Title: Intestinal infection results in impaired lung innate immunity to secondary respiratory infection.

Subtitle: Lung immunity after gut inflammation.

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Abstract

Pneumonia and diarrhea are among the leading causes of death worldwide, and epidemiological studies have demonstrated that diarrhea is associated with an increased risk of subsequent pneumonia. Our aim was to determine the impact of intestinal infection on innate immune responses in the lung. Using a mouse model of intestinal infection by *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium), we investigated how infection in the gut compartment can modulate immunity in the lungs and impact susceptibility to bacterial (*Klebsiella pneumoniae*) challenge. We found alterations in frequencies of innate immune cells in lungs of intestinally-infected mice compared to uninfected mice. On subsequent challenge with *K. pneumoniae* we found that mice with prior intestinal infection have higher lung bacterial burden and inflammation, increased neutrophil margination, and neutrophil extracellular traps (NETs), but lower overall numbers of neutrophils, compared to mice without prior intestinal infection. Together, these results suggest that intestinal infection impacts lung innate immune responses, most notably neutrophil characteristics, potentially resulting in increased susceptibility to secondary pneumonia.

Author summary

Infections of the lung and gut are among the leading causes of death worldwide. Human studies have shown that children with diarrhea are at higher risk of subsequent lung infection. How intestinal infections impact lung immunity is not well known. In the present study, we reveal that bacterial infection of the intestinal mucosa may compromise lung immunity, offering new insights into increased susceptibility to respiratory infections. We found that upon respiratory infection, mice with prior intestinal infection are more moribund and despite having higher bacterial burden, they show reduced numbers of neutrophils in the lung compared to mice without prior intestinal infection. We also found excessive neutrophil extracellular traps formation in the lungs of mice with prior intestinal infection, providing evidence of increased pulmonary tissue damage. Collectively, these data identify a direct link between pulmonary and enteric infection and suggests gut infections impair neutrophils responses in the lungs.

Introduction

Diarrhea and pneumonia are among the leading causes of death worldwide. In children alone, these diseases combine to kill ~1.4 million each year, with the majority of these deaths occurring in lower and middle-income countries [1]. Epidemiological studies have shown that children are at an increased risk of pneumonia following a diarrheal episode [2-5]. However, the immunological mechanisms behind an increased susceptibility to such secondary respiratory infections are not well understood.

Although the gastrointestinal and respiratory tracts have different environments and functions, there is emerging data showing cross-talk between these two mucosal sites in chronic inflammatory diseases such as inflammatory bowel disease (IBD), asthma, and chronic obstructive pulmonary disease [6-10]. Additionally, there is emerging evidence that the intestinal microbiota plays a role in host defense against bacterial pneumonia [11, 12]. The gastrointestinal and respiratory tracts share the same embryonic origin and have common components of the mucosal immune system such as an epithelial barrier, submucosal lymphoid tissue, the production of IgA and defensins, toll-like receptor expression, and the presence of innate lymphocytes and dendritic cells [13]. Leukocytes circulate between peripheral tissue, lymphatics, and blood, to survey for foreign antigens. Notably, several innate-like leukocytes, such as Mucosal Associated Invariant T (MAIT) cells, invariant Natural Killer T (iNKT) cells, gamma delta T (γδ T) cells, innate lymphoid cells (ILCs), dendritic cells (DC) and neutrophils, have the capacity to circulate between tissues, and play important roles in both respiratory and intestinal tract immunity [14-17].

It is anticipated that organs with the same embryonic origin share the same pathological specificities in a disease, for example, an increased number of inflammatory cells have been reported in bronchoalveolar lavage (BAL) fluid [18] and sputum [19] of IBD patients. How intestinal infection impacts immunity in the lung is not known. In this study, we examine the impact of intestinal infection on the immune response in the lungs of mice using *Salmonella enterica* serovar Typhimurium *(S.* Typhimurium), an established model of intestinal infections. We found that mice infected with *S.* Typhimurium have increased susceptibility to respiratory *Klebsiella pneumoniae*

infection compared to mice without prior intestinal infection. Prior intestinal infection modulated effector cells of innate immunity in the lung, contributing to respiratory immune dysregulation and a higher *K. pneumoniae* bacterial burden. This study furthers our understanding of the gut-lung immunological crosstalk, and begins to define the mechanisms of increased susceptibility to secondary pneumonia following diahrreal infections.

Results

1. Mice with prior *S.* Typhimurium intestinal infection have increased lung bacterial load, sickness score, and susceptibility to respiratory *K. pneumoniae* infection.

To test whether prior intestinal infection increases the susceptibility to respiratory *K. pneumoniae* infection, we evaluated survival, body weight loss, sickness score, and bacterial burden in lungs post *K. pneumoniae* challenge. Compared to *S.* Typhimurium infected mice, which had a survival rate of 90-100%, and *K. pneumonia* infected mice, which had a survival of 60-80%, mice with prior *S.* Typhimurium intestinal infection had a survival rate of 0-30% at 120 hours post *K. pneumoniae* infection (Fig 1A). Interestingly, although there were no statistically significant differences in body weight loss between mice with or without *S.* Typhimurium infection with *K. pneumoniae* challenge (Supplementary Figure S1), mice with prior intestinal infection had a higher sickness score (Fig 1B). This sickness scoring system included hunched posture, ruffled fur, decreased movement and altered respiratory rates and quality of breaths (Fig 1C). Of note, mice with only *S.* Typhimurium intestinal infection showed no weight loss and had reduced sickness scores. When bacterial burden was evaluated at 18 and 33 hours post *K. pneumoniae* infection, we found significantly higher lung bacterial burden in mice with prior intestinal infection compared to mice without prior intestinal infection (Fig 1D and 1E). We did not find any *S.* Typhimurium bacterial burden in lungs of mice with intestinal infection.

2. Mice with prior *S.* Typhimurium intestinal infection have increased lung inflammation from subsequent respiratory *K. pneumoniae* infection.

We next examined the degree of lung inflammation by histological analysis of tissue sections from all four groups of mice. Histopathological analysis revealed mixed interstitial inflammatory consolidations in mice with *K. pneumoniae* respiratory infection and increased microabcess formation with pyknotic neutrophils in mice co-infected with *S.* Typhimurium and *K. pneumoniae* (Fig 2A). Uninfected mice and mice with intestinal infection showed normal alveolar and interstitial lung histology (Fig 2A). Upon challenge with *K. pneumoniae* respiratory infection, mice with prior *S.* Typhimurium intestinal

infection also showed marked intravascular clustering of polymorphonuclear neutrophils (PMNs), with increased margination, necrotic cluster formation and extravasation (Fig 2B and Supplementary Figure S2). Intravascular neutrophil clustering was noticeably absent in the other treatment groups. Furthermore, lung sections from mice with intestinal infection, and mice with both intestinal and respiratory infection, showed scattered microthrombi in capillary-sized vessels whereas mice with *K. pneumoniae* respiratory infection (with no prior intestinal infection) showed no microthrombi formation (Supplementary Figure S3).

3. Mice with prior *S.* Typhimurium intestinal infection have altered lung cytokine profiles after *K. pneumoniae* challenge.

The delicate balance between pro- and anti-inflammatory cytokines is crucial in containing pathogens and maintaining tissue repair and homeostasis in the lung [11]. Prior studies have shown the importance of cytokines such as IFN-γ in recruitment of neutrophils to the lung tissue [20, 21]. We investigated whether intestinal infection affected cytokine production in lung homogenates, and whether prior intestinal infection affected this cytokine response to intranasal *K. pneumoniae* challenge. Our results indicate that compared with the uninfected control group, *S.* Typhimurium infected mice had significantly higher lung levels of IFN-γ, MCP-1 and IL-1β (Fig 3 A,B and C). While Thy1-expressing natural killer (NK) cells [22] and NKp46^{+} ILC3 [23] cells are commonly thought as the sources of IFN-γ, there is a mounting evidence that neutrophils are a prominent cellular source of IFN-γ during the innate phase of *S*. Typhimurium-induced colitis [24]. It is possible that large number of primed neutrophils traffick to lungs after intestinal infection, contribute to cytokine production and increases the potential for neutrophil-mediated pathology or NET formation upon secondary infection [17]. Furthermore, following *K. pneumoniae* challenge, mice with prior intestinal infection had higher levels of IFN-γ and lower levels of GM-CSF cytokine production in lung homogenates compared to those without prior intestinal infection (Fig 3A and D). Levels of IL-23, IL-1α, TNFα, IL-12p70, IL-10, IL-6, IL-27, IL-17A and IFN-β were not significantly different between mice with and without prior intestinal infection (Supplementary Figure S4).

4. Mice with prior *S.* Typhimurium intestinal infection have lower number of neutrophils in lung after *K. pneumoniae* challenge.

We next examined the impact of intestinal infection on innate cellular responses in the lung, and also its effect on such responses to *K. pneumoniae* respiratory challenge. We analyzed changes in major innate lung leukocytes (plasmacytoid dendritic cells (pDCs), monocyte-derived dendritic cells (moDCs), CD103+ DCs, neutrophils, alveolar macrophages (AMs) and interstitial macrophages (IMs)) pre- and post-*K. pneumoniae* challenge in mice infected with *S.* Typhimurium. Consistent with previous studies [9], we observed rapid and robust recruitment of neutrophils to the lungs at 18 hours following *K. pneumoniae* infection compared to uninfected controls. Interestingly, mice with prior intestinal infection had significantly lower frequencies and total number of lung neutrophils following *K. pneumoniae* challenge compared to *K. pneumonia* infected mice (Fig 4A and Supplementary Figure 5A). Furthermore, results indicated that frequencies of pDCs increased and moDCs decreased in the lungs of *S.* Typhimurium intestinally infected mice compared to uninfected controls (Fig 4B and C). Following intranasal Klebsiella challenge, we found a marked increase in frequencies of pDCs and significantly lower frequencies of moDCs in mice with prior intestinal infection compared to those without prior intestinal infection (Fig 4B and C). No significant differences were observed in frequencies of $CD103^+$ DCs (Fig 4D), AMs and IMs (Fig 4E and F) between *K. pneumoniae* infected mice with and without prior intestinal infection. Total numbers of pDCs, moDCs, $CD103^+$ DCs, AMs or IMs were not different in mice with prior intestinal infection compared to those without prior intestinal infection (Supplementary Figure S5 B-F). We also found higher numbers of neutrophils, pDCs and IMs and lower numbers of $CD103^+$ DCs in mice with only intestinal infection compared to uninfected mice (Supplementary Figure S5 B-F).

We also investigated innate-like T cells including mucosal-associated invariant T (MAIT) cells, invariant natural killer T (iNKT) cells and γδ T cells as they are known to play an important role in bacterial infections [10]. No differences were observed in percentage frequencies of iNKT cells, MAITs or γδ T cells between mice with and without prior intestinal infection (Supplementary Figure S6). Furthermore, when complete blood

counts were assessed with a Hemavet analyzer, no significant differences were observed in circulating neutrophils, lymphocytes, monocytes, eosinophils, basophils and platelets between mice with and without prior intestinal infection (Supplementary Figure S7).

5. Mice with prior *S.* Typhimurium intestinal infection induces widespread NETosis after *K. pneumoniae* challenge.

Recent studies have revealed that excessive NET formation plays a role in pathogen-associated lung injury, including in models of bacterial pneumonia [25-28]. Our histopathological analysis revealed clusters of pyknotic neutrophils and thrombus formation in mice with prior intestinal infection (Fig 2 and Supplementary figure 3). It is known that neutrophils constitute the main cellular component of thrombi, and mainly participate in thrombosis by releasing NETs [29]. Moreover, we detected higher levels of IFN-γ in mice with prior intestinal infection compared to mice without prior intestinal infection (Fig 3), and IFN-γ can promote NET formation by neutrophils [30]. We therefore explored whether prior intestinal infection induces NET formation. We examined citrullinated histones and myeloperoxidase (MPO) in lung tissues of each experimental group by immunofluorescence. As expected, significantly higher number of NETs (p = 0.0006) were detected in mice with prior intestinal infection compared to those without prior intestinal infection (Fig 5 A and B), as demonstrated by the presence of extracellular DNA overlaid with citrullinated histone 3 and MPO (Fig 5A). Mock-infected lungs did not show any staining for citrullinated histone 3 or MPO (Fig 5A). We detected NETs in lung tissues of mice with *S.* Typhimurium intestinal infection compared to uninfected control or *K. pneumoniae* infected alone, and there was no significant difference between these groups (Fig 5B).

Discussion

We found that bacterial intestinal infection in mice adversely impacts immunity in the lung, increasing susceptibility to secondary respiratory infection. We show that mice with prior intestinal *S.* Typhimurium infection have higher lung bacterial burden and sickness scores after subsequent *K. pneumoniae* challenge compared to mice without prior *S.* Typhimurium infection. This finding was associated with changes in innate cellular responses, most notably those of neutrophils, which were decreased in the parenchyma, clustering in the lung vasculature, and associated with increased NETosis in those with secondary infection. As neutrophils are essential for pulmonary clearance of bacterial infections such as *K. pneumoniae* [31], it is possible that intestinal infection impairs the recruitment and function of lung neutrophil responses against *K. pneumoniae*, leading to an inability to clear the lung bacterial infection, thereby worsening airway disease.

In addition to epidemiological studies showing a higher susceptibility to pulmonary infections after intestinal infection in children [2-5], and that intestinal diseases are often associated with pulmonary disorders [32-34], the immunological crosstalk of the lung-gut axis is not well understood. In the context of IBD, it has been proposed that intestinal inflammation and increased cytokine levels create conditions favorable for neutrophil margination onto the lung endothelium [35, 36]. When the lung encounters a secondary insult, neutrophil recruitment, activation and extravasation could mediate lung tissue injury and IBD-induced respiratory diseases [17, 37]. Our knowledge of how intestinal infection impacts the recruitment, extravasation and function of neutrophils in tissues outside of the intestine is limited. Studies have shown that GM-CSF plays an important role in neutrophil accumulation [38, 39], and GM-CSF is protective in preclinical models of pneumonia-associated lung injury [40, 41]. We found a significantly lower lung GM-CSF response to *K. pneumoniae* infection in mice with prior intestinal infection, which may decrease the lung's ability to recruit circulating neutrophils, resulting in an increased susceptibility to infection. In line with this, we also detected increased NETosis and microthrombosis in *K. pneumoniae* infection in mice with prior

intestinal infection as compared to mice without prior intestinal infection. It is likely that NETs and NET-associated factors, including histones and granule proteases, mediate vascular and tissue injury and contribute to microthrombosis [42-45]. The activation of NETosis also causes changes in neutrophil morphology including cell membrane rupture and neutrophil death [46]. We speculate that prior intestinal infection induces suicidal NETosis contributing to lower numbers of viable neutrophils in lungs. Future studies examining the mechanisms governing neutrophil activity, and activation of NETosis, in lung after intestinal infection are warranted.

Nonconventional T lymphocytes including MAIT cells, *i*NKT cells and γδ-T cells, have tissue-homing properties, and have been implicated in protection against respiratory bacterial infections [15]**.** Here, we found no differences in frequencies or number of these cells between the groups tested. Likewise, except for IFN-γ we did not find any differences in cytokines that have been implicated in lung defense against bacterial pathogens, including IL-17A [47], TNF-α [48] and IL-10 [49, 50]. Both a protective [51] and detrimental [52] role of IFN-γ has been reported against bacterial infections, and we found significant increase in lung IFN-γ production in mice with intestinal infection. The importance of IFN-γ production in our model is unclear and merits investigation. We observed higher frequencies of pDCs in lungs of mice with prior intestinal infection, and this may be related to the role of respiratory pDCs in tissue repair [53]. Alternatively, accumulation of pDCs in lung post intestinal infection may have involvement in the initiation of inflammation and antigen-specific T cell responses [54]. Moreover, frequencies of moDCs, which may contribute to control of secondary respiratory infection, was decreased in mice with prior intestinal infection [55, 56].

Our study has several limitations. Firstly, we did not account for the effect of the pre-existing intestinal microbiota; however, all mice were purchased from the same source, and were cohoused prior to infection. Secondly, we did not evaluate serum levels of IL-6, TNF-α, IFN-γ and VEGF [35, 36] and neutrophil chemokines such as keratinocyte-derived chemokine (KC) [57], macrophage inflammatory protein 2 (MIP-2) [58], CXC

receptor 2 (CXCR2) and CXC ligand 5 (CXCL5) [59] which would further our understanding of lung neutrophil trafficking following intestinal inflammation. Lastly, we have not investigated the role of innate lymphoid cells, which have shown to be recruited from gut to the lungs in response to inflammation [60, 61] [62].

In conclusion, the present study demonstrates that infection in the gut adversely impacts immunity in the lung and reveals potential mechanisms of immunological crosstalk between the lung and gut during enteric infection. While epidemiological studies have demonstrated this lung-gut association, we provide here novel findings that intestinal infection modulates neutrophil and cytokine responses in the lung, resulting in an increased susceptibility to a secondary pneumonia challenge. These data have the potential to inform efforts to prevent and treat respiratory infections in those with intestinal infection or inflammation.

Materials and Methods

Bacterial strains

Salmonella enterica serovar Typhimurium (provided by Dr. June Round) was grown overnight in Luria Broth (LB) supplemented with 100 µg/ml ampicillin and *K. pneumoniae* (subsp. pneumoniae (Schroeter) Trevisan (ATCC® 43816™), serotype 2) was grown overnight in Tryptic Soy Broth (TSB) at 37°C in a shaking incubator. Overnight cultures were diluted 1:10 in fresh medium and sub-cultured for 4 h under mild aeration. Bacteria were washed twice in phosphate-buffered saline (PBS) and then suspended in 1 ml PBS. *S.* Typhimurium culture (OD_{600} = 0.1) was further diluted 1:10^4 in PBS and given to mice in 100 µl volume. *K. pneumoniae* (OD_{600} = 0.4) culture was further diluted 1:10 and given to mice in 50 µl volume. Bacterial colony forming units (CFUs) were confirmed by culturing on LB + ampicillin agar plates for *S.* Typhimurium and MacConkey agar plates for *K. pneumoniae*.

Mice and inoculations

Six to eight week old female C57BL/6J wild type mice were obtained from Jackson Laboratories. All animals were maintained and experiments were performed in accordance with University of Utah and Institutional Animal Care and Use Committee (IACUC) approved guidelines (protocol no. 17-01011). The animals were kept at a constant temperature (25°C) with unlimited access to pellet diet and water in a room with a 12 h light/dark cycle. All animals were monitored daily and infected animals were scored for the signs of clinical illness severity [63]. Animals were ethically euthanized using CO_2.

For the experiments, all mice were pretreated with streptomycin as described previously [64]. Briefly, water and food were withdrawn 4 hours before oral gavage treatment with 7.5 mg of streptomycin in 100 µl HBSS. Afterward, animals were supplied with water and food ad libitum. At 20 h after streptomycin treatment, water and food were withdrawn again for 4 hours before the mice were infected with 10^4 CFU of serovar Typhimurium (oral gavage of 100 µl suspension in PBS) or treated with sterile PBS (control). (Note: we selected this dose of *S*. Typhimurium based on titration experiments

showing that at this dose, bacteria could be cultured from stool but not from lungs). Thereafter, drinking water and food ad libitum was offered immediately. Six days post infection (p.i.), mice were sacrificed by CO_2 asphyxiation, and lungs were removed for analysis.

For *K. pneumoniae* challenge experiments, six days post *S.* Typhimurium infection, isoflurane anesthetized mice were inoculated intranasally with ~ 10^{10} CFU *K. pneumoniae* in 50 µl volume. Inoculated mice were observed until fully recovered from anesthesia and euthanized at 18 h post infection for *K. pneumoniae* bacterial load enumeration and innate immune response assessment. For survival studies, to reduce animal pain and respiratory distress, *K. pneumoniae* dose was reduced to 10^5 CFU .

Lung Histology

After sacrifice, mouse lungs were infused with 10% neutral buffered formalin via the trachea, fixed in formalin overnight, dehydrated in 70% ethyl alcohol and embedded in paraffin. 4 µm sections were stained with haematoxylin and eosin (H&E) and analysed by a board certified anatomic pathologist (A.H.G.). Samples were blinded prior to histopathologic analysis.

Lung Neutrophil Extracellular Trap Assessment

Paraffin imbedded mouse lung were cut at 8 µm thickness on a microtome. Sections were deparaffinized and rehydrated following xylene and decreasing concentration of ethanol washes. Heat induced epitope antigen retrieval of lung sections were processed in a 2100 Retriever Thermal Processor (Electron Microscopy Sciences, 210050) containing Citrate buffer pH 6.0 solution (abcam, ab93678). Sections were incubated for 10 minutes with 0.1% Triton-X-100 and blocked with 10% Donkey Serum for 1 hour at RT. Antibodies for citrullinated Histone H3 (Abcam, ab5103) and Myeloperoxidase (R&D Systems, AF3667) were incubated at 1:100 dilution in 10% donkey serum overnight at 4°C. After washing sections with PBS, secondary rabbit and goat antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 546, respectively, along with DAPI nuclear stain were incubated on sections for 90 minutes at 4°C. Sections

were washed and coverslips were adhered with aqueous mounting medium (Dako, S3025). Images were acquired on an Olympus FV3000 Confocal Laser Scanning Microscope at 20X and 60X magnification. FluoView software (Olympus) and ImageJ Fiji (NIH) were used for image processing and analysis. We quantified NET formation on the images using a standardized grid system as previously described [65]. Briefly, we used ImageJ software to place a standardized grid on randomly selected high-power field images (n=5 field images/sample). The number of times that any NET crossed a grid line was tallied.

Lung mononuclear cell isolation

For lung digestion and preparation of single cell suspensions, lungs were perfused using 5 mL sterile PBS, aseptically harvested from euthanized mice and kept in RPMI with 10% Fetal Bovine Serum (FBS). Lungs were dissociated using the mouse Lung Dissociation Kit (Miltenyi Biotec) and the gentleMACS Dissociator (Miltenyi Biotec) as per manufacturer's instructions. After dissociation, cells were passed through a 70 µm cell strainer and washed with RPMI with 10% FBS. Red blood cells were lysed with red blood cell lysis buffer. Lung mononuclear cells were then washed twice in RPMI with 10% FBS before use in subsequent experiments.

Bacterial Load quantification

K. pneumoniae bacterial load was determined by plating ten-fold serial dilutions of the lung homogenates onto MacConkey agar plates (Sigma-Aldrich). The plates were incubated at 37°C overnight before bacterial CFUs were determined by colony counts.

Mouse inflammation quantification

Lungs homogenates were filtered on 70 µm cell strainers and centrifuged at 300 × g for 5 min. Supernatants were stored at -80°C for cytokine content analysis. Lung cytokine levels were assessed from the supernatant samples via LEGENDplex kit (mouse inflammation panel 13-plex; BioLegend) per manufacturer's instructions. Cytokine levels were acquired using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA), and analyses were performed using LEGENDplex data analysis software (BioLegend).

Tetramer and antibody surface-staining of lung single cell suspensions

From each group of animals, 1-2 million cell aliquots were prepared and stained with the fixable viability dye eFluor^{TM} 780 (eBioscience) for 15 min at room temperature (RT) to exclude dead cells from analysis. The cells were washed with PBS + 2% FBS and incubated with anti-mouse CD16/CD32 Fc Block antibody (BD Biosciences, USA), for 20 min at 4 °C. Cells were then stained for 30 min at RT with appropriately diluted PE conjugated MR-1-5-OP-RU tetramers or α-GalCer (PBS-44)–loaded CD1d tetramer conjugated to APC, anti-CD3-FITC (Biolegend), anti-CD161-BV510 (Biolegend), anti-CD49b-BV711 (BD), anti-TCRγδ-PE-Cy7 (Biolegend), anti-TCRβ-BV421 (Biolegend), anti-CD45R-PE-Cy5 and anti-CD44-BV650 (Biolegend). To evaluate different antigen presenting cells, after Fc Block incubation, cells were also surface stained with anti-CD45 AF700 (Biolegend), anti-CD11b-FITC (Biolegend), anti-CD11c-PE Cy-7 (BD), anti-Siglec-F- BV711 (BD), anti-CD64-BV605 (Biolegend), anti-CD24-PE (Biolegend), anti-Ly-6G-PerCP-Cy5.5 (Biolegend), anti-CD103-BV510 (Biolegend), anti-mPDCA-1 APC (Miltenyi Biotec) and anti-MHC II BV421 (Biolegend) for 30 min at 4 °C. A total of 10^6 gated events per sample were collected using the BD Fortessa flow cytometer (Becton Dickinson, San Diego, CA), and results were analyzed using FlowJo 10.4.2 software.

Statistical analysis

GraphPad Prism 6 software was used for statistical analysis. The Mann-Whitney *U* test was used for comparison of continuous variables between uninfected and *S.* Typhimurium infectd group and between mice with prior intestinal infection and without prior intestinal infection. Results were presented as mean ± standard deviation, and $p < 0.05$ was considered statistically significant.

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Supporting information

Supplemental Figure S1: No differences in body weight loss between mice with or without *S.* Typhimurium prior infection post *K. pneumoniae* challenge . Percent of initial body weight, statistical analysis was performed by fitting a mixed-effect model followed by Tukey's multiple comparisons test.

Supplemental Figure S2: No neutrophil margination and necrotic clusters were detected in mice with intestinal *S.* Typhimurium infection (S) and mice with *K. pneumoniae* respiratory infection (K). Representative images (200x magnification) of lung sections stained with hematoxylin/eosin. Data represents cumulative results of two independent experiments (n = 2 for UI, n = 10 for S group, n = 9 for K group).

Supplemental Figure S3: Pulmonary microthrombi in S and S+K treated mice. Lung sections from S and S+K mice show scattered thrombus formation in capillary-sized vessels (black arrows), readily identified at the periphery of the lungs. H&E, 400x magnification. Data represents cumulative results of two independent experiments (n = 2 for UI, n = 10 for S group, n = 9 for K group and n = 8 for S+K group).

Supplemental Figure S4: Lung cytokine profiles showing no differences between the groups tested. Levels of lung inflammatory cytokines (A) IL-23, (B) IL-1α, (C) TNF-α, (D) IL-12p70, (E) IL-10, (F) IL-6, (G) IL-27, (H) IL-17A, (I) IFNβ were assessed 18 hours post *K. pneumoniae* challenge via bead-based LEGENDplex mouse inflammation panel 13-plex assay. Data represents one experiment for uninfected mice (n =6) and *S.* Typhimurium infected mice (n =6) and cumulative results of three independent experiments for K (n = 18) and S+K group (n = 19). Error bars represents mean ± SD and significance was determined by Mann-Whitney tests.

Supplementary Figure S5: Mice with prior intestinal infection have lower number of total neutrophils in lungs post intranasal challenge with *K. pneumoniae.* Using flow cytometry, total numbers of (A) neutrophils, (B) lung plasmacytoid dendritic cells (pDCs) and (C) monocytic dendritic cells (moDCs), (D) CD103+ DCs, (E) alveolar macrophages (AMs) and (F) interstitial macrophages (IMs) were evaluated between uninfected mice (UI), *S.* Typhimurium infected mice (S), *K. pneumoniae* infected mice (K) and both *S.* Typhimurium and *K. pneumoniae* infected mice (S+K). Data represents cumulative results of five independent experiments (n = 22 for UI, n = 24 for S, n = 25 for K and n = 22 for S+K). Error bars represents mean ± SD and significance was determined by Mann-Whitney tests.

Supplemental Figure S6: **Mice with prior *S.* Typhimurium intestinal infection have no differences in *i*NKT, MAIT and TCRγδ cells in response to respiratory *K. pneumoniae* infection.** Using flow cytometry, percentage frequencies of (A) *i*NKT cells, (B) MAIT cells and (C) TCR γδ cells were evaluated between uninfected mice (UI), *S.* Typhimurium infected mice (S), *K. pneumoniae* infected mice (K) and both *S.* Typhimurium and *K. pneumoniae* infected mice (S+K). Data represents cumulative results of five independent experiments (n = 22 for UI, n = 24 for S, n = 25 for K and n = 22 for S+K). Error bars represents mean ± SD and statistical significance was determined by using Mann-Whitney tests.

Supplemental Figure S7: **Mice with prior *S.* Typhimurium intestinal infection have no differences in blood leukocytes and platelets in response to respiratory *K. pneumoniae* infection.** Using Hemavet analyzer, percentage frequencies of (A) neutrophils, (B) lymphocytes, (C) monocytes, (D) eosinophils, (E) basophils and blood platelet counts were assessed between *K. pneumoniae* infected mice (K) and both *S.* Typhimurium and *K. pneumoniae* infected mice (S+K). Data represents results of one experiment (n = 4 for K and n = 4 for S+K). Error bars represents mean ± SD and statistical significance was determined by using Mann-Whitney tests. Data were analyzed by t-test and presented in mean ± SD.

Fig 1

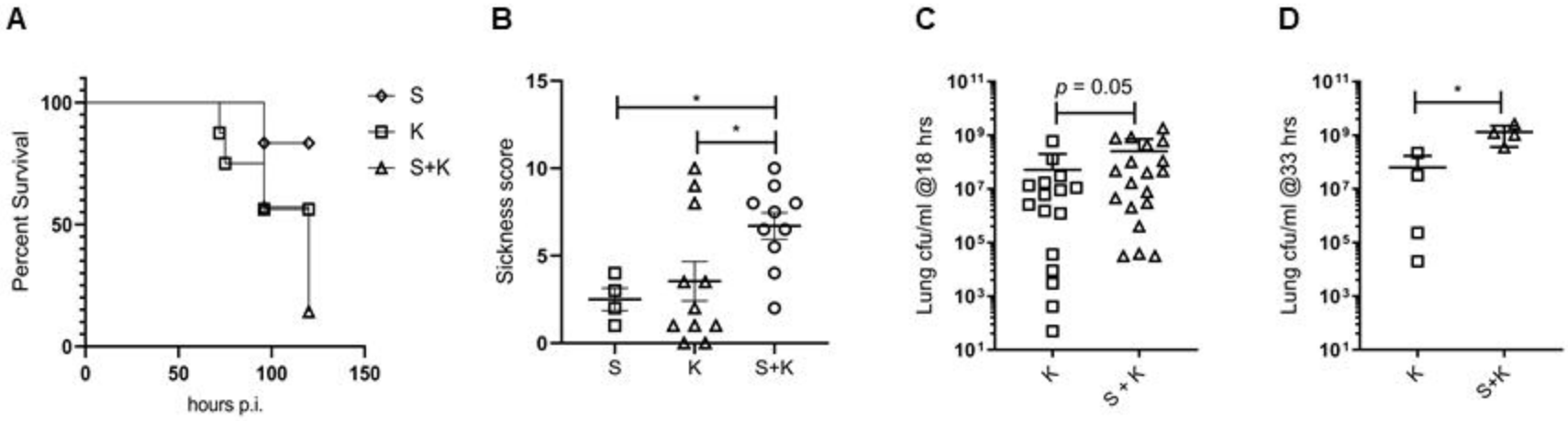

Fig 1: Mice with prior *S.* Typhimurium intestinal infection have increased susceptibility to respiratory *K. pneumoniae* infection. (A) Kaplan-Meier survival curves of mice infected with *S.* Typhimurium intestinal infection (S), *K. pneumoniae infection* only (K) and mice with prior *S.* Typhimurium intestinalinfection and challenged with *K. pneumoniae* infection (S+K). Statistical analysis was performed using log-rank (Mantel-Cox) test, p = 0.07 and log-rank test for significant trend, p = 0.02 (B) sickness score plots where data represents cumulative results of two independent experiments (n = 4-6 for S, n = 11 for K and n = 10 for S+K) and mean ± SD, * denotes $p < 0.05$. Statistical analysis was performed using Kruskal-Wallis test followed by Dunn's multiple comparison test. (C) *K. pneumoniae* bacterial load determined in lungs at 18 hours post *K. pneumoniae* infection in both K and S+K groups Data represents cumulative results of three independent experiments (n = 16 for K and n = 19 for S+K). (D) Lung bacterial loads also determined at 33 hours in both groups (n = 4 for each group). For (C) and (D) data represents mean ± SD and statistical analysis was determined using Mann-Whitney test, * denotes $p < 0.05$.

Fig 2

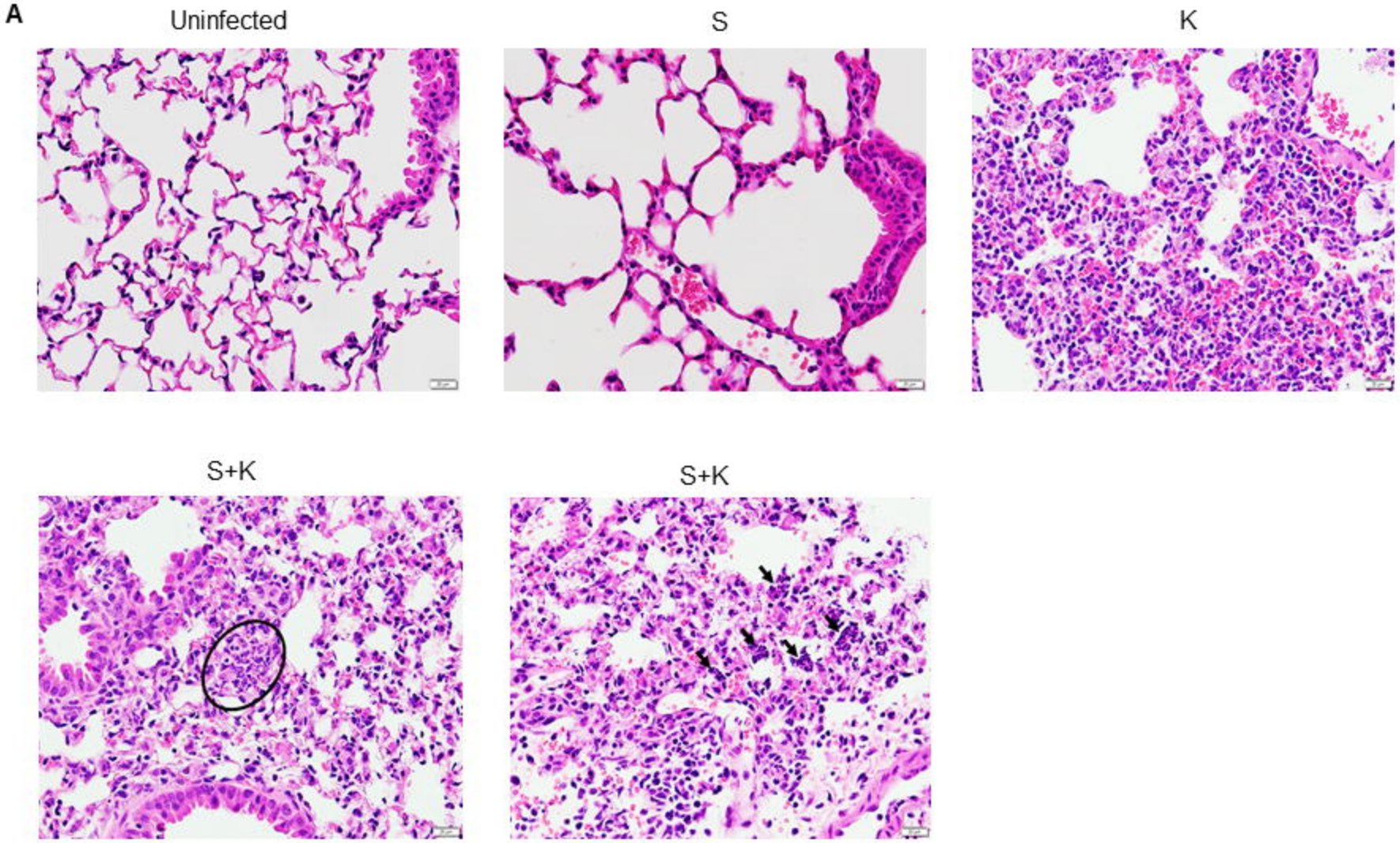

Fig 2

B

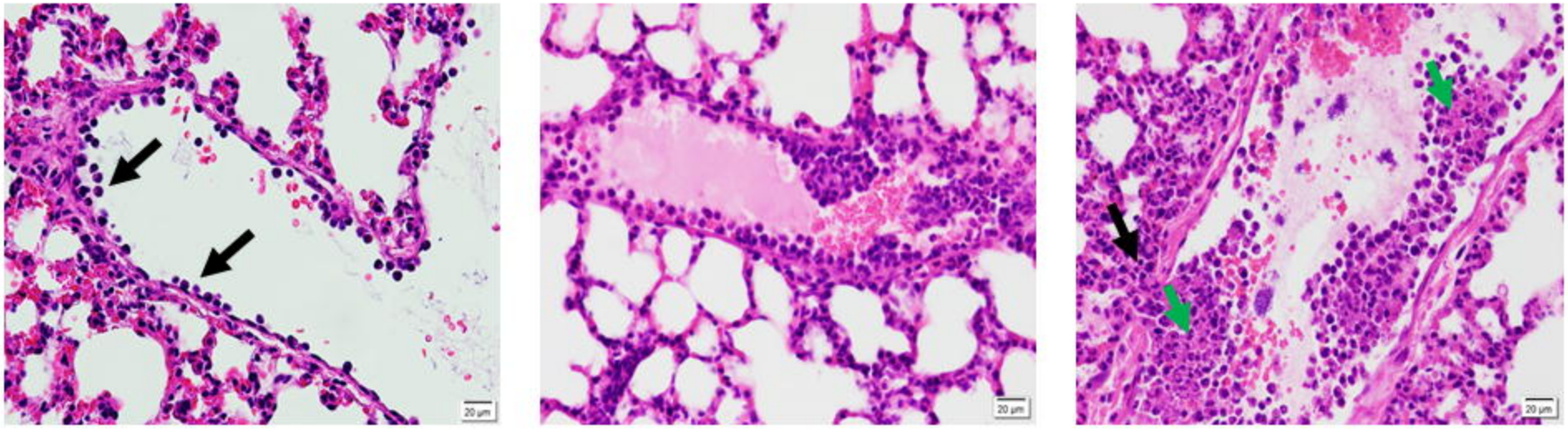

Fig 2: Mice with prior *S.* Typhimurium intestinal infection have increased lung inflammation from subsequent respiratory *K. pneumoniae* infection, characterized by microabcess, pyknotic neutrophils and margination, compared to those with no prior intestinal infection. (A) Representative images (400x magnification, scale bar = 20 μm) of lung sections stained with hematoxylin/eosin, normal lung in uninfected mice and in mice with intestinal infection (S). K mice show mixed interstitial inflammatory consolidations. Microabcess (circled) and clusters of pyknotic neutrophils (arrows) are apparent in mice with prior intestinal *S.* Typhimurium infection and challenged with *K. pneumoniae* infection (S+K).
(B) Representative images (H & E, 400x magnification, scale bar = 20 μm) of lung sections of S + K infected mice. Lung vessels in S + K infected mice showing neutrophil margination (left, black arrows), margination and clustering (middle), necrotic clusters (right, green arrow) and extravasation (right, black arrow). Data represents cumulative results of two independent experiments (n = 2 for UI, n = 10 for S group, n = 9 for K group and n = 8 for S+K group).

Fig 3

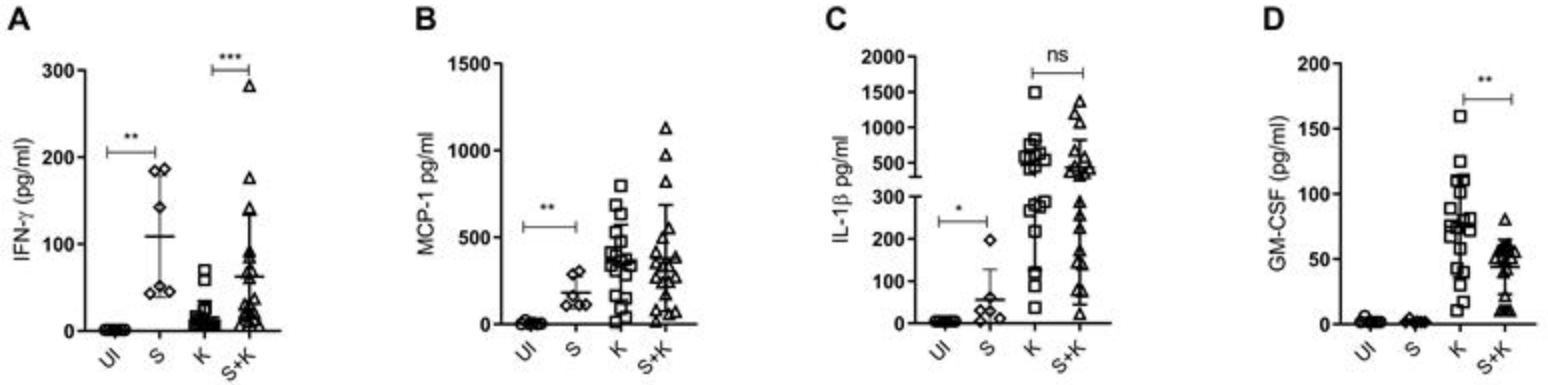

Fig 3: Mice with prior *S.* Typhimurium intestinal infection have altered lung cytokine profiles, and altered cytokine responses to respiratory *K. pneumoniae* infection, compared to those with no prior intestinal infection. Levels of lung inflammatory cytokines (A) IFN-γ, (B) MCP-1, (C) IL-1β and (D) GM-CSF were assessed 18 hours post *K. pneumoniae* challenge via bead-based LEGENDplex mouse inflammation panel 13-plex assay. Data represents one experiment for uninfected mice (n =6) and *S.* Typhimurium infected mice (n =6) and cumulative results of three independent experiments for K (n = 18) and S+K group (n = 19). Error bars represents mean ± SD and significance was determined by Mann-Whitney tests.

Fig 4

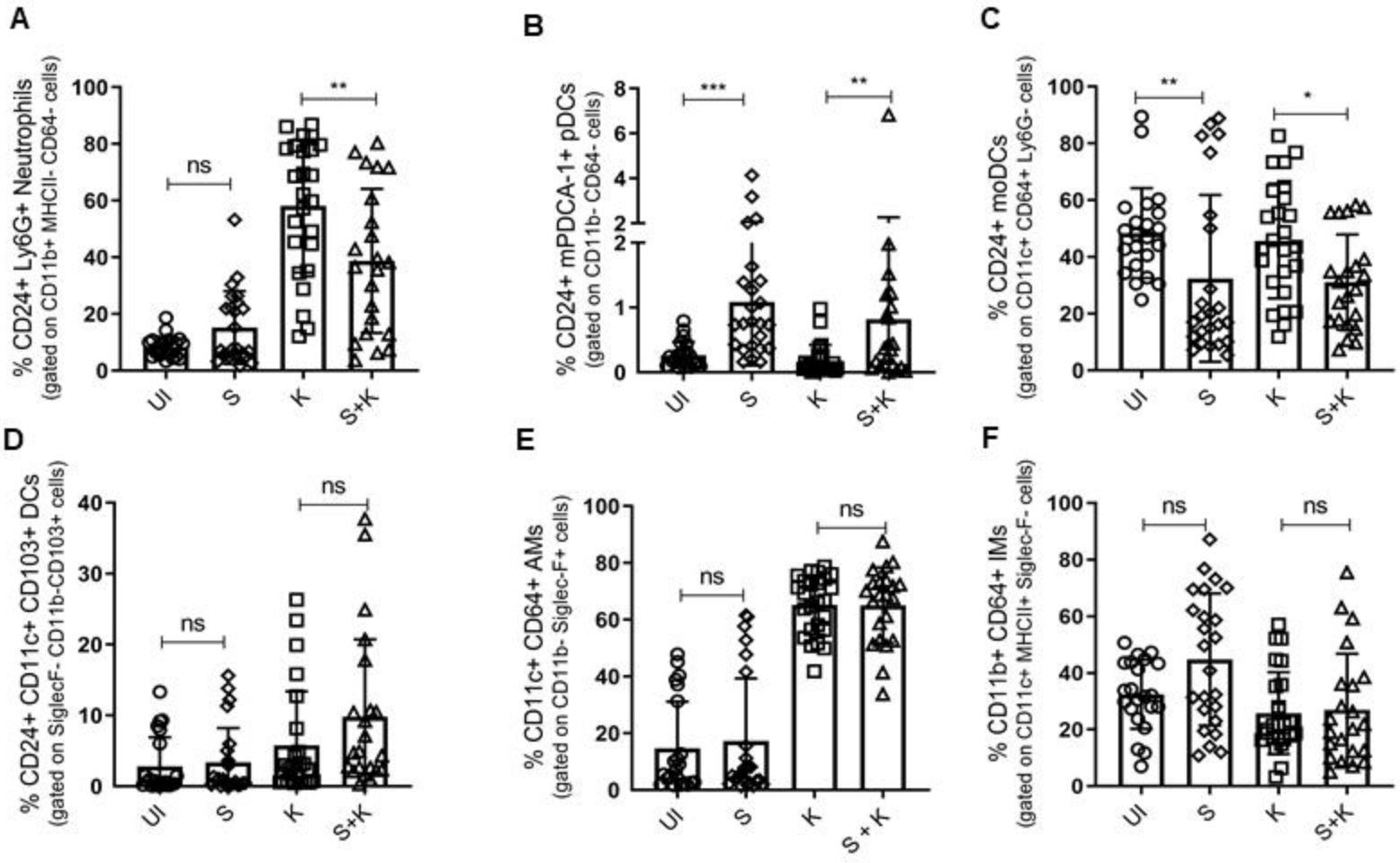

Fig 4: **Mice with prior *S.* Typhimurium intestinal infection have altered frequencies of innate cell types in the lung, and altered lung innate cellular responses to respiratory *K. pneumoniae* infection.** Using flow cytometry, percentage frequencies of (A) neutrophils, (B) lung plasmacytoid dendritic cells (pDCs), (C) monocytic dendritic cells (moDCs), (D) CD103+ DCs, (E) alveolar macrophages (AMs) and (F) interstitial macrophages (IMs) were evaluated between uninfected mice (UI), *S.* Typhimurium infected mice (S), *K. pneumoniae* infected mice (K) and both *S.* Typhimurium and *K. pneumoniae* infected mice (S+K). Data represents cumulative results of five independent experiments (n = 22 for UI, n = 24 for S, n = 25 for K and n = 22 for S+K). Error bars represents mean ± SD and statistical significance was determined by using Mann-Whitney tests.

Fig 5

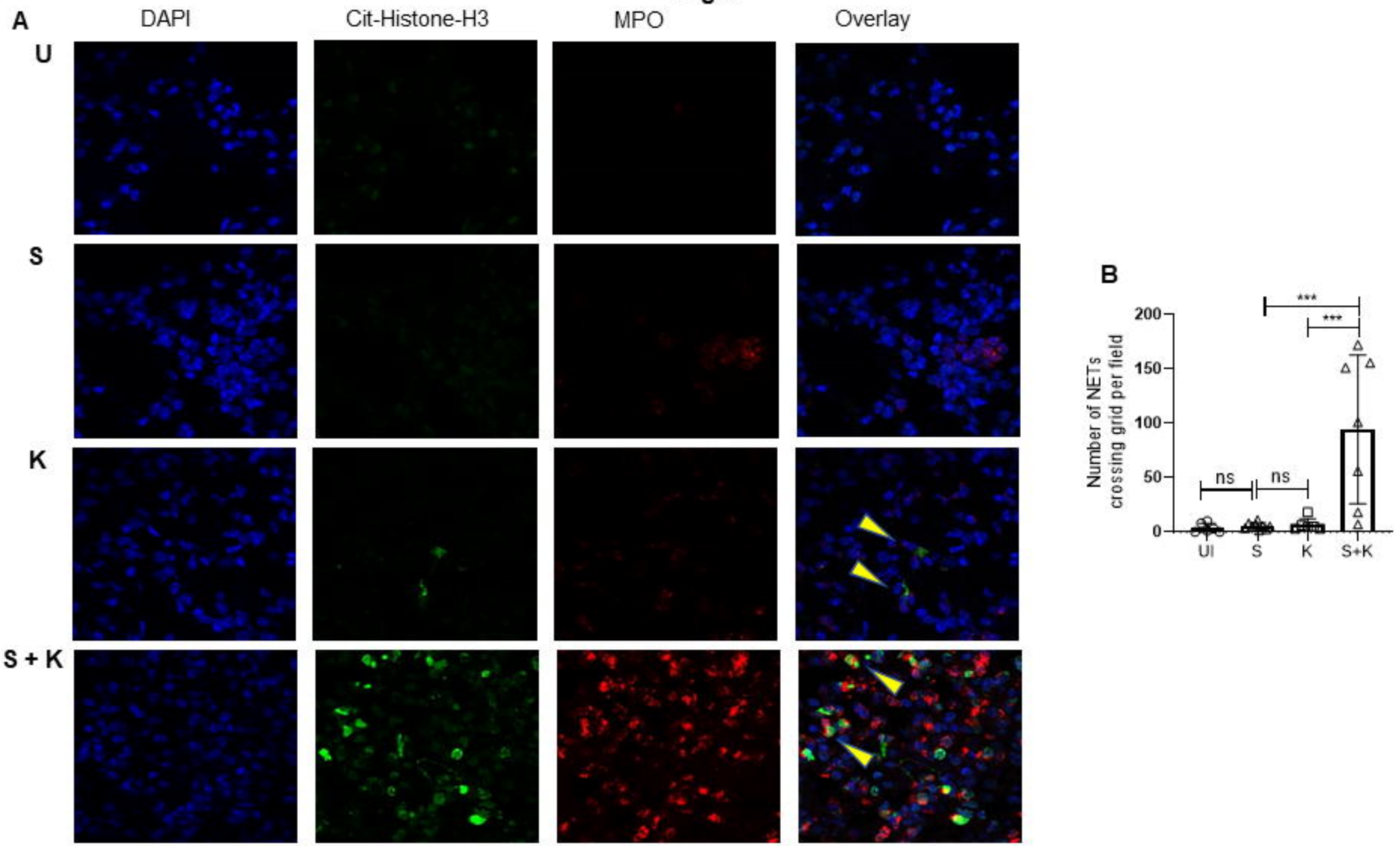

Fig 5: **Mice with prior *S.* Typhimurium intestinal infection have increased NETosis in lungs from subsequent respiratory *K. pneumoniae* infection, compared to those with no prior intestinal infection.** (A) Using Olympus FV3000 Confocal Laser Scanning Microscope , NET formation was assessed in all four groups and images were taken at 60X magnification. Blue fluorescence, nuclear DNA; green fluorescence, NET-associated citrullinated histone H3; red fluorescence, myeloperoxidase (MPO); NETs, yellow arrows in overlay. (B) Bar graph showing number of NETs crossing grid per field. One-way ANOVA with Tukey's post hoc testing. *** denotes $P < 0.001$.