Impaired Innate Mucosal Immunity in Aged Mice Permits Prolonged *Streptococcus pneumoniae* Colonization

Cassandra L. Krone, Krzysztof Trzciński, Tomasz Zborowski, Elisabeth A. M. Sanders, Debby Bogaert

Pediatric Immunology and Infectious Diseases, University Medical Center Utrecht, Utrecht, The Netherlands

Streptococcus pneumoniae is a frequent asymptomatic colonizer of the nasopharyngeal niche and only occasionally progresses toward infection. The burden of pneumococcal disease is particularly high in the elderly, and the mechanisms behind this increased susceptibility are poorly understood. Here we used a mouse model of pneumococcal carriage to study immunosenescence in the upper respiratory tract (URT). Nasal mucosa-associated lymphoid tissue (NALT) showed increased expression of Toll-like receptor 1, interleukin-1 β , NLRp3 inflammasome, and CCL2 in naive elderly compared to young animals. This suggests an increased proinflammatory expression profile in the NALT of aged mice at baseline. Simultaneously, we observed a more tolerogenic profile in respiratory epithelia of naive elderly compared to young adult mice with upregulation of the NF- κ B pathway inhibitor peroxisome proliferator-activated receptor gamma (PPAR γ). After nasal instillation of pneumococci, pneumococcal colonization was prolonged in elderly mice compared to in young adults. The delay in clearance was associated with absent or delayed upregulation of a proinflammatory mediator(s) in the NALT, diminished influx of macrophages into the URT niche, and absent downregulation of PPAR γ in respiratory epithelium, accompanied by diminished expression of cathelicidin (CRAMP) at the site of colonization. These findings suggest that unresponsiveness to pneumococcal challenge due to altered mucosal immune regulation is the key to increased susceptibility to disease in the elderly.

Streptococcus pneumoniae is a frequent but transient commensal of the human upper respiratory tract (URT) that may grow out and progress toward respiratory and invasive disease. Pneumococcal disease disproportionably affects the very young and the elderly; while immature immunity is thought to be responsible for increased susceptibility in young children, the factors behind the high susceptibility of the elderly are poorly understood. The increased risk of the elderly (defined as persons age 65 or older) for community-acquired pneumonia (CAP) is independent of health status and comorbidities (1). The microbial etiology of CAP is similar in all adults with *S. pneumoniae* as the primary bacterial cause (2). In high-income countries, despite access to high-quality health care, mortality due to CAP in the elderly still ranges from 15 to 30% and can double among residents of nursing homes (3–5). Continuous increases in life expectancy leading to a growing population of elderly people underline the public health importance of understanding the basis of this major health problem (5).

The increased risk of infectious diseases in the elderly has been attributed to age-associated deterioration of the immune system, called immunosenescence; however, the exact nature of these defects in immune responses has not been fully elucidated, and immunosenescence of the mucosal system of the URT in particular is still poorly explored (6). Hinojosa et al. (7) have recently shown an association of decreased protein levels of Toll-like receptor 1 (TLR1) and TLR2 in lung homogenates and increased levels of proinflammatory cytokines at baseline in elderly mice with higher mortality in a model of invasive pneumococcal pneumonia. These data suggest that there are innate defects in the lungs of elderly mice that likely contribute to increased risk and severity of pneumonia. Furthermore, TLR1 and -2 are crucial for clearance of pneumococcal colonization in mice, (8), but their contribution to immunosenescence in the URT is still unknown. Other groups have also sought to elucidate the mechanisms of immunosenescence of the innate compartment; however, most of these studies were performed *in vitro* (9–12) using primary cells known to

change phenotype *ex vivo* (e.g., macrophages) (13) and/or used antigens such as lipopolysaccharide (LPS) that are relevant for Gram-negative bacteria but not for Gram-positive bacteria such as *S. pneumoniae*. Furthermore, *in vivo* infection models (7, 14, 15) and *in vitro* work using alveolar macrophages (14, 15) are not informative regarding the prerequisite colonization state before pneumonia develops.

The innate cellular effectors mediating clearance of pneumococcal colonization in young adult mice have recently been described by Zhang et al. (16); the results indicate a major role for monocytes/macrophages in initiation and progression of URT clearance and provided further evidence that neutrophil influx alone is insufficient (17, 18). It has also been demonstrated that the inflammasome NLRp3 is activated by pneumococcal pneumolysin upon intranasal inoculation with *S. pneumoniae*, leading to secretion of interleukin-1 β (IL-1 β) by several cell types. Furthermore, NLRp3 is also required for bacterial clearance in a mouse pneumonia model (19). In addition, NOD2 and other pathogen-associated molecular pattern (PAMP) molecules synergizing with the inflammasome are crucial for IL-1 β processing and secretion (20). IL-1 β in turn can synergize with cathelicidin to induce the production of other cytokines such as CCL2 (21), which via NOD2 recognition of pneumococcal peptidoglycan has been identified as a key part of the recruitment of monocytes/macrophages to the URTs of mice and therefore crucial for pneu-

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Address correspondence to Debby Bogaert, d.bogaert@umcutrecht.nl.

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mucococcal clearance (22). All of these components correspond to a healthy immune response to *S. pneumoniae* colonization. We therefore hypothesize that one or more of these components might be affected in the elderly as part of immunosenescence, contributing to increased susceptibility to respiratory infections.

Since asymptomatic colonization of the URT is an important prerequisite to pneumococcal disease (23), we studied local innate immune responses in elderly mice for possible defects that might specifically impair clearance of *S. pneumoniae* from the upper respiratory niche. As yet, this is the only *in vivo* study to address immunosenescence of the URT related to clearance of a respiratory pathogen, and the information has the potential to further our understanding of this highly important, predisease carrier state in the elderly.

MATERIALS AND METHODS

Ethics statement and animals. Animal experiments were performed in accordance with the Dutch Animal Experimentation Act and European Union directives 86/609/CEE and 2010/63/EU related to the protection of vertebrate animals used for experimental or other scientific purposes. The experimental protocols were approved by the Committee on Animal Experiments of the University of Utrecht (DEC 2009.II.10.107, 2010.II.07.125, and 2011.II.11.175). C57BL/6 female specific-pathogen-free mice were purchased from Harlan (Venray, The Netherlands). For the purpose of this study 3- to 4-month-old mice were defined as young adult and 18- to 23-month-old mice as elderly. Animals with noticeable tumors were excluded from experiments, and animals with neoplasm detected in postmortem examination were excluded from analysis. Mice were euthanized with 120 mg/kg pentobarbital (Veterinary Department Pharmacy) administered intraperitoneally to induce respiratory arrest. Blood samples were taken via postmortem cardiac puncture.

Bacterial strains. *Streptococcus pneumoniae* strain 603 is a serotype 6B clinical isolate that has been previously used in murine models of pneumococcal colonization (24). This strain was mouse passaged, grown to mid-log phase in brain heart infusion (BHI) broth, aliquoted, and stored frozen in 10% glycerol at -80°C . Prior to animal inoculation, bacterial cells were thawed, washed twice with saline, and resuspended to 5×10^8 CFU/ml, and titers in cell suspensions were determined by plating 10-fold dilutions of inoculum on blood agar plates supplemented with gentamicin (SB7-Gent; Oxoid). An autolysin-negative variant of the unencapsulated *S. pneumoniae* strain Rx1 (Rx1 Δ ply) (24) and the unencapsulated variant of strain 603 (603 Δ cps) (25) were used for functional assays.

Mouse colonization experiments. Mice were inoculated intranasally, as described previously, with $10 \mu\text{l}$ of bacterial suspension containing approximately 5×10^6 CFU (24). In colonization experiments that followed, 5 to 10 mice were sacrificed per time point. From each animal $500 \mu\text{l}$ of retrotracheal nasal lavage fluid was collected with saline, followed by nasal tissue harvest. Tissue samples were crushed in 1 ml of phosphate-buffered saline (PBS), stored on ice, and processed within 3 h. Samples were vortexed vigorously and plated in serial dilutions onto SB7-Gent medium in order to quantify *S. pneumoniae* presence in the URT. Per mouse, CFU counts from the lavage fluid and nasal tissue were summed. Blood and lungs of all mice were cultured to check for the presence of *S. pneumoniae*.

Live sampling. Repeated live sampling was performed twice a week for four weeks following inoculation. Briefly, mice were restrained in a 50-ml conical tube with the end cut off, allowing access to the nose, and held inverted over a sterile petri dish. Ten-microliter volumes of ice-cold saline were applied to each nostril, and droplets sneezed out by the animal onto the dish were recovered by washing the plate with $500 \mu\text{l}$ saline. Samples were stored on ice and cultured on SB7-Gent plates to detect the presence of pneumococci.

Cell preparation. Upper respiratory tract phagocytes were collected from mice as previously described, with some modifications (16). Briefly,

a nasal lavage was performed with calcium- and magnesium-free PBS supplemented with 1% bovine serum albumin (PBS+BSA), collecting the first $500 \mu\text{l}$ of fluid exiting the nares. Sixty microliters of lavage fluid was used for pneumococcal CFU counts. The remaining fluid was centrifuged at 1,200 rpm ($\sim 300 \times g$) for 10 min at 4°C . Supernatant was removed and frozen at -80°C . The cell pellet was resuspended in $100 \mu\text{l}$ of PBS+BSA. Single-cell suspensions were obtained from nasal mucosa-associated lymphoid tissues (NALTs) or spleens as described previously (26). If necessary, red blood cells were lysed using ACK lysing buffer (BioWhittaker).

Histopathology. Craniums for histopathological examination of nasopharynxes were prepared as described previously (8) and processed at our Pathology Department. Craniums were embedded in paraffin and sectioned sagittally or cranially, and slides were stained either with hematoxylin and eosin (H&E) or by the Gram staining method. Images were taken with a Nikon Eclipse E800 microscope with a Nikon DXM1200 digital camera using the Nikon ACT-1 software version 2.70 (Nikon Netherlands, Lijnden, The Netherlands) at similar anatomical positions. Images were cropped using Adobe Photoshop CS3; NALT images were all cropped to the exact same size, i.e., $4 \text{ by } 6 \text{ in}$.

NALT quantification. Before sections were taken for quantification experiments, we took multiple series of coronal sections from 2 mice to determine the general shape of the NALT and the optimal distance from the eyes to take sections. Once this was determined, all sections were taken at the same distance from the eyes and several sections were taken to ensure reproducibility. To quantify morphological changes within the NALT in response to pneumococcal colonization, pictures of individual NALTs were taken under identical magnification and microscope settings. One or two mice per time point were used, each with a left and a right NALT that were treated as individual NALTs. Pictures were reviewed with FIJI open-source software (<http://fiji.sc/Fiji>). The area of the NALT was measured with FIJI and converted to a score on a scale from 1 to 10. FIJI area measurements ranged from $32,000 \mu\text{m}^2$ to $120,000 \mu\text{m}^2$; surface areas were coded by assigning 0.5 points to each $5,000 \mu\text{m}^2$ above $30,000 \mu\text{m}^2$ with a maximum of 9 points ($115,000$ to $120,000 \mu\text{m}^2$). To measure NALT cellular infiltration, pictures of individual NALTs were randomized and scored in a blinded manner by two independent observers using a 1-to-10 score scale. Naïve young-adult mouse NALTs, used as the control, were set at a score of 5 points for infiltration. Remaining pictures of NALTs were blinded and put in groups of visual comparable infiltration. These groups were ordered from least to most infiltrated and given a score from 1 to 10 using only whole numbers. Both area and infiltration scores were added per NALT, and the sums were compared with scores observed in naïve mice of corresponding age.

Flow cytometry. Nasal lavages were performed as described above, and samples were pooled from 2, 3, or 5 mice depending on cell counts. Cells were blocked with anti-mouse CD16/CD32 (Mouse Fc Block; BD Pharmingen). A $50\text{-}\mu\text{l}$ cocktail of fluorophore-conjugated antibodies recognizing different cell surface proteins was added and incubated in the dark for 30 min at 4°C , and $200 \mu\text{l}$ of PBS+BSA was added to terminate the reaction. Pellets were collected and washed again with $200 \mu\text{l}$ buffer. Pellets were resuspended in buffer plus 0.5% formalin and subjected in full volume to flow cytometry analysis on a BD FACSCantoII flow cytometer (BD Biosciences). FlowJo version 7.6.3 software (Tree Star, Inc.) was used to analyze flow cytometry data. GR-1^{high} CD11b⁺ F4/80⁺ cells were considered to be neutrophils, and GR-1^{+/−} CD11b⁺ F4/80⁺ cells were considered to be monocytes/macrophages.

Quantitative real-time RT-PCR. NALT dissected from mice (26) was instantly placed into 1 ml of RNA Later (Ambion), and RNA was extracted using the RNAqueous-4PCR kit (Ambion). Upper respiratory tract epithelium RNA was collected after URT retrotracheal lavage with PBS+BSA (as described above) by flushing airways with $500 \mu\text{l}$ lysis/binding buffer from the RNAqueous-4PCR kit (the method was adapted from that described in reference 8). URT samples were determined to be of epithelial origin by reverse transcription-PCR (RT-PCR) using primers specific for keratin 18. RNA quantity and quality for all samples were determined with

a NanoDrop spectrophotometer (260/280 = 1.8 to 2 and 260/30 above 2) and by conventional agarose gel electrophoresis. Mouse universal total RNA (Qiagen) was used as an internal control for PCR.

For NALT and epithelial cells, 1 µg of total RNA was reverse transcribed; for macrophage stimulation assays, 500 ng of total RNA was reverse transcribed using the GeneAmp RNA PCR cDNA synthesis kit (Applied Biosystems) applying random hexamers. Absence of genomic DNA in the samples was determined by omitting reverse transcriptase enzyme in the cDNA reaction and including these samples in the RT-PCR. cDNA was diluted 25× for NALT samples and 5× for epithelial samples, and 10.5 µl was used as the template in a 25-µl mixture with SYBR green PCR master mix (Applied Biosystems) and 0.5 µl primers at a concentration of 250 nM. Amplification was performed on a StepOnePlus (Applied Biosystems) machine under the following conditions: 95°C for 10 min, 95°C for 15 seconds, 60°C for 1 min, and a return to step 2 44 more times. Specificity of the PCR products was assessed by melt curve analysis and running reactions without templates. Amplification efficiency was not equal for all primers, so efficiency per amplicon group was used in determining the amount of transcripts using LinRegPCR software (www.hartfaalcentrum.nl) (27, 28). The transcript amount determined by LinRegPCR for 2 housekeeping genes for each sample was used for normalizing the data. The geometric mean of values for Ywhaz (29) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (8) was used for housekeeping normalization. Primers used are as follows: Krt18 (56), CCL2 (16), CRAMP (57), IL-1β (58), IRAK3 (59), NLRp3 (60), TLR1 forward (7), TLR2 (7); NOD2 forward, CCG CTT TCT ACT TGG CTG TC; NOD2 reverse, AGC CCT GGA TAC ACA GGT TG; PPARγ forward, GCC TGC GGA AGC CCT TTG GT; PPARγ reverse, AAG CCT GGG CGG TCT CCA CT; SIGIRR forward, AGT GTC GGC TGA ACA TGC TG; SIGIRR reverse, GAC ACG TAG GCA TCG TAT AAC TTC; TLR1 reverse, TAT CAG GAC CCT CAG CTT GG.

Neutrophil surface killing assay. Bone marrow cell isolation was performed as described in Boxio et al. (61) with slight modification. Collected bone marrow cells were resuspended in 2 ml PBS+BSA. This suspension was layered over 72%, 64%, and 52% Percoll solution in PBS+BSA in a 10-ml polystyrene tube and centrifuged without braking at 1,500 RPM (~480 × g) for 30 minutes at room temperature. The band between the 64% and 72% layers was collected; cells were washed and quantified by morphology using Cytospin. Opsonin-independent neutrophil killing was measured via surface phagocytosis assays (25, 26) with slight modifications for mouse cells. Briefly, neutrophils were resuspended at a concentration of 2.5×10^6 cells/ml in Hanks balanced salt solution (HBSS) plus 0.1% fetal calf serum (FCS). One hundred replicates of a 10-µl bacterial cell suspension containing on average 25 CFU of the 603 Δcps strain were plated on SB7-Gent plates and allowed to air dry on agar. Twenty microliters of neutrophil suspension was overlaid onto 50 spots of bacteria and allowed to air dry. Plates were incubated overnight at 37°C with 5% CO₂, after which colonies were counted in order to calculate the fraction of pneumococcal cells surviving killing by neutrophils compared to those on control plates without neutrophil overlays.

Macrophage functional assays. Macrophages were derived from the total bone marrow cell population according to previously described procedures (62). Cells were 98% F4/80⁺ and 97% CD11b⁺ by flow cytometry. Macrophages were frozen in liquid nitrogen in aliquots of 3×10^6 cells in fetal bovine serum (FBS) with 10% dimethyl sulfoxide (DMSO).

Mouse bone marrow-derived macrophages (BMDM) were tested for their ability to phagocytose and kill live pneumococci in an assay modified from that described by van Zoelen et al. (30). Briefly, macrophages were thawed and adjusted to a concentration of 2.5×10^6 cells/ml in Dulbecco modified Eagle medium (DMEM)—F-12 with GlutaMAX and 10% FBS. One hundred microliters of cells was added to 1 ml medium in a 12-well tissue culture plate. Cells were allowed to adhere overnight at 37°C with 5% CO₂. The assay was performed both with and without bacterial preincubation in autologous serum. For assays performed with serum, a mouse serum pool was made with sera from 2 to 6 naive mice, and aliquots

were frozen at -20°C. Young adult mouse serum was used with cells from young adult mice and elderly mouse serum for cells derived from elderly mice. Serum and bacteria were incubated on ice for 10 min prior to infection with final volumes of 50 µl serum and 100 µl of pneumococcal cell suspension per well. Adherent monolayers were infected with pneumococcal strain Rx1 Δply at a multiplicity of infection (MOI) of 50, centrifuged at 400 × g for 5 min, and placed at 37°C for 10 to 15 min to allow phagocytosis. After this time, wells were washed 3 times with ice-cold PBS to remove extracellular bacteria, and 1 ml of sterile water was added to half of the wells to lyse macrophages. Lysates were diluted in Dulbecco's PBS (DPBS) and plated onto SB7-Gent to determine the CFU of bacteria inside the macrophages at baseline. One milliliter of culture medium was added to the remaining wells, which were placed back at 37°C for 90 min, after which time medium was removed, cells were lysed, and bacteria were quantified as described above. Macrophage killing was determined based on the reduction in CFU after 90 min compared to CFU at baseline.

For stimulation assays, mouse BMDM cells were thawed, seeded onto 12-well plates as described above, allowed to adhere for 24 to 48 h, and stimulated for either 2.5 or 3.5 h with 100 µl of medium or pneumococcal whole-cell antigen (WCA). WCA was made from ethanol-killed cells of strain Rx1 Δply prepared as described by Malley et al. (24). RNA was harvested from macrophage monolayers (pool of 3 wells each) using the RNeasy RNeasy-4PCR kit (Ambion). Quantitative PCR (qPCR) was performed as described above.

Statistical analysis. Statistical analysis was performed using the graphing software GraphPad Prism version 5.0. Data were tested for normality, and nonparametric tests were used accordingly. The log rank test was used to compare survival-like data of duration of carriage. The paired *t* test was used to compare *in vitro* pneumococcal killing by phagocytes. Kruskal-Wallis tests or two-way analysis of variance (ANOVA) was used to compare group dynamics, and either Dunn's posttests or the Bonferroni correction was used to control for multiple comparisons. Planned single comparisons (e.g., naive young mice compared to naive elderly mice) were tested with Mann-Whitney U tests (with Bonferroni correction as needed).

RESULTS

Delayed clearance of pneumococcal colonization in elderly mice. To investigate whether aging is related to prolonged colonization of the mouse nasopharynx, we studied colonization through time in elderly and young adult mice using a live sampling method allowing continuous monitoring of an individual animal for the presence of *S. pneumoniae* in the URT. Young adult mice began to clear pneumococci by day 14 postinoculation, and by day 21 the presence of pneumococci was undetectable (Fig. 1A). In contrast, elderly mice did not begin to clear pneumococci until day 17, and 50% of the elderly animals remained colonized at the termination of the experiment on day 28. Elderly mice were colonized for a statistically longer period of time than young adult mice (log rank test, $P = 0.0025$), with a median time to clearance of 26 days, versus 19 days for young adult mice.

Next, we tested whether either a higher initial density of pneumococcal presence or a delay in *S. pneumoniae* clearance contributed to the extended carriage duration seen in elderly mice. Mice were intranasally inoculated with *S. pneumoniae*, and the density of pneumococcal colonization was assessed in URT lavage fluid and harvests of nasal tissue collected postmortem. As shown in Fig. 1B, the two groups of mice had equal densities of colonization at 3 days postinoculation. Young adult mice had a significant drop in density of colonization from day 7 on ($P < 0.002$ by the Mann-Whitney test), whereas elderly mice did not have a significant drop in pneumococcal density until day 21 ($P < 0.002$). Two out of 5 elderly mice, compared to none out of five young adult mice, were

FIG 1 Effect of age on duration and density of pneumococcal colonization. Elderly and young adult mice were colonized with a serotype 6B pneumococcal strain. (A) Mice ($n = 10$ each) were live sampled twice a week for 4 weeks. Mice were scored based on presence or absence of pneumococcus in the sample. The median time to clearance for the young adult mice was 19 days compared to 26 days for the elderly mice (log rank test; $P = 0.0025$). Also, 50% of the elderly mice remained colonized at 28 days. (B) Mice were sacrificed after 3, 7, 14, 21, or 28 days. Circles represent young adult mice, and squares represent elderly mice, solid lines are at medians, and the dotted line represents the lowest level of detection. Young adult mice show a gradual decline in colonization density over the first 2 weeks, while elderly mice maintain a stable density of colonization until clearance begins at around day 21. The differences in density between the young adult and elderly mice are statistically significant at all times except at days 3 and 28 postinoculation (Mann-Whitney test with Bonferroni correction, $P < 0.002$ [significant]). In young adult mice, the density of colonization is statistically significantly lower at days 7, 14, 21, and 28 compared to day 3. In elderly mice, the density of colonization is not statistically significantly lower until day 21 compared to day 3. The results represent 5 independent experiments with a combined n of 15 to 20 at each time point except day 28 ($n = 5$).

still colonized at 28 days postinoculation (Fig. 1B). The spread in colonization density within the elderly group could not be attributed or correlated to age (ranging from 18 to 23 months [data not shown]). The aim of our model was to establish asymptomatic colonization in elderly mice, and indeed, no mice showed symptoms of pneumonia, nor were pneumococci recovered from the blood or lungs of any mouse.

Cellular infiltration dynamics in the URT in response to pneumococcal colonization. Previous studies on innate immune

responses to pneumococcal colonization in murine models showed that a switch to a high proportion of monocytes/macrophages infiltrating into the URT at around day 7 was crucial for clearance of pneumococci from the airways (16). In concordance with those results, we observed in young adult mice a significant monocyte/macrophage influx at around day 7 (Fig. 2A) in two independent experiments (ANOVA with Bonferroni posttest, $P < 0.05$). However, in elderly mice the majority of cells infiltrating into the NALT were neutrophils until day 28, when eventually a substantial increase in the presence of monocytes/macrophages was detected, resulting in a significant difference in monocyte/macrophage ratio between young adult and elderly mice at day 7 (ANOVA with Bonferroni posttest, $P < 0.05$). Similar results were obtained by studying the morphology of cells obtained in pooled nasal lavage samples from 5 mice each that were stained with Diff-Quik after Cytospin preparation (data not shown).

NALT dynamics in response to colonization. Due to the apparent different dynamics of cell infiltration in the nasopharynx between young adult and elderly mice upon pneumococcal colonization, we evaluated NALT morphology in naive and colonized animals. As expected, histological examination of NALT showed predominantly lymphocytes with some visible macrophages/dendritic cells. There was a trend toward reduced NALT sizes and cellular density in naive elderly compared to naive young adult mice. After colonization, NALT size (area) and cell density reached their peaks at day 7 postchallenge in young adult mice (Fig. 3A to E), whereas elderly mice responded to pneumococcal colonization with little to no increase in NALT size or cell density until days 14 to 21 (Fig. 3A to E). NALT area and cellular infiltration were quantified by scoring from 1 to 10, and the combined scores are presented in Fig. 3F, clearly showing age-associated differences, although no significance was reached. In both groups, increases in area and infiltration correlated with pneumococcal colonization clearance.

Gene expression in the NALT in response to colonization. Having shown that in elderly mice the duration of colonization was prolonged and correlated with diminished macrophage influx and delayed NALT infiltration responses, we hypothesized that innate inflammatory mediators, including pattern recognition receptor (PRR) expression, would be aberrant already in naive elderly mice or that upregulation might be affected. We therefore studied mRNA expression in the NALTs of naive and colonized mice and focused on immune pathways known to be involved in clearance of pneumococci or other Gram-positive bacteria from the respiratory tract and potentially involved in immunosenescence. TLR1 (31), TLR2 (8, 18, 32), NOD2 (22), NLRp3 (19), IL-1 β (33), and CCL2 (16) have all been demonstrated to be crucial in the innate response to pneumococci. Furthermore, TLR1 (7), TLR2 (7, 34), IL-1 β (34), and macrophage colony-stimulating factor (M-CSF) (35) have been reported to be altered in elderly mouse models of candidiasis and streptococcal infections. At baseline we observed significantly higher expression levels for TLR1, NLRp3, and IL-1 β in naive elderly mice than in naive young adult mice but similar expression levels of TLR2 and NOD2 (Fig. 4). In young adult animals, we observed significant upregulation of TLR1, NLRp3, and IL-1 β at day 3 and of TLR2 and NOD2 at day 7, plus a strong trend of increased CCL2 expression following colonization (Fig. 4). However, in elderly mice no significant upregulation of expression in response to colonization

FIG 2 Age-related differences in phagocyte infiltration and function. (A) Elderly and young adult mice were colonized with serotype 6B and sampled postmortem. Depicted is the ratio of infiltrating macrophages to neutrophils quantified by flow cytometry (mean and range). For days 0, 3, 7, and 14, the results are a compilation from two independent experiments (10 animals per time point in total), and for days 21 and 28, the results are from a single experiment (5 animals per time point). Young mice show a switch in the proportion of the two cell types, i.e., an apparent monocyte influx directly following inoculation with a peak influx at day 7. This correlates with clearance of pneumococcal colonization (depicted in Fig. 1). Elderly mice, however, did not show a marked shift in cell type until day 28, when finally an influx of monocytes/macrophages was detected. *, ANOVA with Bonferroni posttest, $P < 0.05$. (B and C) Pneumococcal killing by phagocytes. Phagocytes were challenged with live, unencapsulated pneumococci. Shown is the percentage of pneumococci surviving for young adult and elderly mice for each assay. No defect in killing is found in elderly neutrophils (B). BMDM of elderly mice, however, kill on average 20% fewer pneumococci than those of young mice (paired t test, $P = 0.051$) (C). (D) Fold induction of CRAMP production by BMDM from young adult and elderly mice after stimulation with pneumococcal WCA for either 2.5 or 3.5 h. Expression in macrophages was measured with qRT-PCR. Macrophages from young adult mice showed an increase in CRAMP production after 2.5 and 3.5 h of stimulation, which was less apparent in macrophages from elderly mice (Student's t test for expression at 2.5 h, $P = 0.059$).

with *S. pneumoniae* was found, although there was a trend toward a delayed upregulation of NOD2 by day 14 (Fig. 4). In addition, we observed a negative correlation between IL-1 β induction (measured by fold change over baseline) and pneumococcal density at 3 days postcolonization and between CCL2 induction and pneumococcal colonization density at day 7 postinoculation (data not shown). There was no difference in expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) between naive and colonized young adult and elderly mice (data not shown).

Gene expression in URT epithelium in response to colonization. Mucosal surfaces are continually colonized by a wide variety of bacteria, and homeostasis in these areas is maintained via pathways unique to the epithelial surface (36, 37). Therefore, we additionally investigated possible age-associated differences in response to URT pneumococcal colonization by studying expression of signaling and effector molecules at the local mucosa. First we assessed expression of the IRAK3, PPAR γ , and SIGIRR

regulatory genes. Expression of IRAK3 and SIGIRR did not differ between age groups at baseline or following colonization (data not shown). For PPAR γ , however, we observed significantly higher expression at baseline and after colonization in elderly compared to young adult mice ($P < 0.0001$ by ANOVA) (Fig. 5). Moreover, we observed a trend toward downregulation of PPAR γ over the first 7 days postcolonization in young adult but not elderly mice. In epithelial cells, important downstream products inhibited by PPAR γ include antimicrobial peptides such as cathelicidin; higher levels of PPAR γ should result in lower levels of transcription of cathelicidin (21, 37). Indeed, we observed decreased expression of mouse cathelicidin CRAMP in the epithelia of elderly mice compared to young adult mice at all time points ($P = 0.001$ by ANOVA). Upon pneumococcal colonization, a strong trend toward CRAMP upregulation was observed in young adult but not elderly mice (Fig. 5). There were no statistical differences seen in gene expression of PRRs in URT epithelium between young adult and elderly mice; however, a trend was observed for young adult

FIG 3 Response of the nasal mucosa-associated lymphoid tissue (NALT) of young adult and elderly mice to pneumococcal colonization. (A to E) Coronal sections were taken of paraffin-embedded young adult (y) and elderly (e) mouse craniums and stained with H&E before inoculation (A) and at 3, 7, 14, and 21 days postinoculation (B to E) with pneumococcal serotype 6B. Pictures are representative of 1 or 2 mice per group per time (2 to 4 NALTs). A clear response to challenge was observed in all young adult mice, whereas more heterogeneity was observed in elderly mice, showing both responders and nonresponders. (F) NALT responses were quantified using a combined score for NALT size (area) and infiltration.

mice to be expressing more NOD2, TLR1, and TLR2 at baseline and more NOD2 after pneumococcal inoculation (data not shown).

Phagocyte function. Neutrophils and monocytes present in the URT are critical effector cells for pneumococcal colonization clearance in mice (16). We therefore studied age-related differences in their function. Since pneumococci downregulate the capsule at the mucosa (38), we used unencapsulated pneumococci for functional assays. Neutrophils isolated from bone marrow of el-

derly mice responded to pneumococcal stimulation at an equal speed and with an equal amount of reactive oxygen species (ROS) as neutrophils isolated from young adult mice (data not shown). Moreover, antibody-independent pneumococcal killing demonstrated by surface phagocytosis (26) did not differ between neutrophils from young adult and elderly mice (Fig. 2B). We also tested bacterial killing by bone marrow-derived macrophages (BMDM) and observed no differences in phagocytosis capacity (data not shown), though we observed an approximately 20%

FIG 4 Gene expression in the NALT in response to colonization with pneumococcal serotype 6B. Circles represent individual young adult mice and squares represent individual elderly mice; lines are at medians. The dynamics of expression are significantly different for TLR1 ($P < 0.0001$), TLR2 ($P = 0.004$), NOD2 ($P = 0.002$), NLRp3 ($P = 0.0002$), and IL-1 β ($P = 0.0001$) by the Kruskal-Wallis test. Individual planned comparisons were tested by the Mann-Whitney test, with a significant P value of <0.0036 according to the Bonferroni correction. Baseline expression levels of TLR1, NLRp3, and IL-1 β are significantly higher in elderly naive mice than in young adult naive mice ($P = 0.0002$, $P = 0.002$, and $P < 0.0001$). After pneumococcal inoculation, young adult mice significantly upregulate 5 genes (trend for CCL2 at day 3, $P = 0.054$), but elderly mice do not (trend for NOD2 at day 14, $P = 0.0425$). *, $P \leq 0.0036$; **, $P \leq 0.002$; ***, $P \leq 0.0002$. Raw expression data per mouse were normalized to two housekeeping genes, those for *Ywhaz* and *GAPDH*.

lower capacity of elderly BMDM than of young adult macrophages to kill pneumococci, which equaled a 1.5-fold-greater amount of bacteria surviving killing by elderly BMDM than of those surviving killing by young adult BMDM ($P = 0.051$) (Fig. 2C). Finally, we measured expression of CRAMP in BMDM stimulated with whole-cell antigen (WCA). BMDM from young adult mice clearly upregulated expression of CRAMP in response to pneumococcal stimulation at either time point, whereas there was

a delay in the response of BMDM from elderly mice (Fig. 2D). Induction of CRAMP expression was lower in elderly than in young adult macrophages ($P = 0.059$).

DISCUSSION

In this study, we showed an impaired innate mucosal immune response to *Streptococcus pneumoniae* colonization in the upper respiratory tracts of elderly mice, which was associated with pro-

FIG 5 Transcriptional regulation in the URT epithelium. There is a significant effect of age on transcription of the NF- κ B inhibitor PPAR γ (two-way ANOVA, $P < 0.0001$) and the murine antimicrobial peptide CRAMP (two-way ANOVA, $P = 0.0013$) at the URT epithelium. At all times tested, elderly mice had significantly higher levels of PPAR γ (Bonferroni posttest, $P < 0.001$), and at day 3 postinoculation, young adult mice expressed significantly more CRAMP (Bonferroni posttest, $P < 0.01$). There are also trends of decreased expression of PPAR γ and increased expression of CRAMP following pneumococcal colonization in young adult mice, which are absent in elderly mice. Raw expression data per mouse were normalized to two housekeeping genes, those for Ywhaz and GAPDH.

longed duration of carriage. Our data suggest that monocyte/macrophage influx is delayed in elderly mice. Moreover, macrophages from elderly mice were less bactericidal and produced less CRAMP than those from young adult mice both at baseline and in response to pneumococcal whole-cell antigen. Furthermore, CRAMP expression was significantly lower in the URTs of aged mice, which was in line with our *in vitro* data with BMDM. The dynamics of neutrophil influx and neutrophil function were similar between age groups. The local lymphoid tissue responses were aberrant in aged mice, with inflammation in the URT already present in naive elderly mice, specifically, high expression of TLR1, NLRp3, IL-1 β , and CCL2. In addition, elderly mice failed to upregulate TLR1, TLR2, NOD2, NLRp3, and IL-1 β in response to colonization. Baseline inflammation in aged mice and failure to upregulate innate response genes could impede signaling for monocyte/macrophage influx and thus explain delayed clearance. Lastly, we found evidence that mediators of tolerance to commensals in the URT were aberrantly expressed in elderly mice, with significant overexpression of PPAR γ that persisted throughout pneumococcal colonization.

There is growing evidence that infiltration of monocytes/macrophages into the URT is the key to innate pneumococcal clearance in mice (16), with neutrophils playing a secondary role (17, 18). Our observation of delayed monocyte/macrophage influx correlating with delayed clearance in elderly mice is in agreement with those earlier studies. Because the products of activated neutrophils can be chemotactic for monocytes/macrophages, we also tested the function of the effector cells after confirming that neutrophil influx by itself seemed adequate (39). We found no functional defects in neutrophils of elderly mice, but BMDM of elderly mice showed lower killing activity against *S. pneumoniae* and macrophages produced fewer transcripts of the mouse cathelicidin CRAMP, both at baseline and in response to pneumococcal antigen. CRAMP, a mouse analog of human cathelicidin, plays a significant role in mucosal clearance of bacteria and stimulation of the immune system (21) and was recently shown to be relevant for natural clearance of pneumococci (40). However, it has been shown that macrophages can change phenotype and function

when cultured *in vitro* (13) and that *ex vivo* adaptations can occur when monocytes from elderly mice are given sufficient quantities of cytokines for macrophage differentiation *in vitro* (41). Therefore, we continued our studies on innate immune function using the NALT and URT mucosal epithelia of mice directly.

The first step of innate immune responses is recognition of pathogens. TLR1 and -2 recognize cell wall-associated polysaccharide of pneumococcus (lipoteichoic acid) (31, 32), and NOD2 recognizes peptidoglycan (22); both types of recognition upregulate inflammatory components, leading to macrophage influx and pathogen clearance. Furthermore, it has been reported in a mouse model of invasive pneumonia that protein levels of TLR1 and -2 are significantly decreased in aged compared to young adult mice following infection (7). We investigated the expression of these PRRs in the URTs of aged mice before and during colonization and found no statistical differences between age groups both before and during colonization in the epithelium. However, we did observe differences in expression in the NALT. In contrast to studies using splenic (9) or peritoneal (12) macrophages, we observed significantly higher expression of TLR1 and a trend in TLR2 in elderly compared to young NALT. However, the failure of elderly mice to upregulate these genes in response to colonization is in line with previous studies showing aberrant responses of TLRs to infection (7). Delayed upregulation of NOD2 also could contribute to the delayed clearance of pneumococci in elderly mice, a finding unique to this study. Since factors other than recognition of danger signals are involved in monocyte/macrophage influx, we investigated expression of other genes downstream of PRRs involved in clearance of pneumococci (16, 19, 33). Again, increased baseline expression of inflammatory components such as inflammasome NLRp3, IL-1 β , and CCL2 was observed in elderly mice, with delayed or no upregulation of those factors upon colonization compared to a marked response in young adult mice. This supports our general observation of (relative) unresponsiveness of elderly mice to pneumococcal challenge. The question remains as to how the increased proinflammatory state at baseline is connected to the absence of or delay in response to pneumococcal challenge.

At mucosal sites, the local lymph nodes are not the only source of immune signaling; the epithelium is also involved in recognition of microbes, signaling, and some responses such as production of antimicrobial peptides (36, 37). Mucosal surfaces of the upper airways are colonized by a variety of bacteria, forming the normal flora of these sites; therefore, the epithelial layer must discriminate between symbiotic colonizers and true pathogens and maintain homeostasis (36). SIGIRR and IRAK3, which are negative regulators of mucosal homeostasis (36), are reported to be increased in elderly mouse models after a Gram-negative stimulus (10, 12), whereas PPAR γ is reported to be expressed similarly in elderly and young macrophages (12). In contrast to the studies performed *in vitro* with other stimuli, we found no differences in expression of SIGIRR or IRAK3 *in vivo* following pneumococcal colonization. Furthermore, we observed significantly higher expression of PPAR γ in elderly mice. On one hand, evidence exists that URT commensals specifically use PPAR γ to promote tolerance and homeostasis (42, 43), but on the other hand, there is also evidence that PPAR γ is controlled by genetic programming to promote longevity in mice (44), and genetics has also been cited as a possible cause for the observed phenomenon of background inflammation in the elderly (called “inflamm-aging”) (45). Thus, it is difficult to ascertain if this increased expression of PPAR γ is the result of lifetime host-pathogen interactions resulting in increased tolerance at the mucosal surface or if this might be the result of counterbalancing the chronic inflammatory mucosal status or even the result of specific genetic programming. However, overexpression of PPAR γ might, at least partially, explain the lack of signaling and upregulation of proinflammatory responses after pneumococcal danger signals are present, as we observed in the NALT.

We also tested a key downstream product of PPAR γ regulation, CRAMP (46). CRAMP expression was indeed significantly lower in aged mice, which was in line with our *in vitro* data with BMDM. Since the function of antimicrobial peptides such as CRAMP is not only to mediate killing of bacteria but also to act as direct chemoattractants to monocytes/macrophages (47), this supports our cytological findings of diminished monocyte/macrophage influx in the upper respiratory tracts of elderly mice following pneumococcal challenge.

Since a high colonization density is linked with pneumonia in humans, we investigated differences in density of colonization between young adult and aged mice (48). Recently Shivshankar et al. suggested that increased pneumococcal ligand expression in lung tissue plays a role in the severity of pneumococcal pneumonia in the elderly (14). However, if this mechanism were involved in pneumococcal colonization, it would affect colonization density in our model, but we did not observe any differences in density of pneumococcal colonization following intranasal challenge between age groups. In humans, the duration of pneumococcal carriage is also associated with incidence of pneumococcal disease (49, 50). Accelerating pneumococcal clearance is therefore also a goal of new vaccines (51); however, success of such prevention strategies in the elderly will require intact innate signaling, including epithelial responses, lymphoid tissue function, and phagocyte function. So far, there is no clear evidence of a longer duration of human carriage in the elderly compared to young adults, although this might be a consequence of scarce longitudinal colonization data for adults and the elderly or of the relatively low sensitivity of conventional detection methods (52–54).

Our data indicate that two major processes seem to be driving immunosenescence in aged mice. First, generalized inflammation that is reported by many to be present systemically in both elderly humans and mice, called “inflamm-aging” (41, 45, 55), is present in the URT as well. Our data support the hypothesis that this background inflammation might, at least partially, be responsible for the lack of recognition of danger signals and therefore the lack of upregulation of many important mediators necessary for pneumococcal colonization clearance (55). Increased duration of colonization is the same phenotype seen in studies where monocyte/macrophage populations are depleted (16), matching the absent/delayed influx of monocytes/macrophages in our elderly mouse model as well. Second, tolerance to commensal bacteria is an important part of human survival. It appears that mediators of tolerance at the URT mucosa, specifically PPAR γ , are indeed increased in aged mice, which will also contribute to absent or delayed upregulation of proinflammatory processes upon pneumococcal challenge. The impairments that we observe in the aged mice are relatively mild but are consistent within the required network of immune signaling, and therefore together they might add up to the eventual clear phenomenon of prolonged pneumococcal colonization in elderly mice. Despite being generally asymptomatic, a lack of clearance of potential pathogens from the URT may lead to increased susceptibility to disease in elderly individuals. Although the data we present here are correlative in nature, this is the first *in vivo* study to investigate age-related defects in the URT related to clearance of a potential pathogen. We focused on the innate responses to colonization for this first study; however, it seems highly relevant for future studies to also investigate adaptive immunity, especially the Th17 pathway in the URT, as activation of this pathway seems to be especially critical for pneumococcal colonization clearance. Further studies on colonization duration in relation to immunosenescence and susceptibility to respiratory disease in the elderly are warranted and necessary for deliberate preventive strategies.

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