

2015

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## Recommended Citation

Linge H, Lee J, Ochani K, Koga K, Kohn N, Ojamaa K, Powell S, Miller E. Age influences inflammatory responses, hemodynamics, and cardiac proteasome activation during acute lung injury. . 2015 Jan 01; 41(4):Article 27 [ p.]. Available from: <https://academicworks.medicine.hofstra.edu/articles/27>. Free full text article.

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Published in final edited form as:

*Exp Lung Res.* 2015 May ; 41(4): 216–227. doi:10.3109/01902148.2014.999174.

## Age Influences Inflammatory Responses, Hemodynamics and Cardiac Proteasome Activation During Acute Lung Injury

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### Abstract

**Background**—Acute lung injury (ALI) is a significant source of morbidity and mortality in critically ill patients. Age is a major determinant of clinical outcome in ALI. The increased ALI-associated mortality in the older population suggests that there are age-dependent alterations in the responses to pulmonary challenge. The objective of this observational study was to evaluate age-dependent differences in the acute (within 6hrs) immunological and physiological responses of the heart and lung, to pulmonary challenge, that could result in increased severity.

**Methods**—Male C57Bl/6 mice (young: 2–3 months, old: 18–20 months) were challenged intratracheally with cell wall components from gram positive bacteria (lipoteichoic acid and peptidoglycan). After 6h, both biochemical and physiological consequences of the challenge were assessed. Alveolar infiltration of inflammatory cells and protein, airspace and blood cytokines, cardiac function and myocardial proteasome activity were determined.

**Results**—In young mice there was a dose dependent response to pulmonary challenge resulting in increased airspace neutrophil counts, lung permeability, and concentrations of cytokines in bronchoalveolar lavage fluid and plasma. A midrange dose was then selected to compare the

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#### COMPETING INTERESTS

The authors have no competing interests

#### AUTHORS CONTRIBUTIONS

Experimental Design of study: HML, KK, KO, NK, SRP, KOj, EJM.

Acquisition of data: HML, JYL, KK, KO.

Analysis and interpretation: HML, JYL, KK, KO, NK, SRP, KOj, EJM.

Writing the manuscript and approving final version: HML, JYL, KK, KO, NK, SRP, KOj, EJM.

responses in young and old animals. In comparison, the old animals displayed increased neutrophil accumulation in the airspaces, decreased arterial oxygen saturation, body temperatures, plasma cytokine concentrations, and a lack of myocardial proteasome response, following challenge.

**Conclusions**—Age dependent differences in the onset of systemic response and in maintenance of vital functions, including temperature control, oxygen saturation and myocardial proteasome activation, are evident. We believe a better understanding of these age-related consequences of ALI can lead to more appropriate treatments in the elderly patient population.

### Keywords

Aging; pulmonary challenge; inflammatory response; myocardial proteasome; acute lung injury; inflammation; neutrophil; physiology; cardiac; proteasome

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## BACKGROUND

Changes in pulmonary physiology, pathology, and function are common in older populations, and lead to altered responses and susceptibility to lung infections. However, in many respects, the reasons for the altered susceptibility remain unclear. Understanding age-related changes within the pulmonary compartment and their impact on cardiopulmonary activity is of critical importance for developing novel therapeutic strategies for the treatment of the elderly patient.

Acute lung injury (ALI), or Acute Respiratory Distress Syndrome as it is now defined in humans<sup>1,2</sup>, is worldwide a major clinical condition affecting approximately 1 in every 1250 individuals annually<sup>3</sup>. Age is a critical determinant in the incidence (16 per 100,000 person-years in young (15–19 yrs of age) and 306 per 100,000 person-years (75–84 yrs of age); and mortality (from 24% (15–19 yrs) to 60 % (>85 yrs))<sup>3</sup>. Although, as the name suggests, the condition has many etiologies, pneumonia and sepsis are leading triggers<sup>4</sup>. ALI is associated with pulmonary endothelial barrier dysfunction<sup>5</sup> the clinical manifestations of which include severe hypoxemia with acute onset and bilateral infiltrates in the absence of heart failure or fluid overload<sup>6</sup>. In severe sepsis, oftentimes, cardiac function is also compromised<sup>7</sup>, which can adversely affect patient survival. Although efforts have been made to develop effective ventilator and fluid management strategies, mortality remains high<sup>8–10</sup>.

*Staphylococcus aureus* infection is a prevalent cause of pneumonia, especially in elderly patients<sup>11</sup> and a common complication of mechanical ventilation<sup>12</sup>. During the progression of infection and pneumonia, ALI and sepsis can develop. Elderly patients are at increased risk of pneumonia due to age-related deterioration of lung function<sup>13</sup>. In addition, studies of critically ill patients have identified advanced age and pulmonary *S. aureus* infection as leading risk factors in sepsis<sup>14</sup>.

To develop a better understanding of the age-dependent changes that occur in the injury phase of ALI, and thereby assist in defining future targets for improved care for elderly patients suffering from severe acute inflammation, we undertook an observational study in a defined model of staphylococcal induced pneumonia. Using this model we compared the acute responses of two different age groups of mice (young: 2–3 months; old: 18–20

months) approximating human age groups of 20 yrs and 55–60 yrs<sup>15</sup>. We evaluated age-associated differences in the local and systemic inflammatory response. Furthermore, since ALI is often accompanied by cardiac dysfunction we also determined age-associated changes in hemodynamics and cardiac proteasome responsiveness.

## METHODS

### Animals Studies

All animal experiments received prior approval by the Institutional Animal Care and Use Committee of The Feinstein Institute. Male C57Bl/6 mice were purchased from Taconic Farms (Albany, NY) at 2 (24±1.3 g) or 12 months of age. The latter were housed under standard conditions until experimentation at 18–20 months of age (35.7±3.1 g).

### Study design

A defined lung model of staphylococcal pneumonia was established in young C57Bl6 mice (2–3 months). Groups of mice were challenged intratracheally with staphylococcal cell wall components, lipoteichoic acid (LTA), and peptidoglycan (PGN). Each group was challenged with a single concentration of the LTA/PGN mixture, with different groups receiving a different concentration so that dose dependent effects on airspace infiltration of inflammatory cells and protein, airspace and blood cytokines, could be evaluated. Having established a dose dependent relationship in the model, we selected a concentration of the LTA/PGN mixture that gave a robust, but not potentially overwhelming, response. Mice from both the young and old (C57Bl6 mice 18–20 months) groups were then challenged with this “mid” dose to evaluate the acute responses of the two groups to the same challenge. Thus, after 6h, both biochemical and physiological consequences of the challenge were assessed evaluating both airspace and systemic inflammation. In addition, assessment of both pulmonary and cardiac function was performed.

Since the ubiquitin-proteasome system plays an important role in cardiac physiology and pathology<sup>16</sup>, and there are age-dependent alterations in proteasome function that may contribute to increased susceptibility to various forms of age-related cardiac dysfunction<sup>17</sup>, we also examined cardiac proteasome activity in the two age groups before and after pulmonary challenge. The measurements in the current study are in accordance with the workshop report issued by the American Thoracic Society “Core” Clinical Critical Care Research Laboratory entitled “Animal Models of Acute Lung Injury”<sup>18</sup>.

### Intratracheal challenge

Young mice (2–3 months) were anesthetized (2% isoflurane with 98% oxygen) and the trachea surgically exposed. LTA and PGN (Sigma, St. Louis, MO) from *S. aureus*, in 50µl sterile saline (0.9% w/v; ambient temperature) was instilled intratracheally using a 29 gauge needle. For dose response determination, groups of young mice (n=6–8 per group) received a low (0.2µg+0.66µg), mid (30µg+100µg) or high (150µg+500µg) dose of LTA and PGN. Sham mice received saline alone. Control mice (n=4) were anesthetized but no surgery was performed. Old mice (18–20 months) received either the mid dose of LTA/PGN (n=7) or saline (n=7). The incision was sutured and the animals recovered on a heated blanket for 20

min. The mice were then maintained at ambient temperature with food and water *ad libitum*. To limit effects on hemodynamics, resuscitation fluids were withheld. The dose of challenge was not adjusted to body weight, ensuring that any age-dependent differences observed were not due to differences in amount of stimulant.

### **Pulse oximetry measurements**

Mice were acclimated to a neck collar clip (three sessions, 5 min each 1–3 days before the experimental protocol). Under anesthesia, the fur around the neck was removed by depilatory cream to allow the probe close proximity to the carotid artery. One day prior to experimentation (at least 4h after recovery from anesthesia), baseline values of arterial oxygen saturation, heart, and respiratory rates were recorded in ambulatory animals using pulse oximetry (Starr Life Sciences Corp., Allison Park, PA). Recordings were repeated 6h after LTA/PGN instillation.

### ***In vivo* hemodynamic analysis**

LV pressures were recorded 6h post instillation of either saline or LTA/PGN. Mice were anesthetized (5 min of isoflurane, 2.5% delivered with 97.5% oxygen) and intubated by tracheotomy and mechanically ventilated with anesthesia using a rodent ventilator (respiratory rate: 170 breaths/min and tidal volume: 170  $\mu$ l; Minivent model 845; Harvard Apparatus, Holliston, MA). Core body temperatures were recorded using a rectal probe (Temp4; Acorn; Oakton, IL) and surgery was initiated when body temperature reached 34°C. The heart was exposed by thoracotomy, and a 1.4-Fr pressure catheter (SPR-671; Millar Instruments, Houston, TX) was inserted into the LV through the apex as we have described previously<sup>19</sup>. LV pressure data from 200–300 cardiac cycles recorded by PowerLab 8/30 acquisition system were analyzed using LabChart Pro software (AD Instruments; Colorado Springs, CO) to determine rates of pressure development, end diastolic pressure (EDP), and heart rate. Mean aortic pressure was measured by advancing the catheter into the ascending aorta.

### **Sample collection**

Mice were euthanized under a surgical plane of anesthesia, 6h post instillation by exsanguination through cardiac puncture. Hearts were rapidly harvested and flash frozen in liquid nitrogen. Bronchoalveolar lavage of the lungs was performed using 3 infusions of 0.6 ml saline each. The returned fluid volume was recorded and did not differ significantly between groups. The bronchoalveolar lavage fluid (BALF) and blood was rendered cell free by centrifugation. BALF and plasma was stored at -80°C until analysis. Total and differential cell counts were performed by light microscopy following Hema-Quick (ThermoFisher, Waltham, MA) staining.

### **Cytokine analysis**

Cytokines (KC, IL-6, TNF $\alpha$ ) were measured using Milliplex technology (Millipore Inc. MO). MCP-1/CCL2 was determined by ELISA (R&D Systems, MN). Total protein content in BALF samples was measured using Coomassie protein reagent (Pierce, Rockford, IL).

## Proteasome activity and adenosine triphosphate (ATP) determinations

Proteasome chymotryptic activity in homogenized myocardium was assayed as previously described using suc-LLVY as substrate<sup>20, 21</sup>. ATP content of myocardium was quantified by a colorimetric assay (Promokine, Heidelberg, Germany).

## Statistical evaluation

Results are reported as mean  $\pm$  standard deviation. Student's t-test was used for comparisons of two groups. Where three or more groups were compared, one-way analyses of variance (ANOVA) with post hoc analysis was used. *p*-values of  $<0.05$  were considered statistically significant. The ratio of the mean of the post-measurement to the mean pre-measurements for each mouse was used, and the analysis was weighted by the product of the pre- and post-measurements divided by the sum of the pre- and post-measurements. The log transformation was used to better meet the assumptions of the ANOVA model. Significant differences between groups were assessed using a post hoc Tukey's HSD test. In the age-related analysis of physiological parameters, two-way ANOVA was used to examine the association between each parameter and challenge and age. The data was processed with the weighting procedure described above.

## RESULTS

### LTA/PGN induces dose dependent neutrophil alveolitis and changes in pulmonary permeability

Pulmonary challenge, with LTA/PGN, induced an acute, dose dependent, pulmonary inflammation within 6h (Figure 1). In the young mice, there were significant increases in the total number of cells in the BALF, due to an influx of neutrophils (Figure 1C). The inflammation was also associated with increased concentrations of total protein in the lavage fluid (Figure 1D). Having established the dose responsive nature of the inflammatory response in the young mice, we then used the mid dose (LTA 30 $\mu$ g + PGN 100 $\mu$ g) to challenge the old mice.

Following challenge with saline alone, the old mice, compared to their young counterparts, had increased total cells (53.6 $\pm$ 13.3  $\times 10^3$  vs. 82.7 $\pm$ 13.2  $\times 10^3$  *p* $<0.01$ ) and macrophages (52.4 $\pm$ 13.5  $\times 10^3$  vs. 74.9 $\pm$ 15.4;  $\times 10^3$ ; *p* $<0.05$ ), but not neutrophils (1.2 $\pm$ 2.4 vs. 7.7 $\pm$ 8.3;  $\times 10^3$ ; *p*=0.09). However, challenge with LTA/PGN in the old mice, resulted in more total cells (Figure 1B) and neutrophils (Figure 1C), than in the same challenge in young mice. Furthermore, the concentration of total protein in BALF was increased in the older animals at baseline (saline challenge), compared to the young animals, and increased further post LTA/PGN challenge, indicative of increased pulmonary permeability.

### Cytokine response in BALF and plasma

Concentrations of cytokines with pro-inflammatory functions IL-6, TNF $\alpha$ , and chemokines with chemotactic activity for neutrophils (KC/CXCL1) or monocytes (MCP-1/CCL2) were measured 6h post instillation in young mice to determine the dose response. In both BALF (Table 1a) and plasma (Table 1b), dose dependent increases were observed for each cytokine with significant changes occurring at the mid and high doses. Compared to mice

that did not undergo surgery, young saline instilled mice had elevated plasma concentrations of KC (200 pg/ml vs. 4000pg/ml,  $p<0.01$ ) and IL-6 (0 vs. 377 pg/ml,  $p<0.01$ ).

In old mice instilled with LTA/PGN, concentrations of KC, IL-6 and TNF $\alpha$ , but not MCP-1, were increased significantly in BALF (Table 1a) whereas in plasma (Table 1b), concentrations of KC, IL-6 and MCP-1, but not TNF $\alpha$  and IL-10, were increased. Notably, plasma concentrations of the neutrophil chemotaxin KC were 2.3-fold lower in the old compared to the young mice after the same challenge. With the exception of MCP-1, which was increased in plasma of old saline instilled mice, there were no differences between age groups in BALF or plasma cytokines in the mice instilled with saline.

Cytokines are key factors in the pathogenesis of pneumonia and compartmentalization of the host response has been demonstrated in both animal and clinical studies<sup>22–25</sup>. Furthermore, the ratio of cytokine concentration between compartments can give valuable information concerning the origin of the cytokine<sup>25</sup> as well predict outcome<sup>26</sup>. Therefore, we examined the ratios of airspace and plasma cytokine concentrations in both young and old animals (Figure 2). The BALF/plasma ratio of both KC and IL-6 were significantly higher in the older animals suggesting a strong response in the airspaces, with a profound neutrophil chemotactic gradient in favor of the alveolae. However, the BALF/plasma ratio of TNF $\alpha$  was significantly lower in the older animals due to a greater increase in airspace TNF $\alpha$  in the younger group.

### Pulse oximetry measurements

Pulse oximetry performed on ambulatory young mice demonstrated a decline in heart rate with the mid (–48.9%) and high dose (–62.0%) challenges. Respiratory rates were significantly reduced in mid (–53.3%) and high dose (–62.3%). Arterial oxygen saturation remained unaltered in young mice at all doses of LTA/PGN. While the O<sub>2</sub> saturation in young mice showed no difference between saline and LTA/PGN instilled mice (4.6% and 1.4% decrease, respectively), old mice were severely affected, decreasing by 14% post challenge (saline instilled mice decreased by 0.8% ( $p<0.0001$ )) (Table 2). Under non-anesthetized conditions, heart- and respiratory rates were significantly decreased by challenge in both age groups and did not differ by age. Arterial O<sub>2</sub> saturation increased when mice were anesthetized with isoflurane delivered with oxygen, and under these conditions no difference in saturation was observed between saline and LTA/PGN instilled mice in either age group (all showing 99–100% saturation).

### Body temperature and hemodynamic analysis

Core body temperature was not altered in young mice under anaesthesia (saline  $35.0 \pm 1.1^\circ\text{C}$  vs LTA/PGN  $35.2 \pm 1.0^\circ\text{C}$ ), whereas it was significantly lower in old mice instilled with either saline ( $32.7 \pm 0.8^\circ\text{C}$ ) or LTA/PGN ( $28.4 \pm 1.2^\circ\text{C}$ ). Heart rates were recorded under anesthesia by non-invasive pulse oximetry (Figure 3A). To standardize LV pressure measurements among groups, the body temperatures of all animals were then brought to  $34^\circ\text{C}$  by external heat support. When heart rate was recorded by using an invasive micro-catheter (Figure 3A), heart rates decreased significantly in the young mice, in response to LTA/PGN, but remained unchanged in the old animals. Except for decreased heart rates,

none of the hemodynamic measurements obtained in the young mice showed significant change following LTA/PGN Challenge (Figure 3B–E). This was in contrast to the old mice in which the rate of maximum and minimum pressure development (max dP/dt, min dP/dt) changed significantly in response to LTA/PGN and re-warming (Figure 3B and C). EDP remained unaltered in both age groups, with or without LTA/PGN treatment (Figure 3D). Mean aortic pressures, indicative of peripheral blood pressure, were significantly higher in the old mice after LTA/PGN challenge and re-warming (from 60.4±9 to 76.9±4.2 mmHg,  $p < 0.05$ ), but remained unaltered in the young mice (Figure 3E). Measurements in saline instilled mice did not differ with age.

### Myocardial proteasome activity and ATP content

To examine the consequences of signaling pathway activation and ability to generate ATP in the myocardium during ALI, we measured proteasome activity and ATP content, respectively. No differences in ATP-dependent activity of the proteasome were observed in the hearts from saline instilled mice of either age group. The ATP-dependent activity of the proteasome in the myocardium of young mice was increased 4.1-fold with LTA/PGN instillation ( $p=0.04$  vs young saline), but remained unaltered in the old mice ( $p=0.66$  vs. old saline) (Figure 4A). Serial addition of exogenous ATP *in vitro*, to activate the proteasome, revealed an age-dependent shift in the total activity from 0–2  $\mu\text{M}$  ATP in the young to 2–5  $\mu\text{M}$  ATP for the old. Specificity of the proteasome chymotryptic measurements was ensured by use of the specific inhibitor lactacystin (data not shown). Endogenous ATP content of the myocardium remained unaltered with challenge in the young mice (1.53±0.28 vs 1.61±0.34 nmol/mg tissue), but increased with challenge and re-warming in the old mice (1.43±0.46 vs 2.16±0.22;  $p<0.01$  Figure 4B).

## DISCUSSION

The elderly population is at increased risk of airway infections, particularly pneumonia that can progress to sepsis and the acute respiratory distress syndrome<sup>1,2</sup>. Under these circumstances, the elderly patient population has a significantly worse prognosis. Furthermore, data from 3147 individuals enrolled in the Sepsis Occurrence in Acutely ill Patients (SOAP)<sup>14</sup> study suggest that predominant characteristics of the group were males (62%) with a median age of 64. The lung was the most common site of infection (68%), with 40% of infections resulting from Gram-positive organisms. To assist in identifying therapeutic targets and provide improved treatment of these patients, we sought to determine possible age associated differences in immunological and cardiopulmonary functions in the early, injury phase, of ALI following pulmonary challenge. For these studies, in an endeavor to recapitulate the clinical situation<sup>14</sup> we used a model in which the lungs of male C57B16 mice, approximating human age groups of 20 yrs and 55–60 yrs, were challenged with Gram-positive cell wall components, and the inflammatory responses monitored. While a recent study questioned the validity of mouse models for studies of inflammation<sup>27</sup> others have reevaluated the same data and suggested that the gene expression patterns in mouse models mimic those in human inflammatory conditions<sup>28</sup>. In addition, a recent review suggested that, while they need to be confirmed in clinical studies, animal studies can reveal

age specific changes that could lead to a better understanding and treatment of older individuals<sup>29</sup>

The pulmonary challenge, consisting of LTA and PGN derived from the cell walls of *S. aureus*, induced a dose-dependent response on cytokine elaboration and cellular migration to the site of insult in young mice. As in our previous studies of acute lung inflammation, we used male C57Bl6 mice<sup>30</sup>. It should be noted that there are physiological changes in the ageing lung (sometimes referred to as senile emphysema) that include dilation of the airways, enlargement of airspaces and decrease in exchange surface area<sup>31, 32</sup>. These changes could alter the way that intratracheal instillates deposit in the lungs of various ages. However, in our previous studies, to visualize distribution of the intratracheally delivered substances into the lungs, Evans Blue was administered intratracheally in a similar manner to that used in this study<sup>33</sup>. These studies showed an even distribution to both lungs, and to the distal parts of the inferior lobes.

In age comparative studies, challenge resulted in 2.3-fold higher plasma concentrations of the neutrophil attracting chemokine KC/CXCL-1 in young mice than in old mice. The higher BAL value combined with the lower plasma concentration of KC/CXCL-1 observed in the old mice creates a steep chemotactic gradient in relation to the alveolar space, potentially increasing neutrophil trafficking towards the lung in an age dependent manner. We have shown previously, in humans, that the concentration of Interleukin-8 (CXCL8; a human homologue of KC) within the airspaces is correlated with the accumulation of neutrophils within the airspaces, and highly elevated concentrations are associated with increased mortality<sup>34</sup>. Indeed, the presence of increased interleukin-8 in the alveolar space precedes the development of acute lung injury<sup>35</sup>. Accordingly, we also found approximately twice the number of neutrophils in the lungs of aged mice following challenge at this time point. A similar finding was reported by Gomez *et al* who, after intraperitoneal injection of lipopolysaccharide (LPS) in mice, observed significantly increased alveolar cell counts in aged mice compared to young counterparts<sup>36</sup>. While poor chemotaxis of neutrophils from old donors has been reported<sup>37</sup>, those studies were performed using human neutrophils isolated from whole blood. In previous publications, we have shown that migration across endothelial-epithelial bilayers mimicking neutrophil diapedesis into the alveolar space during infection, significantly alters neutrophil lifespan and gene expression<sup>38, 39</sup>. This indicates that lung-recruited neutrophils display a different expressional program than those isolated from whole blood, and represents an important consideration when comparing results from different studies. Our conclusion based on our *in vivo* findings takes into account the relationship between local and systemic cytokine concentrations and the chemotactic gradient it creates.

Another indicator of the increased severity in the older animals is the increased IL-6 BAL/plasma ratio, which has been shown in human studies to predict poor outcome<sup>26</sup>. However, the BALF/plasma ratio of TNF $\alpha$  was statistically significantly lower in the older animals. While there were robust increases in TNF $\alpha$  in the BALF of both young and old mice, there were insignificant changes in the plasma of each group. Thus, while the overall change in BAL/plasma ratio may be statistically significant, it may not reflect biological significance. Indeed, since the BAL sampling technique may dilute airspace proteins by around 100-

fold<sup>34</sup> the plasma concentration is only approximately 0.01% of the concentration in the epithelial lining fluid at that time. Thus the predominant response to the challenge is clearly alveolar.

While separate studies in young mice demonstrate that the mid dose did not induce lethality (data not shown), clinical studies suggest increased TNF $\alpha$  in BALF and serum are higher in non-survivors than patients who survive community acquired pneumonia<sup>40</sup>. In the current study we examined the responses at a single time point and with a dose of LTA/PGN which was tolerable both in the young and old animals, and examined the early responses. Interestingly the BALF/plasma ratio of both KC and IL-6 were significantly higher in the older animals suggesting a strong airspace response, with a profound neutrophil chemotactic gradient in favor of the alveolae. However, the BALF/plasma ratio of TNF $\alpha$  was significantly lower in the older animals due to a greater increase in airspace TNF $\alpha$  in the younger group. This suggests that the inflammatory response may not just vary in size and time, but also is different in various compartments. This suggests that future studies should examine both the magnitude of individual compartmental responses and their temporal elaboration.

Maintaining cardiac output is critical for survival during severe inflammation. Age dependent differences in hemodynamic measurements were evident only after LTA/PGN challenge in the old animals. Studies in naive Wistar rats have shown that heart function deteriorates between 24 and 28 months of age due to decreased left ventricle compliance<sup>41</sup>. In contrast, the aged mice used in the current study did not show reduced compliance or dysfunction compared to the young mice at baseline as measured by LV EDP and rates of systolic and diastolic pressure development ( $\pm$ dP/dt) (Figure 3B and C). However, age was an important factor in the hemodynamic response to pulmonary LTA/PGN challenge. Using non-invasive measurements, the key observations were that challenge together with old age was associated with hypothermia ( $28.4\pm 1.2^{\circ}\text{C}$ ) and hypoxemia ( $79.3\pm 5.3\%$ ), although heart rates were significantly lower after challenge in both age groups. This is in contrast to the human symptoms of acute lung inflammation and its possible consequences in ALI where tachycardia and tachypnea are characteristic. Possibly the current time point (6h) and dosage, in fact resembles a further progressed scenario than the hyper-dynamic state often observed in human sepsis.

To perform catheter-based blood pressure measurements, all animals were anesthetized, mechanically ventilated with 97.5% oxygen, and the core body temperature controlled at  $34^{\circ}\text{C}$  using a warming blanket. The effects of the heat adjustment on heart rates are shown in Figure 3A. Surprisingly, the heart rates of old mice, but not young mice, increased to rates observed in saline control animals, possibly as a response to increasing body temperature of the old mice by  $\sim 6^{\circ}\text{C}$ . Hemodynamic measurements made under these conditions, showed that hearts of old mice were hyper-dynamic in response to LTA/PGN instillation (i.e., significantly increased rates of pressure development,  $\pm$  dP/dt; Figure 3). This occurred despite a significant increase in mean aortic blood pressure in the old mice, suggesting peripheral vasoconstriction. Whether these observations support an excessive catecholaminergic response is unclear. However, sympathetic overstimulation is a common characteristic of critical illness that can have adverse effects on the heart producing

tachycardia, myocardial ischemia, and impaired diastolic function<sup>42</sup>. Aging is associated with altered autonomous control of the cardiovascular system and, like critical illness, aging can lead to a heightened sympathetic tone<sup>43</sup>. As reviewed by Court *et al*, systemic sepsis in humans is characterized by a hyper-dynamic circulatory state with high cardiac output in the early stages of disease progression in non-survivors<sup>44</sup>. We observed no mortality in the old mice but did not proceed past 6h with this group.

Proteasomes are large multi-subunit protein complexes responsible for the majority of non-lysosomal proteolysis occurring in cells, and the proteolytic activity is dependent on ATPase activity<sup>20</sup>. One of most important functions of the ubiquitin proteasome system (UPS) is intracellular protein quality control<sup>45</sup>. The UPS also regulates many cell signaling pathways<sup>16</sup>, including those induced by LPS<sup>46</sup> and is also impaired in multiple organs, including the lung and heart, in senescent animals<sup>17, 47</sup>. A subset of proteasomes known as immunoproteasomes regulates antigen presentation and immune function<sup>48</sup>. Cardiac dysfunctions are major complications in patients with trauma, infection, or burns<sup>49</sup>. Since cardiac proteasome functionality may be a limiting factor in maintaining cardiac function during severe inflammation, we assessed proteasome activity. Specifically, we determined the ability of ATP to activate the proteasome to assess the ubiquitin dependent functions of this proteolytic complex<sup>20</sup>. Intracellular ATP levels have been shown to regulate proteasome activity in a bimodal manner<sup>50</sup>. Young mice exhibited increased activation of cardiac proteasome suggesting a response to the enhanced signaling and protein turnover imposed by the inflammatory challenge. We have shown that in response to LPS challenge proteasome activity is markedly increased in murine kidney<sup>51</sup>. Old mice did not exhibit increased activation, suggesting an impaired UPS response which could suggest an impaired immune response and protein quality control. Furthermore, the cardiac proteasomes from old mice required more exogenous ATP to reach the maximum level of activation, suggesting a defective or possibly delayed assembly of the proteolytic subunits. Age dependent impairment of ATP generative pathways has previously been described in hearts of old (22–24m) mice<sup>52</sup>. To determine whether ATP supply was limited in the old hearts, explaining the lack of UPS activation, we next determined myocardial ATP content. The results show that levels in young mice were unaltered by challenge. In contrast, the hearts from old mice responded to the challenge by increased ATP content indicating a sufficient capability to generate ATP. We conclude that the discrepancy in ATP generation and proteasome ATP dependent activity in the old mice suggests a deficiency in ATP utilization by the myocardial UPS. A recent study has suggested interplay between impaired proteasome and increased response to inflammatory mediators<sup>53</sup>. Moreover, inhibition of the proteasome has recently been shown to have detrimental effect in LPS or *S.aureus* superantigen induced systemic inflammation<sup>54</sup>. Further studies in this area are needed to discern the nature of the changes of the changes in the old mice, and whether they are due to altered constitutive- or immune-proteasome<sup>55</sup>. In addition, distinct proteasome subpopulations have been described in patients with acute lung injury<sup>56</sup>. A better understanding of these various types of proteasome, and the changes that can occur may lead to the development of targeted therapeutics to alter specific forms of the proteasome to protect against cardiac dysfunction resulting from infection, particularly in the elderly.

## CONCLUSIONS

We have developed a mouse model of Gram positive non-progressive ALI, which is both dose dependent and associated with relevant inflammatory and physiological changes that are seen in the human acute respiratory distress syndrome. The pulmonary challenge induces age-related increased airspace inflammation and both pulmonary and cardiac dysfunction. The altered cardiac function was associated with altered efficiency of the ubiquitin–proteasome system, which is critical in the regulation of many cellular processes, including the cell cycle, immune responses, apoptosis, cell signaling, and protein turnover under normal and pathological conditions.

Taken together these findings demonstrate clinically relevant age-dependent alterations in the response to pulmonary inflammation that accentuates this patient group as particularly sensitive. Our findings may aid in the development of new targeted therapeutic approaches to acute inflammation in the elderly patient population.

## Acknowledgments

We thank Drs Christine Metz and Xiangying Xue for MCP-1/CCL2 analysis; Dr Andras Divald for assistance in performing proteasome activity assays; and Ms Erica Spessot for assistance with the editing of the manuscript. Financial support for this work was from The National Institutes of Health: NIH R01-HL 081655 (EJM), The Swedish Research Council (HML) and Swedish Society for Medical Research (HML).

## List of Abbreviations

<b>ALI</b>	acute lung injury
<b>ANOVA</b>	one-way analyses of variance
<b>ATP</b>	adenosine triphosphate
<b>BALF</b>	bronchoalveolar lavage fluid
<b>CXCL8</b>	chemokine (C-X-C motif) ligand 8
<b>dP/dt</b>	rate of systolic and diastolic pressure development
<b>EDP</b>	end diastolic pressure
<b>ELISA</b>	enzyme linked immunosorbent assay
<b>IL-6</b>	interleukin-6
<b>KC</b>	chemokine (C-X-C motif) ligand 1
<b>LTA</b>	lipoteichoic acid
<b>LPS</b>	lipopolysaccharide
<b>LV</b>	left ventricular
<b>MCP-1/CCL2</b>	monocyte chemotactic protein 1/ chemokine (C -C motif) ligand 2
<b>PGN</b>	peptidoglycan
<b>Suc-LLVY</b>	<i>N</i> -Succinyl-Leu-Leu-Val-Tyr

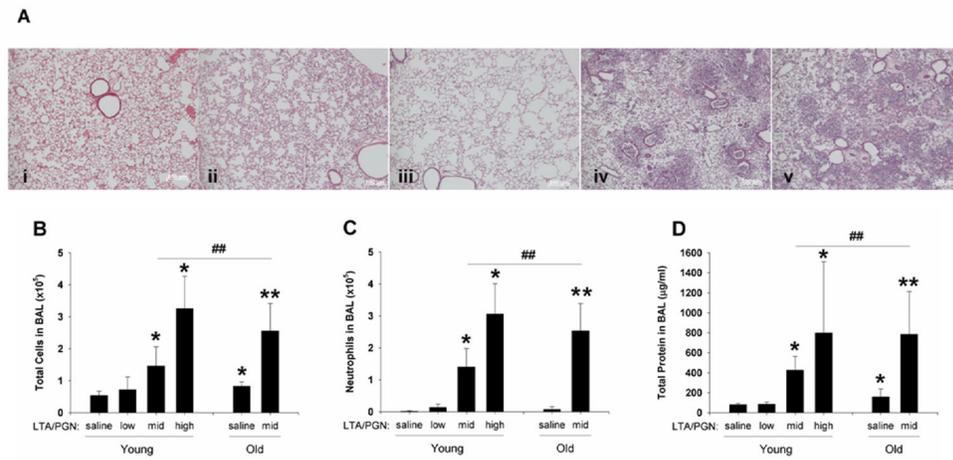
<b>TNF<math>\alpha</math></b>	tumor necrosis factor alpha
<b>Tukey's HSD test</b>	Tukey's Honest Significant Difference test
<b>UPS</b>	ubiquitin proteasome system

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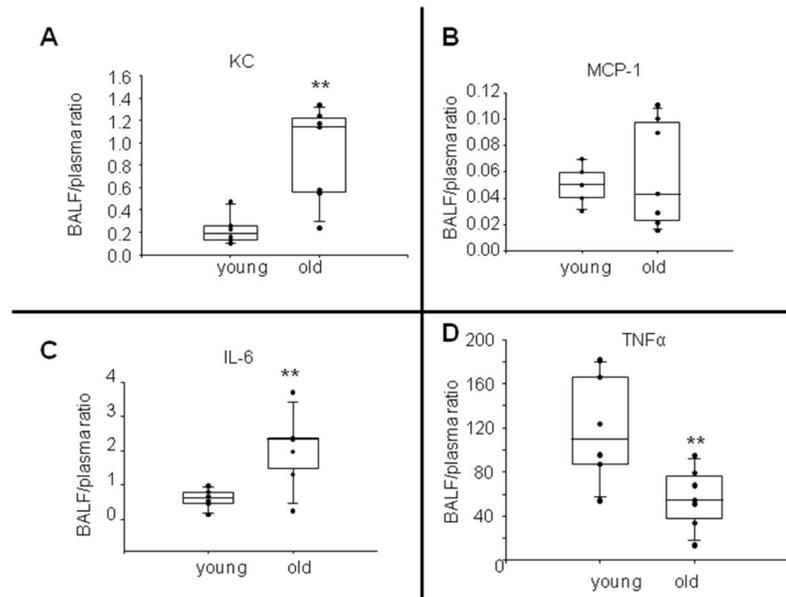
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### Figure 1. Dose dependent inflammatory effects of pulmonary challenge

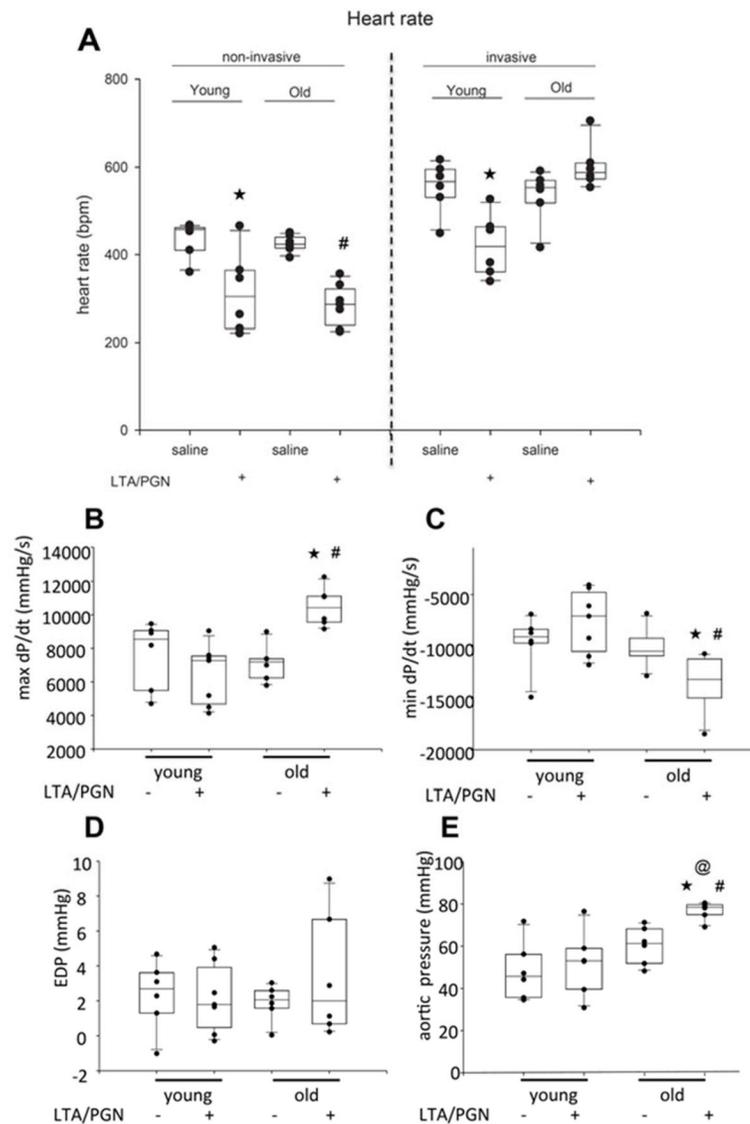
**A)** H&E stained sections of lungs of young mice, fixed at inflation. i) cage controls; or 6h post challenge with ii) saline; iii) low (0.2µg+0.66µg); iv) mid (30µg+100µg) or v) high (150µg+500µg) dose of LTA/PGN. Groups of young mice (n=6) were instilled intratracheally with saline or LTA/PGN (low, mid or high dose), and groups of old mice (n=6) were similarly challenged with either saline or LTA/PGN (mid dose). 6h later the lungs were lavaged, *post mortem*, and the BALF assessed for:

**B)** total cell content; **C)** neutrophils and **D)** total protein. \* p<0.05 vs. young saline, \*\* p<0.05 vs. old saline, ## p<0.05 vs. young mid dose LTA/PGN.



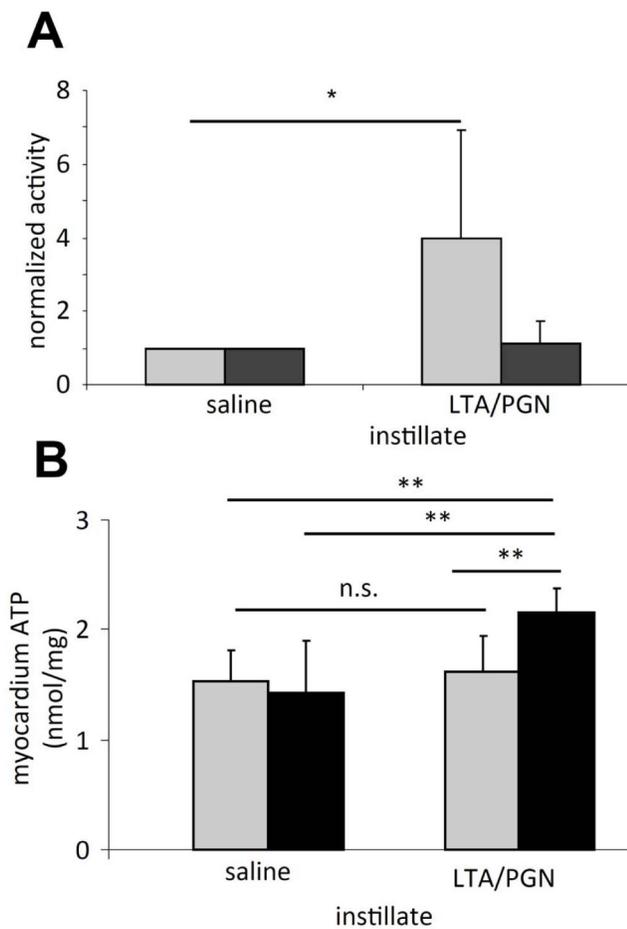
**Figure 2. Relative cytokine concentrations in alveolar and vascular compartments**

To gain better insight into the dynamics between the airspaces, where the challenge occurred and the vascular compartment, BALF/plasma ratios of (A) KC, (B) MCP-1, (C) IL-6, and (D) TNFα concentrations in individual mice challenged with the mid dose of LTA 30μg + PGN 100μg were calculated. Statistically significant differences between the median ratio in young and old animals is indicated \*\* p<0.05



**Figure 3. In vivo hemodynamic studies**

Heart rates (A) were measured in anesthetized mice 6h post instillation of saline or LTA/PGN in young and old mice by pulse oximetry (non-invasive; left side of graph) or micro-catheter (invasive; right side of graph). To standardize measurements all mice were warmed to 34°C prior to hemodynamic measurements. LV pressure measurements were recorded using a micro-catheter inserted through the cardiac apex. Graphs display max dP/dt (B), min dP/dt (C), EDP (D), and aortic mean pressure (E). \*p<0.05 vs. young saline, #p<0.05 vs. old saline, @p<0.05 vs. young mid dose LTA/PGN.



**Figure 4. Age dependent responsiveness in cardiac proteasome activity following pulmonary challenge**

Chymotryptic activity of myocardium proteasomes was measured by suc-LLVY fluorescent substrate. ATP-dependent proteasome activity (total activity minus ATP independent activity; **A**) in the myocardium of young (grey bars) and old (black bars) mice 6h after LTA/PGN installation has been normalized to saline instilled groups. \*  $p < 0.05$  vs. saline. Content of endogenous ATP in mouse myocardium, 6h after either saline or LTA/PGN instillation, was measured by colorimetric assay (**B**). \*\* $p < 0.01$  as indicated.

Table 1

## Cytokine concentrations

Cytokine concentrations in BALF (A) and plasma (B), in young and old mice 6h post intratracheal challenge with either saline or low, mid or high concentrations of LTA+PGN. KC/CXCL1, IL-6, and TNF $\alpha$  were determined by Milliplex technology (Millipore, MO) and MCP-1/CCL2 was determined by ELISA (R&D Systems, MN).

BALF	Young Mice				Old Mice	
	Saline	Low	Mid	High	Saline	Mid
KC (ng/ml)	29 $\pm$ 24	91 $\pm$ 103	3262 $\pm$ 1133*	13322 $\pm$ 13031*	40 $\pm$ 26	5430 $\pm$ 2965* §
MCP-1 (ng/ml)	nd	nd	31 $\pm$ 11*	109 $\pm$ 105*	33 $\pm$ 38	63 $\pm$ 38
IL-6 (ng/ml)	6 $\pm$ 4	30 $\pm$ 31	1790 $\pm$ 789*	4596 $\pm$ 4456*	174 $\pm$ 218	4516 $\pm$ 3513* §
TNF $\alpha$ (ng/ml)	nd	nd	1652 $\pm$ 891*	1288 $\pm$ 620*	nd	2586 $\pm$ 1730§

Plasma	Young Mice				Old Mice		
	Saline	Low	Mid	High	Saline	Mid	High
KC (ng/ml)	3967 $\pm$ 2347	2702 $\pm$ 2301	15820 $\pm$ 9353*	68179 $\pm$ 69178*	2775 $\pm$ 1111	6971 $\pm$ 3809§	
MCP-1 (ng/ml)	270 $\pm$ 166	276 $\pm$ 185	591 $\pm$ 215*	1550 $\pm$ 1199*	733 $\pm$ 241	1207 $\pm$ 340§	
IL-6 (ng/ml)	377 $\pm$ 145	600 $\pm$ 238	2488 $\pm$ 647*	8254 $\pm$ 8161*	264 $\pm$ 111	2689 $\pm$ 1864§	
TNF $\alpha$ (ng/ml)	nd	nd	12 $\pm$ 4*	23 $\pm$ 16*	44 $\pm$ 31	33 $\pm$ 10	

nd not detectable;

\* p<0.05 vs. young saline,

§ p<0.05 vs. old saline

**Table 2**

Oxygen saturation in ambulant mice, pre- and 6hr post pulmonary challenge with the mid dose (LTA 30 $\mu$ g + PGN 100 $\mu$ g) was assessed.

	<b>O<sub>2</sub> saturation (%)</b>	
	<b>0h</b>	<b>6hr</b>
Young Saline	95.8 $\pm$ 1.1	94.6 $\pm$ 2.6
Young LTA/PGN	95.1 $\pm$ 2.5	92.2 $\pm$ 4.1
Old Saline	94.9 $\pm$ 1.7	93.8 $\pm$ 1.7
Old LTA/PGN	93.8 $\pm$ 3.6	79.3 $\pm$ 5.3* #

In the old animals, statistically significant differences occurred between the values 6hr post challenge and their baseline values

\* p<0.005 ; and between old vs. young challenged with LTA/PGN

# p<0.005.