# Neutrophil histamine contributes to inflammation in mycoplasma pneumonia

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**Mycoplasmas cause chronic inflammation and are implicated in asthma. Mast cells defend against mycoplasma infection and worsen allergic inflammation, which is mediated partly by histamine. To address the hypothesis that mycoplasma provokes histamine release, we exposed mice to *Mycoplasma pulmonis*, comparing responses in wild-type and mast cell–deficient $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ (*W-sh*) mice. Low histamine levels in uninfected *W-sh* mice confirmed the conventional wisdom that mast cells are principal sources of airway and serum histamine. Although mycoplasma did not release histamine acutely in wild-type airways, levels rose up to 50-fold above baseline 1 week after infection in mice heavily burdened with neutrophils. Surprisingly, histamine levels also rose profoundly in infected *W-sh* lungs, increasing in parallel with neutrophils and declining with neutrophil depletion. Furthermore, neutrophils from infected airway were highly enriched in histamine compared with naive neutrophils. In vitro, mycoplasma directly stimulated histamine production by naive neutrophils and strongly upregulated mRNA encoding histidine decarboxylase, the rate-limiting enzyme in histamine synthesis. In vivo, treatment with antihistamines pyrilamine or cimetidine decreased lung weight and severity of pneumonia and tracheobronchitis in infected *W-sh* mice. These findings suggest that neutrophils, provoked by mycoplasma, greatly expand their capacity to synthesize histamine, thereby contributing to lung and airway inflammation.**

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Abbreviations used: BAL, bronchoalveolar lavage; BMMC, bone marrow–derived mast cell; $C_t$, cycle threshold; HDC, histidine decarboxylase; HPRT, hypoxanthine guanine phosphoribosyl transferase; PMN, polymorphonuclear neutrophils; *W-sh*, $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$.

Histamine is a major mediator of allergic inflammation (1). This role is supported by several types of evidence, including release of histamine from cells participating in allergic responses, reproduction of features of allergic inflammation by injected or inhaled histamine, reduction of allergic inflammation by histamine receptor antagonists, and more recently by demonstration that mice genetically modified to make less histamine have diminished capacity to develop allergic inflammation (2). Cells of diverse function produce histamine. Traditional major sources are mast cells and basophils, which store histamine in secretory granules, but other leukocytes (3), including platelets (4), have some capacity to produce histamine, as do enterochromaffin-like gastric cells and certain neurons. Foods also contain histamine, occasionally in amounts sufficient to cause histamine poisoning, which resembles conventional anaphylaxis (5). Whatever the biological source, histamine is thought to derive principally from metabolism of the ubiquitous amino acid histidine.

In asthma and other types of allergic inflammation, mast cells and basophils are the postulated major sources of histamine, which is secreted in response to engagement of allergens with surface-bound IgE. Interestingly, most exacerbations of asthma requiring emergency room visits or hospitalization seem to be associated with acute bacterial and viral infection of the respiratory tract rather than exposure to allergens themselves. Prominent among offending bacteria are mycoplasmas (6), which also are linked to first onset of asthma and to chronic persistent asthma in humans (7–9) and in rodent models of allergic airway inflammation (10, 11).

These considerations led us to hypothesize that mast cells are the main source of respiratory

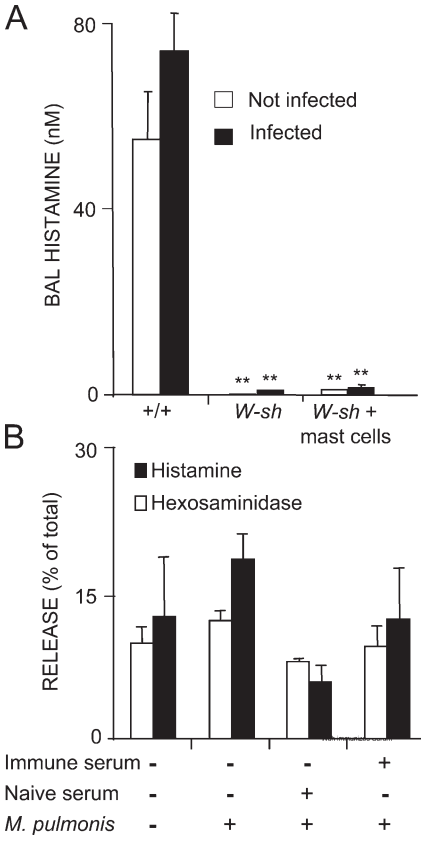


**Figure 1. Mycoplasma does not induce acute release of mast cell histamine.** (A) Histamine levels in BAL supernatants were determined 2 h after airway infection with *M. pulmonis* in +/+ and mast cell–deficient *W-sh* mice and in *W-sh* mice with a super-normal population of intrapulmonary mast cells established by adoptive transfer of +/+ BMMCs. "Not infected" mice were sham infected with sterile broth. Data are mean ± SE; $n = 3$–6 animals per group; **, $P < 0.01$ in comparison with +/+ groups. There were no significant differences between infected and sham-infected results in a given type of mouse. (B) Release of histamine and the secretory granule marker, β-hexosaminidase, was measured in BMMCs. Cells were exposed to serum from mice with high titers of *M. pulmonis*–specific Ig (immune serum), to serum from mice never exposed to mycoplasma (naive serum), and to live *M. pulmonis* in combinations as shown. Control cells were incubated in Tyrode's buffer. Data are mean ± SE; $n = 3$. There were no significant differences between groups.

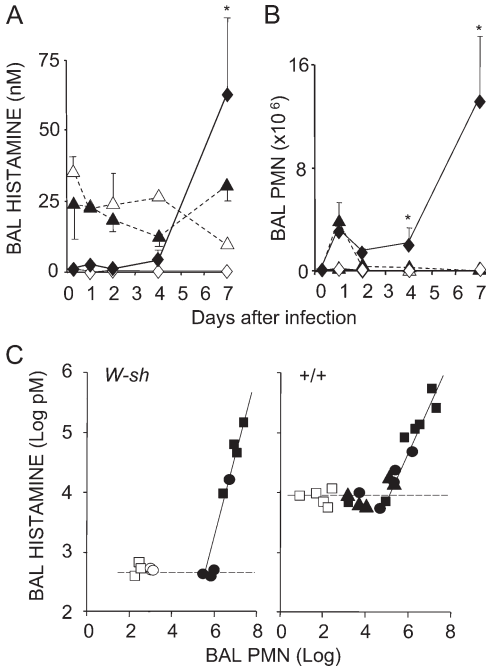


**Figure 2. BAL histamine correlates with PMN levels.** BAL histamine (A) and total PMNs (B) were determined up to 7 d after infection in *W-sh* and +/+ mice and compared with results in sham-infected mice. Closed diamonds, open diamonds, closed triangles, and open triangles represent data from W-sh–infected, W-sh sham-infected, +/+ infected and +/+ sham-infected mice, respectively. Data are mean ± SE; $n = 3$–6 animals per group; *, $P < 0.05$ in comparison with sham-infected *W-sh* values at all time points. In C, concentration of BAL histamine is plotted against total PMNs. Each data point derives from a single BAL sample in a single *W-sh* (left) or +/+ (right) mouse. In the *W-sh* panel, open circles and squares depict samples from mice 4 and 7 d, respectively, after sham infection. Closed circles and squares represent samples from mice 4 and 7 d, respectively, after infection with $0.5 \times 10^6$ CFU of *M. pulmonis*. In the +/+ panel, open squares represent samples obtained 7 d after sham infection. Closed triangles, circles, and squares represent samples obtained 7 d after infection with $0.5 \times 10^6$, $1.0 \times 10^6$, and $2.0 \times 10^6$ CFU, respectively, of *M. pulmonis*. Dotted lines depict approximate baseline histamine concentrations. The intersection of the solid and dotted line identifies the approximate threshold number of BAL neutrophils in an infected mouse needed to generate a detectable rise in histamine above baseline.

tract histamine and that mycoplasma infections of the respiratory tract provoke local histamine release, thereby contributing to allergic and infectious inflammation. To test these hypotheses, the studies described here used mast cell–deficient $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ (*W-sh*) mice to explore sources and release of histamine in acute and chronic respiratory tract mycoplasmosis. These mice are profoundly mast cell deficient but have normal levels of circulating basophils and other leukocytes (12, 13). Their mast cell deficit stems from impaired mast cell expression of c-kit (14), which is essential for mast cell differentiation and survival. In some *W-sh* tissues (not including large airway), mast cell deficiency can be repaired by intravenous adoptive transfer of bone marrow–derived mast cells (BMMCs) in vitro differentiated from wild-type C57BL/6 (+/+) mice (12).

The infectious agent used in these studies is *Mycoplasma pulmonis*, a natural respiratory pathogen of rodents. Like other mycoplasmas, *M. pulmonis* lacks a cell wall and has a small genome (15). In immunocompetent hosts, *M. pulmonis* is mainly an extracellular bacterium that remains limited to the respiratory tract and exhibits an intriguing and incompletely understood capacity to persist in the presence of pathogen-specific antibodies (16). Exposure of a mycoplasma-naive mouse to *M. pulmonis* produces acute tracheobronchitis (and pneumonia with higher level exposures), which subsides to persistent, low-level airway inflammation (17). Its presence is associated

with sustained remodeling of airway epithelium and blood and lymphatic vessels (18) and long-lasting potentiation of neurogenic respiratory inflammation (19). Consequences of respiratory tract exposure to *M. pulmonis* are worse in mast cell–deficient mice than in wild-type mice because mast cell–deficient mice develop a more severe, neutrophilic inflammatory response, which persists rather than subsides in the first few weeks after infection (17).

## RESULTS

### Mast cells are the main source of baseline airway, lung, and serum histamine

As shown in Fig. 1 A and Fig. 2, histamine was consistently very low or undetectable in bronchoalveolar lavage (BAL) fluid from mast cell–deficient *W-sh* mice not exposed to mycoplasma. This was also true of histamine in lung homogenates and serum of uninfected *W-sh* mice, as shown in Fig. 3 A. Given that *W-sh* mice have normal numbers of basophils (13), which also contain histamine, these findings suggest that mast cells are the principal source of histamine in the airway lumen, lung tissue, and serum of healthy mice. The magnitude of the contribution varied, with these data suggesting that mast cells contribute 99, 65, and 89% of airway lumen, lung tissue, and serum histamine, respectively, in +/+ animals. Adoptive transfer of +/+ BMMCs into *W-sh* mice did not increase levels of BAL histamine (Fig. 1 A), despite the fact that lung parenchymal mast cell and histamine content were higher than in +/+ mice (unpublished data). In this regard it may be important that adoptive transfer of BMMCs does not restore mast cell populations to the larger airways and trachea, which could be a source of BAL histamine in healthy +/+ mice. Alternatively, most histamine in epithelial lining fluid under baseline conditions may filter from serum.

### Mycoplasma does not induce acute histamine release into the airway lumen

As shown in Figs. 1 A and Fig. 2, *M. pulmonis* did not increase the concentration of BAL histamine between 2 h and 4 d after infection. Although BAL histamine levels were somewhat higher at 2 h than at later times, there was no difference in this regard between mice undergoing sham versus *M. pulmonis* infection; therefore, these differences are not attributable to infection. Furthermore, Fig. 1 A reveals that the *W-sh* mice "stocked" with adoptively transferred BMMCs to super-normal levels still do not react acutely to the introduction of mycoplasma into the airways, providing additional evidence that mast cells do not release histamine in response to *M. pulmonis* in vivo.

### Mycoplasma does not release histamine from cultured mast cells

Fig. 1 B reveals that BMMCs do not respond directly to *M. pulmonis* by releasing histamine or the granule marker β-hexosaminidase. They also do not respond to the combination of mycoplasma and immune serum, which contains

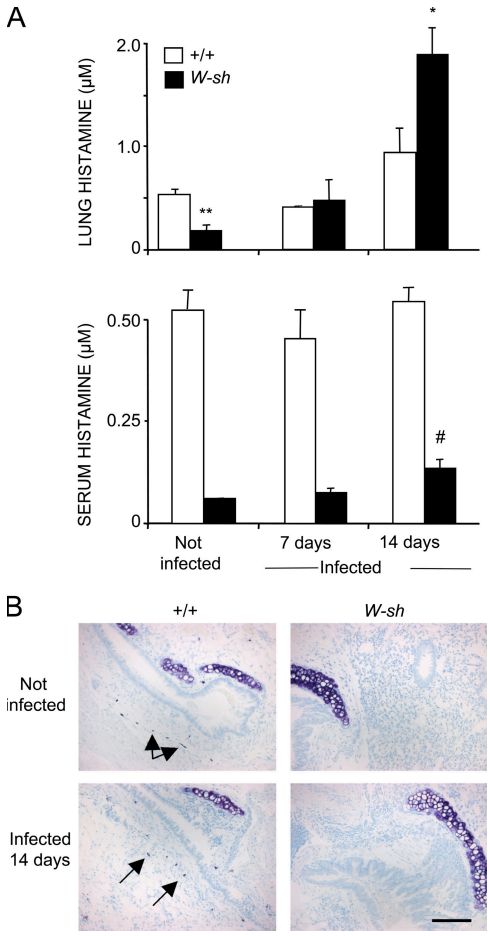


**Figure 3. Increases in lung and serum histamine levels in mycoplasma-infected *W-sh* mice are not caused by induction of mast cells.** (A) The top graph compares histamine concentrations in lung homogenates of uninfected +/+ and mast cell–deficient *W-sh* mice with those in the same types of mice 7 and 14 d after infection with *M. pulmonis*. The bottom graph compares levels of serum histamine in the same groups of mice. Data are mean ± SE; $n = 4$–6 animals per group; *, $P < 0.05$ in comparison with +/+ mice infected 14 d; **, $P < 0.01$ in comparison with +/+ mice not infected; #, $P = 0.05$ in comparison with *W-sh* mice not infected. (B) These representative micrographs compare toluidine blue–stained lung and airway sections in sham- and mycoplasma-infected +/+ and *W-sh* mice. Arrows indicate some of the metachromatically staining mast cells in +/+ sections. Bar, 135 μM.

mycoplasma-specific IgM, IgG, and IgA but not IgE (17). Positive control experiments revealed that $Ca^{2+}$ ionophore A23187 (3 μM) released 28 ± 2% of β-hexosaminidase. Total histamine content of BMMCs was 0.636 ± 0.016 $\mu g/10^6$ cells.

### Mycoplasma increases BAL and lung histamine in mast cell–deficient mice with tracheobronchitis and pneumonia

Fig. 2 A reveals a large increase in BAL histamine 7 d after *M. pulmonis* infection in *W-sh* mice. This increase matched or exceeded the level in infected +/+ mice and was 125 times higher than the level in sham-infected *W-sh* mice.

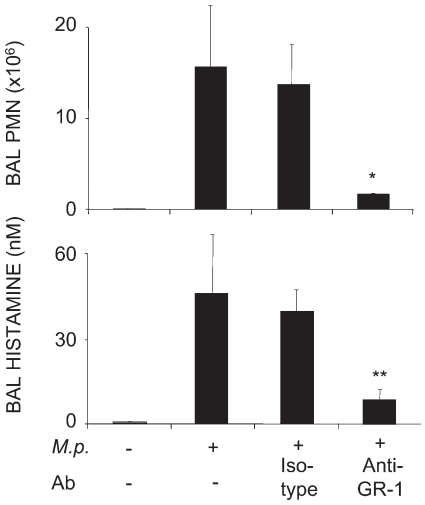


**Figure 4. Anti-PMN antibodies decrease BAL PMNs and histamine levels proportionately in mycoplasma-infected, mast cell–deficient mice.** *W-sh* mice were injected with control (Isotype) or PMN-directed anti-GR-1 mAb 5 d after infection with *M. pulmonis* (*M.p.*). Levels of PMN and histamine in BAL were determined 2 d after mAb injection. "Uninfected" mice were sham-infected. Data are mean ± SE; $n = 3$–4 animals per group; *, $P < 0.05$ and **, $P < 0.01$ compared with isotype control groups.

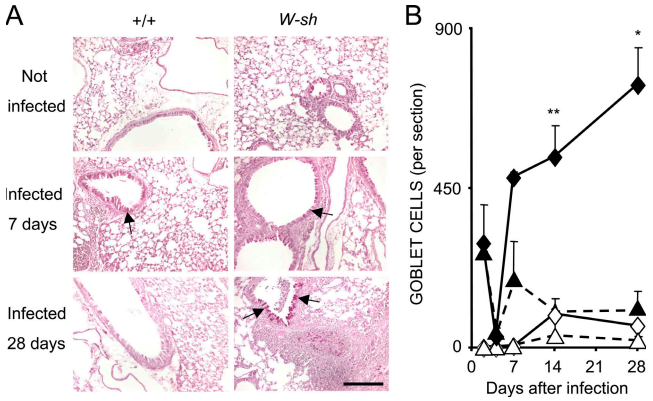


**Figure 5. Neutrophilic tracheobronchitis is associated with airway epithelial remodeling in mycoplasma-infected mice.** (A) These representative micrographs of periodic acid–Schiff and hematoxylin-stained airway sections compare histopathology in +/+ and *W-sh* mice Bar, 180 μM. Infected *W-sh* mice developed more severe and persistent neutrophilic inflammation accompanied by goblet cell hyperplasia, as reflected by increased numbers of periodic acid–Schiff–positive airway epithelial cells (arrows). Control mice were sham infected with saline 7 d before harvest. (B) Airway goblet cell numbers were quantitated in tissue sections from mycoplasma- and sham-infected mice. Symbols represent the following: closed diamonds, mycoplasma-infected *W-sh*; closed triangles, mycoplasma-infected +/+; open diamonds, sham-infected *W-sh*; open triangles, sham-infected +/+. Data are mean ± SE; $n = 4$–5 animals per group; *, $P < 0.05$ and **, $P < 0.01$ compared with infected +/+.

Fig. 2 B reveals that the increase in BAL histamine in infected *W-sh* mice parallels an increase in BAL polymorphonuclear neutrophils (PMNs), suggesting PMNs as a potential source of the histamine. Fig. 2 C confirms this suggestion by showing close correlation between histamine concentration and neutrophil number in paired samples of BAL. This correlation is seen in +/+ and *W-sh* samples, with BAL histamine rising to 50-fold above baseline in +/+ specimens, despite higher baseline concentrations of BAL histamine in +/+ versus *W-sh* samples. The threshold number of BAL PMNs for producing a detectable rise in histamine above baseline was between $10^5$ and $10^6$ cells. As shown in Fig. 3, histamine content also rose in lung tissue extracts 7 d after infection and further increased at 14 d. Thus, mycoplasma greatly increased the concentration of free histamine in the airway lumen and total histamine in lung extracts at later time points. These increases were associated with tracheobronchitis and pneumonia in infected *W-sh* mice, as seen in Fig. 3 B and see Fig. 5 A.

### Mycoplasma increases serum histamine in *W-sh* mice

As shown in Fig. 3 A, serum histamine concentrations in uninfected and infected *W-sh* mice were low compared with all groups of +/+ mice. However, there was a twofold increase in histamine level 14 d after infection in *W-sh* mice, suggesting that the more dramatic rise in lung histamine content influenced serum concentrations. The disparity between lung and serum in the extent of the rise suggests that the increase in lung histamine was largely local and not caused by a systemic rise in histamine production.

### Depleting PMN in infected *W-sh* mice decreases BAL histamine

As revealed by the data in Fig. 4, treatment of mycoplasma-infected *W-sh* mice with anti–Gr-1 decreased BAL PMN and histamine content by ~88 and 79%, respectively. These proportionate drops are consistent with PMNs being the major source of histamine in infected animals.

### Lung inflammation and airway remodeling are severe and persistent in infected *W-sh* mice

As shown in Fig. 3 B and Fig. 5 A, *M. pulmonis*–infected *W-sh* mice developed severe and progressive tracheobronchitis and pneumonia. In contrast, infected +/+ mice developed mild and transient lung and airway inflammation, which mostly resolved by 7 d after infection. Inflammatory cells in the lumen and alveoli were almost exclusively PMN. The chronic inflammatory process in *W-sh* mice was accompanied by progressive airway remodeling as reflected by goblet cell hyperplasia (quantitated in Fig. 5 B). In contrast, goblet cell hyperplasia in +/+ mice was milder and more ephemeral. Control mice did not develop inflammation or remodeling between 2 and 28 d after sham infection. There were no significant differences in goblet cell hyperplasia in mice treated with the histamine H1 receptor antagonist pyrilamine or the H2 antagonist cimetidine singly or in combination, compared with responses in mice treated with saline (unpublished data).

### Mast cells and basophils are not sources of mycoplasma-induced increases in histamine in *W-sh* mice

As shown in Fig. 3 B, mast cells were present in airway walls of +/+ mice, with and without infection. However, no mast cells were noted in *W-sh* mice in the presence or absence of infection, bronchitis, and pneumonia. These findings suggest that local mast cell hyperplasia in infected *W-sh* mice does not explain the observed rise in airway and lung histamine. As noted above, *W-sh* mice injected with BMMCs developed mast cell hyperplasia in lung parenchyma but did not develop mast cells in large airway walls. This finding indicates that our histochemical approaches can detect mast cells in lungs of *W-sh* mice when they are present. As shown by flow cytometry data in Fig. 6 A, lungs of uninfected and infected *W-sh* and +/+ mice contained small but distinct populations of $CD131^+/IgE^+/c\text{-}kit^{lo}$ cells with surface marker and morphology consistent with basophils. These populations separated cleanly from the PMNs predominating in lungs of infected *W-sh* mice. Similar results were obtained when $F_c\varepsilon RI\alpha$ was used as a marker instead of CD131 (unpublished data). As revealed in Fig. 6 B, the absolute number of basophils was significantly lower in *W-sh* mice 7 and 14 d after infection than in sham-infected mice. Therefore, changes in basophil numbers do not explain the observed increases in lung histamine.

### Mycoplasma induces PMN histamine production and release

As seen in Fig. 7 and Fig. 8 A, direct exposure of purified, BM-derived, mycoplasma-naive PMNs to *M. pulmonis* dramatically increased total histamine content compared with cells not exposed to mycoplasma. This effect of mycoplasma was dose responsive and was observed with live or heat-killed organisms, although response to the latter is muted compared with the response to live organisms. There was no difference in the extent of increase in PMNs from *W-sh* and +/+ mice (9.1- and 10.3-fold, respectively). Levels of histamine similar to those in mycoplasma-exposed naive BM PMNs were observed in purified, lung BAL-obtained PMNs natively exposed to *M. pulmonis* in infected *W-sh* and +/+ mice. Histamine content of BAL PMNs was measured 1 and 7 d after infection in *W-sh* mice but on day 1 only in +/+ mice (in which inflammation had subsided by day 7 to the point that few PMNs could be retrieved by BAL). There were no significant differences in histamine levels in *W-sh* mice on day 1 versus 7. Mycoplasma itself was not a source of histamine. Fig. 8 B also demonstrates that mycoplasma stimulates histamine release from naive PMNs. Preparations of BM and lung PMNs in these experiments were at least 98% pure, with no observed contamination with mast cells, basophils, or eosinophils. These data suggest that PMNs are a source of

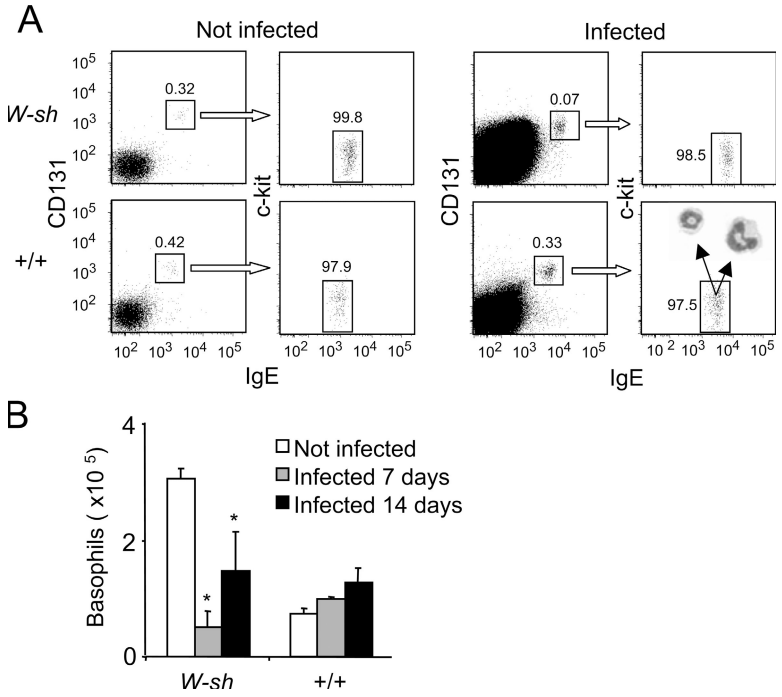


**Figure 6. Flow cytometry reveals that changes in basophil numbers do not account for increases in histamine in mycoplasma-infected mice.** (A) These representative plots compare surface expression of CD131 (β chain common to receptors for IL-3, IL-5, and GM-CSF), IgE, and c-kit in lung cells from +/+ and *W-sh* mice. These data are from mice studied 14 d after sham or mycoplasma infection. The percentage of cells in specific gates (boxed) is indicated. Basophils are CD131 and IgE positive but express little or no c-kit, distinguishing them from mast cells. In lungs from all types of mice, the $CD131^+/IgE^+$ subset (gated as noted) was a small but distinct population of extractable cells, consisting almost entirely of $c\text{-}kit^-$ and $c\text{-}kit^{lo}$ cells. As shown in the lower right panel inset, these cells feature basophilic granules with segmented nuclei. (B) The number of basophils per lung 7 and 14 d after infection was calculated by multiplying total cells by the fraction of $CD131^+/IgE^+$ cells. Data are mean ± SE; $n = 3$ animals per group; *, $P < 0.05$ in comparison with uninfected *W-sh*.

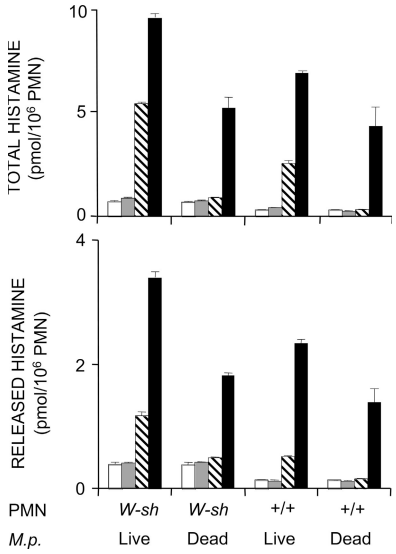


**Figure 7. Histamine production and release is stimulated by live or dead mycoplasma and is dose sensitive.** *M. pulmonis (M.p.)*–stimulated production of histamine was assessed in PMN purified from BM of +/+ and *W-sh* mice. PMNs and live or heat-killed mycoplasma were cocultured for 200 min using PMN to *M. pulmonis* CFU ratios of 1:1 (gray bars), 1:10 (hatched bars), and 1:100 (black bars), followed by measurement of total and released histamine. White bars show total and released histamine in PMNs incubated alone, without mycoplasma. Data are mean ± SE; $n = 3$; $P < 0.001$ for each 1:100 value versus PMN alone control values, for *W-sh* 1:10 live *M. pulmonis* versus PMN alone and versus *W-sh* 1:100, for +/+ 1:10 live *M. pulmonis* versus PMN alone and versus +/+ 1:100; $P < 0.01$ for total histamine *W-sh* dead *M. pulmonis* 1:100 and 1:10 versus PMN alone, and for 1:10 versus 1:100.

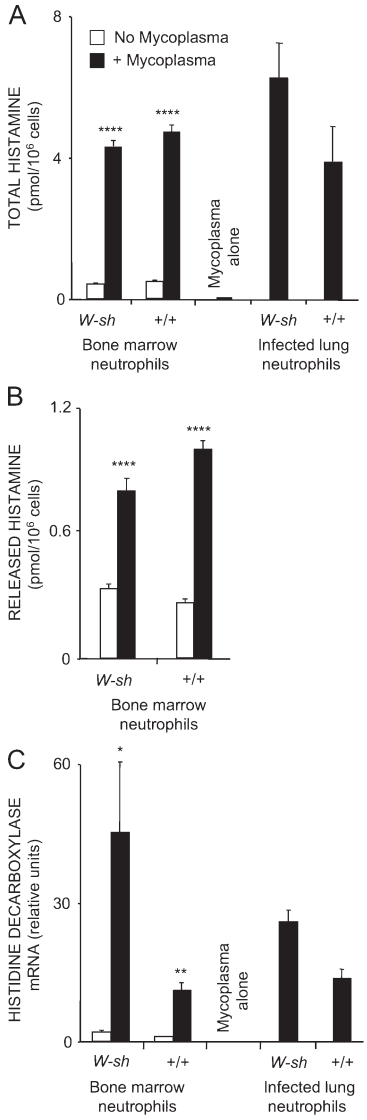


**Figure 8. Mycoplasma increases PMN histamine production and HDC transcripts in vitro and in vivo.** (A) Total histamine production was measured in BM PMNs purified from mycoplasma-naive +/+ and *W-sh* mice and incubated with live *M. pulmonis* in a ratio of 1 neutrophil per 10 CFU. Histamine content also was measured in mycoplasma alone and in PMN purified from *M. pulmonis*–infected lungs of +/+ and *W-sh* mice 1 and 7 d after infection, respectively. (B) Histamine released outside of the cell during incubation with mycoplasma was measured in PMN purified from mycoplasma-naive +/+ and *W-sh* mice. (C) Real time RT-PCR was used to measure expression of mRNA encoding HDC, the rate-limiting enzyme in endogenous production of histamine. Cell sources and conditions are as in A. Results are expressed relative to levels of mRNA encoding HPRT. Data are mean ± SE; $n = 3$–5 animals per group; *, $P < 0.05$, **, $P < 0.01$, and ****, $P < 0.0001$ compared with no mycoplasma control.

histamine, that mycoplasma exposure strongly enhances histamine production and release, and that PMNs do not differ strongly in these respects in *W-sh* and +/+ mice.

### Mycoplasma increases PMN transcripts encoding histidine decarboxylase

As shown in Fig. 6 C, *M. pulmonis* strongly augmented levels of histidine decarboxylase (HDC) mRNA in naive, BM-derived PMNs. PMNs retrieved from infected animals by BAL had high levels of HDC relative to levels in naive cells. These findings suggest that increased expression of HDC accounts for increased histamine production by PMNs in mycoplasma-infected mice. As in the histamine measurements, HDC mRNA content was measured on day 1 and 7 in PMNs from *W-sh* mice, without finding a significant difference between values at the two time points.

### Antihistamines attenuate inflammation in infected *W-sh* mice

As shown in Fig. 9, treatment of *W-sh* mice with H1 or H2 antihistamines (pyrilamine or cimetidine) decreased the ratio of lung to body weight, and also pneumonia severity, in organs harvested 10 d after infection with mycoplasma compared with results in control mice injected with saline. The ratio of lung to body weight rises in infiltrative lung diseases and in lung-selective edema. The change in lung to body weight ratio in response to antihistamine was more dramatic than the change in pneumonia grade, suggesting that part of

the antihistamine effect on the ratio was caused by a decrease in lung edema. The combination of H1 and H2 antihistamine also attenuated these endpoints, although the differences were not significant. As also found in a prior study using the same level of exposure to *M. pulmonis* (17), several infected control animals (3 out of 8 in this study) died before scheduled lung harvest. On the other hand, there were no deaths (0 out of 8) in the cimetidine-treated group, and only 1 out of 8 and 1 out of 6, respectively, in pyrilamine alone and cimetidine plus pyrilamine groups. Therefore, antihistamines may also protect from death from mycoplasma in this model.

## DISCUSSION

In this study, exploring mycoplasma-induced inflammation in mast cell–deficient mice unmasked a previously underrecognized contribution of PMNs to local production of histamine. In the context of the hypotheses tested by these studies, some findings were contrary to expectation and others were entirely unanticipated, thereby yielding fresh insights regarding the nature of inflammation in chronic tracheobronchitis and pneumonia. At the start of these studies, the main hypothesis was that *M. pulmonis* (which is a common respiratory tract pathogen in wild populations of rodents) degranulates mast cells, thereby boosting local inflammation and linking infectious and allergic inflammation in the airway. Although we found no evidence of direct stimulation of mast cells by mycoplasma, our results show that there may indeed be a link between mycoplasma infection and allergic inflammation. However, this link is forged in part by direct, inducible expression and release of the classic allergic inflammatory mediator, histamine, from an unexpected source, PMNs. Some implications of these findings are discussed further below.

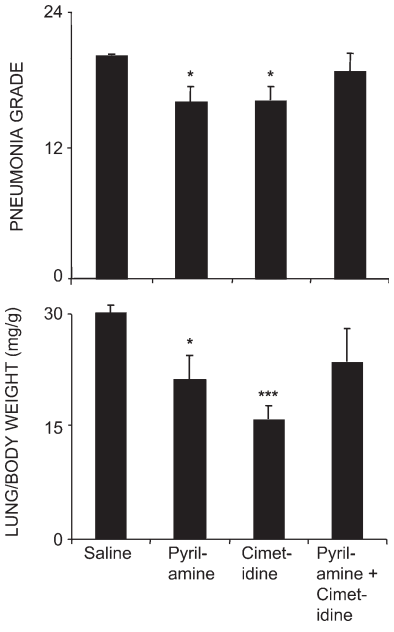


**Figure 9. Antihistamines attenuate pneumonia and lung to body weight ratio in mycoplasma–infected, mast cell–deficient mice.** *W-sh* mice were treated daily with H1-selective antihistamine (pyrilamine), H2-selective antihistamine (cimetidine), both antihistamines in combination, or saline. Lungs harvested 10 d after infection and 7 d after starting antihistamine treatment were weighed and subjected to histopathological scoring for pneumonia (maximal possible score is 26). The top panel shows pneumonia grades in each group. The bottom panel shows ratio of lung weight (mg) to body weight (g). Data are mean ± SE; $n = 5–8$ animals per group; *, $P < 0.05$ and ***, $P < 0.001$ compared with saline control.

Because of the focus on comparing sources of histamine in wild-type and selectively mast cell–deficient mice, these studies establish definitively that mast cells are the chief source of lung and airway histamine at baseline, that is, in mice without respiratory tract inflammation. Although a mast cell origin for respiratory tract histamine will not surprise many investigators, these findings for the first time reveal the extent of the contribution. Levels of free histamine in the airway lumen, as assessed by content in BAL supernatants from +/+ and *W-sh* mice, are almost entirely (99%) contributed by mast cells. Given that BAL histamine does not increase in mice after parenchymal lung mast cell populations are augmented by adoptive transfer of BMMCs, it is likely that much or most free airway histamine in healthy mice originates from blood or plasma, like many components of epithelial lining fluid. In turn, most plasma histamine likely was produced in multiple organs. When studied in vitro, mast cells from a variety of sources exhibit a baseline level of histamine release without specific stimulation (20, 21), as also seen in our BMMC data in Fig. 1 B. This study's comparisons of serum histamine levels in +/+ and *W-sh* mice suggest that the vast majority of serum histamine is from mast cells, a possibility raised previously by studies of IgE-dependent anaphylaxis in mast cell–deficient $Kit^{W}/Kit^{W\text{-}v}$ mice (22). The failure of BMMCs adoptively transferred to the lung to increase BAL histamine levels (as revealed by data in Fig. 1 A), despite a large increase in histamine content of whole lung extracts, is consistent with a plasma origin of most BAL histamine in healthy mice.

Our study does not support our original hypothesis that *M. pulmonis* degranulates mast cells acutely because live organisms did not affect cultured mast cells and did not increase histamine levels in BAL at early time points after infection in vivo. However, it is possible that the baseline levels of histamine prevented detection of acute, low-level release from mast cells. Unexpectedly, BAL and lung histamine levels rose progressively in *W-sh* mice at later time points, especially 1–2 wk after infection when levels came to equal or exceed those in +/+ mice. The histopathological analysis established that these increases were not caused by the appearance of mast cells in *W-sh* mice and coincided with progressive worsening of neutrophilic tracheobronchitis and pneumonia. Similarly, we cannot attribute the large increases in *W-sh* histamine to recruitment of basophils, which decreased in number as inflammation progressed, as shown by FACS. Indeed, the principal source of histamine appeared to be PMNs, which

populated the airway and alveolar lumen in large numbers, and which tracked well with the rise in histamine as revealed by comparing the panels in Fig. 2. As also noted in our prior work with this model (17), the severity of neutrophilic lung and airway inflammation was greater in *W-sh* than in +/+ mice. The difference was especially large by 1 wk, when inflammation in +/+ mice had substantially resolved but in *W-sh* mice continued to worsen.

The basis of the difference in neutrophilic response between +/+ and *W-sh* mice is not clear. In part it may be because of the higher mycoplasma burden developing in *W-sh* mice in the first few days of infection. Later, when mycoplasma burden becomes similar in both types of mice, greater neutrophilia in *W-sh* mice may be caused by loss of a suppressive effect of histamine on PMN influx, as suggested by exaggerated PMN flux into a skin pouch model of inflammation in mice lacking HDC (23). If this is the case, it is conceivable that if not for the production of histamine by PMN, the neutrophilia would be even more exaggerated in *W-sh* mice. Despite differences between +/+ and *W-sh* mice in the magnitude of the neutrophilic response to a given dose or burden of *M. pulmonis*—and notwithstanding higher baseline BAL histamine levels in +/+ mice tending to mask the PMN contribution to histamine at lower levels of neutrophilic inflammation—the present data (Fig. 2 C) establish that airway histamine levels rise markedly in +/+ mice when neutrophil numbers comparable to those seen in mice are provoked by larger initial inocula of mycoplasma. Thus, a major neutrophil contribution to airway histamine is not a phenomenon limited to mast cell–deficient mice. In this regard, the lack of an intrinsic difference between +/+ and *W-sh* mice in vivo is underscored by the absence of major differences between naive PMN from *W-sh* and +/+ mice in mycoplasma-stimulated histamine synthesis and release in vitro. Greater histamine content in *W-sh* mice at the lower mycoplasma dose ($0.5 \times 10^6$ CFU) was more likely caused by higher numbers of mycoplasma-stimulated PMNs than to intrinsic differences in *W-sh* and +/+ neutrophil responsiveness to mycoplasma. Furthermore, the increase in local production of histamine in *W-sh* mice infected for 2 wk was sufficiently great to increase histamine levels in serum. This independently supports the validity and importance of the increases in lung and airway histamine. The elevations of serum histamine in *W-sh* mice, however, remained well below levels in +/+ animals, with or without infection, providing further evidence that extra-pulmonary sources (perhaps most notably mast cells in the skin) make the largest contribution to circulating levels of histamine in +/+ mice.

The marked increase in airway epithelial remodeling in infected *W-sh* mice versus infected +/+ mice, as reflected by goblet cell hyperplasia, also probably relates to more severe and persistent infection in *W-sh* mice (17). Unlike effects of live mycoplasma on PMN production of HDC mRNA and histamine, effects on epithelial remodeling probably were not caused by live mycoplasma and its products interacting alone with respiratory epithelium and goblet cell precursors. This is because epithelial remodeling, and mycoplasma-stimulated tracheal angiogenesis and lymphangiogenesis, occur only in the context of an inflammatory response and development of mycoplasma-specific antibodies and immune complexes (16). Indeed, transfer of immune serum to B cell–deficient mice reconstitutes mycoplasma-induced remodeling (16). The higher burdens of bacterial antigen and higher titers of mycoplasma-specific antibodies in *W-sh* mice (17) presumably increase the formation of antigen–antibody complexes and inflammation in the airway, thereby augmenting antibody-dependent goblet cell hyperplasia.

These results provide the first evidence that mycoplasma stimulates PMN production of histamine. The significance of this novel finding is not so much that PMNs and other leukocytes can be sources of histamine, because past studies suggested that BM-derived cells other than mast cells and basophils can produce small amounts of this mediator (3, 24, 25). Rather, the significance lies in direct stimulation of PMNs by a common respiratory pathogen strongly inducing PMN production of histamine, and also in the unexpected magnitude of the effect on histamine levels in airway lumen, lung tissues, and serum. The finding of mycoplasma-induced increases in histamine content of PMNs purified from BM further argues that internalization of histamine from other sources is not a likely explanation of our findings.

Our data also allow a quantitative comparison of histamine levels in PMNs versus mast cells. As shown in Fig. 7, highest expression of PMN histamine ($\sim$10 pmol/$10^6$ cells = $\sim$1.1 ng/$10^6$ cells) was seen in PMNs purified from *W-sh* BM. Similar levels ($\sim$6.4 pmol/$10^6$ cells = $\sim$0.70 ng/$10^6$ cells) were seen in PMNs from infected lung, as shown in Fig. 8 A. On the other hand, unstimulated PMNs contained only $\sim$0.04 ng/$10^6$ cells. This compares to $\sim$0.64 μg of histamine per $10^6$ BMMCs. Based on these data, there is a 580–910-fold difference between the histamine content of BMMCs and mycoplasma-stimulated PMNs, compared with a 16,000-fold difference between BMMCs and unstimulated PMNs. It is possible that the histamine content of BMMCs does not accurately reflect that of lung and airway mast cells. Nonetheless, the estimates of histamine content obtained by assaying purified PMNs from infected lung fit well with the hypothesis that PMNs are the major source of BAL histamine in the independent measurements shown in Fig. 2. Total histamine content of the 2.4 ml of BAL fluid from infected *W-sh* mice 7 d after infection was $\sim$16 ng compared with the $\sim$9 ng expected to lie within the observed $\sim 13 \times 10^6$ PMNs (based on $\sim$0.7 ng/$10^6$ cells observed in PMNs purified from infected lungs), with the difference being accounted for mainly by extracellular, secreted histamine. Thus, in healthy uninflamed lung, which contains resident mast cells but very few PMNs or basophils, mast cells are by far the major source of extracellular and stored histamine. This conclusion receives strong support from Fig. 1, which shows data obtained very early after infection, before major neutrophilic inflammation has had an opportunity to develop. However, in the setting of established mycoplasma-induced tracheobronchitis

and pneumonia, the number of PMNs relative to mast cells becomes so great (easily a several thousandfold difference in the airway lumen) that PMN production of histamine, augmented by exposure to bacteria, is substantial, even surpassing the mast cell contribution. This previously unsuspected capability of PMNs was uncovered fortuitously by examining the phenomenon in mast cell–deficient mice. Thus, PMNs become a major source of airway histamine by two principal mechanisms: (a) ramping up histamine production in each cell in response to bacteria, and (b) increasing their numbers to achieve overwhelming numerical superiority over other histamine-producing cells.

The finding that indices of tracheobronchitis and pneumonia in mast cell–deficient mice are diminished by treatment with pyrilamine or cimetidine suggests that neutrophil-derived histamine is proinflammatory in this model. The magnitude of this effect may be underestimated, because more mice died in the untreated control group before lung harvest than in antihistamine-treated groups, thereby precluding assessment of inflammation in the sickest mice. This effect may have prevented the observed reduction in indices of lung inflammation from achieving statistical significance in the group treated with the combination of pyrilamine and cimetidine. In any case, further investigations are needed to determine whether these observations in mycoplasma-infected mice apply to other microbes and other mammals. If human PMNs respond similarly, the histamine produced could contribute to worsening of allergic symptoms in acute respiratory infections, which are associated with PMN influx into the airways and with most exacerbations of asthma severe enough to require emergency treatment or hospitalization (26–29). These findings also suggest a mechanism for some of the inflammation and bronchoconstriction in chronic purulent respiratory tract infections and noninfectious neutrophilic inflammation, as in cystic fibrosis (30, 31) and chronic, cigarette-associated bronchitis (32).

Finally, our studies suggest that the mechanism of the increase in histamine production by naive PMNs in vitro involves induction of transcription of the gene encoding the rate-limiting enzyme in endogenous synthesis, HDC. Similar elevations of HDC transcript levels were found in PMNs purified from airways of infected mice. Thus, this mechanism for stimulated histamine production appears to apply in vivo and in vitro. Furthermore, the magnitude of the increase in HDC mRNA parallels that of the increase in histamine production. Thus, the mechanism of the mycoplasma effect on PMNs is likely to involve increased production of HDC, rather than an alternative mechanism, such as slower rates of histamine degradation. This conclusion is consistent with studies suggesting that leukocytes infiltrating foci of allergic inflam mation express HDC transcripts by in situ mRNA hybridization and also contribute to increased HDC activity (25). Further studies will be required to establish the nature of the signals passed between mycoplasma and PMN, and to dissect intracellular pathways leading to an increase in HDC transcription.

In conclusion, this study shows that PMNs can become a major source of histamine in airway mycoplasma infection, which increases histamine production by up-regulating transcripts encoding the rate-limiting enzyme in histamine production. Thus, histamine is a mediator shared by allergic and infectious varieties of airway inflammation.

## MATERIALS AND METHODS

**Animals.** Mast cell–deficient C57BL/6 *W-sh* mice were provided originally by Peter Besmer (Memorial Sloan-Kettering Institute, New York, NY). Wild-type C57BL/6 $Kit^+/Kit^+$ (+/+) mice were purchased from Charles River. Mice were housed under specific pathogen-free barrier conditions as described (12, 17) and were infected at 8–10 wk of age, except in experiments involving mast cell–reconstituted and control animals for which 18-wk-old mice were used. The University of California at San Francisco Institutional Animal Care and Use Committee approved all experimental procedures.

**Mycoplasma infection, PMN depletion, and BAL.** For most experiments, *W-sh* or +/+ mice were anesthetized intramuscularly with ketamine and xylazine and infected intranasally with $5 \times 10^5$ CFU of *M. pulmonis* strain UAB CT7 (25 μl in each nostril) as described (17). For certain other experiments, +/+ mice were infected with higher doses of mycoplasma ($1.0 \times 10^6$ or $2.0 \times 10^6$ CFU). Serum was obtained from animals before sacrifice. Selective neutropenia was induced by i.p. injection of 250 μg of anti–Gr-1 RB6-8C5 mAb (BD Biosciences) 5 d after infection. Control mice received no mAb or were injected with rat IgG2b κ isotype control mAb (A95-1; BD Biosciences). Mice were killed 2 d after mAb injection. To obtain samples of airway epithelial lining cells and fluid, a 22-gauge catheter was inserted into exposed tracheal lumen. BAL samples were collected in three 0.8-ml PBS washes per mouse. Supernatants were stored at –80°C until use. Cell pellets were collected for PMN purification or flow cytometry as described below.

Tissue mast cell and goblet cell staining. After sacrifice, lungs were excised, immersed in 4% paraformaldehyde, and fixed overnight. Specimens were embedded in paraffin and cut into 5-μm sections. Deparaffinized sections were stained with hematoxylin and eosin for general observation or with 0.5% acidified toluidine blue or periodic acid–Schiff reagent to identify mast and goblet cells, respectively. All goblet cells in each section were counted. To assess effects of histamine receptor antagonists on mycoplasma-induced goblet cell hyperplasia, mice were injected i.p. with 20 mg/kg of pyrilamine (H1 antagonist), 40 mg/kg of cimetidine (H2 antagonist), or saline (control) daily for 7 d, starting 30 min before infection of +/+ mice with $0.5 \times 10^6$ *M. pulmonis*.

**Mast cell culture and degranulation assays.** Cells from femoral BM of 5–7-wk-old +/+ mice were cultured in IL-3–containing medium as described (12) for 4–8 wk to generate BMMCs of >95% purity as assessed by the presence of metachromatic granules in toluidine blue–stained cells. BMMCs ($10^5$/well) were incubated at 37°C for 2.5 h or overnight with 1:5 dilutions of serum from mycoplasma-naive mice or from infected +/+ mice 24 d after infection, when high titers of *M. pulmonis*–specific Ig have developed (17). After incubation, cells were washed in cold $Ca^{2+}$- and $Mg^{2+}$-free Tyrode's buffer and then incubated with $10^6$ CFU of *M. pulmonis* at 37°C for 1 h in Tyrode's buffer containing $Ca^{2+}$ and $Mg^{2+}$. Cell supernatants and pellets were collected. Pellets were resuspended, washed three times, and sonicated. Samples were stored at –80°C before hexosaminidase and histamine assays. Incubation of BMMCs with ionophore A23187 (3 μM) was used as a positive control for degranulation. Cell viability was assessed by exclusion of vital dye (trypan blue).

**Purification of BAL and BM-derived PMNs and exposure to mycoplasma.** PMNs from BAL and BM were isolated and purified from *W-sh* and +/+ mice as described (33). Purity was established by flow cytometry with FITC-conjugated Gr-1 antibody staining and by direct visualization

after cytostaining with Diff-quik (American Scientific Products). Purified BAL PMNs were suspended ($10^7$ cells/ml), sonicated, and frozen for later assays of HDC mRNA and histamine content (see below). Naive BM PMNs were assessed for histamine production and release in a 96-well plate assay. Cells were incubated at 37°C for 200 min at a density of $2.5 \times 10^6$ or $10^7$ cells/ml with PBS or live *M. pulmonis* (10 CFU per neutrophil). Dose–response experiments were performed with PMN to *M. pulmonis* ratios ranging from 1:0 to 1:100, using live or heat-killed (65°C, 30 min) organisms. Cells were centrifuged after incubation. Supernatants were collected. Pellets were washed three times, collected, and sonicated. All samples were stored at −80°C until use.

**Measurement of HDC mRNA.** Total RNA from *M. pulmonis*–stimulated PMN purified from mycoplasma-naive BM or from BAL of infected mice was prepared using Qiagen RNeasy kits and incubated with DNase (Promega) to remove residual genomic DNA. Expression of mRNA was quantified by real time RT-PCR. Primer probe sets were purchased from MWG Biotech. Oligonucleotide sequences and expected amplimer length were as follows: HDC (primers 5′-TGAGGAAGACAAGCAACAGG-3′ and 5′-GCCTGTCAAATGCACAGACT-3′; probe 5′-CGTTGCACAG-ACAAACACAGGCA-3′ with 5′ carboxyfluorescein and 3′ black hole quencher-1; amplimer 83 bp); endogenous control hypoxanthine guanine phosphoribosyl transferase (HPRT) (primers 5′-AGGTTGCAAGCTTG-CTGGT-3′ and 5′-TGAAGTACTCATTATAGTCAAGGGCA-3′; probe 5′-TGTTGGATACAGGCCAGACTTTGTTGGAT-3′with5′6-carboxy-2', 4, 4', 5′, 7, 7'-hexachlorofluorescein; amplimer 123 bp). Both amplifications were performed simultaneously for 40 cycles of 95°C for 15 s and 60°C for 15 s on a Chromo 4 PTC-200 thermal cycler (MJ Research; Bio-Rad Laboratories) using Superscript III Platinum RT-PCR kits (Invitrogen). Results were expressed as cycle threshold ($C_t$) values and quantitated by a comparative approach. Differences between naive and mycoplasma-exposed samples were expressed as fold change of HDC $C_t$ relative to HPRT $C_t$ in each sample using the formula $2^{-\Delta\Delta Ct}$, where $\Delta C_t = C_{tHDC} - C_{tHPRT}$ and $-\Delta\Delta C_t = \Delta C_{tMycoplasma} - \Delta C_{tNaive}$. Real-time PCR data from one preparation of mycoplasma-naive PMNs was chosen arbitrarily as a control for interassay (plate-to-plate) variation. The observed ratio of HDC $C_t$ to HPRT $C_t$ of this sample was assigned a value of 1 for each multiwell plate and used to normalize the test data from other samples in each plate.

**Measurement of β-hexosaminidase and histamine.** Release of β-hexosaminidase from BMMCs was measured spectrophotometrically using *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma-Aldrich) as described (34). Histamine concentrations in lung homogenates, BAL, BMMCs, and PMNs were determined by Immunotech ELISA (Beckman Coulter).

**Flow cytometry.** Antibodies were purchased from BD Biosciences unless otherwise indicated. For studies on cells obtained by BAL, the following antibodies were used: FITC or PE-conjugated Mac-1 and FITC-conjugated Gr-1; PE-conjugated CD3, PE-conjugated F4/80, TC-conjugated CD4, CD8, and B220 (Caltag Laboratories); rat anti–mouse monoclonal antibody CD16/32 was used to block nonspecific binding. Cells were analyzed by FACSCaliber flow cytometer (BD Biosciences). Cells double-positive for Mac-1 and Gr-1 were considered to be PMNs. To estimate PMN number in mice treated with anti–GR-1, forward and side scatter characteristics also were used. To obtain tissue cells for analysis by flow cytometry, lungs were perfused via cardiac ventricles with 10 ml of cold PBS, cut into small pieces, and minced through a 70-μm strainer (BD Biosciences Discovery Labware) as described (35). Resulting cells were washed with PBS/2% FCS. Resuspended cell pellets were incubated for 10 min with anti-CD16/CD32 before adding the appropriate antibody combinations. Cells were stained with APC-Alexa Fluor 750 anti-CD4 (Caltag Laboratories), APC anti–c-kit, PE anti-CD131, biotin anti-FcεRIα, biotin anti-IgE, PE anti-FcεRIα (eBioscience), and streptavidin PerCP-Cy5.5. Exclusion of 4', 6-diamidino-2-phenylindole (Roche Applied Science) was used to assess cell viability. Samples were analyzed on a LSR II flow cytometer (BD Biosciences).

**Adoptive transfer of mast cells into mast cell–deficient mice.** In vitro–differentiated 5–6-wk-old BMMCs were injected i.v. into 5-wk-old *W-sh* mice ($10^7$ cells/mouse). Mice were used in experiments 12 wk after adoptive transfer, by which time mice establish large populations of mast cells in the lung parenchyma and small airways (12).

**Grading effects of antihistamines on pneumonia.** *W-sh* mice infected intranasally with M. pulmonis ($5 \times 10^5$ CFU) were injected i.p. with 40 mg/kg pyrilamine (H1-selective antihistamine), 60 mg/kg cimetidine (H2-selective antihistamine), both drugs, or saline. Drugs were given daily from day 3 after infection until day 10, when lungs were harvested, weighed, and subjected to histopathological grading of pneumonia severity as described (17).

**Statistical analysis.** Results are expressed as the mean ± SE. Data were compared by *t* test with $P < 0.05$ considered significant.

We thank Sukhvinder Sidhu for help in goblet cell staining and Anthony Cruz for assistance in analyzing flow cytometry data.

Contributions of X. Xu, N.P. Killeen, D. Zhang, and G.H. Caughey to this work were supported by National Institutes of Health (NIH) grant HL024136. X. Xu also was supported by the Diamond Family Foundation and by an Elizabeth Nash memorial fellowship from Cystic Fibrosis Research, Inc. P.J. Wolters was supported by NIH grant HL075026. H. Zhang and C.A. Lowell were supported by NIH grants AI065495 and AI068150.

The authors have no conflicting financial interests.

**Submitted: 9 June 2006**
**Accepted: 20 November 2006**

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