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Mast Cells Play an Important Role in *Chlamydia pneumoniae* Lung Infection by Facilitating Immune Cell Recruitment into the Airway

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Mast cells are known as central players in allergy and anaphylaxis, and they play a pivotal role in host defense against certain pathogens. *Chlamydia pneumoniae* is an important human pathogen, but it is unclear what role mast cells play during *C. pneumoniae* infection. We infected C57BL/6 (wild-type [WT]) and mast cell-deficient mice (Kit^{W-sh/W-sh} [Wsh]) with *C. pneumoniae*. Wsh mice showed improved survival compared with WT mice, with fewer cells in Wsh bronchoalveolar lavage fluid (BALF), despite similar levels of cytokines and chemokines. We also found a more rapid clearance of bacteria from the lungs of Wsh mice compared with WT mice. Cromolyn, a mast cell stabilizer, reduced BALF cells and bacterial burden similar to the levels seen in Wsh mice; conversely, Compound 48/80, a mast cell degranulator, increased the number of BALF cells and bacterial burden. Histology showed that WT lungs had diffuse inflammation, whereas Wsh mice had patchy accumulations of neutrophils and perivascular accumulations of lymphocytes. Infected Wsh mice had reduced amounts of matrix metalloproteinase-9 in BALF and were resistant to epithelial integral membrane protein degradation, suggesting that barrier integrity remains intact in Wsh mice. Mast cell reconstitution in Wsh mice led to enhanced bacterial growth and normal epithelial integral membrane protein degradation, highlighting the specific role of mast cells in this model. These data suggest that mast cells play a detrimental role during *C. pneumoniae* infection by facilitating immune cell infiltration into the airspace and providing a more favorable replicative environment for *C. pneumoniae*. *The Journal of Immunology*, 2015, 194: 3840–3851.

Chlamydia pneumoniae, an obligate intracellular pathogen, is responsible for up to 10–20% of cases of community-acquired pneumonia and is associated with many chronic inflammatory disorders, including atherosclerosis, asthma, and Alzheimer's disease (1–6). Most people will become seropositive for *C. pneumoniae*, and the titer of anti-*C. pneumoniae* Ab increases with age, indicating continued exposure and immune response throughout life (1–7). Thus, understanding the

mechanism of *C. pneumoniae*-induced inflammatory responses of the host remains an important endeavor. We and other researchers reported that *C. pneumoniae* is recognized by the pattern recognition receptors, such as TLR2 and TLR4, which signal through MyD88 and Trif, and the NOD-like receptors, Nod1 and Nod2, which signal through Rip2 (8–11). More recently, we and other investigators showed that *C. pneumoniae* activates the NLRP3 inflammasome directly (12–14), resulting in mature IL-1 β production, which is critical for proper immune responses against *C. pneumoniae* infection (14, 15). In the airway, *C. pneumoniae* first infects alveolar macrophages and airway epithelial cells, which, in turn, secrete proinflammatory cytokines and chemokines that result in an influx of inflammatory cells, such as monocytes, macrophages, and neutrophils (10, 16, 17). One report observed that *C. pneumoniae* infection could induce human mast cells (MCs) to produce cytokines in vitro and that a pulmonary *C. pneumoniae* infection in mice led to MC degranulation in vivo (18).

MCs are best known for playing a key role in allergy, anaphylaxis, and host defense against helminth parasites by releasing chemical mediators, such as histamine, leukotrienes, and MC proteases (MCPTs). Recently, it was reported that MCs can recognize bacterial pathogens through TLRs and NOD-like receptors, produce proinflammatory cytokines (19–21), and play an important role in host defense against bacteria (12, 22–29). MCPTs, such as tryptase and chymase, degrade many proteins, including extracellular matrix (30, 31). In addition, chymase (MCPT4) was reported to activate matrix metalloproteinase (MMP)-9 by cleaving a specific site of the catalytic domain of MMP-9 (32–34); chymase inhibitors reduce both the pro- and active forms of MMP-9 and attenuate its enzymatic activity in vivo (35, 36). MMP-9 is of particular interest in pulmonary infections because it is necessary

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Abbreviations used in this article: BALF, bronchoalveolar lavage fluid; BMMC, bone marrow-derived MC; IFU, infectious unit; MC, mast cell; MCPT, MC protease; MMP, matrix metalloproteinase; TREM-1, triggering receptor expressed on myeloid cells 1; Treg, regulatory T cell; Wsh, Kit^{W-sh/W-sh}; WT, wild-type.

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for both neutrophil infiltration into the lung and airspace (35, 37) and intratracheal migration of dendritic cells (38, 39). Furthermore, MMP degrades claudins, adhesion molecules important in cell–cell tight junctions, during influenza-induced lung injury (39–41). In the lung, several claudin family members are expressed, including claudin-5 and -18, which are components of alveolar tight junctions (37, 38, 42); their degradation is important for paracellular permeability and cell transmigration (40, 43).

In the current study, we found that MCs were required for normal immune cell infiltration into the airspaces during a *C. pneumoniae* lung infection in mice. MC deficiency resulted in faster bacterial clearance and reduced lung inflammation. Prevention of MC degranulation phenocopied MC deficiency, whereas pharmacological induction of MC cell degranulation resulted in greater inflammatory responses to *C. pneumoniae* infection. MC-deficient mice had less MMP-9 secretion and activation, as well as less degradation of claudin-5 and -18, suggesting that MCs are required for the opening of tight junctions to allow immune cells to infiltrate into the airspaces during a bacterial infection. Finally, MC reconstitution in Wsh mice resulted in greater bacterial growth, increased MMP-9, and degradation of claudin-5. These data suggest an unappreciated role for MCs as gatekeepers to facilitate immune cell infiltration into the airspace by regulating tight junction opening during *C. pneumoniae* lung infection, where immune cell infiltration into host lungs is required for normal *C. pneumoniae* propagation. Thus, unlike most other infections in which MCs are protective, MCs appear to play an important role in the pathogenesis of *C. pneumoniae* infection in mice.

Materials and Methods

Animals and reagents

C57BL/6 (wild-type [WT]) and Kit^{W-sh/W-sh} (Wsh) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Eight- to ten-week-old female mice were used in all experiments in this study. All experiments were done according to Cedars-Sinai Medical Center Institutional Animal Care and Use Committee guidelines. Cromolyn and compound 48/80 were purchased from Sigma-Aldrich (St. Louis, MO).

C. pneumoniae infection and sample preparation

C. pneumoniae CM-1 (American Type Culture Collection, Manassas, VA) was propagated in HEP-2 cells, as previously described (8, 44). Both HEP-2 cells and *C. pneumoniae* aliquots were confirmed as being *Mycoplasma*-free by PCR. Mice were infected with either 2×10^6 or 5×10^6 infectious units (IFU) *C. pneumoniae* by inoculating intratracheally. Bronchoalveolar lavage fluid (BALF) was collected by injecting 0.5 ml PBS containing 5 mM EDTA. BALF was separated into supernatant and cells by centrifugation. Supernatant was used for cytokine and chemokine measurements and Western blotting. Cells were smeared on glass slides and stained by modified Wright–Giemsa staining (Diff-Quick; Fisher Scientific, Pittsburgh, PA) to determine subtypes of leukocytes by their cellular and nuclear characteristics. Lungs were homogenized with 1 ml ice-cold sucrose–phosphate–glutamate medium for cytokine and chemokine measurements and bacterial burden quantification.

Bacterial burden quantification

HEP-2 cells were infected with *C. pneumoniae* in lung specimens for bacterial burden quantification, as described previously (45, 46). Briefly, HEP-2 cells were incubated with diluted lung suspensions or cell lysates in the presence of 1 μ g/ml cycloheximide and 10 μ g/ml gentamicin containing RPMI 1640 medium. Centrifugation was performed for 1 h at 800 $\times$ g, and cells were placed in an incubator (37°C, 0.5% CO₂). After 72 h of culture, cells were washed with PBS, fixed with methanol, and stained with FITC-conjugated anti-*Chlamydia* genus-specific mAb (Pathfinder *Chlamydia* Culture Confirmation System; Bio-Rad, Hercules, CA), according to the manufacturer's instructions. *C. pneumoniae* inclusions in cells were counted by fluorescence microscopic analysis.

MC reconstitution

Bone marrow cells from C57BL/6 mice were cultured in RPMI 1640 medium supplemented with 10% FBS and 20% WEHI-3–conditioned media for 6–8 wk to differentiate into bone marrow–derived MCs (BMMCs). BMMCs were confirmed as c-kit⁺ Fc ϵ RIa⁺ by flow cytometry. Six-week-old Wsh mice received 5×10^6 BMMCs via tail vein injection. Twelve weeks after reconstitution, mice were sacrificed, and tissues and peritoneal lavages were collected and assessed for reconstitution of MC deficiency.

Flow cytometry analysis

Lungs were digested enzymatically to isolate leukocytes in the tissue for flow cytometric analysis, as previously described (17, 47). Briefly, lungs were digested at 37°C for 20 min in HANKS buffer containing 40 U/ml Liberase and 50 U/ml DNase I (both from Roche Diagnostics, Indianapolis, IN). Cells were filtered through a 70- μ m cell strainer (BD Biosciences, San Jose, CA). Erythrocytes were obviated by RBC Lysis Buffer (eBioscience, San Diego, CA). Single cells were stained with the following Abs: F4/80 Ab, CD11c Ab, Ly6G Ab, CD11b Ab, triggering receptor expressed on myeloid cells 1 (TREM-1) Ab, CD4 Ab, CD44 Ab, c-kit Ab, IgE Ab, Lamp1 Ab, and CD62L Ab. For lung single-cell differentials, cell types were identified as neutrophils (CD11c[–] CD11b⁺ Ly6G⁺), alveolar macrophages (CD11b⁺ CD11c⁺ F4/80⁺), and T cells (CD3⁺) with either CD4 or CD8 positivity. MCs were identified as side scatter^{high} and c-kit⁺ IgE⁺. MC degranulation was evaluated by surface LAMP1 staining. For intracellular *Chlamydia* staining, cells were permeabilized using a Cytofix/Cytoperm Kit (BD Biosciences) and stained with anti-*Chlamydia* LPS mAb (Accurate Chemical and Scientific, Westbury, NY) and PE-conjugated anti mouse. Flow cytometric analysis was performed using a CyAn flow cytometer (Beckman Coulter) and analyzed using Summit software (Dako, Carpinteria, CA).

Evaluation of vascular permeability

Mice received 25 mg/kg Evans blue dye via tail vein injection 2 h before lung harvest. Lungs were homogenized with 1 ml PBS, centrifuged, and pellets were incubated with 500 ml formamide at 60°C for 18 h. The supernatant was measured at absorbencies of 620–740 nm. For airway permeability, mice were injected with Evans blue dye, as above, and sacrificed 2 h later, and BALF was obtained. The supernatant was measured at absorbencies of 620–740 nm.

Histopathological analysis

Lungs were fixed in formalin buffer and embedded in paraffin for histological analysis. Paraffin-embedded lungs were cut and stained with H&E. Immunohistochemistry was performed using anti-CD3 Ab (clone 2G6V6) prediluted from Ventana Medical Systems (Tucson, AZ), anti-CD20 Ab (goat polyclonal; Santa Cruz Biotechnologies, Dallas, TX), and anti-Ly6G Ab (clone 1A8; eBioscience). Sections incubated with isotype-control Ab and HRP-conjugated secondary Ab were used as negative controls. Staining was developed with either 3,3'-Diaminobenzidine substrate alone or, in some cases, 3,3'-Diaminobenzidine substrate plus cobalt and nickel chloride (Roche Diagnostics). Images were taken using either a Keyence BZ-9000 microscope or an Olympus BX-51 microscope.

ELISA

The concentration of cytokine in BALF and lung homogenates was determined using an OptEIA Mouse IL-6 ELISA Set (BD Biosciences) and Mouse IL-12p40, Mouse IFN- γ ELISA Kit (eBioscience), and DuoSet Mouse MIP-2 and Mouse KC (R&D Systems, Minneapolis, MN). These assays were performed according to the manufacturers' instructions.

Western blotting

Western blotting was performed as described previously (10, 48, 49). Briefly, lungs from *C. pneumoniae*-infected mice were homogenized and lysed in PLC lysis buffer containing proteinase inhibitors (Protease Inhibitor Cocktail; Sigma-Aldrich). Lung cell lysates and BALF were boiled in sample buffer, and proteins were separated with SDS-PAGE. Separated proteins were transferred to BioTrace polyvinylidene difluoride membrane (Pall, Pensacola, FL). Membranes were probed with anti-mouse claudin-5 Ab (mouse polyclonal), anti-human claudin-18 Ab (rabbit polyclonal; both from LifeSpan Biosciences, Seattle, WA), and anti-mouse MMP-9 Ab (rabbit polyclonal; Abcam, Cambridge, MA), followed by HRP-conjugated goat anti-mouse or anti-rabbit Ab (Thermo Fisher Scientific, Rockford, IL). Chemiluminescence was developed using an enhanced Immuno-Star WesternC Kit and documented using ChemoDoc XRS+ (both from Bio-

Rad). Densitometry was performed using Image Lab software (ver 3.0; Bio-Rad) and was normalized to β -actin.

Statistics

Data are presented as mean $\pm$ SD. For survival studies, significance was evaluated by the Fisher exact test. Statistical significance was evaluated using the Mann–Whitney test. For experiments in which more than two samples were analyzed, one-way ANOVA with the Tukey post hoc test were used to assess statistical significance between groups.

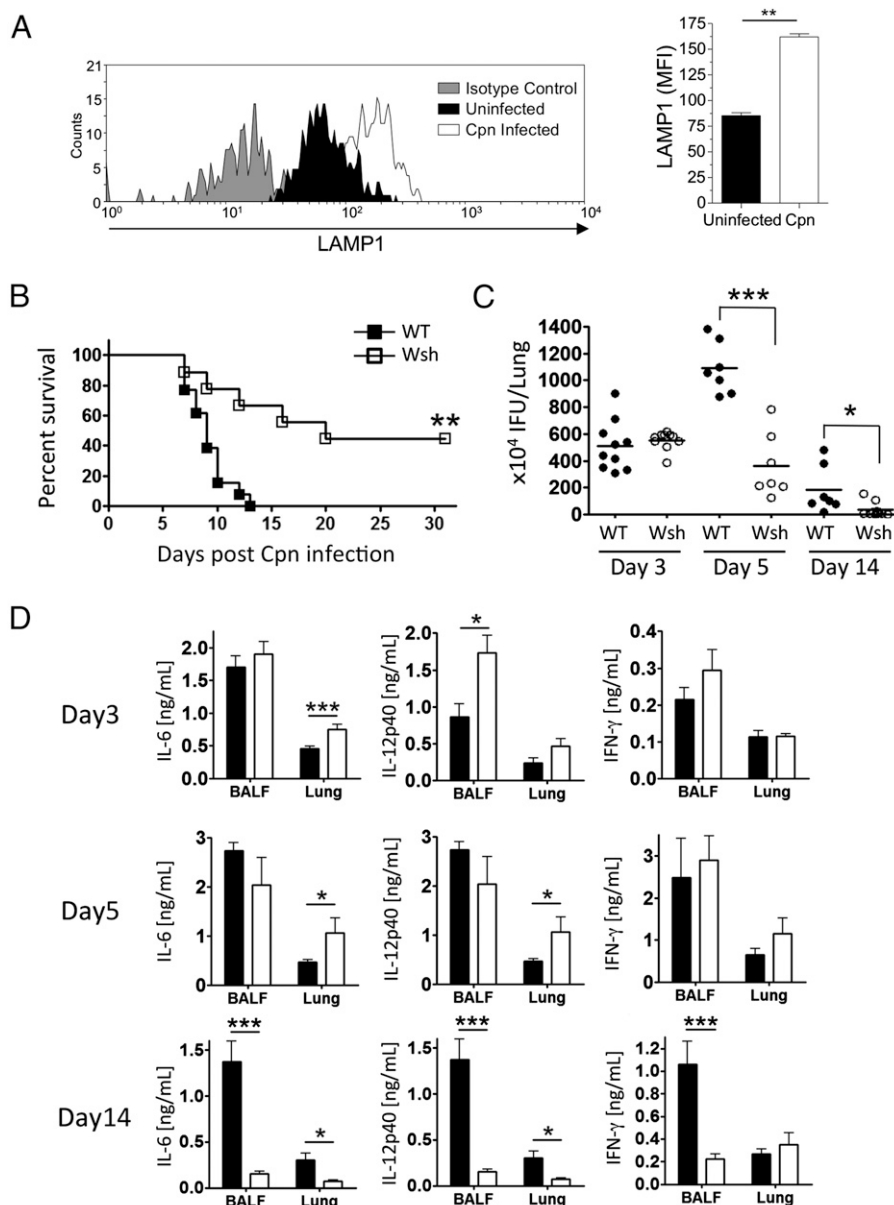
Results

MC-deficient mice are protected from *C. pneumoniae* lung infection-induced mortality

Although MCs were reported to be activated during *C. pneumoniae* infection, these data were obtained several weeks after inoculation (18). Therefore, we infected WT mice (2×10^6 IFU) with *C. pneumoniae* and sacrificed them 4 d postinfection. Using flow cytometry, we identified MCs in the lung and did not find a significant change in their numbers at this time (Supplemental Fig. 1A). However, using LAMP1 as a marker for degranulation (50, 51), we found that MCs appeared to be activated during

C. pneumoniae infection, because LAMP1 surface staining increased in these MCs (Fig. 1A). To investigate the role that MCs might play during *C. pneumoniae* infection in mice, we used Wsh mice, which are deficient for MCs. WT and Wsh mice were infected with *C. pneumoniae* (5×10^6 IFU/mouse) intratracheally and monitored for mortality. All of the WT mice died from severe *C. pneumoniae* lung infection by 14 d postinfection, whereas $\sim 50\%$ of the Wsh mice survived until the end of the experiment (day 31) (Fig. 1B), suggesting that the lack of MCs provide protection from *C. pneumoniae* infection. To understand this unexpected observation, we assessed the bacterial burden in the lung of *C. pneumoniae*-infected WT and Wsh mice at days 3, 5, and 14 post-*C. pneumoniae* infection (Fig. 1C). We used a lower dose of infection for the rest of these studies compared with the mortality experiment (2×10^6 versus 5×10^6 IFU). There was no significant difference in bacterial number between WT and Wsh mice at day 3, but at days 5 and 14 there was a significant decrease in *C. pneumoniae* IFU in Wsh mice compared with WT mice. We next investigated proinflammatory cytokines (IL-6, IL-12p40, and IFN- γ) that are known to be responsible for *C. pneumoniae*

FIGURE 1. Wsh mice are protected from *C. pneumoniae*-induced mortality and *C. pneumoniae* propagation in the lung compared with WT mice. **(A)** WT mice were infected with *C. pneumoniae* (2×10^6 IFU) or mock infected and sacrificed 4 d later. Single cells from the lung were prepared as described in *Materials and Methods*. MCs were identified as side scatter^{high} c-kit⁺ IgE⁺ (Supplemental Fig. 1A). Surface expression levels of LAMP1 were assessed by flow cytometry and reported as the mean fluorescence intensity (MFI). **(B–D)** WT and Wsh mice were infected with *C. pneumoniae* intratracheally (5×10^6 IFU for mortality and 2×10^6 for others). **(B)** *C. pneumoniae*-induced mortality was observed by 31 d post-*C. pneumoniae* inoculation. **(C)** *C. pneumoniae* IFU in the lung from *C. pneumoniae*-infected WT and Wsh mice was quantified as described in *Materials and Methods*. **(D)** BALF was collected from euthanized mice, and the lung was perfused with cold PBS and homogenized. The experiment in (A) was performed one time ($n = 5$). The experiment in (B) was performed twice, and the data were pooled together (total $n = 13$ [WT]; $n = 10$ [Wsh]). The experiment in (C) and (D) was performed three times, and the data were pooled together ($n = 10$ [WT and Wsh] day 3; $n = 7$ [WT and Wsh] day 5; $n = 7$ [WT], $n = 10$ [Wsh] day 14). Cytokine concentration in BALF and lung homogenates was detected by ELISA. Data are mean $\pm$ SD. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, Mann–Whitney test.



clearance. Wsh mice had increased IL-6 in the lung and increased IL-12p40 in BALF early in infection (day 3) (Fig. 1D). Similarly, IL-6 and IL-12p40 also were increased in the lungs at day 5 in Wsh mice compared with WT mice. However, this was reversed by day 14, and Wsh mice had significantly less IL-6 and IL-12p40 in both BALF and lung compared with WT mice. Interestingly, there was no difference in the amounts of IFN- γ between Wsh and WT mice at 3 and 5 d postinfection. IFN- γ is a key mediator of *C. pneumoniae* clearance and the prevention of persistent infection (12, 22–29). However, similar to IL-6 and IL-12p40, IFN- γ concentrations were significantly diminished in BALF at 14 d postinfection in Wsh mice. Although the reduction in cytokines at day 14 in Wsh mice was likely due to the decreased bacterial burden, we did not find this correlation at 5 d postinfection when Wsh mice had increased cytokines, yet a significant decrease in *C. pneumoniae* IFU (Fig. 1C, 1D).

MCs enhance immune cell infiltration into the airway

We hypothesized that the number of macrophages and neutrophils would be increased in Wsh mice compared with WT mice because

we observed that *C. pneumoniae* clearance was faster and IL-6 and IL-12p40 concentrations were elevated in Wsh mice compared with WT mice at an early time point in infection. However, there was no difference between Wsh and WT mice on day 3, but there was a significant reduction in the BALF total cell number in Wsh mice compared with WT mice at both 5 and 14 d postinfection (Fig. 2A). We next determined the inflammatory cell composition in BALF for Wsh and WT mice. We found that, on days 5 and 14, the reduction in cell numbers in BALF could be accounted for largely by the significant decrease in neutrophil numbers, along with a reduction in macrophages on day 14 (Fig. 2B, Supplemental Fig. 2A). Next, we analyzed immune cell infiltration in the lung tissue. Surprisingly, in contrast to BALF data, total cell number in the lung was significantly increased in Wsh mice compared with WT mice at day 3; a similar trend was noted at day 5 (Fig. 2C). Macrophage and neutrophil numbers were significantly increased in the lungs at 3 and 5 d postinfection in Wsh mice compared with WT mice (Fig. 2D, Supplemental Fig. 2B–D). There were no significant differences in the numbers of lymphocytes between Wsh and WT mice (Fig. 2D), including

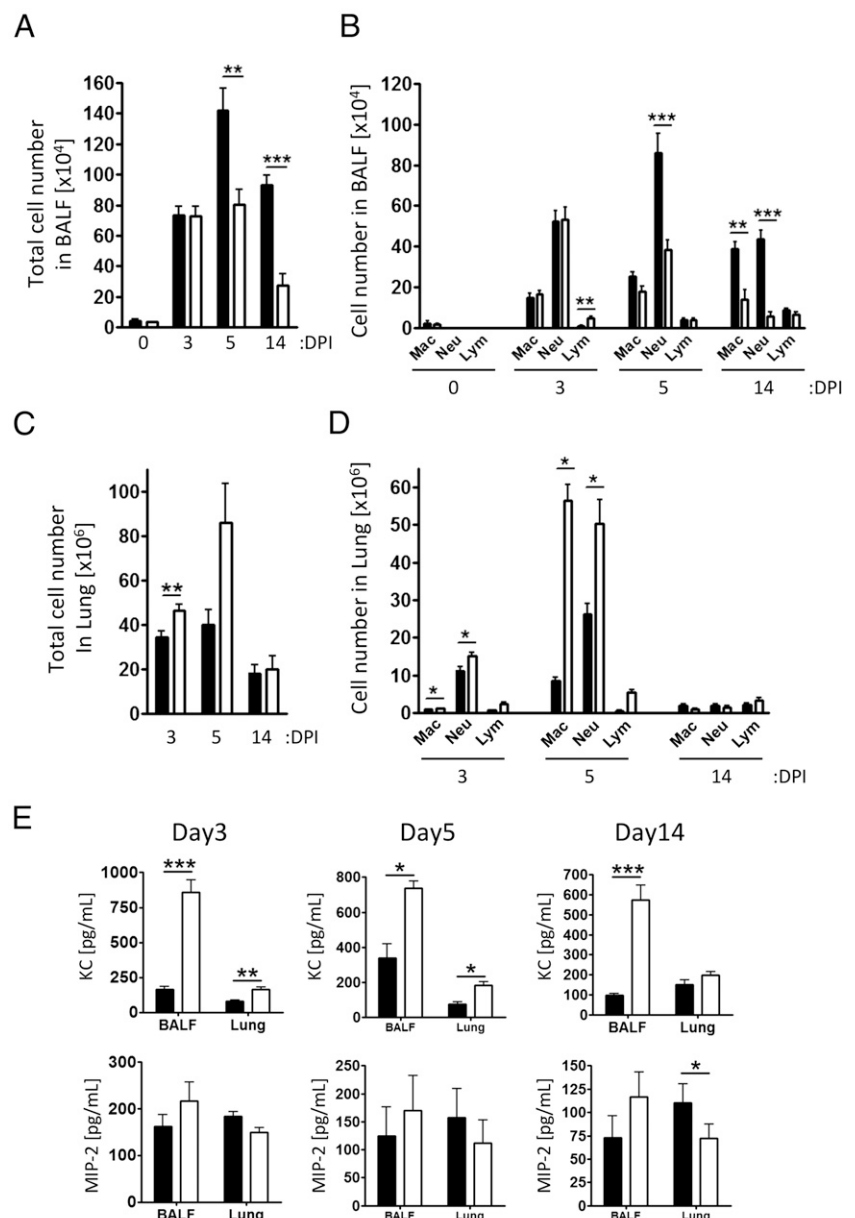


FIGURE 2. Wsh mice displayed reduced inflammatory cell infiltration into the airspace compared with WT mice. WT (filled bars) and Wsh (open bars) mice were infected with 2×10^6 *C. pneumoniae* intratracheally. BALF was collected from euthanized mice on 3, 5, and 14 d post-*C. pneumoniae* propagation. Total (A) and differential (B) cell counts in BALF. Cell types in BALF were determined by modified Wright–Giemsa staining. (C and D) Single cells from lung were collected through enzymatic procedure and analyzed by flow cytometry. (E) Chemokine concentrations in BALF and lung homogenates were determined by ELISA. This experiment was performed three times, and the data were pooled (total $n = 7$ [WT and Wsh] day 0; $n = 10$ [WT] and $n = 8$ [Wsh] day 3; $n = 9$ [WT and Wsh] day 5; and $n = 7$ [WT] and $n = 9$ [Wsh] day 14). Data are mean $\pm$ SD. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, Mann–Whitney test.

the number of regulatory T cells (Tregs) (Supplemental Fig. 2E). To corroborate the differences that we found between BALF cells and lung tissue cells in Wsh mice, we measured the concentration of neutrophilic chemokines, KC and MIP-2, in BALF and lungs of WT and Wsh mice (Fig. 2E). Although the number of neutrophils in BALF from Wsh mice was reduced, there was significantly more KC in both BALF and lung homogenates at 3 and 5 d postinfection, as well as in BALF at 14 d postinfection (Fig. 2E). There was no difference in MIP-2 concentrations between Wsh and WT mice on days 3 and 5 postinfection. To assess whether vascular permeability was affected, we injected Evans blue dye into *C. pneumoniae*-infected mice. Importantly, the vascular permeability was not altered in Wsh mice during *C. pneumoniae* infection (Supplemental Fig. 3A, 3B). Although there was no difference in the amount of Evans blue dye in the lungs of WT and Wsh mice, we also measured the amount of dye present in BALF. Similar to the cellular-infiltration data, there was less Evans blue dye in BALF from Wsh mice compared with WT controls (Supplemental Fig. 3C). Collectively, infiltrating immune cells into the airway were decreased in Wsh mice compared with WT mice, whereas immune cells in the lung were increased. Although pulmonary vascular permeability appeared to be normal, airway permeability was not. At the same time, although KC concentrations were increased in both BALF and lung homogenates in Wsh mice, this did not result in increased neutrophils in BALF.

Chlamydial burden is significantly reduced in macrophages and neutrophils in MC-deficient mice

C. pneumoniae is an obligate intracellular bacteria and needs host cells to replicate. *C. pneumoniae* replicates mainly in macrophage/monocytes and neutrophils and, to a lesser degree, in epithelial

cells (10, 49, 52, 53). We analyzed the presence of intracellular *C. pneumoniae* by flow cytometry to determine which cells were infected by *C. pneumoniae* in MC-deficient mice. On day 3, we did not find any significant differences in *C. pneumoniae* staining in BALF or lung cells between WT and MC-deficient mice (data not shown). This was not unexpected, because both mice had similar bacterial burdens at that time (Fig. 1B). Intracellular *C. pneumoniae* staining was mainly restricted to macrophages at this time (BALF and lungs). However, although intracellular macrophage *C. pneumoniae* staining was identical at day 3, macrophages in the lungs of WT mice had an increased *C. pneumoniae* burden (Fig. 3A) compared with Wsh mice. By day 5, there was a significant decrease in the amount of intracellular *C. pneumoniae* staining in Wsh macrophages and neutrophils in both BALF and lung cells compared with WT mice (Fig. 3B). These data suggest that *C. pneumoniae* infected and replicated in macrophages during the first 3 d and then infected newly infiltrating macrophages and neutrophils. However, perhaps with the reduction in macrophages and neutrophils infiltrating the airways of Wsh mice, *C. pneumoniae* replication was limited by the lack of sufficient numbers of host immune cells.

MC degranulation is required for C. pneumoniae-induced immune cell infiltration into the airspace

Because MCs seemed to be required for proper immune cell infiltration into the airways of *C. pneumoniae*-infected mice, we next investigated the role of MC degranulation in this process. To address this question, we used cromolyn, a MC degranulation inhibitor (52, 54). WT mice were treated with cromolyn (3 mg/kg body weight) intratracheally 1 d prior to and 1 d after *C. pneumoniae* inoculation (Fig. 4). Similar to *C. pneumoniae*-infected

FIGURE 3. *C. pneumoniae*-positive macrophages and neutrophils were reduced in Wsh mice compared with WT mice. WT and Wsh mice were infected with 2×10^6 IFU *C. pneumoniae* intratracheally. Mice were sacrificed either 3 or 5 d post-*C. pneumoniae* infection. BALF cells and lung cells were collected at day 3 (A and B) or day 5 (B and D) and stained with anti-*C. pneumoniae*, anti-CD11c, anti-CD11b, anti-F4/80, and anti-Ly6G for flow cytometric analysis, as before. (A) Representative graphs for *C. pneumoniae* staining in gated populations (see Supplemental Fig. 1 for gating strategy). (B) The percentage of *C. pneumoniae*-positive cells in BALF and lung at day 3. (C) Representative graphs for *C. pneumoniae* staining in gated populations on day 5. (D) The percentage of *C. pneumoniae*-positive cells (Cpn⁺) in BALF and lung at day 5. In (A) and (B), the experiment was performed twice ($n = 5$ [WT] and $n = 6$ [Wsh]). In (C) and (D), the experiment was performed twice ($n = 4$ [WT] and $n = 4$ [Wsh]). Data shown are a representative experiment. Data are mean $\pm$ SD. * $p < 0.05$, Mann-Whitney test.

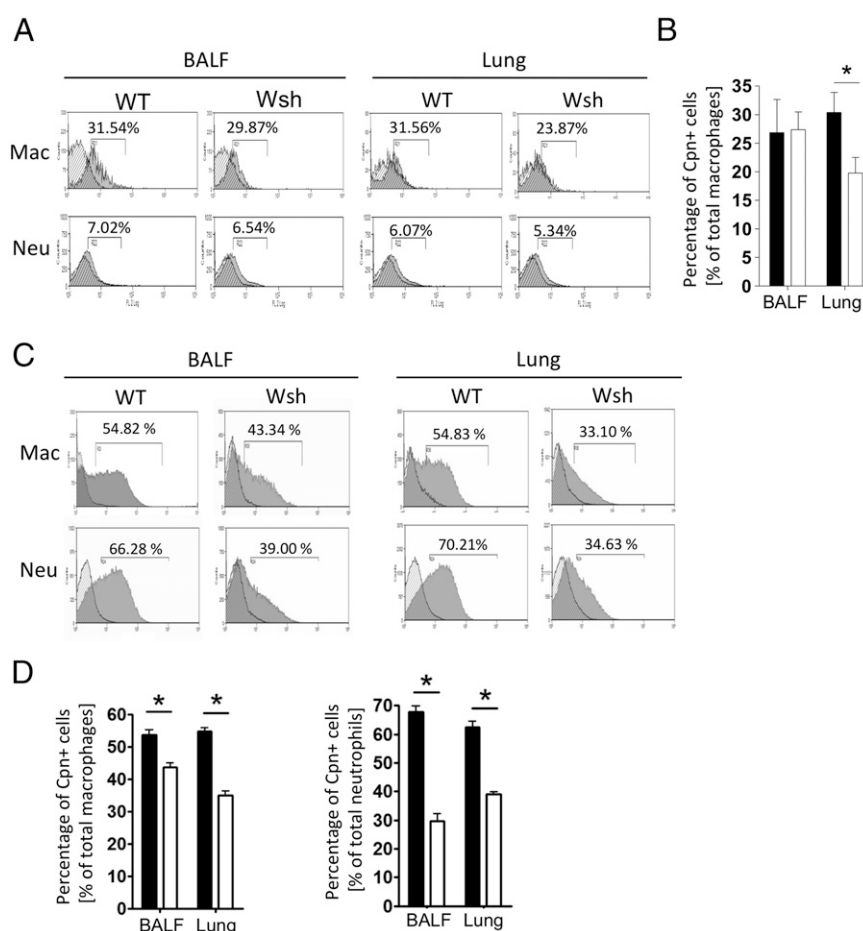
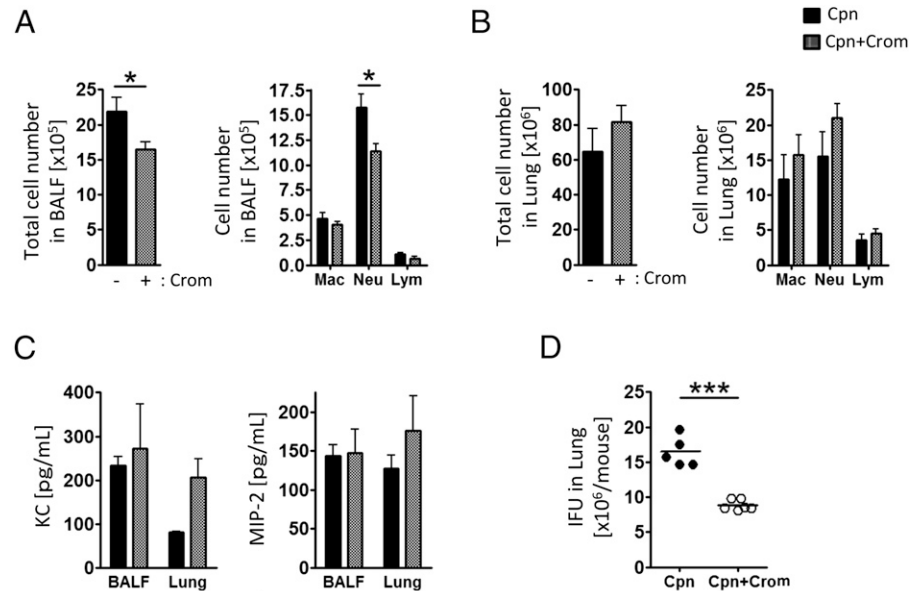


FIGURE 4. Cromolyn reduces inflammatory cell infiltration into the airspace and bacterial number in the lung. WT mice were treated with cromolyn (3 mg/kg body weight) before and after *C. pneumoniae* infection (2×10^6 IFU) and sacrificed 5 d postinfection. **(A)** Total and differential cell counts in BALF. Cell types in BALF were determined by modified Wright–Giemsa staining. **(B)** Single cells from lung were collected through enzymatic procedure and analyzed by flow cytometry for differential cell counts. **(C)** Chemokine concentrations in BALF and lung homogenates were determined by ELISA. **(D)** *C. pneumoniae* IFU in the lungs was quantified as described in *Materials and Methods*. For (A)–(D), the experiment was performed twice ($n = 5$ [Cpn] and $n = 6$ [Cpn + Crom]). Data shown are a representative experiment. Data are mean $\pm$ SD. * $p < 0.05$, *** $p < 0.001$, Mann–Whitney test.



Wsh mice, cromolyn-treated mice displayed reduced total BALF cell number and neutrophil number in BALF (Fig. 4A), as well as relatively increased total cell number in the lung (Fig. 4B). KC and MIP-2 were not significantly different between the groups that were treated or not with cromolyn (Fig. 4C). Strikingly, *C. pneumoniae* IFU was reduced significantly in cromolyn-treated mice (Fig. 4D).

Because our data suggested that MC degranulation is required for proper immune infiltration into the airways, we hypothesized that inducing excess degranulation pharmacologically would have the opposite effect, with greater numbers of cells in the BALF and an increased bacterial burden. We used compound 48/80, an MC degranulation inducer, to investigate the role of MC degranulation

in *C. pneumoniae* lung infection (40, 54). WT mice were treated with compound 48/80 (2 mg/kg body weight) intratracheally 2 d after *C. pneumoniae* inoculation, followed by sacrifice on day 5. As predicted, compound 48/80-treated mice displayed increased total cell numbers in BALF (Fig. 5A), whereas the total cell number in the lung was not changed (Fig. 5B). Compound 48/80-treated mice also had increased KC in BALF and MIP-2 in the lung homogenates compared with nontreated mice (Fig. 5C). Finally, *C. pneumoniae* IFU were significantly increased in compound 48/80-treated mice compared with control mice (Fig. 5D). These data suggest that MC degranulation plays a role in immune cell infiltration of the airway and is beneficial for *C. pneumoniae* replication.

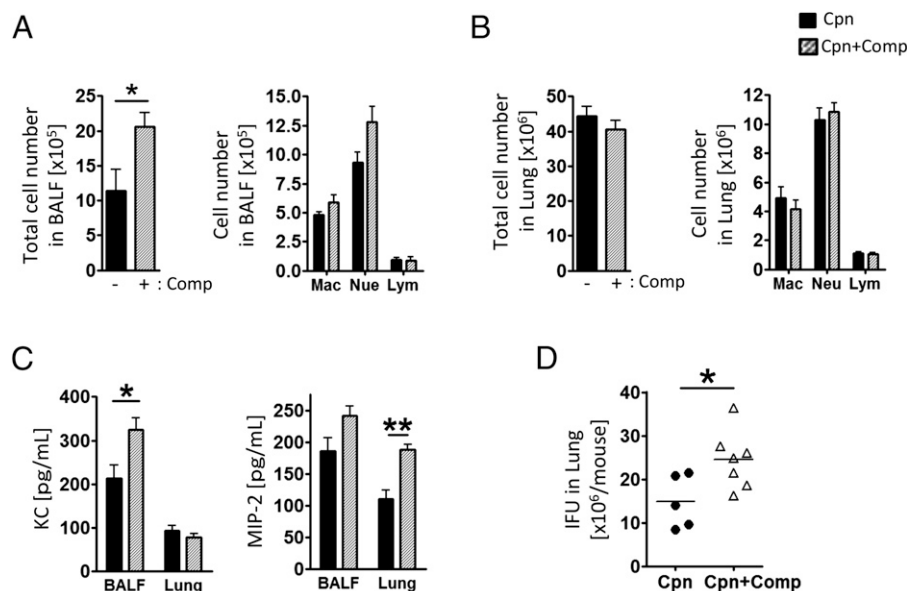


FIGURE 5. Compound 48/80 increased inflammatory cell infiltration into the airspace and bacterial number in the lung. WT mice were treated with compound 48/80 (2 mg/kg body weight) 2 d post-*C. pneumoniae* infection and sacrificed on day 5. **(A)** Total and differential cell counts in BALF. Cell types in BALF were determined by modified Wright–Giemsa staining. **(B)** Single cells from lung were collected through enzymatic procedure and analyzed by flow cytometry. **(C)** Chemokine concentrations in BALF and lung homogenates were determined by ELISA. **(D)** *C. pneumoniae* IFU in the lung from *C. pneumoniae*-infected WT and Wsh mice was quantified as described in *Materials and Methods*. For (A)–(D), the experiment was performed twice ($n = 5$ [Cpn] and $n = 7$ [Cpn + Comp]). Data shown are a representative experiment. Data are mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, Mann–Whitney test.

MC-deficient mice have impaired immune cell distribution in the lung during *C. pneumoniae* infection

Next, we observed the histology of lung tissue from *C. pneumoniae*-infected WT and Wsh mice. At day 3 postinfection, there were no obvious differences between WT and Wsh mice (data not shown). However, at both days 5 and 14 we found that infiltrating cells had accumulated in the perivascular regions of Wsh lungs, whereas in WT mice the immune cells were more evenly distributed (Fig. 6A). Also at days 5 and 14, Wsh lungs displayed patchy inflammatory lesions in lung parenchyma, whereas WT lung inflammation was much more diffuse (Fig. 6A). The perivascular accumulations in Wsh mice contained large numbers of B and T cells (Fig. 6A). Although these structures looked superficially like inducible BALT, their appearance by 5 d postinfection precludes them from being normal inducible BALT, because they generally are a product of a long-term immune response. Additionally, these accumulations did not provide any advantage of acquired immunity later, such as *C. pneumoniae*-specific IgG production or effector memory T cell numbers in draining lymph nodes and IFN- γ production in spleen (data not shown).

Neutrophil staining revealed a patchy distribution in Wsh lungs compared with WT lungs, which showed a very diffuse staining pattern (Fig. 6B), although the neutrophils were not quite as clustered as were the T and B cells. These data suggest that MCs play a role in regulating inflammatory cell distribution in the lung and infiltration into airways.

MC-deficient mice are defective in claudin degradation during *C. pneumoniae* lung infection

Next, we investigated the role that claudins might play in the defective immune cell infiltration into airways of Wsh mice. Claudins are important components of tight junctions, and their degradation is required for paracellular permeability of fluid, as well as immune cells (40). We analyzed claudin-5 and -18 protein levels in the lungs of WT and Wsh mice by Western blotting. When infected with *C. pneumoniae*, the lungs of Wsh mice displayed significantly reduced claudin degradation (both claudin-5 and -18) in comparison with WT mice (Fig. 7A), although there was no difference in the amounts of claudin proteins in the lungs of uninfected WT and Wsh animals (Fig. 7A). Because claudins are degraded by MMP-9 in vivo (40, 55, 56), we assayed the presence of MMP-9 in BALF of infected WT and Wsh mice and found that secreted MMP-9 was indeed reduced in Wsh BALF compared with WT BALF (Fig. 7B). MCs are known to secrete MMP-9 (32, 55, 56). During degranulation, MCs also release chymase, a major MMP-9 activator in vivo (30, 32). Thus, it is possible that, during a *C. pneumoniae* infection in mice, MCs release and activate MMP-9 (most likely via chymase), which then degrades claudin-5 and -18 in epithelial tight junctions and allows infiltrating immune cells into the airspaces.

In addition to claudin degradation, TREM-1 was reported to be required for neutrophil transepithelial migration in the lung (25, 29, 30). However, we did not find any significant difference in TREM-1 cell surface expression between WT and Wsh neutrophils in BALF or lung during infection (data not shown).

FIGURE 6. Wsh mice display patchy inflammation, accumulated T and B cells perivascularly, and accumulated neutrophils in the lung during *C. pneumoniae*-induced lung inflammation. WT and Wsh mice were infected with *C. pneumoniae* intratracheally. Lungs were harvested for H&E staining and immunohistochemistry for B cells and T cells on days 5 and 14 (**A**) and for neutrophils on day 5 (**B**). Arrows denote neutrophil staining. This experiment was performed three times (total $n = 7$ [WT and Wsh] day 5, $n = 7$ [WT], and $n = 10$ [Wsh] day 14). Scale bar, 0.5 mm.

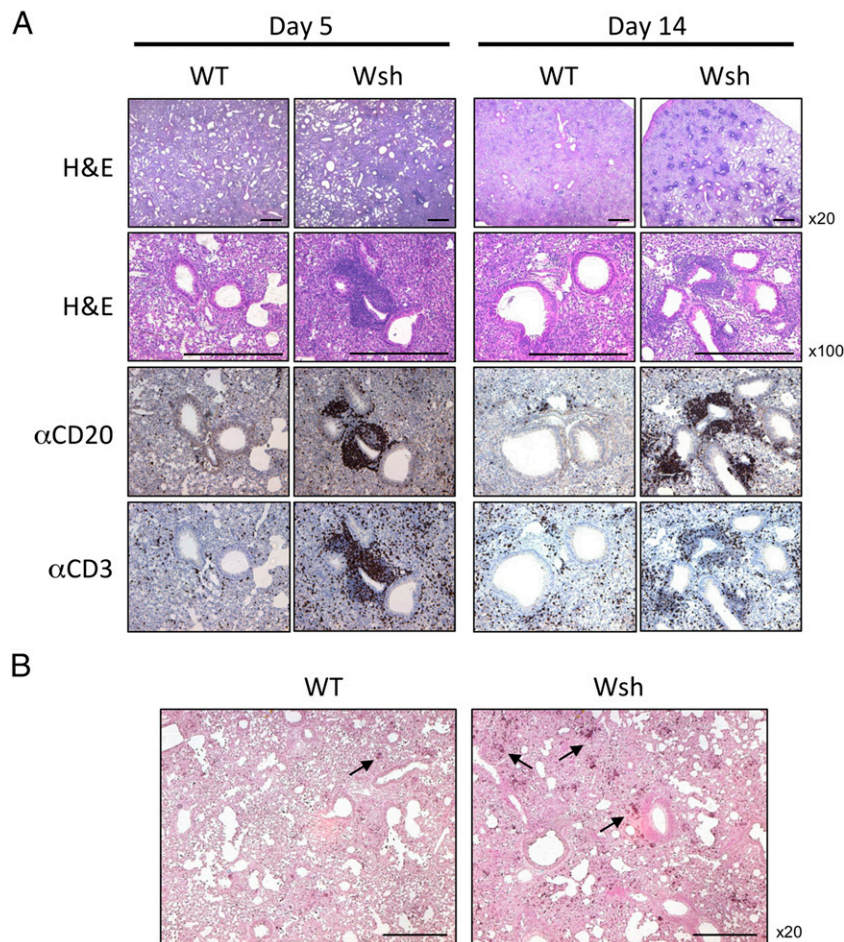
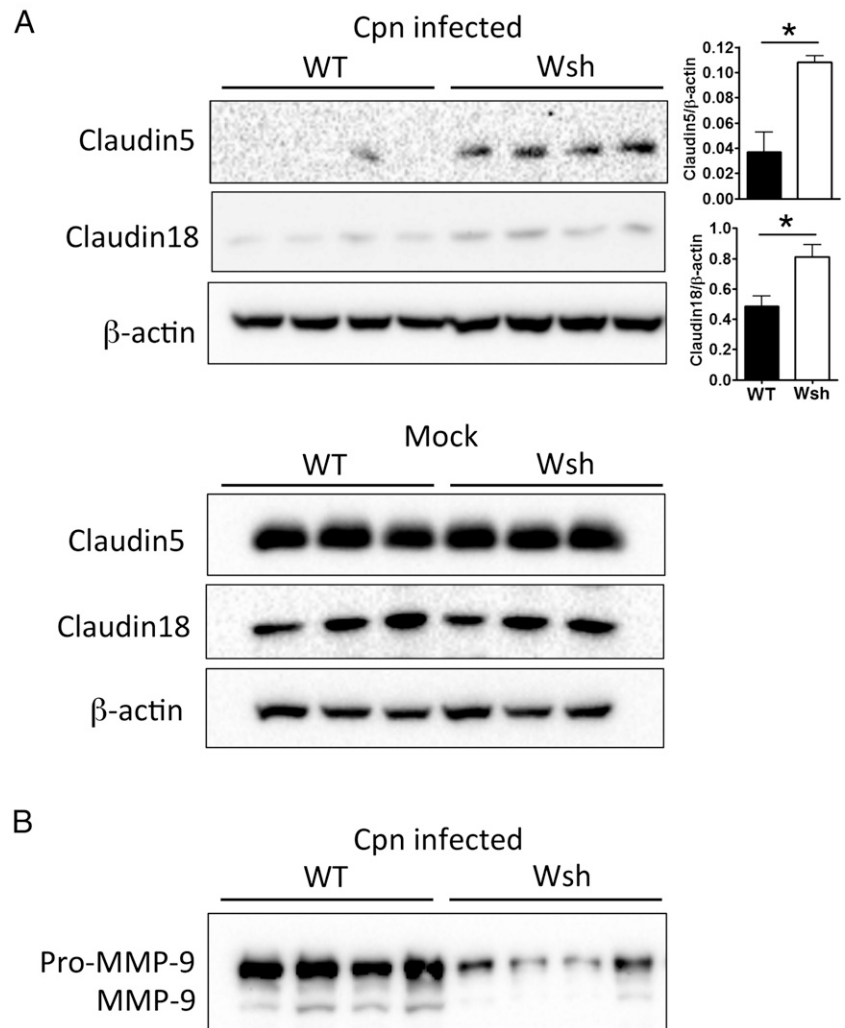


FIGURE 7. Cell–cell tight junction molecule degradation was reduced in Wsh lung compared with WT lung during *C. pneumoniae* lung infection. WT and Wsh mice were infected with 2×10^6 IFU *C. pneumoniae* intratracheally and sacrificed on day 5. **(A)** Lung cell lysates were separated by SDS-PAGE, and immunoblotting was performed with the indicated Abs. The representative Western blots show relative amounts of claudin-5 and -18 in the lung from naive and *C. pneumoniae*-infected WT and Wsh mice. **(B)** BALF was separated by SDS-PAGE, and immunoblotting was performed with anti-MMP-9 Ab to determine secreted MMP-9 in BALF. In (A), the experiment was performed three times ($n = 5$ [WT] and $n = 6$ [Wsh]). In (B), the experiment was performed twice ($n = 4$ [WT] and $n = 4$ [Wsh]). Data shown are a representative experiment. Data are mean $\pm$ SD. * $p < 0.05$, Mann–Whitney test.



MC reconstitution restores WT phenotype to Wsh mice

Wsh mice have an inverted *Kit* gene upstream of the normal gene that leads to a selective reduction in Kit expression and resulting MC deficiency. However, these mice also exhibit other abnormalities, including splenic myeloid and megakaryocytic hyperplasia (57). Therefore, to assess the MC deficiency phenotype in Wsh mice, we adoptively transferred WT MCs into Wsh mice and infected them with *C. pneumoniae*. Reconstitution with WT MCs resulted in an increased bacterial burden, similar to WT mice (Fig. 8A). Additionally, histopathology revealed a diffuse immune cell staining in MC-reconstituted mice that was similar to that seen in WT mice (Fig. 8B). These data demonstrated that the difference in bacterial burden between Wsh and WT mice is solely due to the lack of MCs and is not related to any other underlying features of Wsh mice. MC reconstitution also restored claudin-5 degradation and MMP-9 secretion and activation (Fig. 8C), highlighting the critical role of MCs in these processes during *C. pneumoniae* infection.

Discussion

In this study, we investigated the role that MCs play during *C. pneumoniae* infection of the lung in mice. Using MC-deficient mice, we were surprised to discover that Wsh mice were protected from *C. pneumoniae* infection, with decreased mortality, reduced pulmonary inflammation, and lower bacterial titers. These observations were juxtaposed with a decrease in immune cell infiltrates

into the airspaces, as well as an increase in immune cells into the lungs. Interestingly, although there was no difference in vascular permeability between Wsh and WT mice in the whole lung, there was less Evans blue dye detected in BALF of Wsh mice compared with WT mice. Histopathology of the lungs revealed Wsh mice to have patchy inflammation with large immune cell clusters in a perivascular distribution, whereas WT mice had a much more diffuse staining pattern. Pharmacological manipulation of MCs with cromolyn, a MC stabilizer, phenocopied MC deficiency with decreased inflammation and bacterial burden, whereas compound 48/80, which induces MC degranulation, exacerbated infection with increased inflammation and bacterial burden. The results of these pharmacological interventions suggest that MC degranulation is important in the response to *C. pneumoniae* infection and, possibly, following bacterial persistence. MC deficiency also coincided with a lack of claudin-5 and -18 degradation in the lungs during *C. pneumoniae* infection, with reduced amounts of MMP-9 in BALF, suggesting that MCs may be required for the opening of tight cell junctions to allow infiltrating immune cells access to the airspaces. Critically, MC reconstitution in Wsh mice led to normal bacterial growth, claudin-5 degradation, and increased MMP secretion.

Although it is clear that MCs play a critical role in immune cell recruitment to the airspaces during *C. pneumoniae* infection, further investigation is needed to identify how these MCs are activated. In our model, it is likely that MCs are not directly ac-

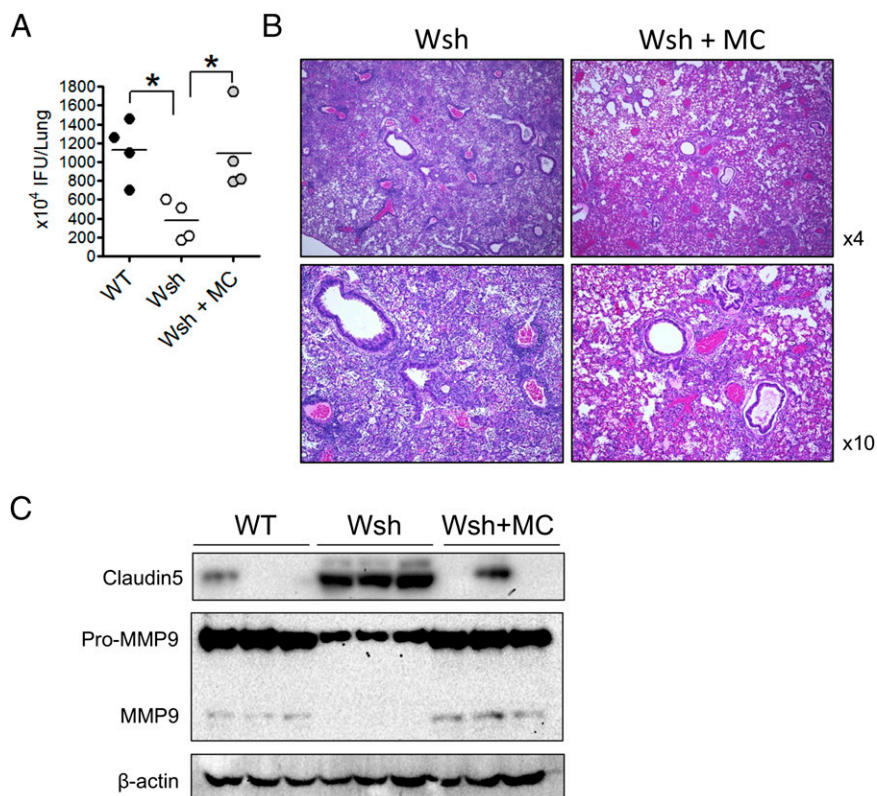


FIGURE 8. MC reconstitution in Wsh mice restores normal responses to *C. pneumoniae* infection. **(A–C)** Wsh mice were given 5×10^6 of MCs i.v. and allowed to reconstitute for 12 wk. Then the mice were infected with *C. pneumoniae* and sacrificed 5 d later. **(A)** Lung *C. pneumoniae* IFU was quantified at day 5 postinfection. **(B)** H&E staining. **(C)** Western blot analysis of claudin-5 and MMP-9 in the lung. Data are mean $\pm$ SD. * $p < 0.05$, one-way ANOVA and Tukey post hoc test.

tivated by *C. pneumoniae*, but rather by cytokines produced by immune cells in response to *C. pneumoniae* infection. Peritoneal MCs exposed to *C. pneumoniae* directly were not activated (data not shown), suggesting that a secondary mechanism is at play. Indeed, MCs were shown to have far-reaching effects, with systemic MC activation playing a critical role in local inflammatory responses (58). However, a previous publication found that human MCs could produce cytokines in response to *C. pneumoniae* directly (in vitro) and that, in a pulmonary *C. pneumoniae* infection in mice, MCs were degranulated at a greater rate (18). Thus, it may be possible that *C. pneumoniae* infection can induce cytokine production directly, as well as indirectly induce degranulation. Indeed, we found evidence for MC degranulation 4 d after *C. pneumoniae* infection based upon increased LAMP1 detection on the surface of MCs in the lung.

In recent studies, MCs have emerged as an important cell type in both viral and bacterial infections. *Francisella tularensis* can activate MCs via TLR2, which results in enhanced macrophage killing of *Francisella* by secreting IL-4 (25, 29, 59). TNF- α produced by MCs plays a critical role in neutrophil recruitment to the lungs during *Klebsiella pneumoniae* infection (28, 59). MC-deficient mice are also more susceptible to *Mycoplasma pneumoniae* infection, with greater bacterial burden and mortality (28). Dietrich et al. found that MCs are primed to respond to both Gram-positive and Gram-negative bacteria by the secretion of proinflammatory cytokines (12). However, unlike these studies, we found that MC-deficient mice were protected from mortality and had a reduced *C. pneumoniae* burden in the lungs. This is somewhat similar to the role that MCs play during influenza A virus infection in mice. MC-deficient mice were protected from body weight loss and inflammation when infected with H1N1 influenza A virus (14, 15). However, despite the lack of inflammation, there was no difference in viral titers in that model. It might come from the difference in target cells between *C. pneu-*

moniae and H1N1 influenza A, which infects epithelial cells more than macrophages (60). Thus, although there seems to be a general consensus that MCs induce a potent inflammatory response to infection, depending on the organism, this inflammation can be beneficial, damaging, or, as in our study, lead to unproductive inflammation and greater bacterial growth. Indeed, in a recent study, MC-deficient mice were protected from *Streptococcus pneumoniae* pneumonia, although this was independent of MC degranulation (61). *C. pneumoniae* requires host cells for productive replication. Initial pulmonary infection occurs mainly in alveolar macrophages and, to a lesser degree, in epithelial cells. In our study, MCs facilitated the influx of neutrophils and other macrophage/monocytes into the airspaces where the bacterial progeny of the first round of replication could infect them, leading to a longer and more damaging infection. Indeed, it was reported previously that neutrophil depletion promotes bacterial clearance during *C. pneumoniae* lung infection (10, 16, 17) and that neutrophils are a critical site of *C. pneumoniae* replication (62, 63), again suggesting that neutrophils play an important role as an infectious target of *C. pneumoniae*.

The key to the protection found against *C. pneumoniae* infection in MC-deficient mice was primarily the lack of immune cell recruitment to the airspaces, despite increased chemokine production. In other models, MCs were found to be important in immune cell recruitment. In the experimental autoimmune encephalomyelitis model of multiple sclerosis, MCs in the dura mater and pia mater exacerbate the inflammation by facilitating neutrophil recruitment and promoting blood brain barrier and cerebral spinal fluid–blood barrier breakdown to allow immune cells access to the CNS (19–21). Additionally, in a *Pseudomonas* LPS model of pulmonary inflammation, MCs mediated the influx of neutrophils into the lung (12, 24–29). One possible mechanism for the control of neutrophil infiltration into the lungs was by controlling TREM-1 expression on neutrophils. TREM-1 was found to be required for

transepithelial migration of neutrophils (30). However, we did not find any differences in TREM-1 expression in neutrophils in MC-deficient mice, indicating that the regulation of immune cell infiltration by MCs in *C. pneumoniae* infection is independent of TREM-1. Additionally, MC-deficient mice displayed abnormal T and B cell distribution in the lung later in the course of *C. pneumoniae* infection, suggesting that MCs regulate neutrophil transmigration, as well as the migration of other cells during infection.

MCs can make and secrete chymase, a critical protease that can activate many other cellular products by their cleavage (32). One such downstream product is MMP-9, an important metalloproteinase that is responsible for cleaving many surface exposed cellular proteins (35). In our study, we found that MMP-9 was present in BALF of WT infected mice, and it was drastically reduced in MC-deficient mice. One function of MMP-9 is to break down tight junctions, allowing cellular infiltration (35). Claudins are a group of extracellular adhesion proteins responsible for tight junction formation (38), and MMP-9 was shown to degrade claudins in epithelial tight junctions to allow transepithelial migration of immune cells (39, 40). Our data clearly showed that, during *C. pneumoniae* infection in mice, both claudin-5 and -18 were degraded in the lungs and that Wsh mice were protected from this degradation. Reconstitution of MCs led to the restoration of claudin-5 degradation and MMP-9 secretion concomitantly with an increased bacterial burden, similar to WT mice. Therefore, we propose that the lack of immune cell infiltrates into the airways in MC-deficient mice during *C. pneumoniae* infection is due to the inability of the host to break down the epithelial cell tight junctions as a consequence of the lack of MC-derived MMP-9. Although a disrupted chemokine gradient could also explain the reduction in immune cell infiltrates into the airspaces, there was actually increased KC in BALF and lungs, suggesting that this is not the case. Although migration of cells into the airspaces was inhibited in MC-deficient mice, the clusters of B and T cells were more pronounced than those of neutrophils, as seen by histology. It is possible that the neutrophils achieved a slightly more diffuse staining pattern compared with that of B and T cells because they can secrete their own MMP-9 (37, 64, 65), which may allow them to migrate slightly more interstitially than the B and T cells. It is also possible that neutrophilic MMP-9 is necessary for claudin degradation. However, because there are actually more neutrophils in the lungs of infected Wsh mice, this seems less likely. Alternatively, it could be that the neutrophils are not activated, but this would still place them downstream of MCs in the immune response.

One caveat to our experiments is the use of the Wsh mouse model. Several recent studies found that, in some cases, MC deficiency was not the reason for the observed phenotype; the mutation itself affected other developmental pathways (43). Of potential concern was the finding that these mice may have differential Treg numbers and activity. In one study investigating oral tolerance, both Wsh and *Kit^{W/W^v}* mice had increased numbers of Tregs that were not affected by MC reconstitution (44). Another study found an opposite result, with greatly reduced Treg numbers, leading to greater inflammation in Wsh mice (45). However, in our experiments we did not find any difference in Tregs in the lungs of our infected WT and MC-deficient mice (Supplemental Fig. 2E). It also was reported that Wsh mice frequently develop myeloid and megakaryocytic proliferation in the spleen and bone marrow, resulting in neutrophilia, basophilia, increased MC progenitor numbers in the spleen, and elevated platelet numbers in the blood (47). Nevertheless, our study demonstrated that Wsh mice had less neutrophil migration in alveoli. Furthermore, pharmaco-

logical intervention of WT mice during *C. pneumoniae* infection phenocopied MC deficiency (cromolyn) or led to greater inflammation and greater bacterial burden (compound 48/80) (Figs. 4, 5). Finally, MC reconstitution restored phenotypic features of *C. pneumoniae* infection, indicating that it is unlikely that our observations regarding the use of Wsh mice are due to any other potential inherent defect in these mice and are due, in fact, to the lack of MCs and their responses during *C. pneumoniae* infection. Finally, although certain MC-deficient mice (i.e., *WBB6F1-Kit^{W/W^v}*) were reported to have associated neutropenia, the mice that we used (WT and Wsh) do not; instead, they have increased numbers of neutrophils, which we found in the lungs (66). Thus, although we do not believe that intrinsic PMN alterations affected our observations on the role of MCs, we cannot completely rule out this possibility.

C. pneumoniae remains an enigmatic infectious organism with associations to many important human diseases. Understanding this unique organism's complex lifecycle and the immune system's response to infection is a high priority. In this study, we found that the normal immune response is actually beneficial to *C. pneumoniae* infection by providing it with host cells to infect and propagate. Without the presence of MCs, inflammation was mild, mortality was reduced, and *C. pneumoniae* was cleared rapidly from the hosts. Not only do these studies provide important new paradigms for how we understand infections, they may provide new avenues of treatments and protective strategies. Additionally, although MCs have long been considered to primarily play a role in Th2-mediated diseases, they were revealed to be major players in both inflammatory disorders and infections. Our findings place MCs as critical gatekeepers during a pulmonary bacterial infection, facilitating immune cell transepithelial migration in the lungs into the airways and, thus, provide a new target for intervention strategies.

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Disclosures

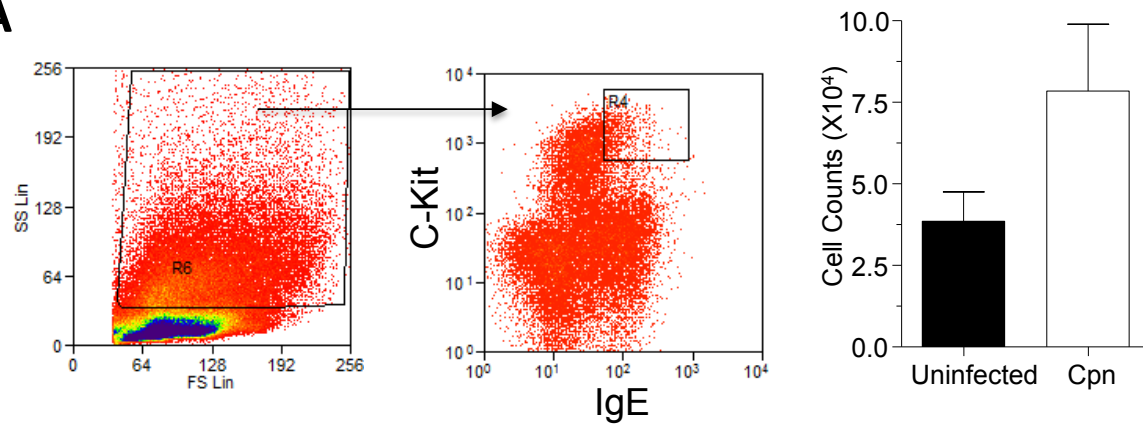
The authors have no financial conflicts of interest.

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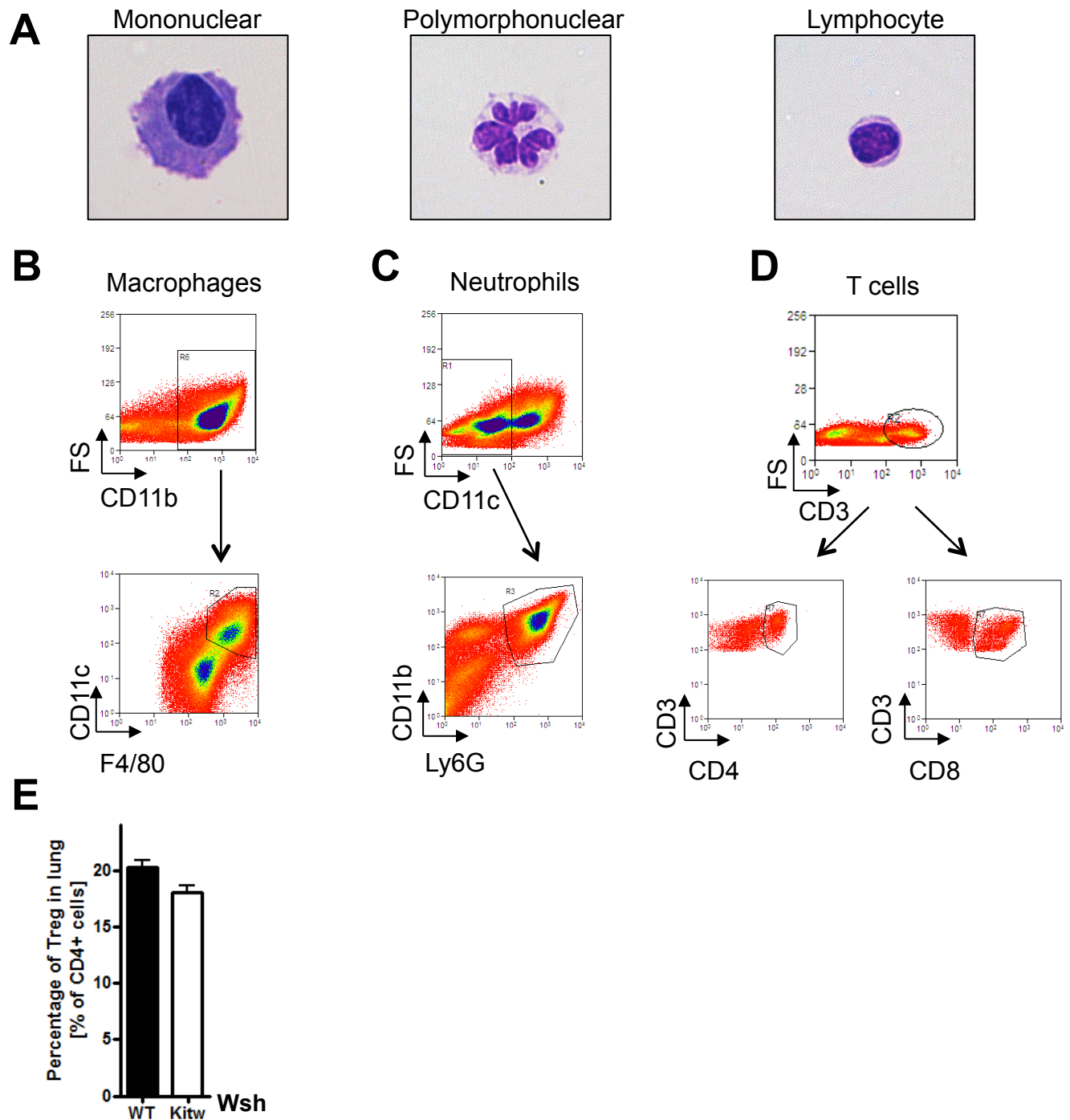
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A

Supplementary Figure 1. Mast cells in WT mice. Single Lung cells were prepared as described in Material and Methods. A) Mast cells were identified as side scatter high and double positive for c-kit and IgE on the surface (n=5). Data are mean $\pm$ SD. Significance of differences was determined by the Mann-Whitney test.



Supplementary Figure 2. Giemsa-Wright staining. Flow cytometry gating schemes for lung immune cells. Treg numbers in Wsh mice. A) BALF cells were stained using Giemsa-Wright staining. Representative images are shown at 400x. B-D) Single Lung cells were prepared as described in Material and Methods. B) Alveolar macrophages were gated as CD11b⁺ CD11c⁺ F4/80⁺. C) Neutrophils were gated as CD11c⁻ CD11b⁺ Ly6G⁺. D) T-cells were gated as CD3⁺ and either CD4⁺ or CD8⁺. E) Cells were stained intracellular Foxp3 following cell surface CD4 and CD25 staining and analyzed by flow cytometry. n=5. Data are mean $\pm$ SD. Significance of differences was determined by the Mann-Whitney test.

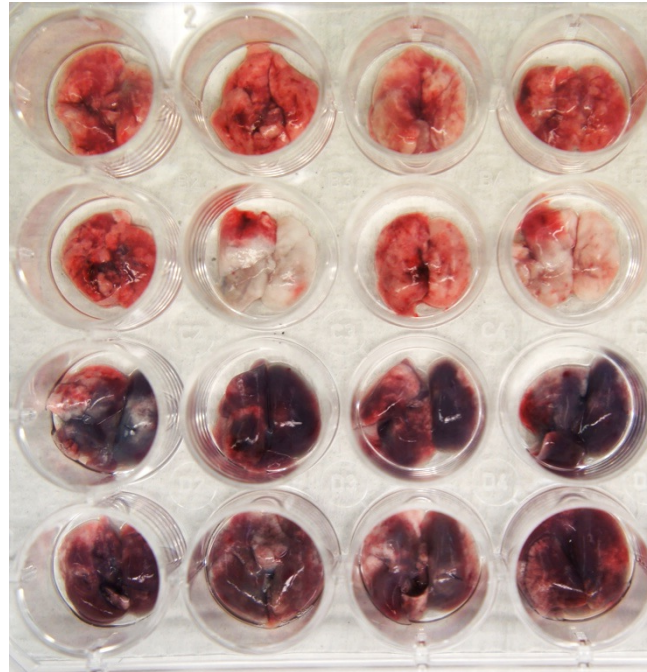
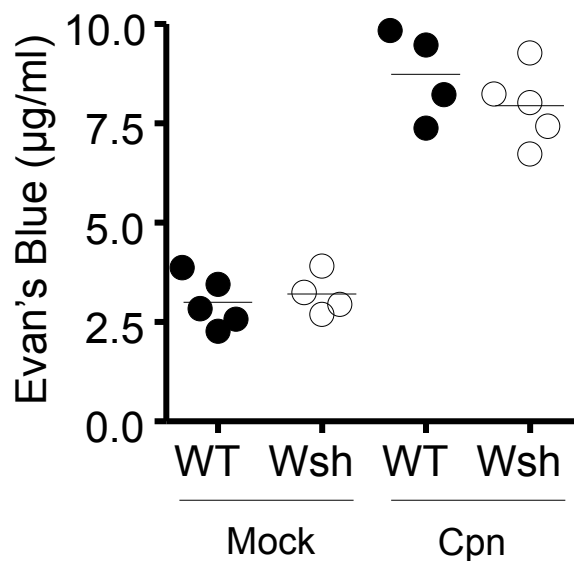
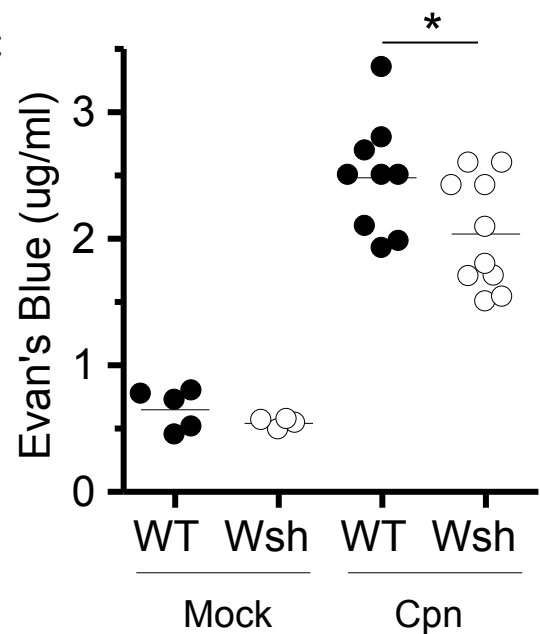
A

WT + Mock

Wsh + Mock

WT + Cpn

Wsh + Cpn

**B****C**

Supplementary Figure 3. Vascular permeability was not altered in Wsh during Cpn infection in the lung, but is in the BALF. (A) Mice were infected with Cpn (2×10^6 IFU). Days 5 after infection, 25 mg/kg Evans Blue was injected i.v. 2 hrs before lung harvest. (B) Whole Lungs were homogenized with 1 mL PBS, then were centrifuged. The pellets were incubated with 500 mL formamide at 60 ° for 18 hrs. The supernatant was measured at absorbencies 620 nm – 740 nm. $n=4\sim5$. (C) Bronchoalveolar lavage was performed and Evan's blue content was measured as above. Shown is the pooled data from two individual experiments. * $p<0.05$, unpaired Student's t-test.