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Decreased NLRP3 Inflammasome Expression in Aged Lung may contribute to Increased Susceptibility to Secondary *Streptococcus pneumoniae* Infection

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Abstract

Post-viral pneumococcal pneumonia is a leading morbidity and mortality in older patients (> 65 years of age). The goal of our current study is to understand the impact of chronological aging on innate immune responses to a secondary, post viral infection with *Streptococcus pneumoniae*, a causative agent of bacterial pneumonia. Using aged murine models of infection, our findings demonstrate increased morbidity and mortality in aged mice within 48 hours post secondary *S. pneumoniae* infection. Increased susceptibility of aged mice was associated with decreased TLR1, TLR6, and TLR9 mRNA expression and diminished IL1 β mRNA expression. Examination of NLRP3 inflammasome expression illustrated decreased NLRP3 mRNA expression and decreased IL1 β production in aged lung in response to secondary *S. pneumoniae* infection.

1. Introduction

Secondary pneumococcal infections are a leading cause of community-acquired pneumonia, sepsis, and death in older patients (> 65 years of age), with *Streptococcus pneumoniae* being the most causative organism (Heron 2011; McBean and Hebert 2004; Sousa and others 2013). Previous studies have illustrated an increase in hospitalization rates for pneumonia, with significant increases occurring among older adults diagnosed with chronic cardiac disease, chronic pulmonary disease, or diabetes mellitus (Fry and others 2005). It is estimated that older patients, account for the most serious cases of pneumococcal infections with the majority of direct medical costs as well as the highest rate of hospitalizations, number of days hospitalized, emergency department visits, outpatient visits, and deaths (Heron 2011; Huang and others 2011).

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It has been well established that the process of chronological aging affects various components of the immune response, leading to impaired host defense, defective vaccine responses, and a significantly higher risk of elderly persons developing life-threatening bacterial infections (Miyashita and others 2012; Pawelec and others 2005; Weng 2006). Due to increased prevalence of comorbidities in older persons, impaired adaptive immune responses to vaccination, and the pervasiveness of antibiotic resistant bacterial strains, there is a pressing need to understand the molecular mechanisms that underlie these impairments and develop cutting-edge therapies that specifically target and amplify innate immune responses in older persons (Simonsen and others 2009).

The NLRP3 inflammasome is a multiprotein complex consisting of the nucleotide-binding domain leucine-rich repeat containing (NLR) family member NLRP3, the adaptor protein ASC, and the cysteine protease caspase 1 (Agostini and others 2004). The NLRP3 inflammasome can activate caspase 1 in response to cellular danger, resulting in the processing and secretion of proinflammatory cytokines IL1 β and IL18 (Kanneganti and others 2006; Mariathasan and others 2006; Martinon and Tschopp 2007). A diverse array of stimuli can activate the NLRP3 inflammasome including both pathogen-associated molecular patterns (PAMPs) and endogenous host-derived molecules indicative of cellular damage (Nakahira and others 2011; Petrilli and others 2007). Recent work has illustrated the importance of caspase 1 dependent responses, mediated by the NLRP3 inflammasome, in modulating innate immunity to *S. pneumoniae* (Fang and others 2014; Karmakar and others 2015; Koedel and others 2002; Mariathasan and others 2006; McNeela and others 2010; Mitchell and Mitchell 2010; Shoma and others 2008; Witzernath and others 2011).

Our previous findings demonstrate that the NLRP3 inflammasome is needed for protection and activation of the inflammasome is decreased and/or delayed in aged lung during influenza infection (Stout-Delgado and others 2012). To expand upon these findings, as detailed in the findings of our current study, we examined the impact of chronological aging on inflammasome expression and production of IL1 β in response to a secondary post influenza *S. pneumoniae* infection. Using murine models of infection, our findings demonstrate increased morbidity and mortality in aged mice within 48 hours post secondary *S. pneumoniae* infection relative to young mice. Increased susceptibility of aged mice was associated with decreased TLR1, TLR6, and TLR9 mRNA expression and diminished IL1 β mRNA expression compare to young controls. Examination of NLRP3 inflammasome expression illustrated decreased NLRP3 mRNA expression and decreased IL1 β production in aged lung in response to secondary *S. pneumoniae* infection when compare to young.

2. Materials and Methods

2.1. Mice

Young (2 months) and aged (19 months) male and female BALB/c mice were purchased from the NIA rodent facility (Charles River Laboratories). Upon receipt, mice were handled under identical husbandry conditions and fed certified commercial feed. Body weights were measured daily and mice were humanely euthanized if they lost more than 15% of their starting body weight. The IACUC at Weill Cornell Medical College approved the use of

animals in this study. No animals were used in the study if they had evidence of skin lesions, weight loss, or lymphadenopathy.

2.2. Pathogen propagation and culture

Influenza A virus (A/PR/8/1934(H1N1)) (PR8) was purchased from ATCC and grown in MDCK cells (ATCC, Manassas, VA) as previously described (Szretter and others 2006). *Streptococcus pneumoniae* (ATCC 6303, ATCC, Manassas, VA) was grown on 10% sheep blood agar plates (BD Biosciences, San Jose, CA) overnight or for 4–24 hours in brain heart infusion (BHI) broth (BD Biosciences). Colony forming units (CFU) were assessed by dilution of samples in BHI and titers were determined by colony counts X dilution.

2.3. In vivo procedures

As detailed in Figure 1A: Primary influenza viral infection (INF + PBS): All mice were anesthetized with isoflurane (5% for induction and 2% for maintenance) prior to intranasal instillation with 1×10^3 PFU of PR8 (50 μ L volume in PBS). On day 14-post influenza instillation, mice received an intranasal instillation of 50 μ L of PBS. Our previous work has illustrated that there are undetectable levels of influenza present in aged lung at day 14 post infection (Stout-Delgado and others 2012). Primary *S. pneumoniae* infection (PBS + *S. pne*): All mice were anesthetized with isoflurane (5% for induction and 2% for maintenance) prior to intranasal instillation with 1×10^3 CFU of *S. pneumoniae* (ATCC 6303) (50 μ L volume in PBS). Secondary *S. pneumoniae* infection (INF + *S. pne*): All mice were anesthetized with isoflurane (5% for induction and 2% for maintenance) prior to intranasal instillation with 1×10^3 PFU of PR8 (50 μ L volume in PBS). On day 14-post influenza instillation, mice received an intranasal instillation of 50 μ L of 1×10^3 CFU of *S. pneumoniae* (ATCC 6303) (50 μ L volume in PBS). As previously described, the following clinical scores were assigned: 0=normal, 1=slightly ruffled, 2=ruffled fur, 3= ruffled fur and inactive, 4=hunched/moribund, and 5= dead (Dimmock and Marriott 2006; Stout-Delgado and others 2012).

2.4. RNA purification and real time PCR

RNA was extracted from lung tissue at 24 hours post-secondary control (influenza + PBS) or *S. pneumoniae* (influenza + *S. pneumoniae*) infection using previously published methods (Stout-Delgado and others 2012). QuantiTect Primer Assays were used to assess gene expression (Qiagen). All reactions were performed in triplicate and an endogenous control was used to ensure experimental reproducibility. Relative levels of messenger RNA (mRNA) were calculated by the comparative cycle threshold method and either β -actin mRNA levels were used as the invariant control for each sample. Briefly, for these calculations, we used young or aged lung from influenza infected mice (day 14 post infection) at 24 hours post PBS instillation (as detailed in Figure 1A) as the calibrator. mRNA expression values in young and aged lung in response to secondary *S. pneumoniae* infection are relative to age-matched calibrator samples.

2.5. ELISA

Culture supernatants and lung homogenates were analyzed for IL1 β and TNF α production using ELISA kits purchased from eBioscience (San Diego, CA) per manufacturer's instructions. Changes in 450nm absorbance and correction at 570nm were assessed using GloMax Multi-Detection System (Promega, Fitchburg, WI). Results were analyzed using Graph Pad Prism software (San Diego, CA).

2.6. Statistical Analysis

Survival analysis between groups was calculated using the Mantel Cox test. Comparison of groups was performed using a two-tailed t-test, one-way or two-way ANOVA, when appropriate. Samples obtained were normally or approximately normally distributed. All samples were independent and contained the same sample size for analysis. Variances of the populations were equal. All data were analyzed using GraphPad Prism software. Statistical significance was considered by a p value < 0.05.

3. Results

3.1. Increased Morbidity and Mortality in Aged Mice in Response to Secondary *S. pneumoniae* Infection

It is well established that the elderly have increased morbidity and mortality to primary *Streptococcus pneumoniae* infections (de Cunto Brandileone and others 1998; Kurtti and others 1997). Given the prevalence for post-viral pneumonia in the elderly, our experiments were designed to examine the impact of aging on NLRP3 inflammasome expression in response to a secondary infection with *S. pneumoniae*. Based on our previous work, we chose to use *S. pneumoniae* ATCC 6303, a highly virulent type 3 strain of *S. pneumoniae* commonly associated with an increased relative risk of death in older persons (Martens and others 2004; Mitzel and others 2014). As illustrated in Figure 1A, young (2 months of age) and aged (19 months of age) adult mice were instilled with saline or influenza (PR8). Mice were monitored for the duration of influenza infection and on day 14, a time point in which influenza is no longer detectable in both young and aged lung, mice received an instillation of PBS or *S. pneumoniae* (Figure 1A) (Stout-Delgado and others 2012). When compared to young, in response to secondary *S. pneumoniae*, there was a significant increase in baseline and pathogen-associated morbidity in aged mice (Figure 1B: 0hr P=0.0005, 24hr P=0.0013, 48hr P=0.0073). In addition, when compared to young, there is a significant increase in weight loss (Figure 1C: P<0.0001) and mortality (Figure 1D: P<0.0001) in aged mice in response to secondary *S. pneumoniae* infection. In response to secondary *S. pneumoniae* infection, there were also significantly higher bacterial titers present in aged lung (Figure 1E, Young vs. aged: PBS + *S. pneumoniae*, P=0.0028 and INF + *S. pneumoniae*, P=0.0001).

3.2. TLR Signaling in Aged Lung in Response to Secondary *S. pneumoniae* Infection

To gain a better understanding of how the process of chronological aging might contribute to increased susceptibility of older persons to secondary *S. pneumoniae*, we first examined the impact of aging on the activation of pathogenic recognition receptors. Previous work has illustrated an important role for toll like receptors (TLR) in the innate immune response to *S.*

pneumoniae and dysregulated TLR signaling in aged lung contributes to increased susceptible to infection (Albiger and others 2007; Boyd and others 2012; Branger and others 2004; Hinojosa and others 2009; Knapp and others 2004; Nguyen and others 2015). We isolated lung from young and aged mice at 24 hours post secondary *S. pneumoniae* infection and examined TLR mRNA expression by real time PCR. Changes in mRNA expression were relative to PBS instilled age-matched lung at day 14 post influenza infection (Figure 2). When compared to young, we detected a significant decrease in TLR1, TLR6, and TLR9 mRNA expression in aged lung in response to secondary *S. pneumoniae* infection (Figure 2A: TLR1 P=0.0011, TLR6 P=0.0223, and TLR9 P=0.0041). Similar expression of TLR2, TLR4, and MYD88 mRNA expression was detected in both young and aged lung tissue (Figure 2A).

Previous work has illustrated that in response to streptococcal peptidoglycan NOD-like receptor (NLR), NOD2, plays an important role in the production of inflammatory mediators, such as TNF α , IL1 β , and IL6 (Chamaillard and others 2003; Davis and others 2011). Given the importance of the NOD1/NOD2 pathway on innate immune responsiveness to multiple pulmonary pathogens, we next examined if changes in NOD1, NOD2, or RIP2 mRNA expression might be associated with increased morbidity and mortality of aged mice to a secondary infection with *S. pneumoniae*. There was decreased upregulation of NOD1 and RIP2 mRNA expression in aged lung relative to young control (Figure 2B: NOD1 P=0.0201, RIP2 P=0.0455). When compared to young, there was a significant upregulation in NOD2 mRNA expression in aged lung in response to secondary infection with *S. pneumoniae* (Figure 2B: NOD2 P<0.0001). We next evaluated the impact of chronological aging on inflammatory cytokine expression in response to secondary infection with *S. pneumoniae*. Expression of IL6 and TNF α mRNA was augmented in both young and aged lung, with significantly higher levels TNF α expression being detected in aged lung relative to young control (Figure 2C: P=0.0089). While the increase in pro-IL18 mRNA expression in lung was similar, pro-IL1 β mRNA expression was significantly decreased in aged lung in response to secondary *S. pneumoniae* infection when compare to young (Figure 2C: P=0.0169). To expand these findings, we examined TNF α expression in lung homogenates collected from young and aged mice in response to secondary *S. pneumoniae* infection. When compared to young, there is a significant increase in TNF α production in aged lung in response to a secondary *S. pneumoniae* infection (Figure 2D: P=0.0284).

3.3. NLRP3 mRNA Expression in Response to Secondary *S. pneumoniae* Infection is diminished in Aged Lung

Based upon these findings, we next evaluated the impact of chronological aging on NLRP3 inflammasome expression during secondary *S. pneumoniae* infection. Despite similar pro-caspase 1 and ASC mRNA upregulation, there is a significant decrease in NLRP3 mRNA expression in aged lung in response to a secondary infection with *S. pneumoniae* when compared to young (Figure 3A: P=0.001). Given these findings, we next examined the impact of decreased NLRP3 mRNA expression on IL1 β production. At 24 hours post secondary *S. pneumoniae* infection, there was a similar detectable increase in IL1 β production in serum collected from both young and aged mice (Figure 3B). While IL1 β production was also detectable in lung homogenates, when compared to young, expression

in aged lung at 24 hours post secondary infection was markedly reduced (Figure 3C: $P < 0.05$).

4. Discussion

At present, very little is known regarding the role of the NLRP3 inflammasome in mediating innate immune responses to a secondary *S. pneumoniae* infection. The results of our current study demonstrate that there is increased morbidity and mortality of aged mice in response to secondary post-viral *S. pneumoniae* infection. In addition, there is an age-associated decrease in TLR1, TLR6, and TLR9 mRNA expression that may contribute to decreased pro-IL1 β expression, and diminished IL1 β production in aged lung in response to secondary *S. pneumoniae* infection.

While previous work has illustrated impaired innate immune responses in aged lung to primary influenza or *S. pneumoniae* infection, very little is known how the process of chronological aging contributes to increased susceptibility to secondary bacterial infections. Results of our current study illustrate that there is decreased TLR1, TLR6, and TLR9 mRNA expression in aged lung in response to secondary *S. pneumoniae* infection. It is possible that in agreement with previously published work, a chronic inflammatory environment contributes to changes in TLR expression and dysfunction (Hinojosa and others 2009). Despite similar levels of influenza clearance by day 14 post influenza, upregulation of TLR expression may remain significantly altered in aged lung and thereby contribute to dysregulated innate immune responses to secondary pathogenic stimuli (Stout-Delgado and others 2012). An alteration in the level of TLR expression and function can result in abnormal innate signaling and associated inflammatory responses. It is evident by the results of our current study that a previous viral infection can significantly impact the responsiveness of innate immune responses to the next infectious stimuli. Based upon our previous work, it is plausible that the increased length of influenza infection in aged lung might influence NLRP3 inflammasome responsiveness to secondary pathogenic stimuli (Stout-Delgado and others 2012).

Dysregulated regulation of proinflammatory cytokine production can result in detrimental innate immune signaling in aged lung, thereby contributing to increased morbidity and mortality in response to infectious stimuli. Results of our study illustrate that TNF α expression is significantly upregulated in aged lung in response to a secondary infection with *S. pneumoniae*. While essential for bacterial clearance, overly heightened expression of TNF α can contribute to increased neutrophil recruitment and activation. Excessive and sustained TNF α expression may also contribute to increased lung damage and downstream imbalances in cytokine responses. Future work to understand the impact of increased TNF α expression in aged lung on neutrophil activation and function in response to secondary pneumococcal infection will need to be performed.

Recent work, using a model of pneumococcal meningitis, has illustrated that the NLRP3 inflammasome can contribute to increased host pathology instead of pathogen protection and clearance (Hoegen and others 2011). Our recent work has illustrated that in response to heightened ROS and DNA damage in response to bleomycin sulfate instillation, aged

macrophages can contribute to harmful NLRP3-mediated inflammation and development of pulmonary fibrosis (Stout-Delgado and others 2016). Similarly, in the presence of high levels of free fatty acids, excessive NLRP3 activation can contribute to increased morbidity in response to *S. pneumoniae* (Moon and others 2016). NLRP3 inflammasome responses are tightly regulated and the balance between harmful and beneficial responses to secondary pathogenic stimuli has not been fully elucidated. Similar to other innate immune pathways, excessive inflammasome activation in response to a secondary infection with *S. pneumoniae* can be detrimental to the host, resulting in extensive tissue damage. It is plausible, that the threshold for early IL1 β production in response to a secondary pathogen may be significantly altered in aged lung. Future work will need to examine the impact of location, magnitude, the context of inflammasome activation, and the impact of chronological aging and primary influenza infection on these responses.

It is important to note that our study investigated a highly virulent serotype 3 strain of *S. pneumoniae* that has been shown to have a higher incidence of occurrence in older adults. Given the divergent nature and secretion of pneumolysin by different *S. pneumoniae* strains, it will be of great interest to investigate if specific changes in TLR mRNA expression post influenza infection are conserved in response to multiple *S. pneumoniae* serotypes. These avenues of discovery may aid in the development of targeted therapeutics that can improve innate antibacterial responses against multiple pathogens.

The usage of antibiotics is essential for the management of pneumococcal infections. A systematic review of twenty antibiotic therapy studies illustrated antibiotic treatment initiated within four to eight hours of hospital arrival resulted in improved survival (Lee and others 2016). Results from our current study illustrate pneumococcal disease severity in aged is rapid and is associated with enhanced bacterial growth in lung. Given these findings, future studies to understand the impact of antibacterial therapies on post viral pneumococcal disease progression in aged lung will need to be performed.

It is important to note that gene expression patterns detailed in the results of our current study are representative of whole lung tissue and thereby, reflect a heterogeneous cell population present in the lung in response to a secondary *S. pneumoniae* infection. Neutrophil recruitment and activation in response to macrophage and epithelial cell cytokine and chemokine production is essential to the control of bacterial replication. The results of our current study illustrate decreased inflammasome gene expression and diminished IL1 β production in aged lung in response to a secondary bacterial infection. As resident and infiltrating macrophages play an important role in the lung's innate host defense against *S. pneumoniae* infection, future work will be pursued to examine the impact of primary influenza infection on cellular migration to lung and the role of chronological age of these frequencies.

In sum, findings of our current study demonstrate a potential mechanism by which an age associated decrease in NLRP3 inflammasome expression and IL1 β production contributes to increased morbidity and mortality in response to secondary pneumococcal infection.

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Abbreviations

S. pne	Streptococcus pneumoniae
NLRP3	NACHT LRR, and PYD domains-containing protein 3
ASC	apoptosis-associated speck-like protein containing a CARD
NOD	nucleotide-binding oligomerization domain-containing protein
RIP2	receptor-interacting serine/threonine-protein kinase 2
TLR	toll like receptor

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Highlights

- Our current study was designed to understand the impact of chronological aging on innate immune responses to a secondary, post influenza infection with *Streptococcus pneumoniae*, a causative agent of bacterial pneumonia.
- Increased susceptibility of aged mice to a secondary *S. pneumoniae* infection was associated with decreased TLR1, TLR6, and TLR9 mRNA expression and diminished IL1 β mRNA expression.
- Examination of NLRP3 inflammasome expression illustrated decreased NLRP3 mRNA expression and decreased IL1 β production in aged lung in response to secondary *S. pneumoniae* infection.

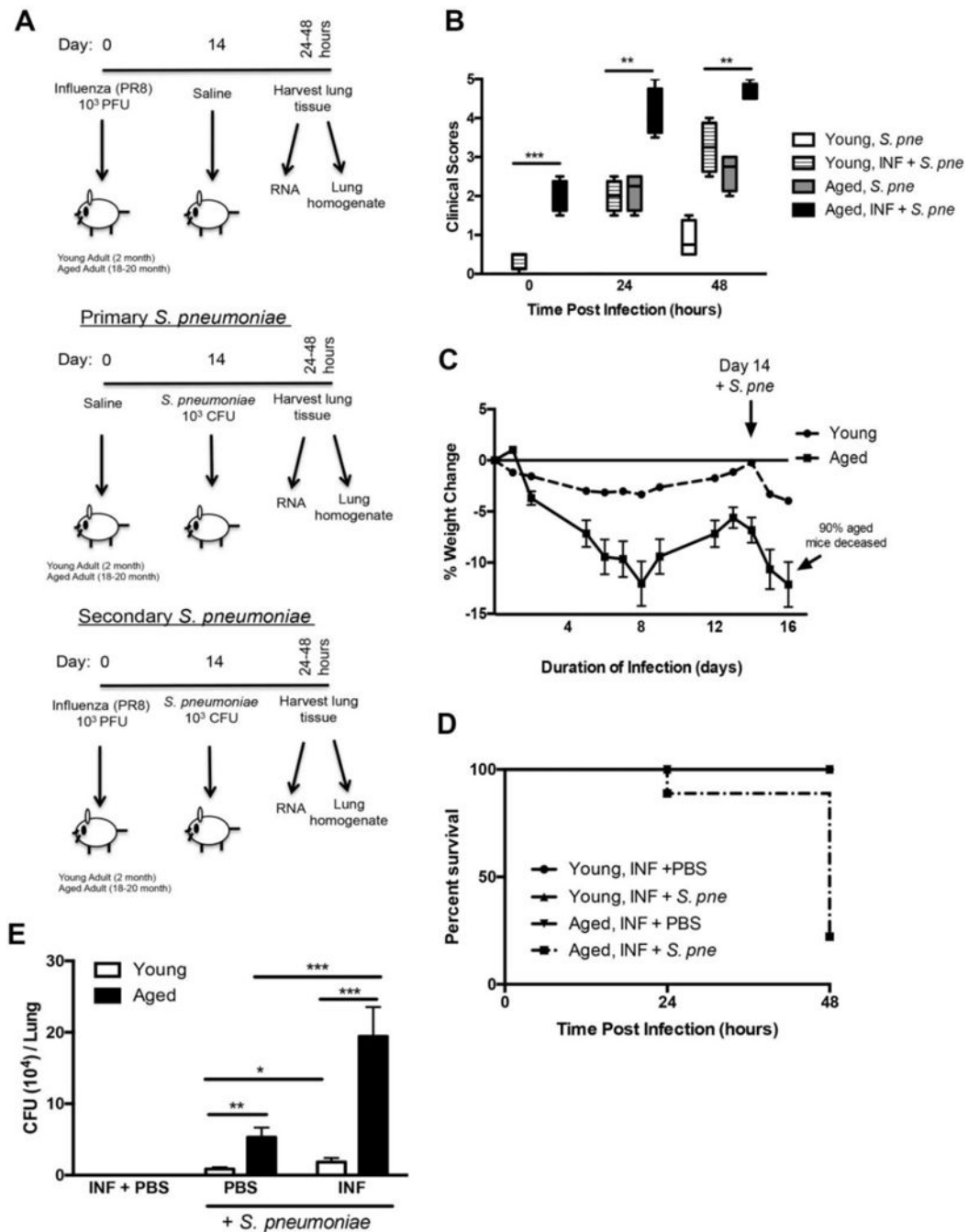


Figure 1. Increased Morbidity and Mortality of Aged Mice in Response to Secondary *S. pneumoniae* Infection

(A) Experimental layout of primary influenza (day 0 instillation with 10³ PFU influenza A/PR/8/34 H1N1 (PR8) followed by day 14 instillation with PBS), primary *S. pneumoniae* (day 0 instillation with PBS followed by day 14 instillation with 10³ CFU of ATCC 6303), and secondary *S. pneumoniae* (day 0 instillation with PR8 10³ PFU followed by day 14 instillation with 10³ CFU of ATCC 6303) infection in young (2 months) and aged (19 months) adult male and female BALB/c mice. (B) Clinical scores (0hr: P=0.0005, 24hr: P=0.0013, and 48hr: P=0.0073), (C) weight changed (P<0.0001), and (D) survival

($P=0.0004$) in young and aged mice in response to secondary *S. pneumoniae* infection. (E) Bacterial titer in lung was assessed in lung homogenates collected at 24 hours post infection (primary *S. pneumoniae* (PBS + *S. pne*): $P=0.0028$, secondary *S. pneumoniae* (INF + *S. pne*): $P=0.0001$, young primary vs. secondary *S. pneumoniae*: $P=0.0461$, and aged primary vs. secondary *S. pneumoniae*: $P=0.0006$). Similar results were obtained from at least two or more independent experiments with an $N=5$ per experiment. Data are expressed as the mean \pm SEM.

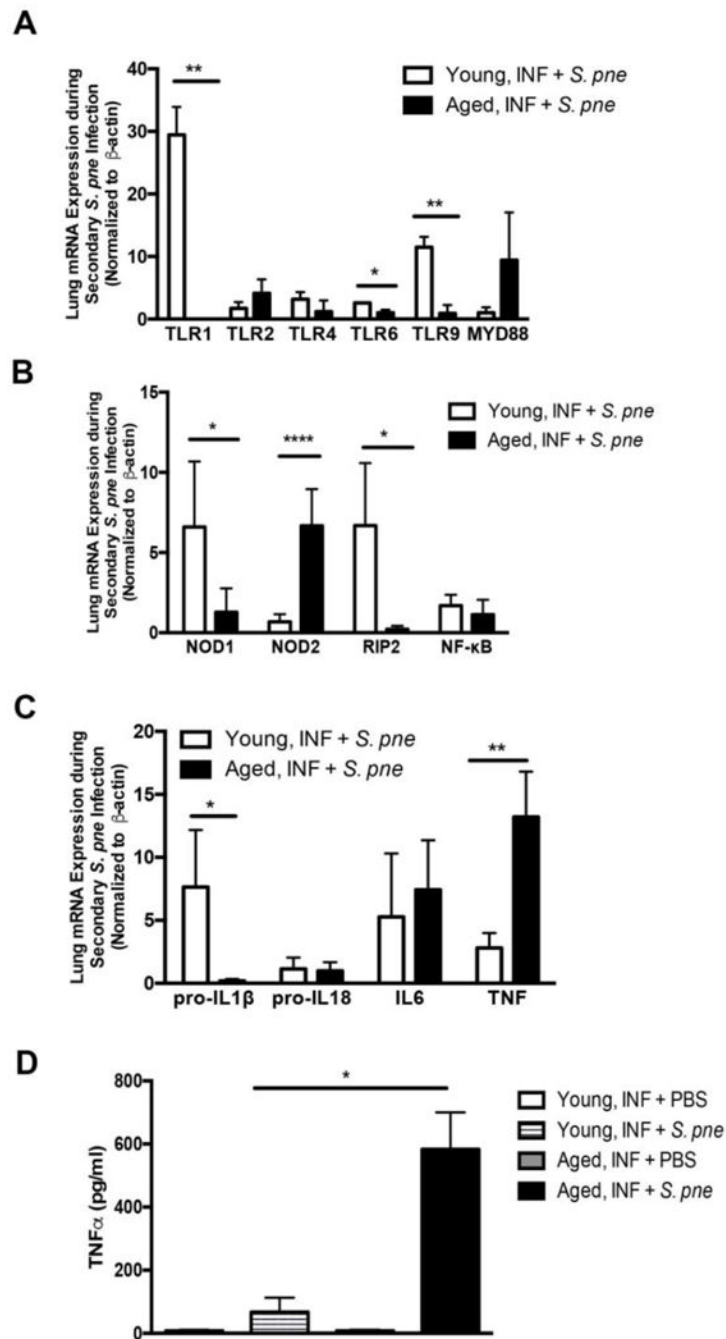


Figure 2. Dysregulated TLR Signaling in Aged Lung in Response to Secondary *S. pneumoniae* Infection

(A–C) Young (2 months) and aged (19 months) male and female BALB/c mice received influenza (day 0: PR8, 10^3 PFU-INF) prior to secondary instillation with *S. pneumoniae* (day 14, 10^3 CFU, ATCC 6303- *S. pne*). Lung tissue was collected at 24 hours post-secondary *S. pneumoniae* infection. Young and aged lung from influenza-infected mice (day 14 post infection) at 24 hours post PBS instillation were used as calibrator samples. mRNA expression values in young and aged lung in response to secondary *S. pneumoniae* infection are relative to age-matched calibrator samples. (A) Changes in lung mRNA expression of

TLR1 (P=0.0011), TLR2, TLR4, TLR6 (P=0.0223), and TLR9 (P=0.0041) in young and aged lung was assessed by real time PCR. (B) Changes in mRNA expression of NOD1 (P=0.0201), NOD2 (P<0.0001), RIP2 (P=0.0455), and NF- κ B at 24 hours post infection were assessed by real time PCR. (C) Inflammatory cytokine expression was investigated in young and aged lung and changes in pro-IL1 β (P=0.0169), pro-IL18, IL6, and TNF (P=0.0089) were assessed by real time PCR. (D) TNF α production in lung homogenates was assessed at 24 hours secondary instillation with PBS (day 0 instillation with influenza followed by day 14 instillation with PBS: INF + PBS) or *S. pneumoniae* (day 0 instillation with influenza followed by day 14 instillation with *S. pneumoniae*: INF + *S. pne*) (P=0.0284). Similar results were obtained from at least three or more independent experiments with an N=5 per experiment. Data are expressed as the mean \pm SEM.

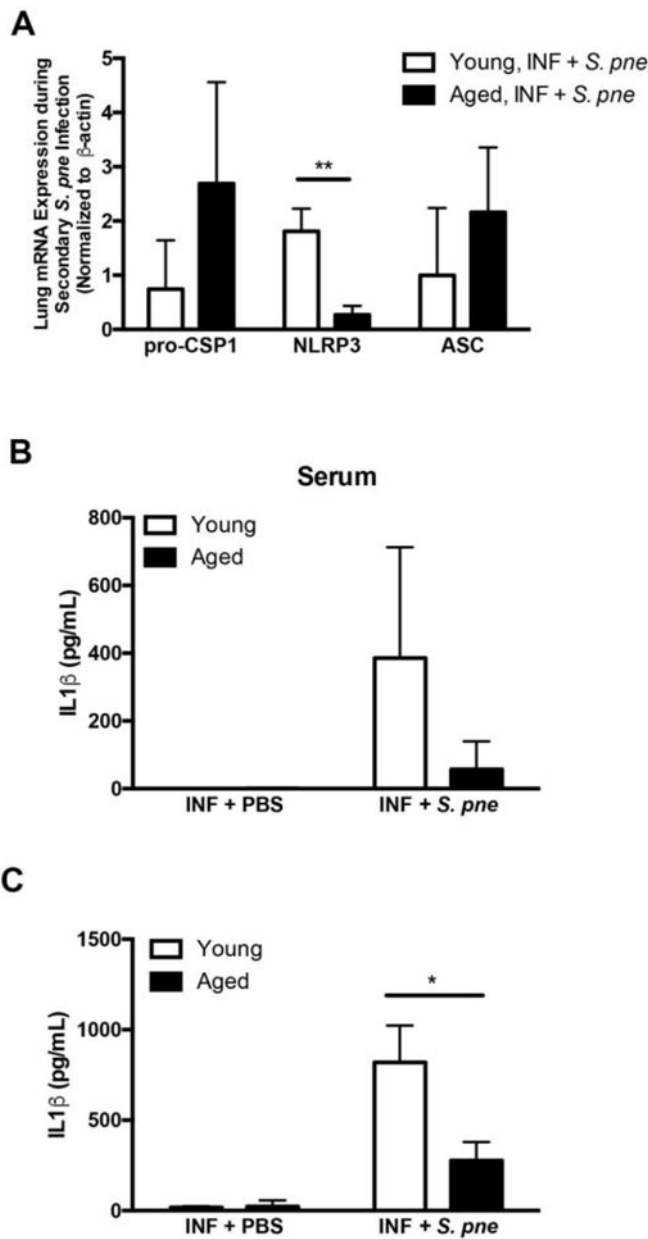


Figure 3. NLRP3 mRNA Expression in Response to Secondary *S. pneumoniae* Infection is diminished in Aged Lung
 (A–C) A–C) Young (2 months) and aged (19 months) male and female BALB/c mice received influenza (day 0: PR8, 10^3 PFU-INF) prior to secondary instillation with *S. pneumoniae* (day 14, 10^3 CFU, ATCC 6303- *S. pne*). Serum and lung tissue was collected at 24 hours post-secondary *S. pneumoniae* infection. Young and aged lung from influenza-infected mice (day 14 post infection) at 24 hours post PBS instillation were used as calibrator samples. mRNA expression values in young and aged lung in response to secondary *S. pneumoniae* infection are relative to age-matched calibrator samples. (A) Changes in lung mRNA expression of pro-caspase 1, NLRP3 ($P=0.001$), and ASC in young and aged lung was assessed by real time PCR. IL1 β production in (B) serum and (C) lung

homogenates ($P=0.0147$) was assessed by ELISA. Similar results were obtained from at least three or more independent experiments with an $N=5$ per experiment. Data are expressed as the mean \pm SEM.

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