Experimental Gerontology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

Decreased NLRP3 inflammasome expression in aged lung may contribute to increased susceptibility to secondary *Streptococcus pneumoniae* infection

Soo Jung Cho[a], Maria Plataki[a], Dana Mitzel[b], Gena Lowry[b], Kristen Rooney[a], Heather Stout-Delgado[a,*]

[a] Department of Medicine, Division of Pulmonary and Critical Care Medicine, Weill Cornell Medicine, New York, NY, United States
[b] Lovelace Respiratory Research Institute, Albuquerque, NM, United States

A B S T R A C T

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 h post-secondary *S. pneumoniae* infection. Increased susceptibility of aged mice was associated with decreased TLR1, TLR6, and TLR9 mRNA expression and diminished IL1β mRNA expression. Examination of NLRP3 inflammasome expression illustrated decreased NLRP3 mRNA expression and decreased IL1β 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 et al., 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 et al., 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 et al., 2011).

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 et al., 2012; Pawelec et al., 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 et al., 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 et al., 2004). The NLRP3 inflammasome can activate caspase 1 in response to cellular danger, resulting in the processing and secretion of proinflammatory cytokines IL1β and IL18 (Kanneganti et al., 2006; Mariathasan et al., 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 et al., 2011; Petrilli et al., 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 et al., 2014; Karmakar et al., 2015; Koedel et al., 2002; Mariathasan et al., 2006; McNeela et al., 2010; Mitchell and Mitchell, 2010; Shoma et al., 2008; Witzenrath et al., 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 et al., 2012). To expand upon these findings, as detailed in the findings

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

* Corresponding author at: Pulmonary and Critical Care Medicine/Weill Cornell Medicine, 1300 York Avenue, Box 96, New York, NY 10065, United States.
E-mail address: hes2019@med.cornell.edu (H. Stout-Delgado).

https://doi.org/10.1016/j.exger.2017.11.010
Received 28 September 2017; Received in revised form 17 November 2017; Accepted 20 November 2017

of our current study, we examined the impact of chronological aging on inflammasome expression and production of IL1β 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 h 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 IL1β mRNA expression compare to young controls. Examination of NLRP3 inflammasome expression illustrated decreased NLRP3 mRNA expression and decreased IL1β 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 > 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 et al., 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 h 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 × dilution.

2.3. In vivo procedures

As detailed in Fig. 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 μL volume in PBS). On day 14-post influenza instillation, mice received an intranasal instillation of 50 μL 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 et al., 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 μL 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 μL volume in PBS). On day 14-post influenza instillation, mice received an intranasal instillation of 50 μL of 1×10^3 CFU of *S. pneumoniae* (ATCC 6303) (50 μL 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 et al., 2012).

2.4. RNA purification and real time PCR

RNA was extracted from lung tissue at 24 h post-secondary control (influenza + PBS) or *S. pneumoniae* (influenza + *S. pneumoniae*) infection using previously published methods (Stout-Delgado et al., 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 h post PBS instillation (as detailed in Fig. 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 IL1β and TNFα production using ELISA kits purchased from eBioscience (San Diego, CA) per manufacturer's instructions. Changes in 450 nm absorbance and correction at 570 nm 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 et al., 1998; Kurtti et al., 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 et al., 2004; Mitzel et al., 2014). As illustrated in Fig. 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* (Fig. 1A) (Stout-Delgado et al., 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 (Fig. 1B: 0 h $P = 0.0005$, 24 h $P = 0.0013$, 48 h $P = 0.0073$). In addition, when compared to young, there is a significant increase in weight loss (Fig. 1C: $P < 0.0001$) and mortality (Fig. 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 (Fig. 1E, Young vs. aged: PBS + *S. pneumoniae*, $P = 0.0028$ and INF + *S. pneumoniae*, $P = 0.0001$).