Reactive oxygen species regulate neutrophil recruitment and survival in pneumococcal pneumonia

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Online Data Supplement

Methods

Histopathology scoring.

Two experienced histopathologists examined tissue sections. All analysis was performed blinded. As a semi-quantitative assessment of neutrophil recruitment lung sections were scored for the presence of areas of consolidation (areas of confluent alveolar neutrophilic infiltration in the alveoli >0.5 mm). A score of zero was assigned if areas of consolidation were <0.5mm and a score of one if areas were >0.5mm. No sections demonstrated significant desquamation of alveolar epithelial cells, desquamation of epithelial cells in the bronchi nor features of rupture of alveolar walls, alveolar collapse, evidence of alveolar haemorrhage or diffuse alveolar damage (defined as one of the presence of hyaline membranes, necrosis of type 1 pneumocytes or endothelial cells, edema, organizing interstitial fibrosis and prominent proliferation of type II pneumocytes) and so semi-quantitative scoring of lung injury was not possible.

Detection of neutrophil apoptosis by Hoechst 33342 staining

BAL was spun at 1000xg at 4°C for 5 mins and cells were preblocked on ice with 1μg Fc-block (BD Biosciences) for 5 mins. Ly6G-PE staining was used to identify neutrophils (E1). Hoechst 3342 (Molecular Probes) was added to give final concentration of 1μg/ml and cells were incubate at room temperature for 10 mins in the dark. Cells were washed in ice cold PBS and re-suspended in ToPro3 in PBS and analysed by flow cytometry (E2).

Neutrophil activation

BAL neutrophil activation was determined by upregulation of surface CD11b and CD18 and shedding of surface CD62L. Cells were blocked with 25μg mouse IgG_1 (Sigma, Poole, UK) for 15 min at 4°C, then incubated with 1μg anti-Ly6G-FITC (neutrophil marker), and 1μg

anti-CD11b-PE, anti-CD18-PE or anti-CD62L-PE (or isotype antibodies) for 15 min at 4°C. Cells were analysed on a FACSCalibur® flow cytometer using CellQuest® software (BD Pharmingen).

Neutrophil elastase activity

Neutrophil elastase activity in BAL was measured by the release of 4-nitroaniline from Ac-Ala-Ala-Ala-pNA (Sigma, Poole, UK) as described previously (E3). One unit of activity was defined as the release of 1nmol 4-nitroaniline/min.

Myeloperoxidase Assay

Lungs were flushed clear of blood, snap frozen in liquid nitrogen and stored at -80°C. Lungs were homogenised in 4ml HTAB buffer (5mg/ml hexadecyltrimethylammonium bromide in 50mM potassium phosphate pH 6.0), sonicated 10 seconds and then freeze/thawed 4 times. The homogenate was centrifuged at 14,000 x *g* for 30 mins at 4°C and the supernatant diluted in equal volume of HTAB buffer. 100µl of diluted supernatant was added to 1.9ml o-dianisidine solution (0.167 mg/ml o-dianisidine hydrochloride, 0.0005% H_2O_2 in 50mM potassium phosphate pH 6.0) and the change of absorbance at 450nm from 30s to 90s measured to give relative myeloperoxidase activity.

Direct ELISA for mouse Secretory Leukocyte Protease Inhibitor (mSLPI)

Mouse SLPI was detected by in-house direct ELISA. In brief, 50µl of mouse BAL (and/or dilutions), and purified mouse SLPI were added to a 96 well immunsorb (Scientific Laboratory Supplies, Coatbridge, UK) plate in duplicate and incubated at room temperature overnight. Plates were then washed three times in 0.05% Tween 20 / PBS before incubating with 2µg/ml biotinylated goat anti-mouse SLPI antibody in PBS (R & D Systems, Abingdon,

UK) (E4) for 2 hours at room temperature. Purified goat IgG was used in parallel to assess non-specific binding which was negligent under these conditions. Plates were then washed three times in 0.05% Tween 20 / PBS before incubating with 0.25µg/ml HRP-streptavidin/PBS (Invitrogen, Paisley, UK) for 20 min. Next plates were washed three times in 0.05% Tween 20 / PBS before adding 100µl of SureBlue™ TMB microwell peroxidase substrate (Insight Biotechnology, Wembley, UK) for up to 30 minutes. Colour reactions were terminated by addition of 100µl of 1M HCl before determination of optical density at 450nm on a Synergy™ HT multi detection microplate reader with Gen5™ software (Fisher Scientific UK, Loughborough, UK).

References

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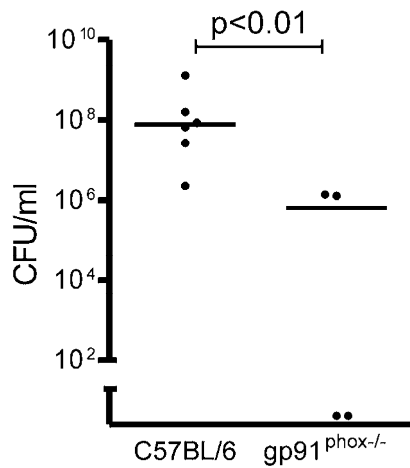

Figure E1. Reduced bacteria in blood from gp91$^{phox-/-}$ mice after intraperitoneal pneumococcal infection. Bacteria in blood from wild type C57BL/6 controls and gp91$^{phox-/-}$ mice 24 hours after intraperitoneal infection with 10^4 CFU type 2 pneumococci, Mann Whitney.

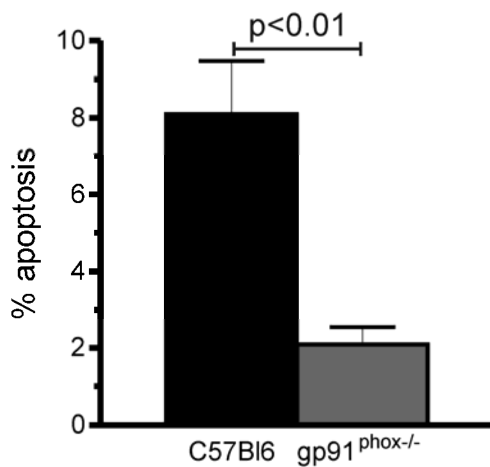

Figure E2. gp91$^{phox-/-}$ mice have reduced numbers of apoptotic neutrophils in the lung after infection with type 2 pneumococci. Percentage neutrophil (PMN) apoptosis (Ly6G$^{+/}$Hoechst^{+}/TO-PRO-3^{-}, flow cytometry) in bronchial alveolar lavage from C57BL/6 (n=3) and gp91$^{phox-/-}$ mice (n=4) 48 hours after intratracheal instillation of 10^7 CFU type 2 pneumococci. Mean + SEM, t-test.

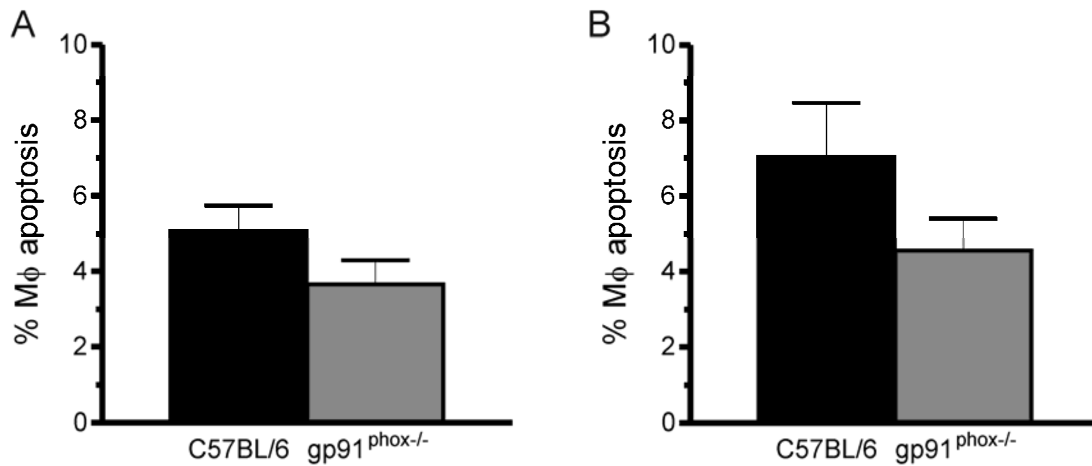

Figure E3. No difference in macrophage apoptosis in the lung after infection with type 2 pneumococci. Percentage macrophage apoptosis (Annexin-V-PE$^+$/TO-PRO-3$^-$, flow cytometry) in bronchial alveolar lavage from C57BL/6 and gp91$^{phox-/-}$ mice A) 24 hours and B) 48 hours after intratracheal instillation of 10^7 CFU type 2 pneumococci, in the same experiments as Figure 5 D) and E) respectively. n=5, Mean + SEM, t-test.

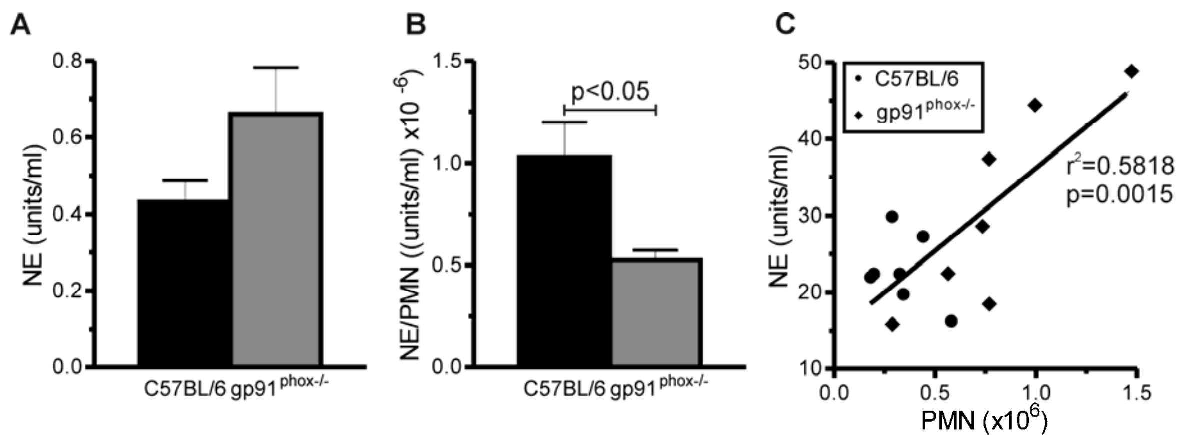

Figure E4. Neutrophil elastase production after infection with type 2 pneumococci. A) Levels of neutrophil elastase (NE) in bronchial alveolar lavage (BAL) from C57BL/6 (n=13) and gp91$^{phox-/-}$ mice (n=12), 24 hours after intratracheal instillation of 10^7 CFU type 2 pneumococci D39. B) Levels of NE normalised to neutrophil numbers (PMN) in a subset of A), n=7. Mean+SEM, Mann Whitney. C) Relationship between NE levels and #PMN in BAL from the same experiments as in B).

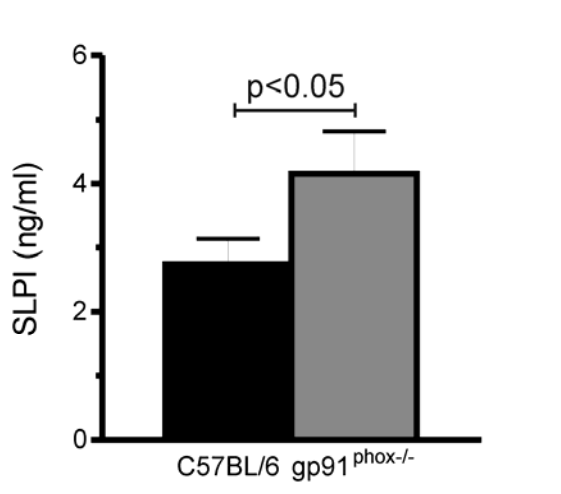

Figure E5. Secretory leucoprotease inhibitor production after infection with type 2 pneumococci. Levels of secretory leucoprotease inhibitor (SLPI) in bronchial alveolar lavage (BAL) from C57BL/6 (n=10) and gp91$^{phox-/-}$ mice (n=7), 24 hours after intratracheal instillation of 10^7 CFU type 2 pneumococci D39, Mean+SEM, Mann Whitney.

Table E1. Infection with pneumococci lacking pyruvate oxidase activity

	C57BL/6	$gp91^{phox-/-}$
Blood (CFU/ml)	$2.7x10^{3*}$ $(0-2.0x10^{8})^{\dagger}$ n=7	$6.7x10^{3}$ $(3.3x10^{2}-3.3x10^{6})$ n=7
Lung (CFU/lung)	$1.5x10^{5}$ $(5.0x10^{3}-8.0x10^{6})$ n=7	$2.7x10^{4}$ $(2.0x10^{3}-1.2x10^{7})$ n=7

* - median; † - interquartile range

CFU – colony forming units

Bacteria in blood and lung homogenates 24hrs post infection with 24 hours after instillation of 10^{7} CFU type 2 pneumococci lacking pyruvate oxidase activity ($SpxB^{-}$)