Distinct phenotypic expression levels of macrophages in neonatal lungs

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Abstract. Alveolar macrophages are the front-line defense against environmental pathogens. However, to the best of our knowledge, differences in function and phenotypic expression levels of macrophages between neonatal and adult lungs have not previously been determined. The present study investigated lung tissues and analyzed blood samples to find cell markers of M1 and M2 macrophages in neonatal and adult rats. Pulmonary sepsis was induced by intrapleural instillation of lipopolysaccharide (LPS; 20 mg/kg) and survival time after administration of LPS was measured. In certain neonates, a selective inducible nitric oxide synthase (iNOS) inhibitor, 1400w, was administered prior to induction of pulmonary sepsis. Compared with adults, fetal and neonatal lung tissues had significantly higher levels of iNOS and CD86 (M1 markers), whereas the expression levels of CD206 and arginase-1 (M2 markers) were lower in the neonatal lung. The circulating cells that co-expressed CD68 (monocytes and macrophages) and CD86 in the blood were also significantly higher in neonates than in adults (25.9 ± 6.6 vs. $11.6\pm2.2\%$; P=0.007. At basal unstimulated conditions, lung tissue concentrations of nitrite and nitrate (NOx) were significantly lower in the neonates than in adults (112.1 ± 55.9 vs. 340.9 ± 124.9 μM/g; P<0.001). However, NOx was increased following administration of LPS. Administration of 1400w suppressed lung tissue levels of NOx and improved the survival time in neonatal rats treated with LPS. The present study demonstrated that M1 is the primary macrophage phenotype in the neonatal lung and that higher iNOS expression levels do not have a protective effect against pulmonary endotoxins in neonates. Overproduction of NO by iNOS in neonatal alveolar macrophages may result in detrimental effects during pulmonary inflammation.

Introduction

Macrophages serve a key role in host immunity against pathogens, such as bacteria and viruses. The primary cell-mediated immune response of macrophages is phagocytosis of pathogens and apoptotic cells, generating phagolysosomes to eliminate cell waste (1). Phenotypically, macrophages are classified into two subtypes; the classic (M1) and alternative (M2) phenotypes (2). M1 macrophages are primarily activated by the release of lipopolysaccharide (LPS) or interferon-g following the invasion of microorganisms (3). During the acute inflammatory phase, activated M1 macrophages release high amounts of interleukin (IL)-12, nitric oxide (NO) and reactive oxygen species (ROS), which results in cytotoxic effects against the invading microorganisms, followed by phagocytosis (4-6). Therefore, M1 macrophages are key macrophages that are polarized during acute inflammation (3). M2 macrophages primarily mediate anti-inflammatory reactions (3). Following activation by anti-inflammatory cytokines, such as IL-4 and IL-13, M2 macrophages release IL-10, which promotes tissue repair and wound healing (4-6). Compared with the M2 subtype, M1 macrophages express higher levels of major histocompatibility complex (MHC) II, CD68, co-stimulatory molecules CD80 and CD86 and inducible nitric oxide synthase (iNOS) (2). The respiratory tract and lung alveoli serve as first-line defense barriers against pathogens and pollutants in the air, and the endogenous defense mechanisms of the respiratory system are essential to maintain the integrity and normal function of the respiratory tract and lung alveoli (6). Notable levels of macrophages are present in alveoli and pulmonary vasculature, serving as the front-line immunity defense barrier for the respiratory system (7,8). However, to the best of our knowledge, the primary subtypes of macrophages present in the lung during basal and acute inflammation, particularly in the developing lung, have not yet been determined. The present study analyzed the phenotypic expression levels of macrophages in the neonatal and adult lung and determined their functional significance in the pulmonary immune defense during the acute phase of endotoxic challenge.

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Materials and methods

Animals. Animal studies were conducted in compliance with the Animal Center of the National Cheng Kung University Hospital (Tainan, Taiwan) and approved by the Institutional of Animal Care and Use Committee (approval no. 107219). A total of 138 (7 fetal, 91 neonatal, 35 adult and 5 aged) rats were used for experimental analysis in this study. Rats (3 days-26 weeks; BioLASCO Taiwan Co., Ltd.) were cared in an animal house with 13 h-light and 11 h-dark cycles at an ambient temperature of 22-25˚C and received standard rodent food and water *ad libitum*. Adult female Sprague-Dawley rats (weight, 220-250 g) were mated with normal males (F:M=2:1). The night after mating was considered as gestation day 0, and the term gestation age was defined as 22 days. Fetuses were harvested at day 21 and neonates were sacrificed within 3 days of delivery. Adult rats were defined as 8-10 weeks old and aged rats were those older than 26 weeks. Animals that were used for mating were not adopted for other experiments. Neonatal, adult and aged rats were sacrificed by intraperitoneal injection of an overdose of pentobarbital (250 mg/kg). Blood samples were collected by direct cardiac puncture and lung tissues were harvested following direct thoracotomy. Plasma and tissue samples were snap frozen at -70˚C for further experiments.

Model of pulmonary infection. Animals were anesthetized with inhaled isoflurane (Piramal Enterprises Ltd.; 2-3 v/v% in oxygen) before induction of pulmonary infection. LPS (20 mg/kg; Sigma-Aldrich; Merck KGaA) was administered by intrapleural instillation into anesthetized rats to induce pulmonary inflammation and lung injury. Animals were allowed to recover from anesthesia on a warm blanket following LPS injection. The activity of animals was observed following LPS challenge and the mortality rate was recorded for up to 10 h following administration of LPS.

Inhibition of iNOS activity. Enzymatic activity of iNOS was inhibited by intraperitoneal injection of 1400w (Sigma-Aldrich; Merck KGaA) (9), which was administered every 6 h for three consecutive doses (10 mg/kg for each dose); the final dose was injected at least 1 h (range, 1-1.5 h) before administration of LPS.

Measurement of NO metabolites and 3-nitrotyrosine (3-NT). Lung tissue was homogenized using a Polytron homogenizer (Thomas Scientific) with protein extraction buffer containing sucrose (0.25 M), EDTA (1 mM), sodium azide (1 mM) and protease inhibitors. Levels of NO in the lung homogenates, as determined by the levels of its metabolites [nitrite/nitrate (NOx)]. Activated macrophages release superoxide and NO, which react to form peroxynitrite, leading to tyrosine nitration (10). 3-NT is considered to be a relatively specific marker of peroxynitrite-mediated oxidative damage (11). Levels of and NOx and 3-NT in lung homogenates were determined using the Griess reagent kit (cat. no. 780001; Cayman Chemical Company) and 3-NT ELISA kit (cat. no. EU2560; FineTest; Wuhan Fine Biotech Co. Ltd.), according to the manufacturer's instructions. Samples analyzed for NOx and 3-NT concentrations were performed in duplicates.

Western blotting. Lung tissue biopsies were minced and homogenized in lysis buffer (M-PER extraction reagent; Thermo Fisher Scientific, Inc.). Samples were centrifuged at 10,000 x g for 5 min at 4˚C and total protein concentration was quantified with a colorimetric assay kit based on the Bradford method (Dye Reagent Concentrate; cat. no. 500-0006; Bio-Rad Laboratories, Inc.). Equal amounts of total protein (50-100 μg) were loaded onto polyacrylamide gels (9-12%). Proteins were then transferred to nitrocellulose membranes by wet transfer. Following incubation in a commercially available chemical-based, protein-free blocking reagent (ImmobilonÒ Block; cat. no. WBAVDCH01; Sigma-Aldrich; Merck KGaA) for 20 min at room temperature, the membranes were incubated overnight at 4˚C with primary antibodies (1:1,000 dilution). The primary antibodies used were: iNOS (mouse monoclonal antibody; cat. no. 610432; BD Transduction Laboratories; BD Biosciences), CD86 (rabbit polyclonal antibody; cat. no. GTX 34569; GeneTex, Inc.), arginase (Arg)-1 (rabbit polyclonal antibody; cat. no. 16001-1-AP; ProteinTech Group, Inc.), CD206 (rabbit polyclonal antibody; cat. no. 18704-1-AP; ProteinTech Group, Inc.), NF-κB (rabbit polyclonal antibody; cat. no. 622602; BioLegend, Inc.) and b-actin (mouse monoclonal antibody; cat. no. MAB 1501; Sigma-Aldrich; Merck KGaA). iNOS and CD86 were defined as cell markers for M1 macrophages; Arg-1 and CD206 were defined as cell markers for M2 macrophages (12,13). After three washes with PBS, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2,000 dilution for goat anti-mouse antibody, cat. no. ab205719; Abcam or 1:2,000 dilution for goat anti-rabbit antibody cat. no. ab205718; Abcam) for 1 h at room temperature. Bands were visualized using enhanced chemiluminescence (Thermo Fisher Scientific, Inc.) and quantified by scanning densitometry (ImageJ v1.48; National Institutes of Health).

Histology and immunohistostaining. Lung tissues were fixed in 10% buffered formal saline for ≥24 h. Biopsies were processed through increasing grades of alcohol and embedded in paraffin wax. Sections of the lung were stained with Harris hematoxylin solution for 10 min at room temperature, followed by counterstaining with eosin-phloxine solution for 1 min at room temperature.

Paraffin-embedded lung tissues were sectioned for immunohistochemical staining. Lung tissue blocks were sectioned at 5 μm thickness on a microtome and the sections were transferred onto glass slides. After being dried overnight at room temperature, the sections were rinsed twice with xylene and tissue sections were dehydrated using various concentrations (100-50% v/v) of ethanol. Endogenous peroxidase tissue activity was blocked by incubating sections in 3% H_2O_2 solution in methanol at room temperature for 10 min. The sections were incubated with retrieval buffer (0.5 mg/ml trypsin in Tris-HCl, pH 8.0) and washed twice with PBS. Following addition of 10% (v/v in PBS), the slides were incubated at room temperature for 1 h. The sections were then incubated with rabbit polyclonal anti-iNOS (1:100 dilution; cat. no. bs-2072R; BIOSS) and CD68 (1:100 dilution; cat. no. BSB2717; Bio SB, Inc.,) antibodies at room temperature for 1 h, followed by incubating with polymer double stain detection system
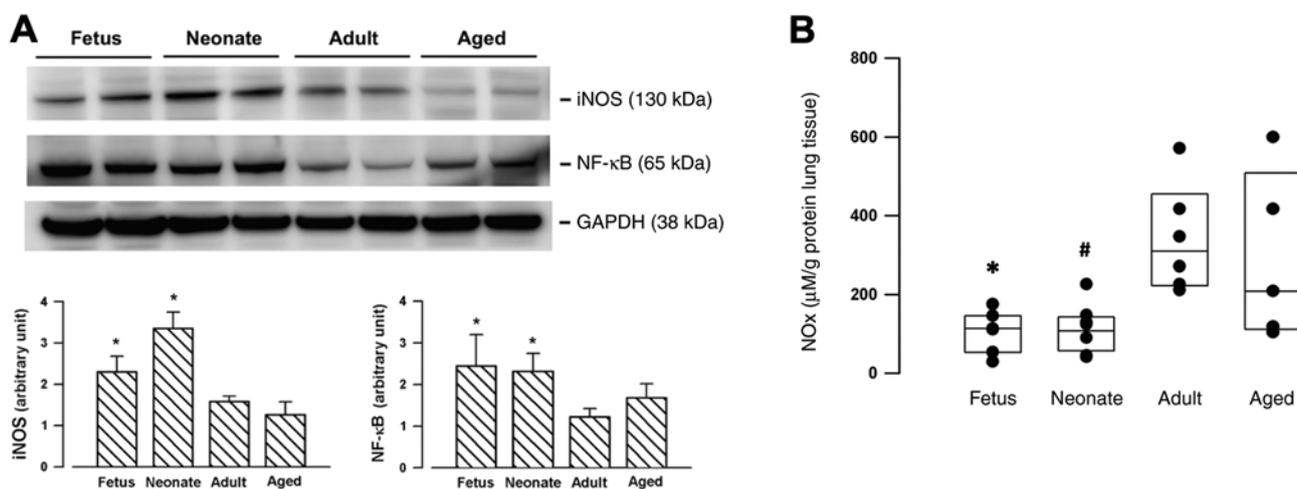
Figure 1. Basal protein expression and activity of iNOS in the lungs of rats at different ages. (A) Expression levels of iNOS and NF-κB in lung homogenates of fetal, neonatal, adult and aged rats. *P<0.05 vs. adults. n=5 animals in each group. (B) Generation of NOx in the lung tissue of fetal, neonatal, adult and aged rats under basal unstimulated condition was analyzed using a Griess reaction assay kit. *P=0.017 vs. adult; #P=0.021 vs. adult (Kruskal-Wallis). n=7 in fetal, n=8 in neonatal, n=9 in adult and n=5 in aged groups. iNOS, inducible nitric oxide synthase; NOx, nitrite and nitrate.

(BioTnA), which contained horseradish peroxidase (HRP) green and 3,3'-diaminobenzidine (DAB) brown chromogen, for another 20 min at room temperature. All procedures were performed according to the manufacturer's instructions. The expression levels of iNOS and CD68 on the lung sections were presented in HRP Green and DAB brown. Hematoxylin and eosin and immunohistochemically stained lung sections were examined under a light microscope (magnification, x100-200; Eclipse E200, Nikon Corporation).

Flow cytometry analysis. Following lysis of red blood cells with the lysis buffer (BD FACS lysing solution; BD Biosciences), the remaining blood cells were washed three times with PBS and resuspended in PBS. For immunofluorescence labeling, the cells were incubated with FITC-conjugated anti-CD86 antibody (1:100 dilution; cat. no. 130-109-180; Miltenyi Biotec) and phycoerythrin-conjugated anti-CD68 antibody (1:100 dilution; cat. no. 130-103-363; Miltenyi Biotec). Following incubation for 30 min at room temperature, cells were washed three times with PBS and specificity of staining was confirmed using equal concentrations of isotype-matched control antibodies. Cell fluorescence was immediately measured and analyzed using a FACSCalibur flow cytometer (BD Biosciences) and CellQuest Pro software (version 6.0; BD Biosciences). Cells that co-expressCD86 and CD68 were defined as M1 macrophages (14).

Statistical analysis. Results are presented as the mean ± SD. Tissue concentrations of NOx and 3-NT were analyzed in duplicates. The number of repeats in each experiment are presented in the figure legends. Differences in survival rates were analyzed using Kaplan-Meier curves and log-rank tests. Data were analyzed using an independent t-test, or Kruskal-Wallis followed by Dunn's post hoc test for multiple group comparisons, as appropriate. P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed using the SigmaPlot v14.0 software (Systat Software, Inc.).

Results

Expression levels and enzymatic activity of iNOS in neonatal lungs. Compared with mature counterparts (adults or aged rats), iNOS expressions were significantly enhanced in the lungs of the younger (fetal and neonatal) rats (Fig. 1A). The transcription factor NF-κB was also upregulated in the fetal and neonatal lungs (Fig. 1A). For the basal unstimulated condition, the enzymatic activity of iNOS in the fetal and neonatal lung, as determined by measuring the concentrations of NOx, was significantly lower than the levels in the adult lung (Fig. 1B).

Characterization of phenotypical subtypes of macrophages in neonatal rats. The phenotypical subtypes of macrophages were characterized by the expression levels of specific cell markers on macrophages. The total protein expression level was determined via western blotting. Expression levels of M1 macrophage markers (i.e. iNOS and CD86) were significantly enhanced in neonate lungs, whereas the expression levels of markers of M2 macrophages (i.e. Arg-1 and CD206) were decreased in neonates compared with adult and aged rats (Fig. 2A). Flow cytometry analysis of blood samples further confirmed that the number of isolated cells co-expressing CD68 and CD86 (M1 phenotype) (15) was significantly increased in the peripheral blood of neonates (Fig. 2B). Immunostaining of serial lung sections demonstrated co-localized expression levels of CD68 and iNOS (Fig. 3), indicating the presence of M1 macrophages in the neonatal lung samples.

Functional significance of enhanced iNOS in neonatal rats. An experimental model of pulmonary inflammation was induced by instillation of LPS into the pleural space of neonatal and adult rats. Compared with adult rats, the time-to-event curve demonstrated that the mortality rate of neonates was significantly increased up to 10 h following instillation of LPS (P<0.001; Fig. 4A). The increase in tissue concentrations of NOx and 3-NT in neonatal and adult lungs following LPS
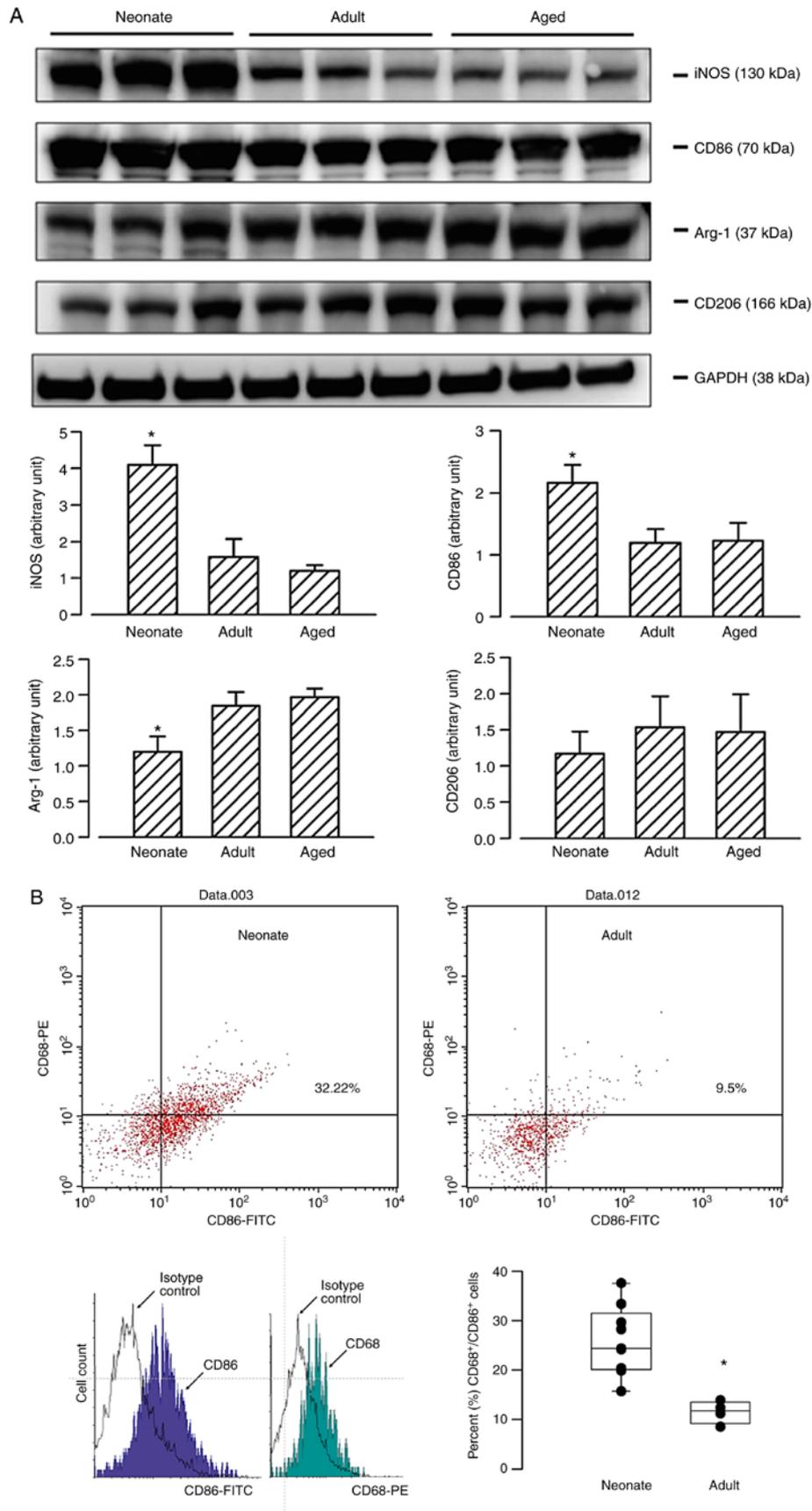
Figure 2. Characterization of macrophage phenotypes in rats at different ages. (A) Expression levels of M1 and M2 markers in the neonatal, adult and aged lung tissues. iNOS and CD86 are markers for M1; Arg-1 and CD206 are markers for M2. *P<0.05 vs. adults (Kruskal-Wallis; n=5). (B) Upper quadrants of flow cytometry diagrams represent the amount of circulating blood cells that co-expressed the M1 marker CD86 (x-axis) and monocyte marker CD68 (y-axis). Representative histograms for isotype controls for CD86 and CD68 staining in total white blood cells (lower left panel). The percentage of CD86+/CD68+ cells in the peripheral blood was significantly higher in neonates than adults. *P=0.007 vs. neonates, assessed using an independent t-test (adult, n=3; neonate, n=9). iNOS, inducible nitric oxide synthase; Arg, arginase.
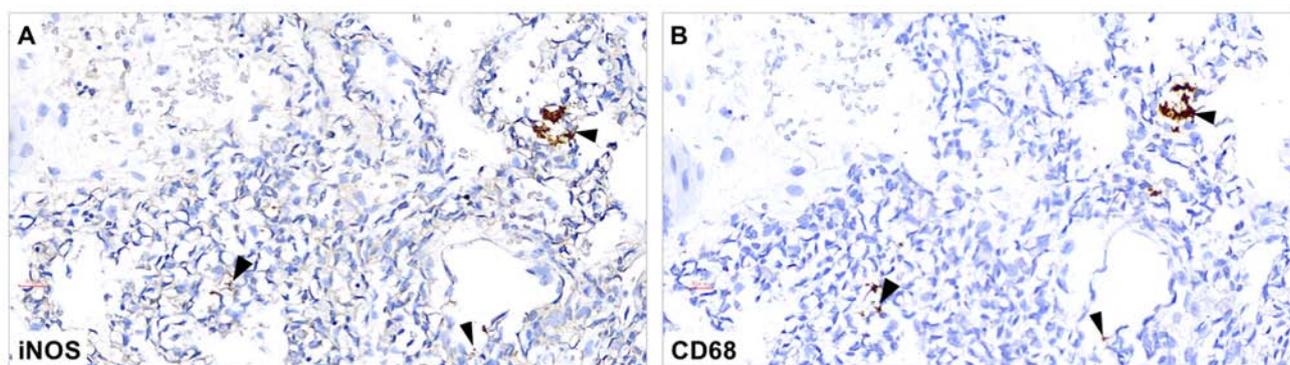
Figure 3. Representative serial lung sections of neonatal rats showing positive immunostaining of (A) CD68 and (B) iNOS. Arrowheads indicate corresponding lung areas that expressed both CD68 and iNOS. Experiments were performed in randomly selected lung sections of neonatal rats (n=5). Magnification, x200. iNOS, inducible nitric oxide synthase.
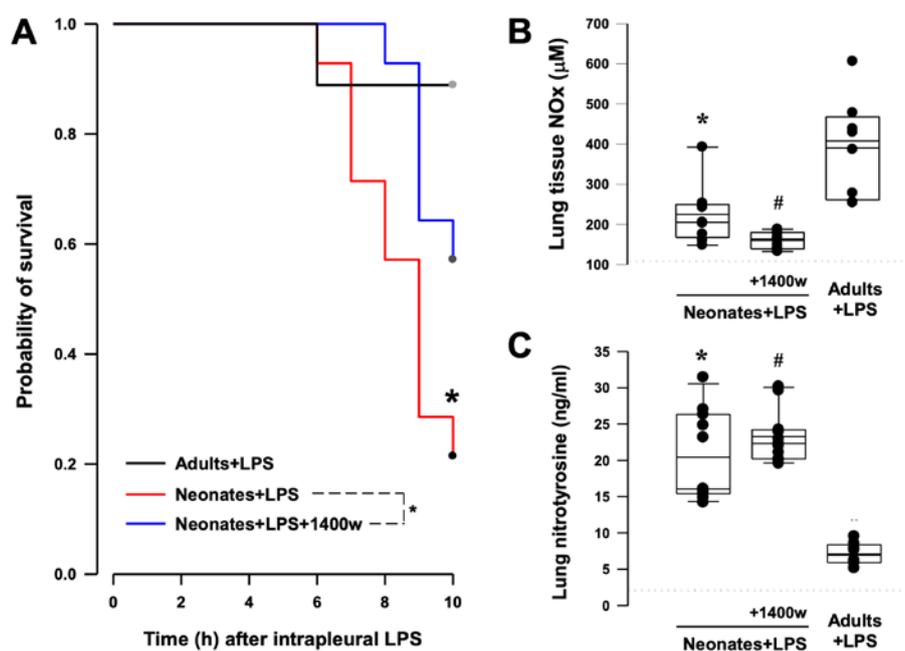

Figure 4. Effect of iNOS activity on the survival following endotoxic-induced lung injury. (A) Time-to-survival curves following intrapleural instillation of LPS in neonatal and adult rats. Compared with adults, mortality in neonates increased up to 10 h following pulmonary sepsis. Pre-treatment with a selective iNOS inhibitor, 1400w, significantly improved the survival time in neonates. *P=0.02 vs. neonates, assessed using the log-rank test. n=9 in adults+LPS, n=14 in neonates+LPS and n=14 in neonates+LPS+1400w groups. (B) Generation of NO following induction of pulmonary sepsis. Lung tissue concentrations of NOx were assessed using a Griess reaction assay kit. NOx levels were significantly decreased in lung homogenates of neonates treated with 1400w. *P=0.043 vs. adults; #P<0.001 vs. adults, assessed via Kruskal-Wallis. n=9 in neonates+LPS, n=9 in neonates+LPS+1400w and n=8 in adults+LPS groups. (C) Formation of 3-NT following induction of pulmonary sepsis. Lung tissue concentrations of 3-NT were measured using a 3-NT ELISA kit. *P=0.003 vs. adults; #P<0.001 vs. adults, assessed via Kruskal-Wallis. n=11 in neonates+LPS, n=11 in neonates+LPS+1400w and n=8 in adults+LPS groups. Dotted lines (B and C) indicate the tissue concentrations in the neonatal lungs at basal unstimulated condition. LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; NOx, nitrite and nitrate; 3-NT, 3-nitrotyrosine.

instillation (Fig. 4B and C) suggested an increase in peroxynitrite-mediated protein nitration. A selective iNOS inhibitor, 1400w, was used to suppress the enzymatic activity of iNOS in the neonates. Compared with the baseline, tissue levels of 3-NT were significantly increased in neonatal lungs after LPS challenge (2.4±0.5 vs. 20.4±6.2 ng/ml, neonatal baseline vs. neonatal LPS; P<0.001; Fig. 4C). Pre-treatment with 1400w significantly improved the overall survival time of animals receiving an intrapleural injection of LPS (P=0.02; log-rank test for Kaplan Meier curves between neonates and neonates pretreated with 1400w; Fig. 4A). The inhibitor significantly suppressed the generation of NOx in the lung (Fig. 4B), but

the formation of 3-NT was not decreased following inhibition of iNOS activity (Fig. 4C). However, neonatal lung generated high concentrations of 3-NT compared with adult lung tissues following intrapleural sepsis (20.4±5.9 vs. 7.1±1.4 ng/ml, respectively; P<0.001; Fig. 4C).

Discussion

Neonates and newborns are vulnerable to pulmonary aspiration or respiratory tract infection. In the USA, respiratory distress is the most common cause of neonatal intensive care unit admission, accounting for the admission of 50% of term

babies and 29% of late pre-term infants, with a higher incidence rate among prematurely born infants (16). Aspiration of meconium-stained amniotic fluid and maternal chorion-amnionitis are common risk factors for neonatal pulmonary infection (16). The host innate immunity in neonates is highly plastic and tolerant due to changes in environment and exposure immediately following delivery from the maternal uterus. This 'quiescent mode' of the innate immune system is essential in neonates as their immune system encounters numerous environmental and physical challenges during early life, and modulates the interactions between the innate immune system and host defense cells, such as macrophages (17).

In human adults, M1 macrophages have been demonstrated to be activated by intracellular pathogens during the acute phase of inflammation, and to promote Th1 polarization of CD4 cells in response to the inflammatory environment dominated by the Toll-like receptor and interferon signaling (18). The polarization of adult macrophages to the M1 phenotype enhances the release of proinflammatory cytokines and C-X-C motif ligand chemokines, thereby activating the Th1 response and complement-mediated phagocytosis (18). Although polarization and phenotypical switching of macrophages have been well-characterized in adults (18), the major subtypes of macrophages in the developing lung during acute inflammation have not been clearly determined. Therefore, the determination of the phenotypical differences in macrophages between neonates and adults is an important research topic, as excessive inflammation reactions are often observed in very young subjects (19). Winterberg *et al* (19) proposed that phenotypical features and functional regulation of macrophages were different in the neonatal peritoneum with or without LPS challenge. In their experiments, peritoneal macrophages from neonatal (age, <24 h) and adult (age, 6 weeks) C57BL/6J mice were isolated and analyzed by high content chipcytometry. This demonstrated that the expression levels of F4/80, MHC-II, CD80 and CD86 (M1 markers) are decreased in neonates, and that the transcriptomes of neonatal and adult macrophages are different both before and after LPS (0, 1, 10 and 100 ng/ml) stimulation. Winterberg *et al* (19) also found that neonatal macrophages secrete higher levels of proinflammatory cytokines following LPS stimulation but exhibit a decreased ability to induce T-cell proliferation. These findings demonstrated that the phenotypical features and function of peritoneal macrophages in neonates are distinct from those in adult mice.

In the present study, cell markers of macrophages and circulating monocyte subtypes in the lung and peripheral blood of fetal and neonatal rats were analyzed. The expression levels of iNOS and CD86 were significantly enhanced, whereas the level of Arg-1 was decreased, in the neonates, which is consistent with the hypothesis that M1 phenotypic macrophages are predominant in the fetal and neonatal lung. Immunohistochemical examination identified co-expression of CD68 and CD86 in the lung parenchyma of neonatal rats, confirming the presence of M1 macrophages in the alveoli. Flow cytometry analysis also confirmed that circulating cells that co-expressed CD68 and CD86 were significantly higher in neonates compared with adults. These results demonstrated that the phenotype of pulmonary macrophages and circulating monocytes in neonatal rats is predominantly of the M1 subtype.

The present study revealed the biological role of enhanced iNOS expression levels in the neonatal lung. High iNOS expression levels in the fetal and neonatal lung were associated with a higher protein level of inducible nuclear factor NF-κB, indicating that iNOS was induced at the transcriptional level. However, the bioavailability of iNOS in the basal unstimulated condition, as measured by NOx levels, was significantly decreased in neonates compared with in adult rats. Since iNOS is an inducible enzyme, lower basal NOx concentrations in the neonatal lung may indicate that neonates are exposed to fewer airborne pathogens than adults. Following endotoxic challenge, the enzymatic activity of iNOS was significantly upregulated in neonatal and adult rats, as demonstrated by the increase in lung tissue levels of NOx. NO released by activated macrophages mediates not only cytotoxic and cytostatic effects against invading microorganisms, but also modulates the activity of T- and B-cells, as well as the recruitment of leukocytes (3). In addition, activated macrophages generate superoxide and ROS via induction of NAPDH oxidase, a process known as respiratory burst (20,21). The reaction between NO and superoxide anions in phagosomes generates an oxidizing intermediate, peroxynitrite (ONOO-), which is responsible for the microbicidal effect exhibited by macrophages (22,23). At the cellular level, ONOO- nitrates protein-bound tyrosine at position 3 to form 3-NT, thereby diminishing the function of proteins (24). Therefore, the formation of 3-NT is considered to be a sensitive *in vivo* biomarker for endogenous ONOO⁻ activity (11). In the present study, the tissue concentrations of 3-NT in the lung were measured to determine the degree of protein nitration in response to LPS-induced pulmonary inflammation. Consistent with the increased production of NOx, formation of 3-NT was also significantly enhanced in the neonatal lung following exposure to LPS. Although the lung concentrations of 3-NT were similar in neonatal and adult rats at basal unstimulated conditions, generation of 3-NT was more significantly increased in neonatal lung tissues than in the adult lung tissues following intrapleural instillation of endotoxin. Previous studies have demonstrated that the cytotoxic response of activated macrophages is directly derived from the generation of intra-phagosomal ONOO⁻, and subsequently the development of protein hydroxylation in the invading pathogens (25,26).

In order to clarify the functional significance of enhanced iNOS expression levels in the neonatal lung, a selective iNOS inhibitor, 1400w, was administered prior to induction of lung inflammation, and the effect on mortality following pulmonary sepsis was recorded. Pre-treatment with 1400w suppressed the enzymatic activity of iNOS and decreased the generation of NOx in the lung tissue. Furthermore, the overall survival rate of neonatal rats was improved up to 10 h following intrapulmonary LPS challenge. The beneficial effect on survival of iNOS inhibition during intrapulmonary sepsis in neonatal rats was consistent with an improved survival rate in iNOS-deficient mice subjected to intraperitoneal sepsis (27,28). Mice with iNOS deficiency are also more responsive to microvascular catecholamine infusion and exhibit a more stable hemodynamic reaction during sepsis (27).

Although suppression of iNOS enzymatic activity improved the survival outcomes of neonatal rats in the present study, the mortality of neonates pretreated with 1400w remained higher than that of their adult counterparts

at 10 h following pulmonary sepsis (42.8 vs. 11.1%). It is notable that the lung tissue level of 3-NT was significantly increased in neonatal rats pretreated with 1400w, despite the suppression of iNOS activity. Uncontrolled or excessive production of ROS and/or ONOO⁻ by tissue macrophages contributes to oxidative damage and nitrosative stress in host organs, thereby exacerbating acute tissue injury or organ dysfunction (29). Since the generation of ROS and reactive nitrogen species by macrophages involves the assembly and membrane migration of NADPH oxidase subunits (23), iNOS inhibition may not be sufficient to suppress excessive formation of ONOO⁻ during sepsis. Therefore, further investigations regarding the underlying mechanisms of other macrophage-mediated cytotoxic and cytostatic effects in the neonatal lung are essential.

The present study was limited, since the functional role of the M2 macrophage subtype was not determined in the neonatal lung. Since M2 macrophages are predominantly anti-inflammatory macrophages that contribute to the late and healing phases of inflammation (30), their function in the relatively sterile neonatal lungs can be less significant than that in the adults. In addition, the effect of 1400w on the survival outcome was only observed up to 10 h following the administration of endotoxin. The longer-term effect of iNOS suppression and the physiological role of M2 macrophages in the late phase of inflammation in neonates is the subject of further investigation. In contrast to the results of the present study in neonatal lungs, Winterberg *et al* (19) demonstrated that the number of peritoneal M1 macrophages in neonatal mice was lower than that in adults. This discrepancy may be associated with the heterogeneity of macrophage populations in different organs or tissues (31). Lastly, although LPS is recognized as the primary pathogen-associated molecule that triggers host innate immune responses of mammalian cells to bacterial invasion, the phenotypical modulation of macrophages in response to the challenge of whole bacteria requires further investigation.

To the best of our knowledge, the present study was the first to demonstrate that M1 is the predominant alveolar macrophage subtype with enhanced expression levels of iNOS in the neonatal lung. However, overproduction of NO and formation of 3-NT during pulmonary sepsis increase the mortality of neonatal rats in the acute inflammatory phase, indicating that high enzymatic activity of iNOS in alveolar M1 macrophages may deteriorate following endotoxin-induced lung injury.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SYF performed the experiments, designed the study, analyzed the data and contributed to manuscript preparation. JLC was involved in animal experiments, data collection and manuscript preparation. MHC and CCH performed western blotting and tissue assays. MWL and CFL conceived and designed the experiments, interpreted the results, analyzed the data and prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Animal Care and Use Committee (approval no. 107219) of the National Cheng Kung University Hospital, Tainan, Taiwan.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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