



# Aging leads to dysfunctional innate immune responses to TLR2 and TLR4 agonists

Kristina L. Bailey<sup>1,3</sup> · Lynette M. Smith<sup>2</sup> · Art J. Heires<sup>1,3</sup> · Dawn M. Katafiasz<sup>1</sup> · Debra J. Romberger<sup>1,3</sup> · Tricia D. LeVan<sup>1,3,4</sup>

Received: 4 September 2018 / Accepted: 24 October 2018 / Published online: 7 November 2018  
© Springer Nature Switzerland AG 2018

## Abstract

**Background** Sepsis is more common in the elderly. TNF $\alpha$  is recognized as an important mediator in sepsis and Toll-like receptors (TLRs) play an important role in initiating signaling cascades to produce TNF $\alpha$ . Little is known about how innate immunity is altered in healthy human aging that predisposes to sepsis.

**Aims and methods** We tested the hypothesis that aging dysregulates the innate immune response to TLR 2 and 4 ligands. We performed whole blood assays on 554 healthy subjects aged 40–80 years. TNF $\alpha$  production was measured at baseline and after stimulation with the TLR2 agonists: peptidoglycan, lipoteichoic acid, Pam3CysK, Zymosan A and the TLR4 agonist lipopolysaccharide (LPS). In a subset of subjects ( $n=250$ ), we measured Toll-like receptor (TLR) 2, 4 and MyD88 expression using real-time PCR.

**Results and discussion** We measured a 2.5% increase per year in basal secretion of TNF $\alpha$  with aging ( $n=554$   $p=0.02$ ). Likewise, TNF $\alpha$  secretion was increased with aging after stimulation with peptidoglycan (1.3% increase/year;  $p=0.0005$ ) and zymosan A (1.1% increase/year  $p=0.03$ ). We also examined the difference between baseline and stimulated TNF $\alpha$  for each individual. We found that the increase was driven by the elevated baseline levels. In fact, there was a diminished stimulated response to LPS (1.9% decrease/year;  $p=0.05$ ), lipoteichoic acid (2.1% decrease/year  $p=0.03$ ), and Pam3CysK (2.6% decrease/year  $p=0.0007$ ). There were no differences in TLR or MyD88 mRNA expression with aging, however, there was an inverse relationship between TLR expression and stimulated TNF $\alpha$  production.

**Conclusions** With aging, circulating leukocytes produce high levels of TNF $\alpha$  at baseline and have inadequate responses to TLR2 and TLR4 agonists. These defects likely contribute to the increased susceptibility to sepsis in older adults.

**Keywords** Aging · Sepsis · Shock · Innate immunity · TLR signaling · Inflammaging · Immunosenescence · TNF $\alpha$

## Introduction

Sepsis is currently the eleventh leading cause of death in the US [1]. Older people have both a higher incidence and a higher mortality than younger patients [2], and older patients make up more than half of all critical care unit admissions in the US [3]. The incidence of severe sepsis increases four-fold after the age of 65 and nearly tenfold for those over the age of 85 compared to younger age groups [4]. Despite these disparities, little is known about how aging changes susceptibility to sepsis. One important component in the defense against infection and sepsis is innate immunity. Innate immunity consists of defense mechanisms that come into play within hours of infection. One important component of innate immunity is the production of inflammatory cytokines such as TNF $\alpha$ . TNF $\alpha$  is produced by many

✉ Kristina L. Bailey  
kbailey@unmc.edu

<sup>1</sup> Division of Pulmonary, Critical Care, Sleep and Allergy, Department of Internal Medicine, University of Nebraska Medical Center, Omaha, NE 68198-5910, USA

<sup>2</sup> Department of Biostatistics, University of Nebraska Medical Center, Omaha, NE 68198, USA

<sup>3</sup> VA Nebraska-Western Iowa Health Care System, Omaha, NE 68105, USA

<sup>4</sup> Department of Epidemiology, University of Nebraska Medical Center, Omaha, NE 68198, USA

circulating leukocytes including: monocytes, neutrophils, and lymphocytes [5].

TNF $\alpha$  plays a central role in the pathogenesis of sepsis [6, 7], and is an early regulator of the immune response. For TNF $\alpha$  to play a beneficial role in sepsis, it must be produced at the right place, at the right time, in appropriate concentrations. Dysregulation of TNF $\alpha$  production has been associated with the development of shock and increased mortality in sepsis [8]. Elevated levels of circulating TNF $\alpha$  have also been measured with aging [9] and may be a marker of frailty [10] and predict mortality [11] in healthy aging. One of the mechanisms of stimulating TNF $\alpha$  production is toll-like receptor signaling.

Toll-like receptors (TLRs) are expressed on multiple cell types including circulating monocytes, neutrophils and other white blood cells. TLR2 recognizes components of the gram-positive bacterial cell wall, while TLR4 recognizes components of the gram-negative cell wall. Both gram-positive and gram-negative bacteria are frequent causes of sepsis in older adults. Once bacterial components are recognized, they trigger a cascade of signaling that ultimately results in the secretion of inflammatory cytokines. Little is known about how this process may be altered in older people. There have been reports of both increased and decreased TLR expression and signaling in older people [12, 13].

In the present study, we tested the hypothesis that aging dysregulates the innate immune response to TLR2 and 4 ligands. We performed whole blood assays at baseline, and after stimulation with TLR2 and TLR4 agonists using blood from 554 healthy younger and older adults. We observed dramatic increase in basal TNF $\alpha$  production in older participants compared to younger participants. This was accompanied by an inability of the older cells to produce an appropriate stimulation of TNF $\alpha$ . These changes potentially have implications in development and outcomes of sepsis in older adults.

## Materials and methods

### Internal review board approval

This project was conducted with the approval of the internal review board from the VA Nebraska Western Iowa Health Care System. All participants underwent an informed consent process prior to participating.

### Study population

Veterans were recruited from primary care clinics at the VA Nebraska Western-Iowa Health Care System Omaha campus. Enrollment began in March 2008 and continued until December 2013, with 554 subjects with complete

data. We enrolled subjects aged 40–80. Those with a history of an infection, or immunosuppression in the previous 3 weeks were excluded. Eligibility information was obtained by self-report and medical chart confirmation. Subject demographics and smoking habits were obtained by in-person and telephone interviews. A participant was considered to be a smoker if they had smoked > 100 cigarettes in their lifetime. Because chronic lung diseases such as chronic obstructive pulmonary disease (COPD) are known to alter systemic inflammation [14], and our study population has a high rate of COPD [15], subjects were tested for lung disease by undergoing spirometry. Those that had a FEV<sub>1</sub>/FVC < 0.7 underwent post-bronchodilator spirometry with 0.083% albuterol. COPD was defined as an FEV<sub>1</sub>/FVC ratio of < 0.70 that was not completely reversible with a bronchodilator.

### Specimen and collection

Blood (10 ml) was collected from participants using venipuncture and heparinized tubes. This blood was used for cell differentials and the whole blood assay. An additional sample (2.5 ml) of unstimulated blood was collected in blood RNA tubes (PAXgene, PreAnalytiX, Hombrechtikon, Switzerland) according to the manufacturer's instructions and frozen at – 80 °C until mRNA extraction.

### Whole blood assay

The whole blood assay was performed as previously described [16], within 2 h of blood collection. Briefly, whole blood (250  $\mu$ l) was stimulated *ex vivo* with RPMI medium (Roswell Park Memorial Institute) only (baseline control), peptidoglycan (10  $\mu$ g/ml), lipoteichoic acid (20  $\mu$ g/ml), lipopolysaccharide (1 ng/ml), Pam3CYSK4 *N*- $\alpha$ -Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-L-cysteine, Palmitoyl-Cys((*RS*)-2,3-di(palmitoyloxy)-propyl)-OH (10  $\mu$ g/ml) or zymosan A (10  $\mu$ g/ml) prepared in RPMI medium (750  $\mu$ l). Blood-to-mediator ratio was 1:4 (v:v) for an assay volume of 1 ml. They were each incubated at 37 °C and 5% CO<sub>2</sub> for 24 h. This time point was chosen based on pilot experiments in our lab and similar published methods [17]. The cell-free supernates were collected and stored at – 80 °C until cytokine ELISAs could be performed.

### Cytokine measurements

TNF $\alpha$  levels were measured using a sandwich ELISA as previously described [18]. Briefly, microtiter plates were coated with mouse anti-human TNF $\alpha$  (R&D Systems, Minneapolis, MN) diluted 1:250. The detection antibody was biotinylated goat anti-human TNF $\alpha$  antibody diluted 1:250 (R&D Systems). The immune complex was visualized using

streptavidin-HRP diluted 1:200 (R&D Systems) followed by TMB/peroxidase substrate (R&D Systems). The TNF $\alpha$  titers were interpolated from a standard curve consisting of serially diluted recombinant TNF $\alpha$ , and the plate was read at 450 nm in an automated ELISA reader (VERSAmax, Molecular Devices, Sunnyvale, CA). Cytokine measurements were normalized for the total number of leukocytes in the whole blood assay.

### RNA extraction and real-time polymerase chain reaction (PCR)

RNA was extracted from unstimulated blood using the Qia-gen RNAeasy kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. The quantity of RNA was measured using spectrophotometry (Nanodrop, Fisher scientific). RNA integrity was measured using the Agilent 2100 Bioanalyzer (Santa Clara, CA). RNA samples with a RNA integrity number (RIN) > 7 were used. Total cDNA was prepared using the Taqman real-time PCR kit (Applied Biosystems, Foster City, CA) according to the manufacturer's directions using 50 ng of RNA template and 2.5  $\mu$ M random hexamers. The real-time PCR reaction mixture consisted of a cocktail of Taqman universal master mix (Applied Biosystems), primers for both the gene of interest (TLR2, or TLR4 or MyD88) and the endogenous control gene 18S ribosomal RNA. The following primer/probe sets for were used: (TLR2: Hs00152932; TLR4: Hs00152939; MyD88: Hs01573837; Applied Biosystems). Reactions were performed in duplicate. Real-time PCR was performed using an ABI Prism 7500 sequence detection system (Applied Biosystems). The PCR conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. The gene of interest was normalized to the housekeeping gene using the following equation:  $2^{-\Delta CT}$ , where  $\Delta CT = CT_{\text{Gene of interest}} - CT_{\text{Housekeeping gene}}$  [19]. With this method, care has to be taken to use the same amount of input cDNA for all samples. We confirmed this by quantitating the mRNA prior to the reaction using spectroscopy. In addition, we also ensured that all housekeeping gene CTs were similar after real-time PCR. If they were not similar, the assay was rerun.

### Statistical analysis

Descriptive statistics are presented for subject characteristics by age categorized at 60 years. Chi square test (or Fisher's exact for small sample situations) and *t* test were used to compare subject characteristics by age group. The relationship between TNF $\alpha$  levels [baseline, stimulated, and the difference ( $\Delta$ ) between stimulated and baseline] and age as a continuous variable was explored in a quantile regression analysis, with TNF $\alpha$  level as the outcome

variable. A log transformation was applied to TNF $\alpha$  levels prior to analysis due to skew. Quantile regression was chosen because baseline TNF $\alpha$  levels could be below the limit of detection and linear regression results may not be valid. Multivariable quantile regression models looked at the effect of age on TNF $\alpha$  adjusting for BMI, sex, education, race, and COPD/smoking status (defined as no COPD/never smoke; no COPD/ever smoke; COPD). These variables were chosen based on the results of the univariate analysis presented in Table 1 as well as other studies showing that education level is correlated with baseline serum TNF $\alpha$  levels [20]. TLR2, TLR4 and MyD88 mRNA levels in whole blood were log transformed and examined for associations with age in unadjusted and adjusted quantile regression models. Interpreting parameter estimates in a linear regression when variables have been log transformed is not straightforward, so we have back-transformed the  $\beta$  coefficients from the univariate and multivariable models for ease of interpretability. Adjustment for multiple comparisons was made using the false discovery rate (FDR) method by Benjamini–Hochberg [21]. Pearson's correlations were performed comparing baseline and stimulated TNF $\alpha$  levels to TLR2, TLR4 and MyD88 mRNA levels. Data analysis was performed with SAS Software version 9.3 (SAS Institute Inc., Cary, NC).

**Table 1** Subject characteristics

	Age < 60 ( <i>n</i> = 134)		Age 60 + ( <i>n</i> = 420)	
	<i>N</i>	%	<i>N</i>	%
Sex*				
Male	125	93	415	99
Female	9	7	5	1
Race*				
White	121	92	405	97
Other	11	8	11	3
Education*				
≤ High school	40	32	192	47
> High school	85	68	215	53
Smoking status*				
Never	29	22	86	21
Former	51	39	260	62
Current	50	38	71	17
COPD				
No	91	67	260	62
Yes	43	32	157	38

\**p* < 0.05

## Results

### Subject characteristics

Seventy-six percent of the population was greater than 60 years of age (Table 1). The population was predominantly composed of white males. Older veterans (> 60 years) had less education, with 47% having education below a high school level, compared to 32% of younger veterans ( $\leq 60$ ). Participants that were > 60 years were more likely to be former smokers (62% vs 39%), while those  $\leq 60$  were more likely to be current smokers (38% vs 17%). The prevalence of COPD among the two age groups was similar.

### Baseline TNF $\alpha$ production is increased with aging

The whole blood assay was used to assess the relationship between TNF $\alpha$  inflammatory responses and aging. Baseline TNF $\alpha$  production was measured in 554 participants. The whole blood assay was incubated for 24 h without stimulation, and TNF $\alpha$  was measured from the supernatants by ELISA. TNF $\alpha$  levels were normalized to leukocytes then log transformed due to the skewed nature of the data. We found that for every 1-year increase in age, we saw a 2.3% increase in TNF $\alpha$  production ( $p=0.03$ ) (Table 2). Likewise, for every 10-year increase in age there was a 23% increase in TNF $\alpha$  production. This increase was 2.5% per year, or 25% per 10 years, after adjustment for body mass index (BMI), sex, race, COPD/smoking status and education ( $p=0.02$ ) (Table 2). We used Spearman's correlations to determine whether baseline TNF $\alpha$  correlated with baseline blood counts. Baseline TNF $\alpha$  production did not correlate with total WBC count ( $r=0.033$ ,  $p=0.44$ ), neutrophil count ( $r=0.001$ ,  $p=0.99$ ), or lymphocyte count ( $r=0.002$ ,

$p=0.96$ ). There was however, a weak correlation between basal TNF $\alpha$  levels and monocytes ( $r=0.103$ ,  $p=0.02$ ).

### Stimulated TNF $\alpha$ production is dysregulated with aging

Whole blood from 554 participants was stimulated with components of the gram-positive bacterial cell wall (TLR2 agonists): peptidoglycan, zymosan, lipoteichoic acid, and *N*- $\alpha$ -palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-L-cysteine, palmitoyl-Cys((*RS*)-2,3-di(palmitoyloxy)-propyl)-OH (Pam3CysK). A component of gram-negative bacterial cell wall (TLR4 ligand), lipopolysaccharide (LPS), was also used. Stimulated TNF $\alpha$  was measured using ELISA after exposure to each stimulus for 24 h.

TNF $\alpha$  levels increased with aging after stimulation with peptidoglycan (Table 2). In the multivariable analysis, there was an increase in TNF $\alpha$  production of 1.3% per 1-year increase in age after stimulation with peptidoglycan ( $p=0.0005$  in the multivariable model), and a 1.1% increase per 1-year increase in age after stimulation with zymosan ( $p=0.03$  in the multivariable model). (Table 2) This increase was slightly less than the increase we measured at baseline. There were no statistically significant changes in TNF $\alpha$  production after stimulation with lipopolysaccharide, lipoteichoic acid, or Pam3CysK, despite an increase in baseline TNF $\alpha$  production. (Table 2) There was no difference with aging in the stimulated levels of TNF $\alpha$  after exposure to lipoteichoic acid ( $p=0.95$ ) and Pam3CysK ( $p=0.80$ ).

The increase in TNF $\alpha$  with aging after stimuli could be driven by the increased basal secretion of TNF $\alpha$  with aging, or increased responses to the stimuli. To determine which of these scenarios was occurring, we analyzed the difference between individual participant basal and stimulated TNF $\alpha$  levels (TNF $\alpha$  after stimulation–TNF $\alpha$  baseline)

**Table 2** Association between ligand-stimulated TNF $\alpha$  secretion and age

Quantile regression of TNF $\alpha$ (pg/ml, normalized to leukocytes)								
Stimuli	Univariate ( $n=554$ )				Multivariable <sup>a</sup> ( $n=554$ )			
	$\beta^b$	95% CI <sup>b</sup>		$p$ value	$\beta^b$	95% CI <sup>b</sup>		$p$ value
None	1.023	1.002	1.046	0.03*	1.025	1.003	1.047	0.02*
Peptidoglycan	1.010	1.002	1.018	0.02*	1.013	1.006	1.020	0.0005 <sup>^</sup>
Zymosan A	1.015	1.004	1.025	0.006 <sup>^</sup>	1.011	1.001	1.022	0.03*
Lipopolysaccharide	1.012	1.001	1.024	0.04*	1.008	0.996	1.020	0.17
Lipoteichoic acid	0.999	0.989	1.009	0.86	1.000	0.989	1.012	0.95
Pam3CysK	0.998	0.982	1.014	0.80	1.002	0.985	1.019	0.80

*Pam3CysK* *N*- $\alpha$ -palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-L-cysteine, palmitoyl-Cys((*RS*)-2,3-di(palmitoyloxy)-propyl)-OH

\*False discovery rate < 0.1

<sup>^</sup>False discovery rate < 0.05

<sup>a</sup>Multivariable models were adjusted for BMI, sex, education, race, COPD/smoking status

<sup>b</sup> $\beta$  coefficients and confidence limits were back-transformed to the original scale

[Delta ( $\Delta$ ) TNF $\alpha$ ] (Table 3). We saw significant decrease in the  $\Delta$ TNF $\alpha$  after exposure to LPS, lipoteichoic acid and Pam3CysK. After adjustment, there was a 1.9% decrease per 1-year increase in age in  $\Delta$  TNF $\alpha$  after stimulation by LPS ( $p=0.05$ ); a 2.1% decrease per year increase in age after LTA stimulation ( $p=0.03$ ) and a 2.6% decrease per year increase in age after Pam3CysK stimulation ( $p=0.0007$ ) (Table 3). There were no differences in  $\Delta$ TNF $\alpha$  with aging after exposure to peptidoglycan ( $p=0.27$ ) or zymosan ( $p=0.59$ ). This suggests that the increased TNF $\alpha$  was driven primarily by the increased baseline TNF $\alpha$  level with aging, and that with aging, there is a diminished capacity to respond to stimulation with LPS, lipoteichoic acid and Pam3CysK.

### Stimulated TNF $\alpha$ production correlates inversely with baseline TLR2 and TLR4 mRNA expression

A subset of participants had mRNA available from whole blood prior to stimulation ( $n=250$ ). This mRNA was assayed for TLR2, TLR4 and MyD88. These are components of the innate immune system that are important in initiating inflammation during sepsis. Baseline expression levels of TLR2, TLR4 and MyD88 were not associated with age in a quantile regression analysis (Table 4).

However, TLR4 mRNA expression was weakly, inversely, correlated with baseline TNF $\alpha$  production ( $p=0.013$ ,  $r=-0.15$ ,  $n=250$ ). Baseline TNF $\alpha$  production was not correlated with baseline TLR2 mRNA expression ( $p=0.09$ ,  $r=-0.12$ ,  $n=250$ ), or MyD88 mRNA expression ( $p=0.058$ ,  $r=-0.12$ ,  $n=250$ ).

Stronger, inverse correlations were observed between TLR2 mRNA expression and stimulated levels of TNF $\alpha$  (Fig. 1). Higher levels of TLR2 mRNA expression correlated with lower TNF $\alpha$  production induced by TLR2 ligands (Fig. 1). This was true for lipoteichoic acid ( $r=-0.26$ ,  $p<0.0001$ ), peptidoglycan ( $r=-0.26$ ,  $p<0.0001$ ), zymosan ( $r=-0.21$ ,  $p=0.0007$ ), and Pam3CysK ( $r=-0.25$ ,  $p<0.0001$ ). Likewise, higher levels of TLR4 mRNA expression correlated with lower levels of TNF $\alpha$  production induced by the TLR4 ligand lipopolysaccharide ( $r=-0.21$ ,  $p=0.0006$ ) (Fig. 2). MyD88 inversely correlated only with TNF $\alpha$  production after peptidoglycan stimulation ( $r=-0.23$ ,  $p=0.0002$ ). Not surprisingly, TLR2, TLR4 and MyD88 mRNA expression were highly positively correlated with each other. TLR2 compared to TLR4 ( $r=0.76$ ,  $p<0.0001$ ); TLR2 compared to MyD88 ( $r=0.64$ ,  $p<0.0001$ ), TLR4 compared to MyD88 ( $r=0.63$ ,  $p<0.0001$ ).

**Table 3** Association between  $\Delta$ TNF $\alpha$  production and age

Stimuli	Univariate ( $n=554$ )				Multivariable <sup>a</sup> ( $n=554$ )			
	$\beta^b$	95% CI <sup>b</sup>	$p$ value		$\beta^b$	95% CI <sup>b</sup>	$p$ value	
Peptidoglycan	0.984	0.965	1.004	0.11	0.989	0.969	1.009	0.27
Zymosan A	0.991	0.973	1.010	0.37	0.994	0.974	1.015	0.59
Lipopolysaccharide	0.990	0.969	1.012	0.36	0.981	0.962	1.000	0.05*
Lipoteichoic acid	0.983	0.967	1.000	0.051	0.979	0.960	0.998	0.03*
Pam3CysK	0.985	0.970	1.000	0.052	0.974	0.960	0.989	0.0007 <sup>^</sup>

$\Delta$ TNF $\alpha$  stimulated TNF $\alpha$  minus baseline TNF $\alpha$ , Pam3CysK *N*- $\alpha$ -palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-L-cysteine, palmitoyl-Cys((*RS*)-2,3-di(palmitoyloxy)-propyl)-OH

\*False discovery rate  $<0.1$

<sup>^</sup>false discovery rate  $<0.05$

<sup>a</sup>Multivariable models were adjusted for BMI, sex, education, race, and COPD/smoking status

<sup>b</sup> $\beta$  coefficients and confidence limits were back-transformed to original scale

**Table 4** There are no associations between TLR2, TLR4 or MyD88 expression and age

	Univariate ( $n=250$ )				Multivariable <sup>a</sup> ( $n=250$ )			
	$\beta^b$	95% CI <sup>b</sup>	$p$ value		$\beta^b$	95% CI <sup>b</sup>	$p$ value	
TLR2 mRNA	1.006	0.998	1.013	0.14	1.002	0.994	1.009	0.7
TLR4 mRNA	1.004	0.996	1.012	0.29	0.998	0.990	1.006	0.68
MyD88 mRNA	1.003	0.997	1.010	0.34	0.998	0.991	1.005	0.55

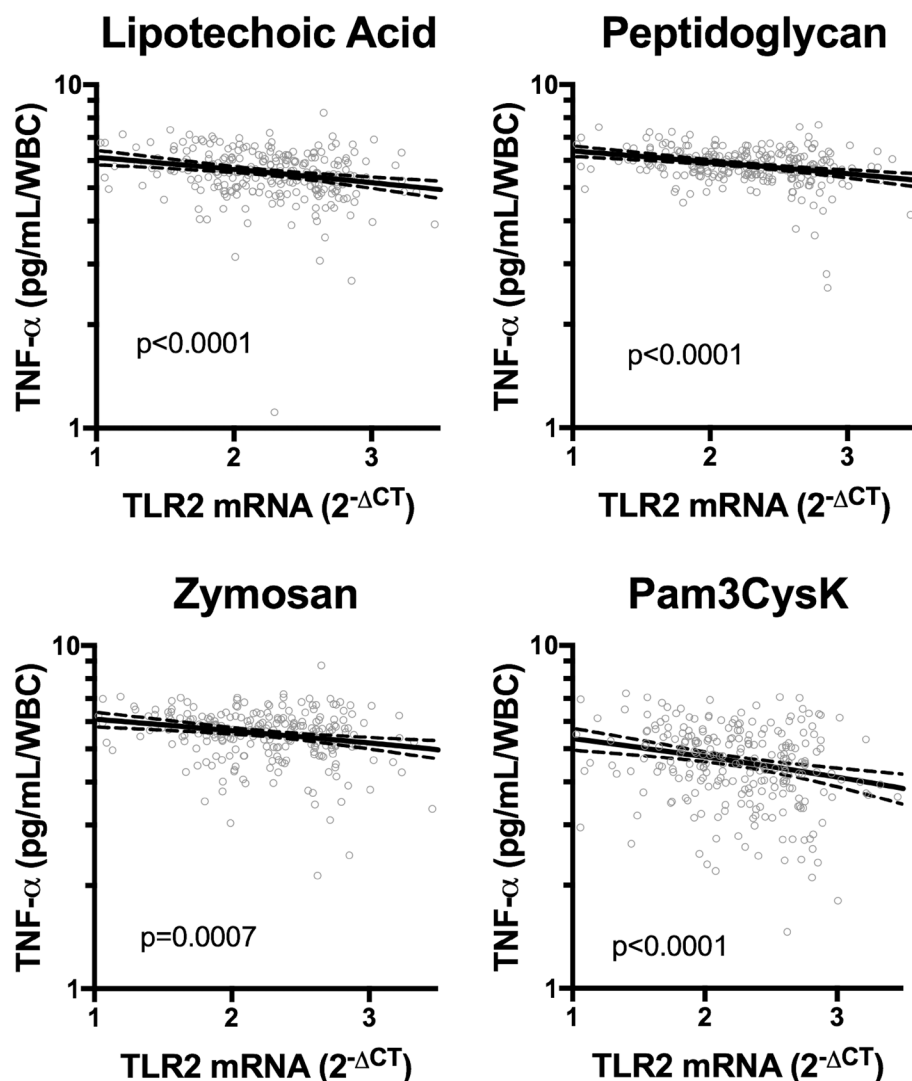
TLR2, TLR4 and MyD88 were measured from a cohort of 250 that had available mRNA

<sup>a</sup>Adjusted for BMI, sex, education, race, COPD/ smoking status combination variable

<sup>b</sup> $\beta$  coefficients and confidence limits back-transformed to original scale



**Fig. 1** TLR2 mRNA expression inversely correlates with stimulated TNF $\alpha$  production. TLR2 mRNA was measured from unstimulated whole blood. TLR2 was normalized to the housekeeping gene 18s RNA using the  $2^{-\Delta CT}$  method. TNF $\alpha$  is measured after stimulation with lipoteichoic acid (20  $\mu$ g/ml), peptidoglycan (10  $\mu$ g/ml), zymosan (10  $\mu$ g/ml), and Pam3CysK (10  $\mu$ g/ml) for 24 h. TNF $\alpha$  (Logged) is reported as pg/ml/WBC. The center line is the line of best fit of a simple linear regression and the dashed lines are the 95% confidence intervals. The reported  $p$  value is whether the slope of the line is significantly non-zero



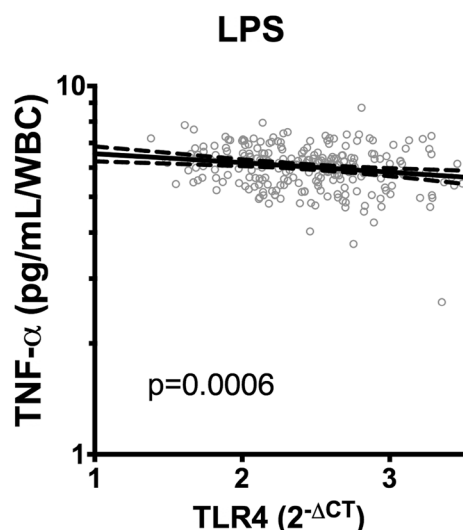
## Discussion

In these experiments, we were able to measure baseline and stimulated TNF $\alpha$  levels from a large cohort ( $n=554$ ) of subjects aged 40–80 years old. We also measured TLR2, TLR4 and MyD88 mRNA levels from a subset of 250 subjects. This large cohort allows us to confidently measure differences in TNF $\alpha$  production with aging in the whole blood assay at baseline and after stimulation with TLR2 and TLR4 agonists.

We observed a significant increase in baseline TNF $\alpha$  in the whole blood assay with increasing age, accompanied by decreased responsiveness to stimuli with age. This pattern of low-grade chronic inflammation (inflammaging) accompanied by the inability to mount an appropriate response to stimuli (immunosenescence) potentially places older sepsis patients at increased risk for morbidity and mortality. Functional, acute inflammation is a beneficial innate immune response that helps protect against invading

pathogens [22]. Chronic, persistent inflammation, on the other hand, can lead to tissue damage [22]. We observed both impaired acute inflammation and increased chronic inflammation in older cells in these experiments.

This pattern of elevated TNF $\alpha$  production by circulating leukocytes at baseline may explain previous reports of elevated serum TNF $\alpha$  in aging [10, 23]. Higher levels of serum TNF $\alpha$  have also been measured in older sepsis patients [24]. Higher plasma levels of TNF $\alpha$  also likely correlate with increased mortality from sepsis [25]. Others have speculated that this increased level of circulating TNF $\alpha$  with aging may be due to prolonged activation of the leukocytes [26], or increased NF $\kappa$ B activity, at baseline [27], an increase in transcription of TNF $\alpha$  due to relative zinc deficiency [28], chronic immune stimulation due to viruses such as Cytomegalovirus [29], changes in gut microbiota leading to increased leakiness and inflammation [30]. Others have suggested that increased serum TNF $\alpha$  seen in aging may be related to increased



**Fig. 2** TLR4 mRNA expression inversely correlates with TNF $\alpha$  production. TLR4 mRNA was measured from unstimulated whole blood. TLR4 was normalized to the housekeeping gene 18s RNA using the  $2^{-\Delta CT}$  method. TNF $\alpha$  is measured after stimulation with lipopolysaccharide (LPS) (1 ng/ml) for 24 h. TNF $\alpha$  (Logged) is reported as pg/ml/WBC. The center line is the line of best fit of a simple linear regression and the dashed lines are the 95% confidence intervals. The reported  $p$  value is whether the slope of the line is significantly non-zero

comorbidities or chronic diseases, rather than aging itself [31].

How older leukocytes respond to TLR ligands has been controversial. Smaller studies with isolated peripheral blood monocytes have shown diminished TNF $\alpha$  production in response to stimuli [32, 33]. In contrast, other studies have shown an increase in TNF $\alpha$  in aged individuals after stimulation [34]. Mononuclear cells from older individuals ( $n = 13$ ) stimulated with PMA responded with increased levels of TNF $\alpha$  compared to younger individuals [34]. Our finding that older cells are not able to produce an appropriate level of TNF $\alpha$  after stimulation with a TLR ligand was powered to try to resolve this controversy.

Likewise, the literature is strikingly variable regarding how TLR expression and signaling in peripheral leukocytes is affected with aging. Increase in TLR2 expression and function has been reported [35], along with decrease in TLR2 [12], as well as no change with aging [33, 36, 37]. Others have also shown diminished TLR function in aged mice [13, 38]. For this reason, we included measurement of TLR2 mRNA in our analysis. The differences in expression likely relate to measurement in different cell types (i.e., peripheral blood monocytes, neutrophils, dendritic cells) and differences in methods of measurement (Western blot, PCR, FACS). Our data showed no change in TLR2 mRNA expression with aging, likely due to the mixture of cells contained in the whole blood assay. In terms of TLR2 signaling, a

defect in TLR2/1-mediated TNF $\alpha$  production in aging has been reported in human neutrophils [32]. In mice, TLR2 function has been shown to be limited at the level of mitogen-activated protein kinases (MAPKs) [13, 39]. Consistent with our findings, TLR4 expression has been reported to be largely unchanged with aging [36, 37], as is MyD88 [36]. Despite no change in TLR4 expression, cytokine production after TLR4 stimulation with LPS has been reported to be increased [40], decreased [13, 41] and unchanged [42] with aging.

In our experiments, we measured an inverse relationship between TLR2 and TLR4 expression and TNF $\alpha$  production. This suggests a defect, not with TLR expression in aging, but impaired downstream signaling. Several signaling molecules downstream of TLRs have been reported to be impaired in aging. For instance, CD80 is decreased in aging PBMCs [43], which leads to impaired TLR signaling. ERK phosphorylation has also been shown to be impaired in peripheral blood monocytes with aging [33]. MicroRNAs may also play a role in dampening the TLR immune response with aging [44]. In mice, the chronic low-grade inflammation leads to an upregulation of A20, a de-ubiquitinase that inhibits TLR signaling [45]. It is also possible that the changes in signaling are mediated by a TLR that we did not measure. For instance, changes in TLR1 or TLR6 could affect TLR2 signaling. Decrease in TLR1 expression in aging has been shown to dampen TLR2 signaling in peripheral blood monocytes [32].

The present study has several limitations. The first is that population studied is primarily male, and over the age of 40. However, these demographics are also consistent with adult sepsis patients in general, with 93% of septic patients over the age of 40 and 69% over the age of 60 [46]. Our study is unique in that it shows that there is a difference between middle-aged people (Age 40–59) and older people (> 60) in terms of innate immunity. Many other studies have looked at the extremes of age. We have shown differences occur in the crucial years between midlife and older age, which likely contribute to the higher incidence of sepsis with aging. Another limitation is that the use of the whole blood assay does not allow us to determine which white blood cells are responsible for the changes in TNF $\alpha$  that we observed. However, it does have the advantage of helping us understand how the cells work together in a more complex, co-culture model. Because aging often induces impairments in intracellular communication [47], this model allows for us to account for these changes. The measurement of TLR expression only by RT-PCR is also a limitation, as is the fact that we measured only TLR2 and TLR4.

In summary, we have demonstrated that peripheral blood leukocytes from older participants have elevated production of TNF $\alpha$  at baseline. Older cells also have difficulty mounting an appropriate TNF $\alpha$  response to stimuli such as TLR

agonists. TLR2, TLR4 and MyD88 mRNA expression do not change with aging, and their expression does not explain the decrease in TNF $\alpha$  production. These data contribute to the growing data explaining the increased incidence, severity and mortality in sepsis in older people.

**Acknowledgements** All authors have read the journal's policy on disclosure of potential conflicts of interest. None of the authors have any financial or personal relationship with organizations that could potentially be perceived as influencing the described research to disclose. All authors have read the journal's authorship statement and agree to the submission of this paper.

**Funding** Funding sources: VA Merit Award (I01CX000434) to TDL; National Institute for Occupational Safety and Health (R01OH008539) to DJR; National Institute on Aging (R01AG053553) to KLB.

## Compliance with ethical standards

**Conflict of interest** On behalf of all authors, the corresponding authors states that there is no conflict of interest.

**Statement of human and animal rights** This project was conducted with the approval of the internal review board from the VA Nebraska Western Iowa Health Care System.

**Informed consent** All participants underwent an informed consent process prior to participating.

## References

- Kochanek KD et al (2016) Deaths: final data for 2014. *Nat Vital Stat Rep* 65:1–122
- Martin GS, Mannino DM, Moss M (2006) The effect of age on the development and outcome of adult sepsis. *Crit Care Med* 34:15–21
- Pisani MA (2009) Analytic reviews: considerations in caring for the critically ill older patient. *J Intensive Care Med* 24:83–95
- Dombrovskiy VY et al (2007) Rapid increase in hospitalization and mortality rates for severe sepsis in the United States: a trend analysis from 1993 to 2003. *Crit Care Med* 35:1244–1250
- Grivennikov SI et al (2005) Distinct and nonredundant in vivo functions of TNF produced by T cells and macrophages/neutrophils: protective and deleterious effects. *Immunity* 22:93–104
- Ceramil A, Beutler B (1988) The role of cachectin/TNF in endotoxic shock and cachexia. *Immunol Today* 9:28–31
- Leon LR, White AA, Kluger MJ (1998) Role of IL-6 and TNF in thermoregulation and survival during sepsis in mice. *Am J Physiol* 275:R269–R277
- Debets JM et al (1989) Plasma tumor necrosis factor and mortality in critically ill septic patients. *Crit Care Med* 17:489–494
- Shurin GV et al (2007) Dynamic alteration of soluble serum biomarkers in healthy aging. *Cytokine* 39:123–129
- de Gonzalo-Calvo D et al (2010) Differential inflammatory responses in aging and disease: TNF- $\alpha$  and IL-6 as possible biomarkers. *Free Radic Biol Med* 49:733–737
- Bruunsgaard H et al (2003) Elevated levels of tumor necrosis factor alpha and mortality in centenarians. *Am J Med* 115:278–283
- Panda A et al (2010) Age-associated decrease in TLR function in primary human dendritic cells predicts influenza vaccine response. *J Immunol* 184:2518–2527
- Boehmer ED et al (2005) Aging negatively skews macrophage TLR2- and TLR4-mediated pro-inflammatory responses without affecting the IL-2-stimulated pathway. *Mech Ageing Dev* 126:1305–1313
- Gan WQ et al (2004) Association between chronic obstructive pulmonary disease and systemic inflammation: a systematic review and a meta-analysis. *Thorax* 59:574–580
- Sharafkhaneh A et al (2010) Burden of COPD in a government health care system: a retrospective observational study using data from the US Veterans Affairs population. *Int J Chronic Obstr Pulm Dis* 5:125
- Harting JR et al (2012) Chronic obstructive pulmonary disease patients have greater systemic responsiveness to ex vivo stimulation with swine dust extract and its components versus healthy volunteers. *J Toxicol Environ Health A* 75:1456–1470
- De Groote D et al (1992) Direct stimulation of cytokines (IL-1 $\beta$ , TNF- $\alpha$ , IL-6, IL-2, IFN- $\gamma$  and GM-CSF) in whole blood. I. Comparison with isolated PBMC stimulation. *Cytokine* 4:239–248
- Wyatt TA et al (2017) Alcohol inhibits organic dust-induced ICAM-1 expression on bronchial epithelial cells. *Safety (Basel)* 3:5
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>(-Delta Delta C(T))</sup> method. *Methods* 25:402–408
- Koster A et al (2006) Association of inflammatory markers with socioeconomic status. *J Gerontol A Biol Sci Med Sci* 61:284–290
- Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Ser B (Methodol)* 57:289–300
- Franceschi C, Campisi J (2014) Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. *J Gerontol A Biol Sci Med Sci* 69:S4–S9
- Frankel JH et al (2018) Age-related immune responses after burn and inhalation injury are associated with altered clinical outcomes. *Exp Gerontol* 105:78–86
- Marik PE, Zaloga GP, I. Norasept II Study Investigators (2001) North American Sepsis Trial, the effect of aging on circulating levels of proinflammatory cytokines during septic shock. Norasept II Study Investigators. *J Am Geriatr Soc* 49:5–9
- Dalli J et al (2017) Human sepsis eicosanoid and proresolving lipid mediator temporal profiles: correlations with survival and clinical outcomes. *Crit Care Med* 45:58–68
- Dunston CR, Griffiths HR (2010) The effect of ageing on macrophage Toll-like receptor-mediated responses in the fight against pathogens. *Clin Exp Immunol* 161:407–416
- Qian F, Montgomery RR (2012) Quantitative imaging of lineage-specific Toll-like receptor-mediated signaling in monocytes and dendritic cells from small samples of human blood. *J Vis Exp* 62:e3741–e3741
- Prasad AS (2014) Zinc: an antioxidant and anti-inflammatory agent: role of zinc in degenerative disorders of aging. *J Trace Elem Med Biol* 28:364–371
- Pawelec G et al (2010) Immunosenescence and cytomegalovirus: where do we stand after a decade? *Immun Ageing* 7:13
- Biagi E et al (2010) Through ageing, and beyond: gut microbiota and inflammatory status in seniors and centenarians. *PLoS One* 5:e10667
- Morrisette-Thomas V et al (2014) Inflamm-aging does not simply reflect increases in pro-inflammatory markers. *Mech Ageing Dev* 139:49–57
- van Duin D et al (2007) Age-associated defect in human TLR-1/2 function. *J Immunol* 178:970–975
- Nyugen J et al (2010) Impaired functions of peripheral blood monocyte subpopulations in aged humans. *J Clin Immunol* 30:806–813



34. Fagiolo U et al (1993) Increased cytokine production in mononuclear cells of healthy elderly people. *Eur J Immunol* 23:2375–2378
35. Simpson JL et al (2013) Influence of age, past smoking, and disease severity on TLR2, neutrophilic inflammation, and MMP-9 levels in COPD. *Mediat Inflamm* 2013:462934
36. Fulop T et al (2004) Signal transduction and functional changes in neutrophils with aging. *Aging Cell* 3:217–226
37. Murciano C et al (2007) In vitro response to *Candida albicans* in cultures of whole human blood from young and aged donors. *FEMS Immunol Med Microbiol* 51:327–335
38. Renshaw M et al (2002) Cutting edge: impaired Toll-like receptor expression and function in aging. *J Immunol* 169:4697–4701
39. Boyd AR et al (2012) Age-related defects in TLR2 signaling diminish the cytokine response by alveolar macrophages during murine pneumococcal pneumonia. *Exp Gerontol* 47:507–518
40. Born J et al (1995) Cytokine production and lymphocyte subpopulations in aged humans. An assessment during nocturnal sleep. *Mech Ageing Dev* 84:113–126
41. Bruunsgaard H et al (1999) Impaired production of proinflammatory cytokines in response to lipopolysaccharide (LPS) stimulation in elderly humans. *Clin Exp Immunol* 118:235–241
42. Roubenoff R et al (1998) Monocyte cytokine production in an elderly population: effect of age and inflammation. *J Gerontol A Biol Sci Med Sci* 53:M20–M26
43. van Duin D et al (2007) Prevacine determination of the expression of costimulatory B7 molecules in activated monocytes predicts influenza vaccine responses in young and older adults. *J Infect Dis* 195:1590–1597
44. Olivieri F et al (2013) Toll like receptor signaling in “inflammaging”: microRNA as new players. *Immun Ageing* 10:11
45. Hinojosa E, Boyd AR, Orihuela CJ (2009) Age-associated inflammation and toll-like receptor dysfunction prime the lungs for pneumococcal pneumonia. *J Infect Dis* 200:546–554
46. Rhee C et al (2017) Incidence and trends of sepsis in US hospitals using clinical vs claims data, 2009–2014. *JAMA* 318:1241–1249
47. Lopez-Otin C et al (2013) The hallmarks of aging. *Cell* 153:1194–1217