Age-Associated Inflammation and Toll-Like Receptor Dysfunction Prime the Lungs for Pneumococcal Pneumonia

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Background. Aging is associated with increased inflammation and risk of community-acquired pneumonia. Streptococcus pneumoniae co-opts the nuclear factor κ B (NFκB)–regulated proteins polymeric immunoglobulin receptor (pIgR) and platelet-activating factor receptor (PAFr) to attach and invade cells. We sought to determine whether aging and chronic inflammation were associated with increased pIgR and PAFr levels in the lungs and increased susceptibility to S. pneumoniae infection.

Methods. Lung protein and messenger RNA levels were quantitated using Western blot and quantitative polymerase chain reaction. NFκB activation was measured by electrophoretic mobility shift assay. Cytokine levels were measured by cytometric bead analysis. To model chronic inflammation, mice were implanted with osmotic pumps that delivered tumor necrosis factor–α.

Results. Aged mice and those infused with tumor necrosis factor–α had increased levels of pIgR and PAFr in their lungs and were more susceptible to S. pneumoniae infection. During pneumonia, aged mice had reduced levels of pIgR and PAFr and less NFκB activation, despite greater bacterial burden. We determined that aged mice had decreased amounts of lung Toll-like receptors 1, 2, and 4 and reduced capacity to respond to S. pneumoniae with proinflammatory cytokine production.

Conclusions. Aged mice and, potentially, elderly humans are more susceptible to pneumonia because of a priming effect of chronic inflammation and Toll-like receptor dysfunction.

Streptococcus pneumoniae (pneumococcus) is the microorganism most frequently associated with community-acquired pneumonia (CAP), causing up to 58% of all cases [1]. It is also a leading cause of invasive bacterial disease (i.e., bacteremia, sepsis, and meningitis) [2–4]. In developed countries, despite aggressive vaccination policies, S. pneumoniae remains a major medical problem in the elderly population. In the United States, it is estimated that >130,000 elderly persons (age, ≥65 years) are affected by pneumococcal pneumonia annually [4–7]. Of importance, despite access to intensive medical care and appropriate antimicrobial therapy, the case-fatality rate among elderly persons is 15%–25% [2, 4, 7, 8]. Thus, elderly persons are at risk of severe pneumonia that frequently results in death.

Cellular inflammation is an integral component of the pneumococcal disease process. Pneumococcal cell wall and the toxin pneumolysin cause inflammation by binding to Toll-like receptors (TLR)–1 and –2 and TLR–4, respectively, on the surface of cells [9, 10]. Binding of these molecules initiates a cell-signaling cascade that activates the transcription factor nuclear factor κ B (NFκB). NFκB is a major regulator of innate immunity, and its activation results in the expression of genes encoding acute phase proteins, in the production and secretion of proinflammatory cytokines, and in increased surface expression of the proteins polymeric immunoglobulin receptor (pIgR) and platelet-activating factor receptor (PAFr), among others [11, 12]. S. pneumoniae attachment is mediated by the bacterial adhesin choline-binding protein A (CbpA) and the cell
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**METHODS**

**Invasive pneumococcal disease.** Young (age, 4–5 months) and aged (age, 19–20 months) Balb/cBy mice were obtained from the National Institute on Aging Aged Rodent Colony at Harlan Sprague Dawley (Indianapolis, IN). *S. pneumoniae* serotype 4 strain TIGR4 [26] was grown in Todd-Hewitt broth or on blood agar plates at 37°C in 5% carbon dioxide. Exponential phase cultures of TIGR4 were centrifuged, washed with sterile phosphate-buffered saline (PBS), and suspended in PBS at a final concentration of $5 \times 10^8$ colony-forming units (cfu) per mL. Mice were anesthetized with 2.5% vaporized isoflurane and 20 µL of the pneumococcal suspension instilled into the left nostril ($1 \times 10^7$ cfu). At the designated times, mice were sacrificed, and tissue samples were collected. Blood samples were obtained by heart puncture, and bacterial titers were determined by serial dilution and plating. Bacterial titers in the lungs were assessed per gram of homogenized tissue. For path-
Elevated levels of polymeric immunoglobulin receptor (pIgR) and platelet-activating factor receptor (PAFr) in the lungs of aged mice. Relative amounts of pIgR (A) and PAFr (B) in lung samples from young (open square) and aged (closed square) mice were determined by quantitative chemiluminescent analysis of Western blots. C, Protein loads were determined to be equal on the basis of subsequent blots for actin. Horizontal bars indicate the median value. Statistical analysis was performed using Student’s t test. Asterisks denote statistical significance (P < .05). Adj vol int, adjusted volume intensity.

Figure 2. Elevated levels of polymeric immunoglobulin receptor (pIgR) and platelet-activating factor receptor (PAFr) in the lungs of aged mice. Relative amounts of pIgR (A) and PAFr (B) in lung samples from young (open square) and aged (closed square) mice were determined by quantitative chemiluminescent analysis of Western blots. C, Protein loads were determined to be equal on the basis of subsequent blots for actin. Horizontal bars indicate the median value. Statistical analysis was performed using Student’s t test. Asterisks denote statistical significance (P < .05). Adj vol int, adjusted volume intensity.

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pneumoniae, pump mice were infected 4 days after surgery, and tissue samples were collected at the end of day 6. All experiments involving mice were performed using Institutional Animal Care and Use Committee–approved protocols.

**Measurement of activated NFkB.** Levels of NFkB activation in the lungs were determined using an electrophoretic mobility-shift assay [29]. Nuclear extracts were prepared from freshly excised lung sections with use of a Nuclear Extraction Kit (Chemicon). To determine the specificity of the electrophoretic mobility-shift assay for p65, excess unlabeled oligonucleotides were added to some samples. Samples were separated on 5% polyacrylamide gels with Tris-borate–EDTA running buffer, the gels were transferred onto Whatman paper, and the bound complexes were visualized and quantitated using a Molecular Dynamics Storm-840 Phosphorimager.

**Cytokine analysis of bronchoalveolar lavage fluid samples.** Mice were infected intratracheally with 100 μL of PBS containing heat-killed bacteria (equivalent to 1 × 10⁷ cfu), recombinant pneumolysin (1 μg/mL; a gift from Tim Mitchell), or purified pneumococcal cell wall (1 × 10⁶ cfu equivalents). Six hours later, mice were sacrificed, and bronchoalveolar lavage fluid samples were collected by lung lavage with 3.0 mL of PBS [30]. Cellular debris was removed from the bronchoalveolar lavage fluid by centrifugation at 14,000 g for 10 min, and the supernatants were aliquoted and frozen at −80°C. A cytometric bead array (BD CBA Mouse Inflammation Kit; BD Biosciences) was used to measure IL-6 and TNF-α levels in the samples.

Flow cytometry was performed using a FACSARia (BD Biosciences) cell sorter and analyzer. Pneumococcal cell wall was collected and purified by the method described by Tuomanen et al [31]. To ensure that the samples were endotoxin free, samples were tested for endotoxin with a Limulus test (Pyrosate; Associates of Cape Cod) immediately prior to their use. Samples were collected at 6 h after challenge to preclude measurement of levels of TNF-α and IL-6 produced by infiltrating neutrophils. Neutrophil influx has been shown to peak 6–12 h after intratracheal challenge with bacterial components [32, 33].

**Quantitative real-time polymerase chain reaction (qRT-PCR).** Total RNA was isolated from lung tissue with use of a Qiagen RNeasy Mini Kit. RNA was DNased using the Turbo DNA-free Kit (Ambion) and was quantitated using a NanoDrop Spectrophotometer (NanoDrop Technologies). The quality and purity of the RNA was confirmed by visual inspection on Tris-borate–EDTA 1.5% agarose gel. With use of a High-Capacity Reverse Transcription Kit (Applied Biosystems), 1 μg of RNA was converted into complementary DNA (cDNA). qRT-PCR was performed using a BioRad Chromo4 Real-Time PCR Detector (BioRad) with Sybr Green Master Mix (Applied Biosystems), 5–10 ng cDNA, and gene-specific primers at a concentration of 300 nmol/L each. A template control was included for each primer set to detect false-positive results arising from the amplification of primer-dimers. Primers were designed using Primer3 software and were checked for single gene amplification using BLAT [34, 35]. Primer sequences were as fol-
Figure 4. Levels of activated nuclear factor κ B (NFkB), polymeric immunoglobulin receptor (pIgR), and platelet-activating factor receptor (PAFr) are lower in aged mice during an infection than in young mice. Relative levels of pIgR (A), PAFr (B), actin (C), and activated NFkB (D) in young and aged mice 2 days after infection with 1 × 10^7 colony-forming units of Streptococcus pneumoniae. Protein levels were determined by quantitative immunoblot analyses, and activated NFkB levels were determined by electrophoretic mobility shift assay. Squares and diamonds indicate the individual protein expression level for each mouse tested. Horizontal bars indicate the median value. Statistical analysis was performed using Student’s t test. Asterisks denote statistical significance (\( *P < .05 \)). Adj vol int, adjusted volume intensity.

loows: glyceraldehyde 3-phosphate dehydrogenase (forward), 5′-CTCATGACCACAGTCCATGC; glyceraldehyde 3-phosphate dehydrogenase (reverse), 5′-CACATTGGGGGTAGGAACAC; PAFr (forward), 5′-AGCAGATGTTGGCTACCAAG; PAFr (reverse), 5′-TGCGCATGCTGTAAAACTTG; pIgR (forward), 5′-CCTCCTCCAGACACACAGCA; pIgR (reverse), 5′-CACATTGGGGGTAGGAACAC; TLR-1 (forward), 5′-GGCAGCAGGTGGAATTGTAT; TLR-1 (reverse), 5′-GGTGCCACAAGATCACCCTT; TLR-2 (forward), 5′-AAGAGGAAGCCCAAAGAAAAGC; TLR-2 (reverse), 5′-CATGCGATGCTGTAAAACTTG; TLR-4 (forward), 5′-GGCAGCAGGTGGAATTGTAT; and TLR-4 (reverse), 5′-AGGCCCCAGATTTGGTCT.

Repeat measures and statistical analyses. For each experiment, at least 2 groups of mice were tested, each with experimental procedures performed at least 1 week apart. Differences in mortality were examined for significance with use of the Kaplan-Meier log-rank test. For all other comparisons, Student’s t test was used.

RESULTS

Aged mice are more susceptible to S. pneumoniae infection and express elevated levels of pIgR and PAFr. Twenty-four hours after intranasal infection with S. pneumoniae, aged mice had >10,000-fold more bacteria in their lung and blood samples than did young mice (figure 1A). Pathological examination of lung sections demonstrated that aged mice had significantly greater lung damage, characterized by vascular congestion, alveolar edema, and infiltration of neutrophils and red blood cells (figure 1B). Increased bacterial blood titers corresponded with an increased rate of mortality among aged mice (figure 1C). Thirty-six hours after infection, all aged mice infected with S. pneumoniae had died; in contrast, all young mice remained alive, and >50% of the young mice survived the study. Therefore, aged mice, similar to elderly humans, were more susceptible to pneumococcal pneumonia than were their young counterparts.

Because aging is associated with chronic low-grade inflammation, we tested for age-dependent changes in the NFkB-regulated proteins pIgR and PAFr. Quantitative immunoblots using whole lung extracts determined that aged mice had 4.7-fold more pIgR and 5.8-fold more PAFr in their lungs than did young mice (figure 2). Although we observed no difference in the levels of pIgR messenger RNA (mRNA; among 5 aged mice and 6 young mice; \( P > .66 \)), using qRT-PCR, we determined that PAFr mRNA levels were increased 2.2-fold in aged mice (among 6 aged mice and 6 young mice; \( P = .04 \)). Therefore, aging was associated with increased lung transcript and protein for PAFr and only protein for pIgR.

Young mice infused with TNF-α express elevated levels of pIgR and PAFr. To test whether systemic low-grade inflammation was sufficient to alter lung pIgR and PAFr levels, osmotic pumps were implanted subcutaneously and delivered 10 ng/h of TNF-α or saline continuously. Figure 3A demonstrates consistent delivery of intact TNF-α by an osmotic pump for up to 6 days, which was the duration of our longest experiment. Mice with saline pumps (n = 4) had undetectable levels of TNF-α (<20 pg/mL) at day 5, whereas those receiving TNF-α (n = 6) had a mean serum value of 27 pg/mL (\( P = .008 \)).
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**Figure 5.** The amount of Toll-like receptors (TLRs) is decreased only in aged mice before and during infection. Relative levels of TLR-1, -2, and -4 proteins were measured in the lungs of healthy (A) and infected (B) young and aged mice 2 days after infection. Squares indicate the individual protein expression level for each mouse tested. Horizontal bars indicate the median value. Protein loads were confirmed to be equal by probing for actin (not shown). Statistical analysis was performed using Student’s *t* test. Asterisks denote statistical significance (*P* = .02). Adj. vol int, adjusted volume intensity.

Prolonged delivery of TNF-α increased lung pIgR and PAFr protein levels 2.4-fold and 1.9-fold, respectively, more than did delivery of saline (figure 3B–D). pIgR mRNA levels remained unchanged (*P* = .197); however, PAFr mRNA levels increased 1.5-fold (*P* = .02). Mice infused with TNF-α were determined to be more susceptible to pneumococcal pneumonia. Two days after challenge, mice infused with TNF-α had 60-fold more bacteria in their lungs and 10-fold more bacteria in their blood (*P* = .053), compared with saline control mice (figure 3E). Thus low-grade inflammation resulting from TNF-α infusion was sufficient to increase lung pIgR and PAFr protein levels and to increase susceptibility to pneumococcal pneumonia.

**pIgR and PAFr levels are lower in aged mice during infection.** Having shown that lung pIgR and PAFr levels were elevated in aged mice, we examined whether increased levels of these proteins were also present during pneumonia. Surprisingly, aged mice infected with *S. pneumoniae* had 40% lower pIgR levels and 42% lower PAFr protein levels than did the infected young mice (figure 4A–C). This reduction coincided with diminished levels of activated NFκB (figure 4D) and no differences in pIgR and PAFr mRNA levels between young and aged infected mice (among 5 young mice and 6 aged mice; for pIgR, *P* = .29; for PAFr, *P* = .08). These findings were unexpected, because aged mice had >10,000-fold more bacteria in their lungs at time of tissue collection (figure 1A), and we expected to observe significant increases in pIgR and PAFr protein levels, mRNA levels, and activated NFκB in aged mice, compared with young mice.

**Aging is associated with decreased TLR protein levels in the lungs.** To determine whether reduced TLR levels might be responsible for the anergic NFκB response, we examined TLR levels in the lungs of young and aged mice. Although no differences were observed between uninfected young and aged mice in mRNA levels of TLR-1, -2, and -4, we determined that aged mice had 52%, 22%, and 68% lower levels of these proteins, respectively, than did young mice (figure 5A). This reduction was also observed in infected aged mice, which had 16%, 28%, and 48% lower TLR-1, -2, and -4 levels, respectively, than did young mice (figure 5B). We subsequently determined that aged mice produced significantly less TNF-α and IL-6 when intratracheally challenged with heat-killed bacteria, recombinant pneumolysin, and purified pneumococcal cell wall (figure 6). Heat-killed bacteria was the least-inflammatory component tested, and age-dependent differences were observed only with regard to IL-6 level (aged mice had 16% lower IL-6 levels than did young mice). Recombinant pneumolysin was a potent TLR stimulus in young mice but not in aged mice. Aged mice produced 5% lower TNF-α levels and 13% lower IL-6 levels than did young mice. Purified cell wall gave similar results; aged mice produced 41% lower levels of TNF-α and 52% lower levels of IL-6 than did young mice. Collectively, these experiments demonstrate that aged mice respond to *S. pneumoniae* with a muted cytokine response. Combined with the previously observed age-dependent decrease in TLR protein levels, these results suggest that age-dependent TLR dysfunction oc-
Figure 6. Aged mice produce less tumor necrosis factor (TNF-α) and interleukin (IL)-6 when challenged with Streptococcus pneumoniae components. Levels of TNF-α and IL-6 in bronchoalveolar lavage fluid (BALF) samples from young and aged mice 6 h after intratracheal challenge with 100 µL of phosphate-buffered saline containing either 1 × 10^7 colony-forming unit (cfu) equivalents of whole heat-killed S. pneumoniae (A; among 4 young mice and 6 aged mice), purified pneumococcal cell wall (B; 1 × 10^9 cfu equivalents; among 7 young mice and 7 aged mice), or recombinant pneumolysin (C; 1 µg/mL; among 5 young mice and 5 aged mice). Statistical analysis was performed using Student’s t test. Asterisks denote statistical significance (P ≤ .05).

Figure 7. Illustration explaining how age-associated inflammation and Toll-like receptor (TLR) dysfunction increase the susceptibility of elderly persons to pneumococcal pneumonia. Underlying disease and chronic age-associated inflammation cause expression of the host proteins polymeric immunoglobulin receptor (pIgR) and platelet-activating factor receptor (PAFr) in the lungs that pneumococcus uses to attach to and invade lung cells. Once the infection is established, TLR dysfunction results in a muted immune response to the bacteria and to development of fulminate pneumonia, which is associated with a high mortality rate.

curs in the lungs and helps to explain the increased susceptibility of aged mice to pneumococcal infection.

**DISCUSSION**

In the elderly population, pneumococcal pneumonia is characterized by its rapid onset, severity, and high case-fatality rate [4–7]. Consistent with reports describing increased levels of activated NFκB in aged tissues [19–21], elevated levels of proinflammatory cytokines in serum and bronchoalveolar lavage fluid samples from healthy older persons [22, 23], and positive regulation of pIgR and PAFr by nuclear localized NFκB [11, 12], we determined that aged mice had increased levels of pIgR and PAFr in their lungs and, moreover, are more susceptible to infection with S. pneumoniae than are young mice. In agreement with findings by Yende et al [36] that have shown that elevated levels of TNF-α and IL-6 are sufficient to increase the risk for CAP, we determined that mice infused with TNF-α were more susceptible to S. pneumoniae than were mice that received saline. Our observation of increased pIgR and PAFr protein levels in these mice provides a molecular mechanism that helps to explain how aging and preinfection inflammation, such as that frequently observed in individuals with underlying morbidities (e.g., cardiovascular disease), increase susceptibility to CAP.

Because of the inflammatory nature of pneumococcal disease, we hypothesized that, during pneumonia, elevated levels of pIgR and PAFr would be present in the lungs of aged mice. Unexpectedly, we observed the opposite and determined that, despite considerably greater bacterial burden, aged mice had lower levels of pIgR and PAFr in the lungs and less NFκB activation. Of importance, young mice infused with TNF-α had normal pIgR and PAFr expression and production, as well as NFκB activation, during infection (data not shown), which suggests that this muted response was not the result of prolonged exposure to proinflammatory cytokines. TLR-1, -2, and -4 detect pneumococcal pathogen-associated molecular patterns and initiate a cell-signaling cascade that activates NFκB. Additional experiments determined that aged mice had significantly reduced protein levels of TLR-1, -2, and -4 in their lungs and produced less TNF-α and IL-6 when challenged with purified pneumococcal components. These observations help to explain the reduced levels of activated NFκB observed in infected aged mice. It is plausible that diminished TLR levels
would lead to reduced cell-signaling, activation of NFκB, and subsequent cytokine production. Reduced cytokine production would further diminish autocrine and paracrine NFκB activation through the TNF-α and IL-6 receptor pathways.

Multiple age-related immune defects have been reported that increase susceptibility to pneumonia, including reduced mucociliary clearance of airborne particles, diminished macrophage function and T-cell activation, and decreased antibody avidity [37–39]. Although age-dependent TLR dysfunction has already been described for peritoneal macrophages from aged C57/Bl6 mice and for peripheral monocytes from healthy elderly persons [40–42], this is, to our knowledge, the first report to indicate that age-dependent TLR dysfunction occurs in the lungs and that it may contribute toward susceptibility to pneumonia. Of interest, elderly persons with pneumonia often have disease presentation that would be considered to be atypical for mature adults. One or more of the 3 classic symptoms of pneumonia (cough, fever, dyspnea) are absent in >50% of elderly persons with pneumonia, and ~10% show no signs of infection, with the exception of confusion or delirium [43]. TLR dysfunction is a possible explanation for why these individuals fail to show overt symptoms of infection, such as fever. Of importance, age-related defects in TLR signaling may be occurring simultaneously with diminished TLR protein levels. Studies have shown a 50% reduction in the expression of protein kinases (e.g., p38 and JNK) and their phosphorylation in peritoneal macrophages isolated from aged mice, compared with young controls [41].

At this time, it is unclear which cells in the lungs experience age-dependent TLR dysfunction. Although published data have shown that macrophages experience age-dependent TLR dysfunction [40–42], the possibility of epithelial cell TLR dysfunction is supported by the fact that we observed these age-dependent changes with use of whole lung extracts, which are composed primarily of nonlymphoid cells. Which lung cells experience age-dependent TLR dysfunction is an important question that remains to be answered. Another important consideration is that changes in pIgR and TLR protein levels were not mirrored by changes in the corresponding mRNA levels. However, multiple studies have shown that the amount of mRNA detected does not always correlate with the observed protein levels [44, 45]. Possible explanations include regulation of protein production by posttranslational modification and age-dependent differences in protein degradation.

Multiple respiratory tract pathogens, including Haemophilus influenzae, Neisseria meningitidis, and Pseudomonas aeruginosa, express ChoP on their surface and use it to bind PAFr [46–48]. Increased lung expression of PAFr as a result of aging and/or chronic inflammation would facilitate the ability of these bacteria to cause respiratory disease. Conflicting reports exist regarding the ability of S. pneumoniae CbpA to attach to mouse pIgR [49, 50]; however, it is undisputed that CbpA binds to human pIgR. Increased levels of pIgR in the lungs of elderly persons would also most likely increase their susceptibility to pneumonia. Finally, age-dependent TLR-1, -2, and -4 defects would impair the ability of aged animals to detect a variety of microorganisms and delay activation of the immune response, again increasing susceptibility to pneumonia. It remains undetermined whether other TLRs are also negatively affected by age in the lungs.

At first glance, age-associated inflammation and TLR dysfunction appear to be paradoxical. However, age-associated inflammation occurs in uninfected aged animals and is a very different physiological condition than that during infection. In figure 7, we propose a model that suggests that age-associated inflammation primes the lungs for S. pneumoniae attachment and facilitates the establishment of lower respiratory tract infection. Subsequently, age-dependent TLR dysfunction inhibits detection of the bacteria and results in a delayed and/or muted immune response. Our finding that age-associated inflammation primes the lungs for infection through pIgR and PAFr, combined with the finding that aged mice experience lung TLR dysfunction, suggests that, in addition to the already described age-associated immune defects, conditions are favorable for the development of S. pneumoniae infection. Because these events occur before the innate immune system is fully engaged, these studies suggest that an infection can become well established before an adequate immune response is initiated.

References


