Mast Cells Protect Mice from Mycoplasma Pneumonia

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Rationale: As the smallest free-living bacteria and a frequent cause of respiratory infections, mycoplasmas are unique pathogens. Mice infected with *Mycoplasma pulmonis* can develop localized, life-long airway infection accompanied by persistent inflammation and remodeling.

Objective: Because mast cells protect mice from acute septic peritonitis and gram-negative pneumonia, we hypothesized that they defend against mycoplasma infection. This study tests this hypothesis using mast cell-deficient mice.

Methods: Responses to airway infection with *M. pulmonis* were compared in wild-type and mast cell-deficient *Kit^{W-sh}/Kit^{W-sh}* mice and sham-infected control mice.

Measurements and Main Results: Endpoints include mortality, body and lymph node weight, mycoplasma antibody titer, and lung mycoplasma burden and histopathology at intervals after infection. The results reveal that infected *Kit^{W-sh}/Kit^{W-sh}* mice, compared with other groups, lose more weight and are more likely to die. Live mycoplasma burden is greater in *Kit^{W-sh}/Kit^{W-sh}* than in wild-type mice at early time points. Four days after infection, the difference is 162-fold. Titers of mycoplasma-specific IgM and IgA appear earlier and rise higher in *Kit^{W-sh}/Kit^{W-sh}* mice, but antibody responses to heat-killed mycoplasma are not different compared with wild-type mice. Infected *Kit^{W-sh}/Kit^{W-sh}* mice develop larger bronchial lymph nodes and progressive pneumonia and airway occlusion with neutrophil-rich exudates, accompanied by angiogenesis and lymphangiogenesis. In wild-type mice, pneumonia and exudates are less severe, quicker to resolve, and are not associated with increased angiogenesis.

Conclusions: These findings suggest that mast cells are important for innate immune containment of and recovery from respiratory mycoplasma infection.

Keywords: angiogenesis; bronchitis; innate immunity; lymphangiogenesis; pneumonia

Mast cells are widely distributed in tissues and are especially prominent near surfaces such as airways exposed to the outside environment. Their strategic positions allow them to act as sentinels of invading parasites and other potential pathogens. Mast cells receive considerable attention for responding to IgE-reactive antigens and for their roles in allergic inflammation. Recent evidence suggests that they also defend against bacteria, protecting from acute septic peritonitis and gram-negative pneumonia (1–4). In this regard, their involvement is an innate response rather than

an adaptive response initiated by acquired, pathogen-specific antibodies. Indeed, mast cells share certain features with classical innate immune cells, such as neutrophils and macrophages. For example, a variety of stimuli other than antigen-bound immunoglobulins activate mast cell release of biologically active products also associated with neutrophils or macrophages. Some of these products (such as cathelicidins) are directly toxic to pathogens (5) and others (such as tumor necrosis factor α [TNF- α]) regulate inflammation and immune function (1, 6–8). Mast cells can be activated by inflammatory peptides and ligands of Toll-like and complement receptors (9–13). Such responses link mast cells to innate immune defense against microbes.

Mycoplasmas are the smallest free-living, self-replicating bacteria and possess unusually small genomes (reviewed in Reference 14). They differ in key ways from other microbes, including the lack of a cell wall. Mycoplasmas are primarily mucosal pathogens, living as extracellular parasites in close association with host epithelial cells, typically in respiratory and urogenital tracts. *Mycoplasma pneumoniae* is a leading cause of childhood and adult tracheobronchitis and pneumonia. Infection with mycoplasma diseases can worsen or even precipitate noninfectious respiratory diseases, such as asthma and chronic obstructive pulmonary disease, in humans (15, 16) and in rodent models (17, 18). Many mycoplasmas infecting humans and other mammals are known for their ability to induce chronic disease in which clearing of the organism is difficult (14). This is partly because mycoplasmas can avoid immune recognition by changing their repertoire of surface antigens. Some mycoplasmas evade immune surveillance by living inside host cells (19). They also can modulate host immune responsiveness and establish persistent infection. *Mycoplasma pulmonis* causes natural murine respiratory disease with manifestations similar to those in humans with *M. pneumoniae* infection (17, 20).

We hypothesized that because lung mast cells help to defend against acute infections with some conventional bacteria, they also defend against acute and chronic mycoplasma infection. In addition, because mast cells promote certain types of angiogenesis and stimulate airway remodeling in chronic allergic inflammation (21–23), we hypothesized that they contribute to mycoplasma-induced remodeling (20, 24). To test these hypotheses, we compared airway responses to *M. pulmonis* in wild-type and mast cell-deficient mice. Some of these results were reported in the form of an abstract (25).

METHODS

Mycoplasma Infection

Mast cell-deficient C57BL/6 *Kit^{W-sh}/Kit^{W-sh}* (Wsh) and wild-type C57BL/6 *Kit⁺/Kit⁺* (+/+) mice were housed as described (26) and studied at 8 to 10 wk of age, except in adoptive transfer experiments in which mice were infected at 17 to 18 wk of age. Mice were inoculated intranasally with 5×10^5 cfu of *M. pulmonis* strain CT7 (27). This dose was established by pilot studies, which detected high mortality in Wsh mice with larger inocula. Selected mice received a smaller inoculum (10^5 cfu). Control mice received sterile broth.

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Gross Observations and Histopathology

After infection, mice were killed at intervals up to 28 d. Endpoints included morbidity, body and bronchial lymph node weight, and pneumonia severity, as assessed by histopathologic scoring (28).

Quantitative Mycoplasma Cultures

Homogenates of infected lungs were serially diluted onto agar plates (17). Colonies were counted after 7 to 10 d.

Bronchoalveolar Lavage

Under anesthesia, a sterile, 22-gauge catheter was inserted into exposed tracheal lumen. Bronchoalveolar lavage (BAL) fluid was collected from three 0.8-ml aliquots of phosphate-buffered saline (PBS) per mouse. Supernatants were stored at -80°C .

Flow Cytometry

Cells disaggregated from bronchial lymph nodes harvested 7 d after infection were washed and incubated with fluorescein isothiocyanate-conjugated anti-Mac-1 and CD69 (BD PharMingen, San Diego, CA), phycoerythrin-conjugated anti-CD8, and Tri-Color-conjugated anti-CD4 and B220 (Caltag, Burlingame, CA). Nonspecific binding was blocked with rat anti-mouse anti-CD16/32 (BD PharMingen). Cells were analyzed with a FACSCaliber flow cytometer (Becton Dickinson, San Jose, CA).

Immunization with Heat-killed Mycoplasma

M. pulmonis organisms (0.5×10^6 or 20×10^6) killed by exposure to 65°C for 30 min were injected intraperitoneally into Wsh and $+/+$ mice. Serum was harvested up to 28 d later.

Measurement of Antimycoplasma Immunoglobulins

Immunoassay plates coated with *M. pulmonis* antigen (2×10^5 cfu/well in 50 mM carbonate buffer, pH 9.6) were incubated overnight at 4°C . Wells were blocked for 2 h with 1% bovine serum albumin (BSA)/PBS. Serum diluted initially 1:20 (for IgG1 and IgG2a), 1:10 (IgA and IgM), and 1:5 or undiluted (IgE), and then serially with PBS/0.05% Tween-20/0.5% BSA was added, followed by 50 μl of PharMingen biotinylated anti-mouse IgG1, IgG2a, IgA, IgM, or IgE (1:2,000). After incubation overnight, 50 μl of alkaline phosphatase-conjugated streptavidin (1:3,000; Jackson ImmunoResearch, West Grove, PA) were added and detected spectrophotometrically at 405 nm using phosphatase substrate (Sigma, St. Louis, MO).

Measurement of Histamine

Histamine concentration in lung homogenates was determined by Immunotech ELISA (Beckman Coulter, Fullerton, CA) according to instructions provided by the manufacturer.

Measurement of Cytokines and Surfactant Proteins

Signals were quantified by densitometry. BAL cytokine and chemokine levels were assayed with ELISA kits for TNF- α , monocyte chemoattractant protein 1 (MCP-1), and interleukin (IL)-6 (BD PharMingen) and macrophage inflammatory protein 2 (MIP-2) (R&D Systems, Minneapolis, MN); these proteins were chosen because they are secreted by mast cells and implicated in responses to infection. Surfactant protein (SP)-A and SP-D collectins enhance phagocytosis, regulate macrophage activity (29), and bind to *M. pneumoniae* (30). We compared SP-A and SP-D in Wsh and $+/+$ mice by dot-immunoblotting of serially diluted BAL supernatants. Anti-SP-A and anti-SP-D were incubated with blots as described (31) and detected with horseradish peroxidase-conjugated anti-rabbit IgG by ECL (Amersham Pharmacia, Piscataway, NJ). Signals were quantified by densitometry.

Mast Cell Culture and Adoptive Transfer

Marrow cells of 5- to 7-wk-old $+/+$ mice were differentiated into greater than 95% mast cells over 4 to 5 wk (3). Wsh mice were tail-vein-injected with 10^7 cells and studied 12 wk later based on data that mast cells populate Wsh lung within 12 wk of injection (26).

Vessel Morphometry

Whole-mounted tracheas excised after infection were incubated with polyclonal anti-LYVE-1 and anti-mouse CD31 to label lymphatic and blood vessels, respectively (27). For tracheas excised from control mice, primary antibody was omitted or nonimmune serum was substituted. Area densities (percentage of total tissue area) of LYVE-1- and CD31-positive vessels in fluorescent images were measured by stereologic point counting of 10 1.7-mm^2 regions/trachea.

Statistical Analysis

Data were compared by *t* test or one-way analysis of variance, with $p < 0.05$ considered significant.

RESULTS

Weight Loss and Death after Infection

To investigate overall severity of mycoplasma-induced illness, we compared body weight and morbidity in age-matched Wsh and $+/+$ mice. Body weight of Wsh and $+/+$ mice is similar before infection and both types of mice lose weight after infection (Figure 1), but Wsh mice lose more and take longer to recover. By 16 d, $+/+$ mice recoup lost weight, whereas Wsh do not recover to baseline by 28 d. Indeed, 3 of 22 infected Wsh (but no $+/+$) mice died. In short-term experiments, Wsh mice with parenchymal and small airway mast cell populations restored and augmented by reconstitution lost weight more quickly and were more likely to die in the first week of receiving 0.5×10^6 cfu of mycoplasma (see Table E1 in the online supplement). However, mast cell-reconstituted mice receiving a fivefold smaller inoculum lost significantly less weight than unreconstituted Wsh mice exposed to a similar inoculum ($p = 0.03$; see Figure E1).

Bronchitis and Pneumonia

To probe the severity of airway and lung inflammation after infection, we compared lung histopathology and pneumonia scores in Wsh and $+/+$ mice. Both types of mice develop neutrophilic pneumonia 2 d after infection (Figures 2A–2F; see also Figure E2). By 4 d, acute inflammation is subsiding in $+/+$ but not Wsh. At 7 d, pneumonia progresses in Wsh mice while leukocytic infiltrates decrease further in $+/+$ mice, with residual inflammation being mainly peribronchiolar and perivascular. Airway exudates are prominent in Wsh but not $+/+$ mice. By 14 d, the progression of pneumonia and exudates in surviving Wsh mice is severe, whereas $+/+$ mice are substantially recovered, with scattered infiltrates persisting at 28 d, while surviving

Figure 1. Body-weight change and mortality after mycoplasma infection in Wsh and $+/+$ mice. In the first 2 wk, infected Wsh mice lose progressively more weight than infected $+/+$ mice. Three infected Wsh mice died at times indicated by arrowheads. After 2 wk, surviving infected Wsh mice start to gain weight. Data are means $\pm$ SEM; $n = 5\text{--}21$.

Figure 2. Lung inflammation after mycoplasma infection in Wsh and +/+ mice. (A–F) Micrographs of lungs harvested 2, 14, and 28 d after *M. pulmonis* exposure (stained with hematoxylin and eosin). (G) Quantitative pneumonia scores of Wsh and +/+ mice. The maximum possible score is 26. Data are means $\pm$ SEM; $n = 4$ –6; ** $p < 0.01$, *** $p < 0.001$ compared with +/+ mice.

Wsh mice exhibit severe organizing pneumonia with airways remaining choked with inflammatory exudates. Quantitative scoring (Figure 2G) confirms these impressions. Thus, bronchitis and pneumonia are more prolonged and severe in Wsh than +/+ mice.

Lymph Node Hypertrophy

To investigate the immune response to mycoplasma, we compared bronchial lymph node weight and cell composition in Wsh and +/+ mice. Bronchial lymph nodes are bigger in Wsh than +/+ mice after infection (Table 1). Wsh lymph nodes are two- to fourfold heavier than +/+ nodes 4 to 14 d after infection. Fluorescence-activated cell sorter analysis of lymph-node cells of mice infected for 7 d reveals similar total cell numbers in uninfected Wsh and +/+ mice, whereas infected Wsh totals are 2.7-fold higher than in infected +/+ mice and nearly fivefold higher than in uninfected mice. Uninfected Wsh and +/+ mice have similar bronchial lymph node populations of CD4⁺ and CD8⁺ T cells and B220⁺ B cells. Seven days after infection, percentages of T cells fall and of B cells rise in Wsh and +/+ mice alike. The proportions of CD8⁺ cells decline in both groups of infected mice but less so in Wsh mice. Thus, lymph nodes in infected Wsh mice are swollen with lymphocytes.

Mycoplasma Burden

To test whether mast cell deficiency impairs antimycoplasma defenses, we compared the number of mycoplasma in Wsh and +/+ lungs, finding 162-fold more live mycoplasma in Wsh than in +/+ mice 4 d after infection (Figure 3A). Despite this difference, there is no evidence of extrapulmonary dissemination as assessed by the absence of live organisms in spleen homogenates. To

determine if mast cells rescue mice from this phenotype, we compared infection in Wsh mice with and without lung mast cell reconstitution in adoptive transfer experiments, finding fewer live organisms in reconstituted mice 7 d after infection (Figure 3B). However, this difference was not statistically significant ($p = 0.2$). A similar trend toward recovery of wild-type phenotype was detected in Wsh, reconstituted Wsh, and +/+ mice 7 d after receiving a smaller (10^5 cfu) inoculum (Figure 3B). Histologic analysis of mice injected with bone marrow mast cells reveals mast cell reconstitution in the parenchyma and small airways, but not in the trachea, in which mast cells remain rare or absent (not shown; see Reference 26). These data suggest that mast cells of large airway in addition to mast cells of small airway and lung parenchyma may be required for full protection from mycoplasma. Histamine levels in lung homogenates suggest that reconstitution yields mast cell levels that are substantially higher than in +/+ mice in lung parenchyma (see Table E1), as was also found in prior work from this laboratory by counting parenchymal mast cells in naive Wsh mice after reconstitution (26).

Pathogen-specific Antibody Responses

Antimycoplasma IgM and IgA appear earlier in Wsh than +/+ mice after infection with live organisms, with no differences in IgG1 and IgG2a (Table 2). These responses differ from those with heat-killed mycoplasma, which yield no difference between Wsh and +/+ mice in antibody development (Table 3), suggesting that humoral responses to mycoplasma antigen are intact in mast cell-deficient mice.

Mycoplasma-induced Changes in Cytokines and Chemokines

To assess whether pulmonary responses to mycoplasma in mast cell-deficient mice are influenced by mast cell-associated

TABLE 1. BRONCHIAL LYMPH NODE WEIGHT AND CELL POPULATIONS 7 d AFTER INFECTION WITH *Mycoplasma pulmonis*

Group	Weight (mg)	Total Cells ($\times 10^5$)	B Cells (%)	T Cells (%)		CD69 ⁺ Activated (%)		Mac-1 ⁺ (%)
				CD4 ⁺	CD8 ⁺	CD4 ⁺	CD8 ⁺	
Wsh infected	32 $\pm$ 7*	148 $\pm$ 28*	56 $\pm$ 1	21 $\pm$ 1	17 $\pm$ 0 [†]	22 $\pm$ 1	8.5 $\pm$ 0.7	1.5 $\pm$ 0.4
+/+ Infected	9 $\pm$ 2	54 $\pm$ 13	51 $\pm$ 3	24 $\pm$ 2	13 $\pm$ 1	27 $\pm$ 2	6.5 $\pm$ 0.6	1.0 $\pm$ 0.1
Wsh uninfected	5 $\pm$ 2	32 $\pm$ 3	34 $\pm$ 5	35 $\pm$ 1	26 $\pm$ 3	19 $\pm$ 0	7.6 $\pm$ 2.5	1.9 $\pm$ 0.2
+/+ Uninfected	1 $\pm$ 0	31 $\pm$ 8	37 $\pm$ 5	34 $\pm$ 1	24 $\pm$ 2	12 $\pm$ 4	6.2 $\pm$ 1.5	1.9 $\pm$ 0.4

Data are means $\pm$ SEM, $n = 3$ –4.

* $p < 0.05$.

[†] $p < 0.01$ in comparison with infected +/+ mice by one-way analysis of variance.

Figure 3. Influence of mast cell deficiency and reconstitution on mycoplasma burden. In studies depicted in (A), Wsh and +/+ mice were killed 4–28 d after infection. Mycoplasma burden is expressed as number of live organisms in lung homogenates ($\log_{10}$ of means $\pm$ SEM of colony-forming units [cfu]). (B) Comparison of lung burdens in Wsh, pulmonary mast cell-reconstituted (Wsh + MC) and +/+ mice after 7 d of infection. The Wsh + MC group was infected 12 wk after adoptive transfer of mast cells. Black and white bars represent data from mice infected with 0.5×10^6 and 10^5 cfu, respectively. $n = 4$ –9 for each group; * $p < 0.05$ compared with infected +/+ mice.

cytokines and chemokines, we compared BAL levels of selected proteins in Wsh and +/+ mice. Early after infection, levels of TNF- α , MCP-1, MIP-2, and IL-6 increase greatly in both Wsh and +/+ mice compared with uninfected mice (Figure 4). By 7 d, levels decline and become indistinguishable from those in uninfected mice but remain high in infected Wsh mice. Thus, these data do not suggest that deficiency of these cytokines is a basis for the differences in responses to mycoplasma in Wsh versus +/+ mice.

Effect of Mycoplasma on Surfactant Collectins

To assess whether increased mycoplasma burden in Wsh mice is due to collectin deficiency, we compared levels of SP-A and SP-D in Wsh and +/+ mice. Levels of SP-A and SP-D increase after infection, especially in Wsh mice, in which SP-D is twice as high as in +/+ mice 4 d after infection (Figure 5). Therefore, the larger mycoplasma burden in Wsh mice is not due to surfactant collectin deficiency.

Effect of Mycoplasma on Airway Vascular Remodeling

To assess the importance of mast cells in mycoplasma-induced vascular remodeling (20, 27, 32), we quantified tracheal angiogenesis and lymphangiogenesis. By 28 d, angiogenesis is greater in infected Wsh than +/+ mice (Figure 6). Infection-induced lymphangiogenesis is more striking. In Wsh mice, lymphatic density increases 6.2- and 7.1-fold, respectively, 14 and 28 d after infection. In +/+ mice, lymphatic density rises progressively and is sustained, even after acute bronchitis and pneumonia subside. In Wsh and +/+ mice alike, lymphatic density is much lower than the blood-vessel density in uninfected mice, but increases to match and eventually greatly exceed blood-vessel density in infected mice. These data reveal that mast cell deficiency does not prevent mycoplasma-induced remodeling of blood and lymphatic vessels and confirm that lymph vessels are more sensitive than blood vessels to mycoplasma-induced remodeling, which persists even at low levels of inflammation.

DISCUSSION

The present study supports a role for mast cells in protecting the respiratory tract from mycoplasma infection. The key findings are that infected mice, when mast cell deficient, lose more weight and are sicker and more likely to die, developing sustained, severe bronchitis and pneumonia. Mast cell-deficient mice also have much higher burdens of mycoplasma organisms early after infection. On the other hand, by 28 d, some of the surviving Wsh mice are regaining weight and showing early histopathologic signs of recovery, suggesting that the combination of mycoplasma and mast cell deficiency may not be uniformly fatal. Certainly the immune defects are not as comprehensive as in

TABLE 2. SERUM MYCOPLASMA ANTIBODY TITERS AFTER INFECTION WITH *M. pulmonis*

Days after Infection	Exposure Group	IgM	IgA	IgG1	IgG2a
4	Wsh + <i>M.p.</i>	0.3 $\pm$ 0.3	ND	ND	ND
	WT + <i>M.p.</i>	ND	ND	ND	ND
	Wsh + medium	ND	ND	ND	ND
	WT + medium	ND	ND	ND	ND
7	Wsh + <i>M.p.</i>	2.1 $\pm$ 0.1*	ND	ND	ND
	WT + <i>M.p.</i>	1.6 $\pm$ 0.1	ND	ND	ND
	Wsh + medium	ND	ND	ND	ND
	WT + medium	ND	ND	ND	ND
14	Wsh + <i>M.p.</i>	2.3 $\pm$ 0.1	1.5 $\pm$ 0.4*	2.4 $\pm$ 0.1	1.5 $\pm$ 0.3
	WT + <i>M.p.</i>	2.5 $\pm$ 0.1	ND	2.8 $\pm$ 0.1	0.7 $\pm$ 0.4
	Wsh + medium	ND	ND	ND	ND
	WT + medium	ND	ND	ND	ND
28	Wsh + <i>M.p.</i>	2.5 $\pm$ 0.1	2.5 $\pm$ 0.1†	2.9 $\pm$ 0.2	2.6 $\pm$ 0.2
	WT + <i>M.p.</i>	2.3 $\pm$ 0.1	1.2 $\pm$ 0.3	2.6 $\pm$ 0.1	2.2 $\pm$ 0.2
	Wsh + medium	ND	ND	ND	ND
	WT + medium	ND	ND	ND	ND

Definition of abbreviations: *M.p.* = *M. pulmonis*; ND = none detected (no IgE was detected in any sample); WT = wild-type (+/+) mice.

Data are means $\pm$ SEM ($n = 5$ –7).

* $p < 0.05$.

† $p < 0.01$ compared with control groups by one-way analysis of variance.

TABLE 3. SERUM ANTIBODY TITERS AFTER IMMUNIZATION WITH HEAT-KILLED MYCOPLASMA

Days after Injection	Exposure Group	<i>M. pulmonis</i> (cfu × 10 ⁶)	IgM	IgG1
7	Wsh	0.5	0.7 ± 0.3	ND
	+/+	0.5	0.3 ± 0.3	ND
	Wsh	20.0	1.6 ± 0.2	ND
14	+/+	20.0	1.9 ± 0.1	ND
	Wsh	0.5	0.3 ± 0.3	ND
	+/+	0.5	0.3 ± 0.3	ND
28	Wsh	20.0	1.3 ± 0.2	0.9 ± 0.4
	+/+	20.0	1.2 ± 0.1	0.3 ± 0.3
	Wsh	0.5	ND	ND
	+/+	0.5	ND	ND
	Wsh	20.0	0.4 ± 0.4	1.3 ± 0.6
	+/+	20.0	0.5 ± 0.3	0.7 ± 0.4

Definition of abbreviation: ND = none detected (no IgG₁, IgG_{2a}, or IgE was detected in any sample).

Data are means ± SEM; n = 3–4 mice/group.

mycoplasma-infected mice with severe combined immunodeficiency, which is associated with polyarthritis and failure to contain mycoplasma infection within the lung (33). However, the pulmonary inflammation and remodeling response in that setting may be less severe because it depends at least in part on the capability of mounting of an immune response. The similarity of Wsh and +/+ mouse antibody responses to heat-killed mycoplasma antigens suggests that deficits in innate rather than adaptive responses underlie the enhanced susceptibility of mast cell-deficient mice to infection. In Wsh mice, the lack of extrapulmonary dissemi-

Figure 4. Effect of mycoplasma infection on bronchoalveolar lavage (BAL) cytokines and chemokines in Wsh and +/+ mice. BAL was obtained 2 and 6 h and 1 to 7 d after infection. Levels of tumor necrosis factor α (TNF- α), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 2 (MIP-2), and interleukin 6 (IL-6) were quantified by ELISA. Data are mean ± SEM; n = 4–6; *p < 0.05, **p < 0.01, ***p < 0.001 compared with infected +/+ mice.

Figure 5. Effect of *M. pulmonis* infection on surfactant collectins in BAL from Wsh and +/+ mice. Levels of surfactant protein (SP)-A and SP-D are shown in the top and bottom panels, respectively. Data are means ± SEM; n = 4–6; **p < 0.01 compared with infected +/+.

nation, which is thwarted in mice with severe combined immunodeficiency by transfer of serum from mycoplasma-infected wild-type mice (33), also argues for intact humoral immunity in our infected Wsh mice. The higher titers and earlier appearance of pathogen-specific IgM and IgA in Wsh mice exposed to live mycoplasma probably are due to the large antigen load imposed by the higher burden of organisms in the first few days of infection. The observed close similarity in numbers of all major immune cell populations (excepting mast cells) in Wsh and +/+ mice before infection (26, 34) also supports a primary contribution of mast cells to the observed differential response to infection. However, it is possible that Wsh mice have an unappreciated defect in immune function that is unrelated to selective lack of mast cells.

Persistence of infection despite development of a pathogen-specific humoral response and poor correspondence between mycoplasma burden and severity of inflammation illustrate the intriguing complexities of this model of “sustained but contained” airway infection. Interestingly, the live mycoplasma burden in +/+ mice catches up with the burden in Wsh mice, even though at that point airway and parenchymal inflammation has fallen to a level that plateaus slightly above the baseline of pathogen-free mice. In mast cell-deficient mice, even though their bacterial burdens are similar to those of +/+ mice at the later time points, inflammation remains profound. This association of equivalent bacterial burdens with major differences in neutrophilic inflammation raises the intriguing possibility that mast cells are antiinflammatory in the context of this chronic infection. Notwithstanding their well-known production of inflammatory mediators, mast cells also secrete antiinflammatory products, including heparin (35), which blunts several manifestations of inflammation when used as a drug (36). Deficiency of histamine in mice lacking histidine decarboxylase in a model of immune complex-induced inflammation also is associated with increased neutrophil infiltration (37). Absence of mast cell histamine may contribute to the exuberant airway neutrophilia in infected Wsh mice. Alternatively, mast cell participation in chronic mycoplasma infection may render the process of killing and controlling infection more efficient, so that fewer neutrophils are needed to achieve a given level of control. In any case, the relation between killing efficiency and neutrophil concentration can be

Figure 6. Effect of mycoplasma infection of Wsh and +/+ mice on tracheal angiogenesis and lymphangiogenesis. Micrographs show whole-mounted tracheas harvested 14 d after infection. Preparations coincubated with anti-CD31 and -LYVE-1 reveal blood and lymph vessels, respectively. Paired images are from the same field. Dark bands correspond to cartilaginous rings. Anti-CD31 also stains leukocytes (dots), which are numerous in Wsh mucosa. These examples reveal increased lymphangiogenesis in Wsh trachea, but little change in blood vessels despite marked increases in adherent and infiltrating leukocytes. Images were subjected to computer-assisted measurement of area density for blood and lymph vessels. Data are means $\pm$ SEM; $n = 4-6$; # $p < 0.05$ compared with infected +/+ mice; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared with corresponding sham-infected mice. Scale bar is 0.5 mm.

complex and not a simple function of the ratio of neutrophils to pathogens (38). Mast cells can bind and respond directly to mycoplasma organisms (39). In this way they may detect and signal the presence of an invading pathogen to leukocytes such as neutrophils and macrophages with the capacity to kill bacteria. Although mast cells have phagocytic potential (40), it is unlikely that they can muster the number of cells needed on the luminal side of the airway to make a major contribution to microbial killing, given that most mast cells are subepithelial in location and that their numbers within the epithelium or lumen are tiny at baseline compared with resident macrophages and are dwarfed by recruited neutrophils soon after onset of infection.

Notwithstanding established links between mast cells and certain types of angiogenesis, we found that lack of airway mast cells does not prevent mycoplasma-induced tracheal angiogenesis or lymphangiogenesis, which is profound in this model. This does not rule out a mast cell contribution to airway vascular remodeling, but does suggest that mast cell deficiency does not affect large airway vessel density in pathogen-free mice and that absence of mast cells cannot offset angiogenic stimuli provided

by the more intense and sustained inflammation in infected mice. Also notable is rather striking asynchrony of angiogenesis and lymphangiogenesis in infected Wsh as well as +/+ mice, with high-level lymphangiogenesis persisting in the latter mice even after inflammation subsides. This suggests that lymphangiogenesis is harder or slower to reverse than angiogenic remodeling, as has been found in a recent study of vascular remodeling in mycoplasma-infected mice (27), or that lymphangiogenesis is more sensitive to persistence of live organisms and low-level inflammation. The present study finds that +/+ mice do not manifest a large angiogenic response to infection. This is consistent with our experience (20, 27), because wild-type C57BL/6 mice are relatively resistant to *M. pulmonis* infection and we reduced the standard inoculum after pilot studies showed that C57BL/6 Wsh mice suffer high mortality when infected with larger doses of live organisms.

The present study reports that Wsh mice have greater hypertrophy of bronchial lymph nodes than do +/+ mice in response to airway infection. This finding differs from a report that mast cell-deficient mice have less lymph node swelling than wild-type mice after paw injections of *Escherichia coli* and that mast cell TNF- α is a determinant of lymph node hypertrophy (7). However, differences between the two studies preclude direct comparison. These include the site of infection, infecting organism, and strain of mast cell-deficient mouse (*Kit^W/Kit^{W-v}* vs. *Kit^{W-sh}/Kit^{W-sh}*). However, the most important difference likely is temporal, with our longer term study allowing mycoplasma burden to become higher in Wsh mice, resulting in greater antigen load, increased trafficking of antigen-presenting cells to lymph nodes, and more vigorous production of B cells expressing mycoplasma-specific immunoglobulins. Much of the antigen presentation and B-cell proliferation can be expected to occur in local and regional lymph nodes; indeed, our analysis reveals that most of the cells contributing to the increase in lymph node cell numbers after infection are B lymphocytes. These findings suggest that although mast cell deficiency may delay or reduce lymph node cell recruitment and proliferation in response to infection acutely, this tendency can be overcome and reversed in a more chronic, severe infection. A role for mast cell TNF- α in promoting chronic lymph node swelling is not supported by the findings in our mycoplasma-infection model given a lack of significant differences between Wsh and +/+ mice in BAL TNF- α levels at most time points and a significant increase in Wsh mice at 7 d. The most probable sources of excess TNF- α in BAL fluid of infected Wsh mice are neutrophils (which are flooding lungs and airways) and to a lesser extent macrophages. IL-6, MIP-2, and MCP-1 levels also are significantly higher in infected Wsh than +/+ mice at 7 d, possibly for similar reasons. As is the case for TNF- α , mast cells can be important sources of IL-6 (3) and MCP-1 (41), but so also can other inflammatory cells, which likely dominate in Wsh mice.

In summary, our results suggest that mast cells are critical for the containment of and recovery from airway mycoplasma infection. Mast cell-deficient mice have no apparent defects in adaptive immune responses but may contribute to innate defenses against this important respiratory pathogen. If this is also the case in humans, than pharmaceutical strategies to eliminate mast cells in severely affected asthmatics may weaken defenses against mycoplasma and lead to exaggerated inflammatory responses to infection.

Conflict of Interest Statement: None of the authors have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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