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Contrasting roles for reactive oxygen species and nitric oxide in the innate response to pulmonary infection with *Streptococcus pneumoniae*

Helen M. Marriott, Paul G. Hellewell, Moira K.B. Whyte, David H. Dockrell *

School of Medicine and Biomedical Sciences, L-Floor, University of Sheffield, Beech Hill Road, Sheffield S10 2RX, UK

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Abstract

The pulmonary innate response to low-dose bacterial challenge requires functioning alveolar macrophages (AM) but also subsequent macrophage apoptosis. To address the role of reactive oxygen species (ROS) and nitric oxide (NO) in AM apoptosis, sub-clinical *Streptococcus pneumoniae* infection was established in gp91$^{phox-/-}$ and inducible NO synthase deficient (iNOS$^{-/-}$) mice. Both AM apoptosis and the number of macrophages containing apoptotic bodies are reduced in iNOS$^{-/-}$ as compared to control or gp91$^{phox-/-}$ mice. iNOS$^{-/-}$ mice recruit neutrophils and generate TNF-α to compensate for impaired AM competence but ROS deficiency has no apparent effect on AM function in this model.

Keywords: Macrophages; Apoptosis; Pneumococci; Mice; Nitric oxide

1. Introduction

Following aspiration of small numbers of bacteria into the distal airways alveolar macrophages play a critical role ingesting and removing bacteria [1]. The fate of these alveolar macrophages has previously been uncertain but we have demonstrated that increased alveolar macrophage apoptosis is a feature of sub-clinical challenge with small inocula of *Streptococcus pneumoniae* [1]. Alveolar macrophage apoptosis allows both clearance of cells that have phagocytosed bacteria and provides a potential source of pneumococcal antigens for presentation by antigen presenting cells, thus linking the innate and adaptive immune responses [2]. Previous studies have demonstrated a link between pneumococcal phagocytosis and killing and induction of apoptosis, suggesting that microbicidal molecules could contribute to the induction of alveolar macrophage apoptosis [3].

Macrophages, like neutrophils, contain the NADPH oxidase complex [4] and generate reactive oxygen species (ROS) following phagocytosis of bacteria [5], although whether ROS generated through the myeloperoxidase system plays a significant role in host defense in macrophages remains unclear [6]. Macrophage-derived ROS induces alveolar macrophage apoptosis in certain settings [7]. Nevertheless, the exact role of ROS in the innate response to pulmonary challenge with pneumococci is undetermined. The major source of nitric oxide (NO) in macrophages is from inducible nitric oxide synthase (iNOS) and this is believed to be of particular importance in the macrophage during infection with intracellular pathogens [6]. NO can mediate apoptosis via a number of mechanisms [8]. We have recently demonstrated that NO also contributes to macrophage killing of pneumococci [9] and NO deficiency has been associated with decreased clearance of bacteria from the lung in iNOS deficient mice [10].

Since both ROS and NO contribute to induction of apoptosis in a variety of settings [8,11] we examined the role of each in the induction of alveolar macrophage apoptosis using well characterised mice that either lack gp91^{phox}, a

* Corresponding author. Tel.: +44 114 2724072; fax: +44 114 2713892.
E-mail address: d.h.dockrell@sheffield.ac.uk (D.H. Dockrell).

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component of the NADPH oxidase system (gp91phox$^{-/-}$ [12]) or iNOS (iNOS$^{-/-}$ [13]). We studied these mice using a low-dose sub-clinical infection model in which alveolar macrophages can clear small numbers of bacteria without recruitment of other inflammatory cells [1]. This model is characterised by alveolar macrophage apoptosis and provides an opportunity to investigate the role of both ROS and NO in alveolar macrophage apoptosis and the clearance of apoptotic cells in a setting where the apoptotic cells are derived solely from alveolar macrophages and not epithelial cells or recruited inflammatory cells [1].

2. Materials and methods

2.1. Animals

iNOS and gp91^{phox} deficient mice backcrossed onto a C57BL/6 background were obtained from Jackson Laboratories (Bar Harbor, Maine, US) and maintained as homozygous colonies. C57BL/6 mice (Harlan, Bicester, UK) were used as wild type controls. Female mice were used throughout. All animal experiments were conducted in accordance with the Home Office Animals (Scientific Procedures) Act of 1986 and received local ethical committee approval.

2.2. Pneumococcal infection model

Infection of mice with 10^4 (or in selected experiments 10^7) colony forming units (CFU) type 1 pneumococci (Statens Serum Institut, Copenhagen, Denmark) or mock infection with PBS was by direct tracheal instillation after anaesthesia with ketamine (100 mg/kg i.p.) and acepromazine (5 mg/kg i.p.) as previously described [1].

2.3. Collection of bronchial alveolar lavage, blood and lungs

Mice were killed by overdose of sodium pentabarbitone and exsanguinated by cardiac puncture. Bronchial alveolar lavage (BAL) was performed as described [1]. The cell differential was determined by review of cytospin preparations, with differentials determined by analysing the number of each cell type in a total population of 300 cells and then determining the percentage for that cell type [1]. Viable bacterial counts in lung were obtained as described [14].

2.4. Detection of apoptosis

Apoptosis detection was by nuclear morphology on cytospin preparations as described [1]. Three hundred cells were analysed per cytospin. The identity of cells as macrophages was confirmed by identifying F4/80 positivity [1,15]. Clearance of apoptotic bodies was estimated by determining the number of alveolar macrophages with internalised apoptotic bodies per 300 cells in Diff-Quik stained cytospins.

2.5. Cytokine production

TNF-α in BAL was measured using an eBioscience mouse TNF-α ELISA Ready-SET-Go reagent set (Insight Biotechnology Limited, Wembley, UK) following the manufacturer's protocols. Limit of detection was 15 pg/ml.

2.6. Statistics

Results are recorded as mean and S.E.M. Statistical testing was performed using ANOVA with Bonferroni's Multiple Comparison Test by Prism 4.0 software (GraphPad Inc.). Differences in microbiologic outcome were assessed using the Mann–Whitney test. Significance was defined as $p < 0.05$.

3. Results

3.1. gp91$^{phox-/-}$ and iNOS$^{-/-}$ mice clear a low-dose pneumococcal challenge from the lung

Instillation of a small inoculum of type 1 pneumococci results in clearance of bacteria from the lung over a 24 h period without demonstrable recruitment of neutrophils or other inflammatory cells at any time point in C57BL/6 mice [1]. When these experiments were repeated with either iNOS$^{-/-}$ or gp91$^{phox-/-}$ mice bacterial clearance was also similar to C57BL/6 mice, confirming both models remained models of sub-clinical resolving infection, Fig. 1. Mice remained well without signs of illness and blood cultures showed no evidence of bacteremia in the majority of mice (data not shown). Examination of cytospins from bronchial alveolar lavage showed no evidence of recruitment of neutrophils or other inflammatory cells (data not shown).

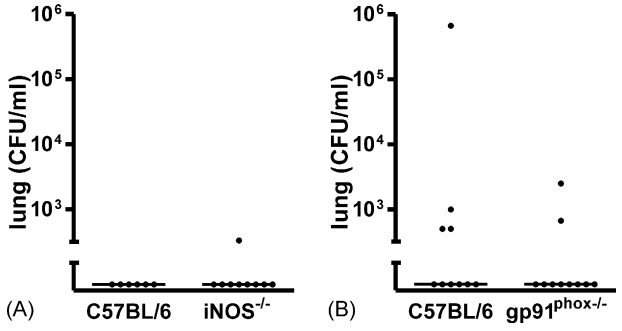

Fig. 1. No difference in bacteria in lungs from iNOS or gp91^{phox} deficient mice after low-dose pneumococcal infection. Bacteria in lung homogenates 24 h after intratracheal instillation of 10^4 CFU type 1 pneumococci in (A) wild type controls (C57BL/6, $n = 6$) and iNOS deficient mice (iNOS$^{-/-}$, $n = 9$) and (B) C57BL/6 mice, $n = 10$ and gp91^{phox} deficient (gp91$^{phox-/-}$, $n = 10$) both from three independent experiments.