cytokines. As discussed in the introduction in more detail, transgenic experiments show that Th2 cytokines such as IL-5 or IL-13 can induce mucus secretion, fibrosis, and AHR [6] [7]. IL-11 is also capable of promoting fibrosis of the airway wall and smooth muscle hyperplasia[2, 3].

Asthma, as an example of a chronic inflammatory disease of the airways, is typically managed by alleviating the symptoms of airway hyperresponsiveness, which

untreated cause physical discomfort, difficulty breathing and even death. It is becoming more and more apparent, however, that asthmatics can have long term physical consequences of their asthma, even if their acute symptoms are successfully alleviated.

The work presented in this thesis directly addresses the mechanism of how chronic inflammation is instigated and how it can lead to tissue remodeling in the airways and disease. The mechanism described may not be unique to the airway and may very well feature in the inflammation-induced remodeling that occurs in cancer, arthritis, and other chronic conditions. The pathway defined illustrates how a confined bacterial infection stimulates adaptive immunity that in turn cooperates with the bacteria to provide continuous recruitment and activation of neutrophils and macrophages to the airway. The inflammatory cells respond by producing soluble factors that directly and indirectly cause vascular, epithelial and general mucosal remodeling. The development or exacerbation of asthma and other chronic diseases often correlates with the presence or persistence of a latent infection. This raises the possibility that the murine mycoplasmosis model may have relevance to human disease beyond the symptoms of remodeling it induces. It is possible that as in mycoplasmosis, an infectious trigger may be causative for the onset and chronic aspects of specific human airway diseases.

Murine mycoplasmosis as a Model system

In addition to its similarity to human diseases, part of the attraction in studying the murine model of respiratory mycoplasmosis is that it is relatively unexplored. From an immunological standpoint, it provides a unique opportunity to study an infectious model that is both chronic and confined to a specific, non-lymphoid tissue. Mycoplasmas

successfully evade both the innate and adaptive immune systems by mechanisms that remain entirely mysterious. More importantly, in the context of this thesis, the immune response against the bacteria causes damage to the host.

Before we began the work presented here, several groups had observed the absence of gross lung lesions in the airways of infected SCID mice, and this was the first suggestion that the airway disease was likely to be immunopathological in nature [48, 105]. Later it was confirmed that in spite of similar numbers of bacteria living in their lungs, and the systemic dissemination of the bacteria throughout the body, lymphocyte-deficient mice did not have the airway disease found in wild-type mouse strains. In chapter 2 and 3, I report on studies of the mechanisms behind lymphocyte dependent remodeling. Several types of tissue changes were initially studied including the cell types and mechanisms driving the remodeling of the airway mucosa. However, my initial emphasis was to focus on the dramatic remodeling that occurs in the airway vasculature of *M. pulmonis*- infected mice. As exciting new discoveries about the process of new lymphatic growth, or lymphangiogenesis, were published and new reagents became available to study this process, it was natural to extend my work on angiogenesis to include lymphangiogenesis. Furthermore, the subject of lymphocyte-mediated angiogenesis and lymphangiogenesis, particularily in the airway, was a largely unexplored area, making it an appealing and exciting focus for research. As shown in this thesis, it is now possible to make connections between specific components of the adaptive immune system, inflammatory leukocytes, and their secretion of products that induce tissue remodeling. Such connections can now be solidified by future studies that

will be aimed at clarifying the significance of individual cell types and their products in distinct remodeling processes.

Summary of key findings

Most of the current information available in the literature regarding tissue remodeling in the airways comes from studies that were focused on the role of key inflammatory mediators in causing specific aspects of pathology. Many of the mediators that have been implicated in airway remodeling such as fibrosis and epithelial hyperplasia are directly produced by, or are under the control of, other factors produced by T cells. The involvement of the immune system in vascular remodeling has mostly been documented as the ability of neutrophils and macrophages to produce pro-angiogenic factors. For this reason, we were intrigued to discover a requirement for B cells in inducing remodeling. The significance of B cells in remodeling became apparent from several findings. First, the analysis of airway vessels and lymphatics in B cell-deficient mice during the chronic phase of infection clearly showed an absence of angiogenesis and lymphangiogenesis in these mice compared to controls. In addition, while some mucosal changes had taken place, the epithelial and smooth muscle hyperplasia characteristic of the mycoplasma-infected airway did not take place without B cells. Finally, the recovery of angiogenesis by the transfer of immune serum into B cell deficient mice showed that Ig, one of the main effector functions of B cells, could substitute for B cells.

One caveat to the finding that B cells were required for remodeling is the discovery that B cell-deficient mice have defective T cell responses (Chapter 4, Figure

1b). The suggestion could be made then that T cells play a prominent role in angiogenesis and B cells have a secondary role. The serum-transfer experiments in which angiogenesis could be recovered by administering immune serum from wild-type mice to B cell-deficient mice argued against this possibility. This experiment only scored endothelial cell proliferation, one aspect of remodeling, and thus could have missed evidence of a T cell contribution to another form of remodeling. A T cell contribution can not be ruled out at this time. The analysis of tracheal whole-mounts from T cell-deficient mice at four weeks post infection show that T cells also played a role in remodeling but a specific contribution by T cells to remodeling, aside from providing help for the T cell-dependent antibody response, has not yet been carefully examined. Because of the ability of T cells to make a host of cytokines, it would not be surprising to find a B cell-independent T cell contribution to mycoplasma airway disease. To address this question, experiments are planned to compare angiogenesis in T-cell deficient mice with T-cell deficient mice that have received immune serum.

The data presented are compatible with a working model in which T cell-dependent *M. pulmonis*-specific antibodies in the form of immune complexes induce the inflammation responsible for angiogenesis and lymphangiogenesis. The potential significance of Ig, specifically in the form of immune complexes, was first realized by visualizing these structures in the airways of wild-type infected tracheas and B-cell deficient mice given immune serum. The real confirmation linking immune complexes to remodeling came from studies using Fc receptor-deficient mice. Fc receptor deficiency completely abolishes the Arthus reaction, a classic model of immune-complex-mediated inflammation, and also interrupts other immune complex mediated

diseases such as rheumatoid arthritis. That angiogenesis and lymphangiogenesis in the airway are dependent on these receptors confirms the reliance of the remodeling mechanism on immune-complex mediated inflammation. One possible role for Fc receptors, would involve stimulation through an Fc receptor expressed by one of the cell types in the airway which in turn might directly trigger the cell to produce pro-angiogenic or lymphangiogenic factors. To address this possibility, it would be helpful to perform experiments in which expression of soluble factors by cells from the wild-type infected airway will be compared to the expression profiles of cells from Fc receptor deficient airways. The observation that Fc receptor deficiency does not completely ablate vascular remodeling suggests other mechanisms must be in place that can send signals through the airway inflammatory cells to induce their production of soluble mediators.

Complement also influenced vascular remodeling though the effect was transient. During the acute phase of infection (i.e. around day 10), mice deficient in C3, the central component of the complement cascade exhibited retarded angiogenesis in their tracheas as observed by the decrease in endothelial cell proliferation measured in comparison to wild-type mice. This contribution seemed only to delay the kinetics of remodeling because by 4 weeks of infection, the lymphangiogenesis and angiogenesis of $C3^{-/-}$ mice looked similar to that of wild-type and the density of the vessels in the tracheas of both types of mice was equivalent. At least one way complement contributes to remodeling is through the stabilization of immune complexes that are essential for inflammation-induced remodeling. In tracheas from C3-deficient mice, the relative density and intensity of staining for IgG immune complexes was consistently diminished at early and late timepoints compared to wild-type mice. C3b can contribute to the stabilization of

these structures by forming C3b2-IgG complexes that can then bind to surfaces and cluster into lattices. If stimulation of key inflammatory cells occurs via these structures, an absence of C3b might result in a delayed development or reduction in the number of these lattices. It might take longer, then, for enough inflammatory cells to accumulate and/or be activated for the production of soluble factors involved in remodeling. (see discussion figure 3) We also have shown that cleavage of C5 into C5a (a potent neutrophil chemoattractant) and C5b is not essential for angiogenesis and lymphangiogenesis because C5-deficient mice have remodeling in their tracheas that is indistinguishable from wild-type.

The majority of the inflammatory cells recruited to the airways in response to mycoplasma infection are neutrophils. Neutrophils produce pro-angiogenic factors in a number of settings as reviewed in the introduction. Therefore, we had expected that C5a might be one important contributor to the process of remodeling. That it is not critical suggests that one or more of the many other neutrophil and macrophage chemoattractants can sufficiently recruit these cells to the airways. The recruitment of these cell types to the site of infection in the absence of immune complexes has the potential to induce remodeling. The observation of transient remodeling at early time-points of infection, focused around occasional areas of infiltrating inflammatory cells in *Rag1*$^{-/-}$ mice, strongly suggested a direct association between these cells and angiogenesis and lymphangiogenesis. We have also been able to provide direct evidence that neutrophils and/or macrophages isolated from the airways of infected mice are induced to produce multiple tissue-remodeling factors including VEGF-A, TGFβ, and FGF-2. In addition, we were able to show that expression of some of these mediators, in particular VEGF-D,

is higher in airway inflammatory cells from wild-type mice than in cells from *Rag1*$^{-/-}$ mice. Consistent with the idea that there were differences in the activation state of neutrophils and macrophages dependent on the presence of adaptive immunity, there were significant changes in cell surface expression of proteins such as CD62L and MHC class II on the leukocytes from wild-type and *Rag1*$^{-/-}$ mice

In spite of the data clearly highlighting qualitative differences between neutrophils and macrophages from airways of wild-type compared to *Rag-1*$^{-/-}$ mice, one fundamental question remains unanswered. Is it the number of inflammatory cells that persist in the airway tissue, or the specific activation state of the cells that is most critical for the induction of remodeling? If the former is true, one would predict that just getting enough inflammatory cells to persist in the airways at any given time would be enough to cause remodeling. The data pertaining to the appearance of transient remodeling lends support here. If it is the latter, then the inflammatory cells would first have to traffic to the airway, but in addition would need specific signals, at least in part through their Fc receptors, in order to sustain production of tissue-changing mediators. Resolving between these two possibilities for remodeling of the blood and lymphatic vessels is an important outstanding issue for future investigation. Another key area of future investigation will be to identify the type of cell(s) that participate in producing VEGF-D, FGF-2, and others. A significant challenge in pinpointing which cell type(s) are producing soluble mediators is the lack of mutant mice that selectively lack neutrophils, macrophages, or other potentially important myeloid cell types such as mast cells. While mast cell -deficient mice do not present with any defects in remodeling (unpublished results), the data is confounded by the significantly larger numbers of cfu's that exist in

the lungs of these mice. One way to get around this issue may be to first tackle the challenge of identifying what pro-angiogenic and lymphangiogenic factors are absolutely essential for airway remodeling. If some of these can be clearly identified, the cell types making them can then be identified. The identification of mediators made by cells in the BAL was a key first step towards this goal. Experiments are now underway to refine this quantitative analysis so that expression can be examined in homogeneous, identifiable populations of cells. In addition, experiments using blocking antibodies specific to these factors will help distinguish which ones are dispensable and which ones are essential for *M. pulmonis* induced remodeling. Answering these questions is central to the long-term goal of identifying targets for the development of treatments that can effectively inhibit tissue remodeling and not just the inflammation surrounding it.

Concluding remarks

From these studies, we propose a model in which the humoral immune response against *M. pulmonis* is important for preventing systemic dissemination of the infection but is ineffective at eliminating the bacteria. As a result of the large numbers of bacteria remaining in the airways, mycoplasma-specific Ig forms immune complexes with the bacteria, providing a platform for the continual recruitment and chronic stimulation of neutrophils and macrophages through their Fc receptors. These inflammatory cells are then capable of producing soluble factors that contribute to angiogenesis and lymphangiogenesis and perhaps other types of remodeling (discussion Figure 1). This mechanism may be expedited by the stabilization of the immune complexes by the complement protein C3b. Inflammatory cells are recruited to the airway because of the

significant numbers of bacteria living there (possibly through stimulation via Toll-like receptors) and make factors that induce tissue changes. Without stimulation from immune-complexes, through Fc receptors, the production of pro-angiogenic and lymphangiogenic factors is not enough or not sustained long enough to perpetuate remodeling (discussion Figure 2).

This thesis had made clear a pathway that starts from an adaptive immune response leads to recruitment of inflammatory cells and results in the accumulation of mediators that can influence tissue remodeling. Some of the key contributors named here here, in addition to the description of the pathway, include the identification of dependency of remodeling on Fc receptors, experimental approaches for monitoring immune-complex induced gene expression in airway inflammatory cells, and the development of a novel system to track T cell responses to mycoplasma infection. Knowledge about Fc receptor dependency will allow for future experiments in which loss of Fc receptor function is induced selectively in specific subsets of myeloid cells. This can be accomplished by using mouse strains that have conditional deletions of the Syk gene. Such experiments could be of great benefit in identifying the cell types that are critical for causing remodeling. The gene expression analysis approach we have developed provides a molecular profile of remodeling that can now be expanded and exploited as a rapid tool for analyzing mice for the presence of remodeling events. Finally, the engineered mycoplasma offer considerable promise for the analysis of how acute versus chronic exposure to an airway pathogen such as M. pulmonis influences the form and effectiveness of the T cell response. In conclusion, this thesis has helped to shed new light on a poorly understood connection between adaptive immunity and tissue remodeling while also providing a substantial foundation for the continued studies on remodeling and basic studies on T cell responses.

Figure 1

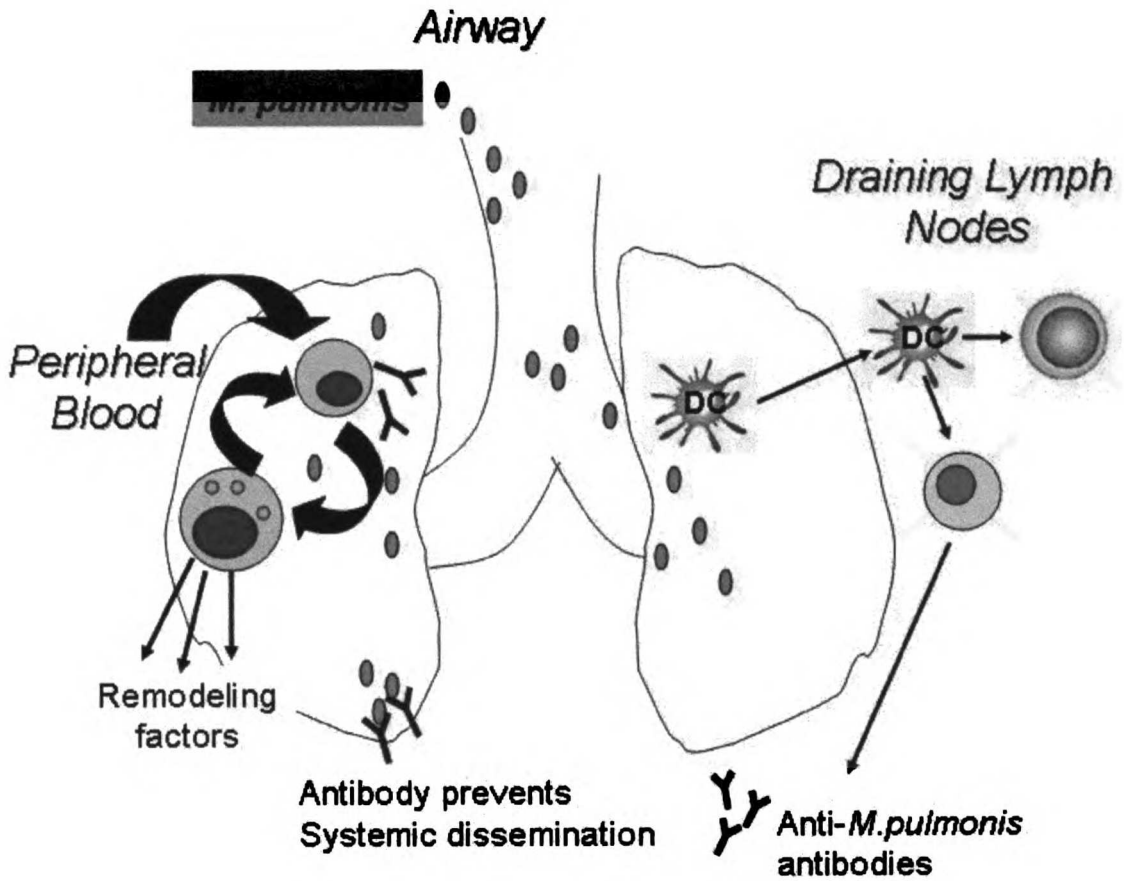
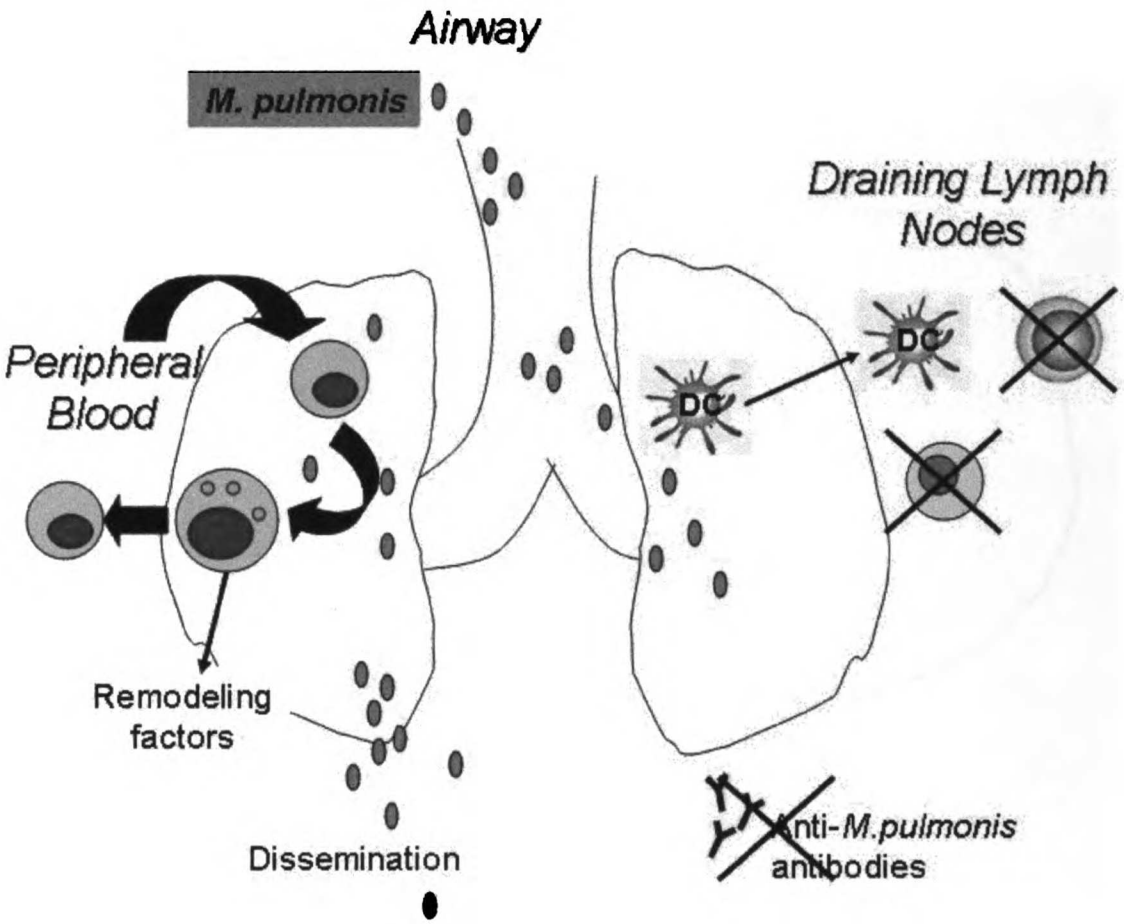

Airway
M.pulmonis
Draining Lymph Nodes
Peripheral Blood
DC
DC
Remodeling factors
Antibody prevents Systemic dissemination
Anti-M.pulmonis antibodies

Figure 2


Airway
M. pulmonis
Draining Lymph Nodes
Peripheral Blood
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Dissemination
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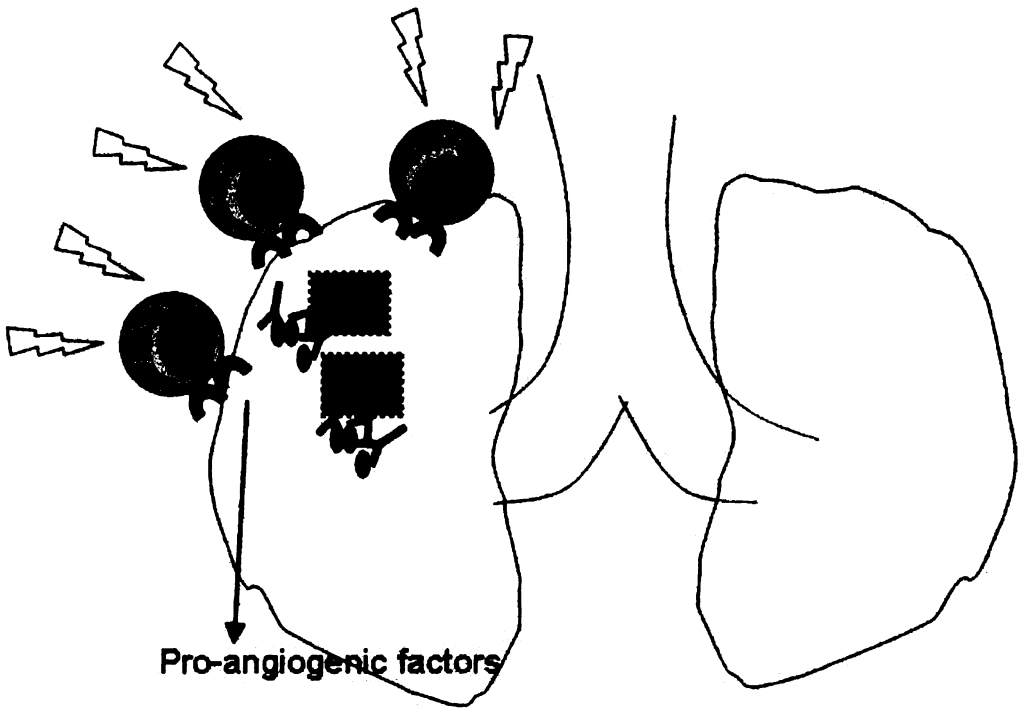
Figure 3



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