Supplementary Methods

Animals

10-12 week old female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). PAFR deficient mice (C57BL/6 background) were a kind gift from Dr. Elaine Tuomanen (1) and IL-1α knockout (KO) mice (C57BL/6 background) were a kind gift from Dr. Yoichiro Iwakura (University of Tokyo) (2). Both strains were bred in house. Mice were housed under specific pathogen-free conditions with a light-dark cycle of 12 hours and *ad libitum* access to food and water. The Animal Research Ethics Board of McMaster University approved all experimental procedures.

Bacterial preparation

Stocks of *S. pneumoniae* P1547, a serotype 6A virulent clinical isolate (3, 4), were streaked on tryptic soy agar (BD Biosciences, Franklin Lakes, NJ) and supplemented with 5% sheep's blood (Cedarlane, Burlington, ON, Canada) and neomycin (Sigma-Aldrich, Oakville, ON, Canada) at 10μg/ml. Single colonies were picked and grown to log-phase in tryptic soy broth (BD Biosciences, Franklin Lakes, NJ) containing neomycin. Colony forming units (CFU) were predicted from the OD value at 600 nm based on a previously generated standard curve. The bacteria were washed 3 times with PBS, re-suspended, and diluted to approximately 10^6 CFU/10μl for nasal inoculation into mice.

Cigarette smoke exposure

Mice were exposed to the mainstream smoke from 12 3R4F reference cigarettes (Tobacco and Health Research Institute, University of Kentucky, Lexington, KY, United States), with filters removed, for 50 minutes, twice daily, at 5 days per week (unless otherwise stated) using a whole body cigarette smoke exposure system (SIU-48, Promech Lab AB (Vintrie, Sweden)) as reported previously (5). Prior to cigarette smoke exposure, mice were acclimatized to the restrainers for 20 minutes on the first day, 30 minutes on the second day, and 50 minutes on the third day. Control mice were exposed to room air only.

Nasal colonization

Mice were room air or cigarette smoke-exposed for one week prior to nasal bacterial colonization. Following cigarette smoke exposure on day 1 of the second week, mice were intranasally inoculated, in the absence of anaesthesia, with 10µl containing the indicated dose (CFU) of *S. pneumoniae*. Mice continued to be cigarette smoke-exposed post-nasal colonization without interruption. Mice were given HydroGel® and placed on heating pads following each cigarette smoke exposure for the first 3 days post bacterial inoculation to facilitate the establishment of nasal colonization.

Assessment of health status and endpoint monitoring

According to the Canadian Council on Animal Care Guidelines, the death of experimental animals is considered unethical. Therefore, mice were subjected to endpoint monitoring based on criteria pre-determined in our Animal Utilization Protocol. Briefly, animals were scored based on

appearance, behaviour, hydration status, and clinical signs, as reported in detail previously (6). Mice were considered at endpoint when presenting with a score higher than 9 or greater than 20% weight loss at two consecutive readings 4 hours apart. Mice reaching endpoint were sacrificed for sample collection for the indicated duration of the survival study. Surviving mice were sacrificed on the indicated end date of the study.

Determination of cellular inflammation and bacterial burden

Mice were sacrificed for the collection of the nasal wash and bronchoalveolar lavage (BAL), as well as the generation of lung, brain and spleen homogenates. Briefly, the trachea was cannulated and nasal wash collected from the nares by instilling 200µl PBS through the trachea. BAL was collected by instilling the left lung lobe with 250µl PBS, followed by 200µl PBS. Total cell numbers in the BAL and blood were determined using a haemocytometer. BAL cytospins and blood smears were generated and stained with Hema 3 (Biochemical Sciences, Swedesboro, NJ). 500 and 200 cells were counted per slide to determine the relative proportion of mononuclear cells and neutrophils in the BAL and blood, respectively.

The unlavaged multi lung lobes, brain, and spleen were homogenized in 1ml of PBS using a Polytron PT 2100 homogenizer (Kinematica, Switzerland) at 21,000-25,000 rpm for 3-6 seconds. Bacterial burden was quantified by plating the nasal wash, BAL, and homogenized tissue on tryptic soy agar supplemented with sheep's blood (5%) and neomycin (10µg/ml).

Pathology

Mice reaching endpoint were anesthetised by intraperitoneal injection with an overdose of pentobarbital. The chest cavity was opened to expose the heart and 50U of heparin was injected into the heart apex. Mice were perfused through the heart with lactated ringer's solution first, then with 10% formalin. The brain and heart were collected and fixed in formalin for at least 24 hours. In a separate experiment, the lungs were removed and inflated with 10% formalin at 30cm H_2O pressure. Tissues were paraffin embedded, sectioned, stained with H&E, and examined by a trained pathologist.

Gram stain

Gram stain was performed on heart microlesions based on the Brown and Hopps method. Briefly, sections were treated with crystal violet, decolorized so that the blue-violet stain from Gram-negative bacteria is removed, and counterstained with Fuchsin to dye Gram-negative bacteria red.

Serum cytokine measurement

TNF-α and IL-6 levels were determined in the serum by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN) as per the manufacturer's instructions.

Real time quantitative PCR

Nasal wash was performed with 500µl lysis Buffer RLT (Qiagen, Mississauga, ON, Canada) as previously described (7). Lung tissue was homogenized in lysis Buffer RLT. RNA was isolated using

RNeasy Mini Kit with optional DNase step (Qiagen). cDNA was synthesized with Superscript II Reverse Transcriptase (Invitrogen, Grand Island, NY) and TaqMan real-time RT-quantitative PCR (qPCR) was performed with the StepOnePlus™ Real-Time PCR System (Life Technologies Inc., Burlington, ON, Canada). *Tnfa*, *Il12p40*, *Cxcl1*, *Cxcl2*, *Ptafr*, and *Il1a* expression were determined using the ΔΔCt method. Target gene expression was normalized to the housekeeping gene GAPDH and expressed as fold change over the relevant control group.

Flow cytometry

Nasal wash samples were stained with fluorescent antibodies including CD45: Pacific Blue, Ly6G: PE, and CD64: APC (eBioscience, San Diego, CA). Cells were stained by incubation with fluorophore-conjugated antibodies after blocking with 2.4G2 antibody (eBioscience) at 4°C and assayed with a BD LSRII flow cytometer on the same day. Data were gathered with FACSDiva software (BD) and analyzed with FlowJo software (TreeStar).

Imaging

Room air or cigarette smoke-exposed mice were anesthetised by intraperitoneal injection with ketamine/xylazine and nasally inoculated with approximately 200μCi in 10μl of ^{99m}Technetium-diethylene triamine pentaacetic acid (^{99m}Tc-DTPA) (Lantheus Medical Imaging, Mississauga, ON, Canada) at a time point corresponding to day 3 post-nasal colonization. A dynamic 2D planar image was acquired for a 20 min period at a rate of one frame per 30 seconds using SPECT (X-SPECT system, GammaMedica-Ideas, Northridge, CA) to observe distribution of the radioligand. The ^{99m}Tc-DTPA images over the 20-minute period were analyzed using Amide medical image

analysis software (Free Software Foundation). A standard region of interest was positioned at the nasal region, and the amount of ^{99m}Tc-DTPA for each 30-second period was measured. The mean count from the nose region of interest was converted to percent of the highest count (obtained in the first frame) for each mouse every 30 seconds and was expressed as percent maximal activity. A whole body scan was acquired at 30 minutes, followed by a whole body CT. Standard regions of interest were positioned on the upper airways and whole body. The upper airway dose of ^{99m}TC-DTPA as a percentage of the whole body distribution was compared between room air and cigarette smoke-exposed mice.

Statistical analysis

Data are expressed as mean ± SEM. GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA) and SPSS Software (IBM, Armonk, NY) were utilized for statistical analyses. Levene's test for equality of variances was used to account for differences in data variability between groups. Independent t tests were used for comparisons between two groups. Log-rank test was used for comparison of Kaplan-Meier survival curves. Differences of $p < 0.05$ were considered statistically significant.

References

1. **Radin JN**, **Orihuela CJ**, **Murti G**, **Guglielmo C**, **Murray PJ**, **Tuomanen EI**. 2005. β-arrestin 1 participates in platelet-activating factor receptor-mediated endocytosis of Streptococcus pneumoniae. Infect Immun **73**:7827–7835.

2. **Horai R**, **Asano M**, **Sudo K**, **Kanuka H**, **Suzuki M**, **Nishihara M**, **Takahashi M**, **Iwakura Y**. 1998. Production of mice deficient in genes for interleukin (IL)-1alpha, IL-1beta, IL-1alpha/beta, and IL-1 receptor antagonist shows that IL-1beta is crucial in turpentine-induced fever development and glucocorticoid secretion. J Exp Med, 1998/06/06 ed. **187**:1463–1475.

3. **Nakamura S**, **Davis KM**, **Weiser JN**. Synergistic stimulation of type I interferons during influenza virus coinfection promotes Streptococcus pneumoniae colonization in mice. J Clin Invest, 2011/08/16 ed. **121**:3657–3665.

4. **Kim JO**, **Weiser JN**. 1998. Association of intrastrain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of Streptococcus pneumoniae. J Infect Dis, 1998/02/18 ed. **177**:368–377.

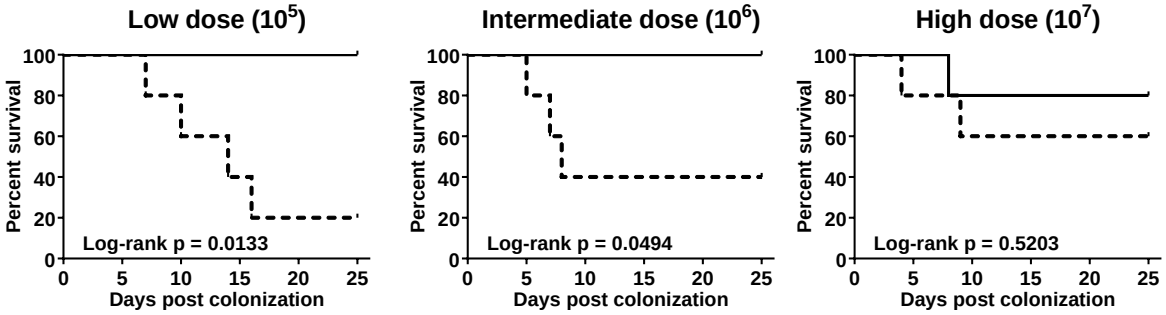
5. **Botelho FM**, **Gaschler GJ**, **Kianpour S**, **Zavitz CC**, **Trimble NJ**, **Nikota JK**, **Bauer CM**, **Stampfli MR**. Innate immune processes are sufficient for driving cigarette smoke-induced

inflammation in mice. Am J Respir Cell Mol Biol, 2009/06/09 ed. **42**:394–403.

6. **Drannik AG**, **Pouladi M a**, **Robbins CS**, **Goncharova SI**, **Kianpour S**, **Stämpfli MR**. 2004. Impact of cigarette smoke on clearance and inflammation after Pseudomonas aeruginosa infection. Am J Respir Crit Care Med **170**:1164–71.

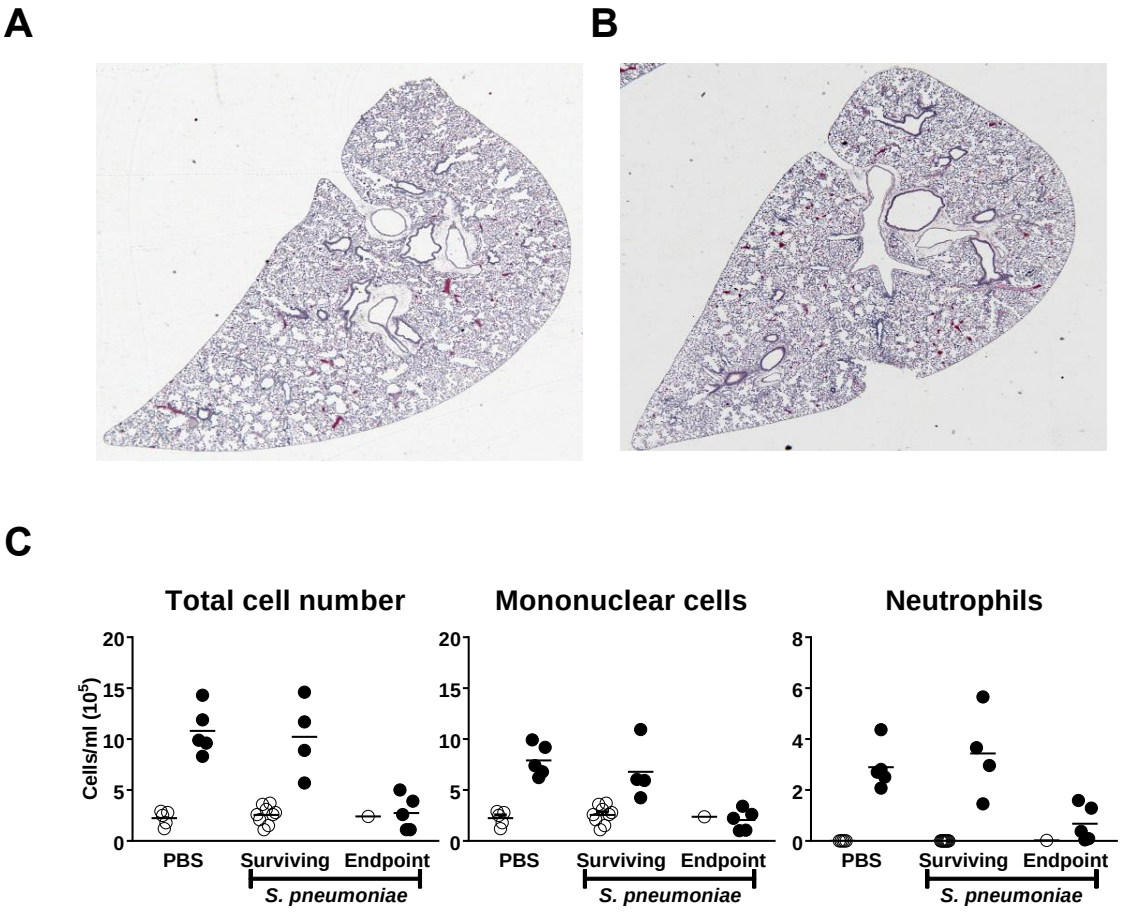
7. **Beisswenger C**, **Lysenko ES**, **Weiser JN**. 2009. Early bacterial colonization induces toll-like receptor-dependent transforming growth factor beta signaling in the epithelium. Infect Immun, 2009/03/04 ed. **77**:2212–2220.

Supplementary Figure 1



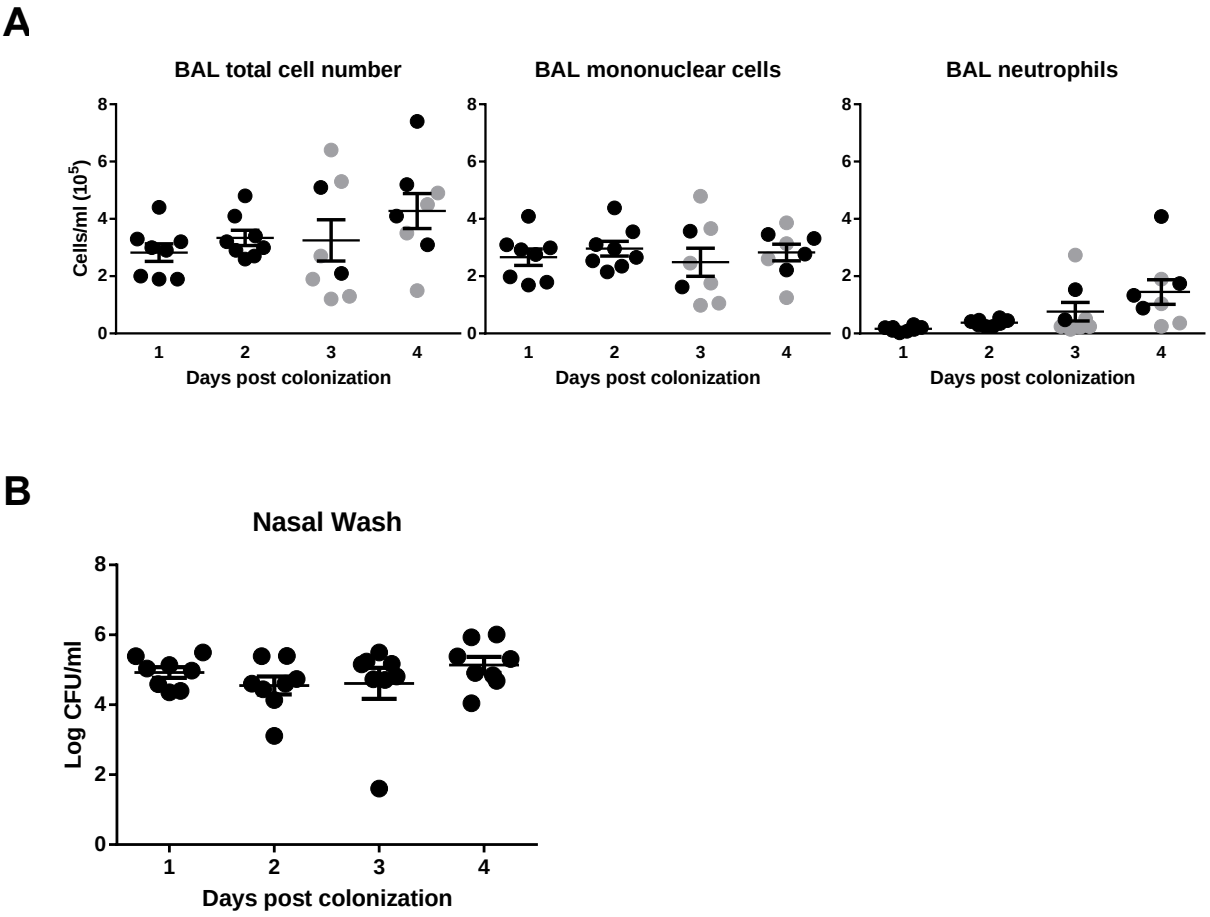
Supplementary Figure 1: Cigarette smoke exposure predisposed to mortality in mice following nasal pneumococcal colonization over a range of initial bacterial inoculation doses. Room air control or one week cigarette smoke-exposed mice were nasally colonized with the indicated dose of *S. pneumoniae*. Mice continued to be cigarette smoke-exposed for 25 days post-nasal colonization and euthanized as they reached endpoint. Survival curves are shown. Control (solid line), smoke (dotted line), n=5 mice per group. Survival curves were compared by log-rank test, $p<0.05$ was considered significant.

Supplementary Figure 2



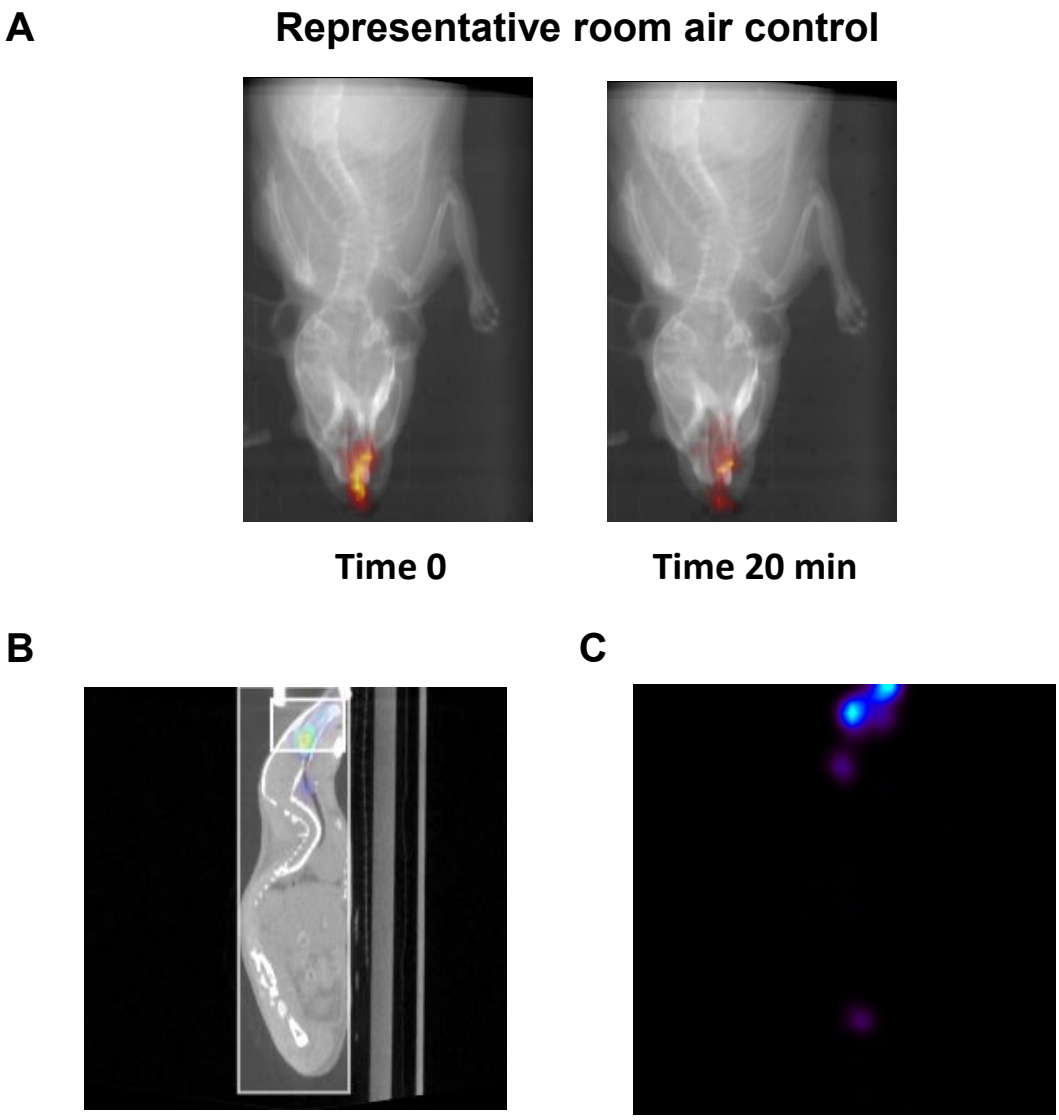
Supplementary Figure 2: Cigarette smoke-exposed mice at endpoint have no increased lung inflammation. Room air control or cigarette smoke-exposed mice were nasally colonized with 10^6 CFU *S. pneumoniae*. Mice continued to be cigarette smoke-exposed for 16 days post-nasal colonization and euthanized as they reached endpoint. The single lung lobe of (A) smoke-exposed mouse at endpoint and (B) smoke-exposed mouse surviving to day 16, were fixed with formalin, sectioned, and stained with H&E. Representative images are shown. (C) Differential cell counts of the BAL from surviving and endpoint mice are shown with that of vehicle control. n=5-10 per group of room air or cigarette smoke-exposed mice.

Supplementary Figure 3



Supplementary Figure 3: Cigarette smoke-exposed mice showed no changes in BAL inflammation or nasal bacterial burden over the course of 4 days despite widespread bacterial dissemination. Cigarette smoke-exposed mice were nasally colonized with 10^6 CFU *S. pneumoniae*. Mice continued to be cigarette smoke-exposed and sacrificed daily for 4 days for the determination of (A) BAL cellular inflammation and (B) nasal wash bacterial burden. Gray circles in (A) indicate cigarette smoke-exposed mice with bacterial burden detected in the BAL, while black circles represent mice with no BAL bacteria. Data are shown as mean ± SEM. n=8 smoke-exposed mice per time point.

Supplementary Figure 4



Supplementary Figure 4: Representative SPECT and CT images of mice following nasal inoculation with ^{99m}TC-DTPA. Room air or cigarette smoke-exposed mice were given ^{99m}TC-DTPA intranasally at a time point corresponding to day 3 post-nasal colonization. (A) Representative images of ^{99m}TC-DTPA activity in the nasal region immediately after inoculation and 20 minutes later. (B-C) A whole body scan was performed at 30 minutes post-^{99m}TC-DTPA administration, followed by a whole body CT scan. (B) CT scan of mouse outlining the regions of interest in the upper airways and whole body. (C) Observed radioactive signal in the whole body 30 minutes following nasal delivery of ^{99m}TC-DTPA. Mouse is in the same orientation as shown in (B).