Effects of macrophage inducible nitric oxide synthase in murine septic lung injury


Centre for Critical Illness Research, Division of Respirology, Departments of Medicine, Physiology, and Pharmacology, London Health Sciences Center and Lawson Health Research Institute, University Of Western Ontario, London, Ontario, Canada

Submitted 7 June 2005; accepted in final form 5 January 2006

Farley, K. S., L. F. Wang, H. M. Razavi, C. Law, M. Rohan, D. G. McCormack, and S. Mehta. Effects of macrophage inducible nitric oxide synthase in murine septic lung injury, Am J Physiol Lung Cell Mol Physiol 290: L1164–L1172, 2006. First published January 13, 2005; doi:10.1152/ajplung.00248.2005.—Inducible nitric oxide synthase (iNOS) contributes importantly to septic pulmonary protein leak in mice with septic acute lung injury (ALI). However, the role of alveolar macrophage (AM) iNOS in septic ALI is not known. Thus we assessed the specific effects of AM iNOS in murine septic ALI through selective AM depletion (via intratracheal instillation of clodronate liposomes) and subsequent AM reconstitution (via intratracheal instillation of donor iNOS+/+ or iNOS−/− AM). Sepsis was induced by cecal ligation and perforation, and ALI was assessed at 4 h: protein leak by the Evans blue (EB) dye method, neutrophil infiltration via myeloperoxidase (MPO) activity, and pulmonary iNOS mRNA expression via RT-PCR. In iNOS+/+ mice, AM depletion attenuated the sepsis-induced increases in pulmonary microvascular protein leak (0.3 ± 0.1 vs. 1.4 ± 0.1 μg EB-g lung−1 min−1; P < 0.05) and MPO activity (37 ± 4 vs. 67 ± 8 U/g lung; P < 0.05) compared with that shown in non-AM-depleted mice. In AM-depleted iNOS+/+ mice, septic pulmonary protein leak was restored by AM reconstitution with iNOS+/+ AM (0.9 ± 0.3 μg EB-g lung−1 min−1) but not with iNOS−/− donor AM. In iNOS−/− mice, sepsis did not induce pulmonary protein leak or iNOS mRNA expression, despite increased pulmonary MPO activity. However, AM depletion in iNOS−/− mice and subsequent reconstitution with iNOS+/+ donor AM resulted in significant sepsis-induced pulmonary protein leak and iNOS expression. Septic pulmonary MPO levels were similar in all AM-reconstituted groups. Thus septic pulmonary protein leak is absolutely dependent on the presence of functional AM and specifically on iNOS in AM. AM iNOS-dependent pulmonary protein leak was not mediated through changes in pulmonary neutrophil influx.

inflammation; vascular permeability; sepsis; reconstitution; acute lung injury

ACUTE LUNG INJURY (ALI) remains a common and important clinical problem, affecting over 150,000 patients annually in the United States and leading to death in 30–40% of patients (1, 18, 40, 51, 58). ALI is characterized by diffuse pulmonary opacities, pulmonary edema, and severe hypoxemia. Pulmonary edema is the result of the influx of inflammatory cells into the lung tissue, which is associated with increased permeability of the alveolar-capillary barrier and the leak of protein-rich fluid into the air spaces.

The most common cause of ALI is sepsis, a systemic inflammatory response to an overwhelming infection (1, 17, 18). Sepsis and ALI are characterized by the activation of a variety of cells, including inflammatory cells such as neutrophils and macrophages, and increased production and secretion of numerous soluble mediators, including cytokines such as tumor necrosis factor (TNF)-α and several interleukins (16, 40, 49, 58). Despite a greater understanding of the pathophysiology of ALI, therapeutic options remain limited to treatment of the underlying disease process, supplemental oxygenation, fluid management, mechanical ventilation, and in some cases activated protein C.

Sepsis and ALI are also associated with significantly increased endogenous nitric oxide (NO) production caused by enhanced expression of the inducible form of NO synthase (iNOS) (15, 44, 45, 61). NO has been implicated in the pathophysiology of ALI in humans and in animal models of ALI (3, 11, 23, 24, 47, 57). However, NOS inhibition with nonisoform-selective inhibitors, such as L-arginine (L-NMMA), in patients with sepsis and ALI has resulted in worse cardiac function and increased mortality (7, 32, 34). On the basis of the increased expression of iNOS in sepsis and ALI, targeted inhibition of iNOS may be more effective in sepsis and ALI. Indeed, we and others have previously shown in murine models of sepsis-induced ALI that the absence of iNOS in “knockout” (iNOS−/−) mice or the selective inhibition of iNOS with N-(3-aminomethyl-L)-arginine (L-NMMA), in patients with sepsis and ALI has resulted in worse cardiac function and increased mortality (7, 32, 34). On the basis of the increased expression of iNOS in sepsis and ALI, targeted inhibition of iNOS may be more effective in sepsis and ALI. Indeed, we and others have previously shown in murine models of sepsis-induced ALI that the absence of iNOS in “knockout” (iNOS−/−) mice or the selective inhibition of iNOS with N-(3-aminomethylbenzyl) acetamide (1400W) in wild-type (iNOS+/+) mice was associated with reduced ALI, as evidenced by decreased microvascular protein leak and bronchoalveolar lavage (BAL) protein and decreased pulmonary oxidant and nitrosative stress (24, 42, 57). However, complete inhibition or deficiency of iNOS does not consistently improve outcome in animal models of sepsis and ALI (13, 24, 26, 39).

We and others have tried to resolve this paradox by creating and studying septic ALI in reciprocal bone marrow-transplanted iNOS chimeric mice, in which iNOS expression can be restricted to discrete cell populations (14, 56, 57). A series of studies supports the concept of cell source-specific effects of iNOS in ALI (42, 43, 56, 57). For example, we showed that pulmonary microvascular protein leak and oxidant stress in septic mice were completely dependent on iNOS presence in inflammatory cells, including neutrophils and macrophages, with no obvious contribution of iNOS in tissue parenchymal cells (42, 57). However, the specific effects of iNOS in alveolar macrophages (AM) in septic ALI have not been addressed or defined.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Thus we hypothesized that AM iNOS-derived NO contributes importantly to septic ALI in mice. We first defined the role of AM in murine septic ALI through characterization of the effects of depletion of AM on septic ALI. Second, we created AM-specific iNOS chimeric mice by reconstituting AM intra-tracheally in AM-depleted iNOS+/- and iNOS-/- mice using AM isolated from either iNOS+/- or iNOS-/- donor mice. The specific role of AM iNOS in septic ALI was then assessed by comparing markers of septic ALI between these AM-depleted or AM-reconstituted, reciprocal AM-specific iNOS chimeric mice.

METHODS

Animal Preparation

Male C57BL/6 mice, iNOS+/- or iNOS-/- (7–8 wk old, 20–25 g; Charles River, St. Constant, Quebec, Canada), were used. All protocols were approved by the institutional animal ethics committee, in accordance with the guidelines of the Canadian Council on Animal Care, and were supervised by a veterinarian.

Mouse Model of Sepsis-Induced Lung Injury

Animals were randomized to sham surgery vs. sepsis induced by cecal ligation and perforation (CLP), as previously described (44, 57). Briefly, under halothane anesthesia, the abdomen was incised and the cecum was exposed and ligated. An 18-gauge needle was used to puncture two holes in the cecum, from which the entire contents of the cecum were exuded into the peritoneum. The cecum was replaced, and the abdomen was closed with 4-0 silk. Sham animals were anesthetized and allowed to recover. Both CLP and sham animals received buprenorphine (0.1 μg/g weight) and were fluid resuscitated with 1 ml of saline subcutaneously. All animals were killed 4 h after CLP by an intraperitoneal injection of pentobarbital sodium (0.1 mg/g body wt). Lung tissue was obtained for assessment of septic ALI.

Assessment of Pulmonary Microvascular Protein Leak

Thirty minutes before death, 0.4% Evans blue (EB) dye (50 mg/kg) was injected into the circulation through the tail vein. After death, the pulmonary circulation was flushed with PBS, and the lungs were removed and snap frozen in liquid nitrogen. The frozen tissue was homogenized in PBS, and EB was extracted into formamide by incubating at 60°C for 16 h. Absorbance of the supernatant was measured at 620 and 740 nm, and pulmonary EB-albumin flux (μg EB·g lung-1·min-1) was calculated as previously described (57).

Assessment of Pulmonary Neutrophil Influx via Myeloperoxidase Activity

Pulmonary myeloperoxidase (MPO) activity was assessed in the lung tissue as the hydrogen peroxide-dependent oxidation of tetramethylbenzidine over 5 min at 37°C as previously described (44, 59). One unit of MPO activity was defined as a change of 1.0 optical density unit (ΔAbs) per minute and expressed as units per gram of lung.

Assessment of Cytokine Levels in BAL Fluid

The lungs were lavaged with three 1-ml aliquots of 0.5 mM EDTA-PBS, and the lavage was stored at −80°C until further analysis. The focus was on a few key cytokines, including TNF-α and IL-1β, early response cytokines important in initiation of ALI, IL-6, a common marker of ALI, macrophage inflammatory protein (MIP)-2, a C-X-C chemokine important in neutrophil recruitment, and IL-10, an anti-inflammatory cytokine. Levels of each cytokine, except for MIP-2, were measured by sandwich ELISA by using color-coded, antibody-coupled microspheres and a secondary phycoerythrin-la-beled antibody, detected by laser spectrophotometry (mouse cytokine lincoplex kit; Linco Research, St. Charles, MO). With the use of this assay, the minimum detectable concentrations were 0.9 pg/ml (TNF-α), 1.6 pg/ml (IL-1β), 0.7 pg/ml (IL-6), and 10.3 pg/ml (IL-10). MIP-2 levels were measured with a commercial sandwich ELISA assay, with a minimum detection limit of 15.6 pg/ml (R&D Systems, Minneapolis, MN).

Assessment of NO Metabolite Levels in Plasma and BAL Fluid

Levels of nitrites/nitrates in plasma and BAL were measured by chemiluminescence, as previously reported (44, 59). In brief, samples were refluxed with vanadium chloride in hydrochloric acid at 90°C to reduce nitrites/nitrates to NO, which was detected by a commercial NO analyzer (270B; Sievers Instruments, Boulder, CO).

Pulmonary iNOS mRNA Levels

Pulmonary iNOS mRNA expression was determined by RT-PCR. Total RNA was extracted from the lung tissue with Trizol reagent. The RNA was reverse transcribed to cDNA by the Moloney murine leukemia virus RT system and amplified by PCR (Progene, Technne, Duxford, Cambridge, UK). Primers used for murine iNOS were (GenBank accession no. M84373) 5’-ACC CCT GTG TTC CAC CAG GAG ATG TTG AA-3’ (forward) and 5’-TGA AGC CAT GAC CTG TCG CAT TAG CAT GG-3’ (reverse). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also amplified as a control (GenBank accession no. M17701): forward primer 5’-AAA GGG CAT CCT GGG CTA CA-3’ and reverse primer 5’-CAG TTG TGG GGG CTG AGT TG-3’. PCR products were separated on a 1.2% agarose gel, and densitometric analysis was done using Gel Pro Analyzer 3.1. Pulmonary iNOS mRNA expression is reported as the ratio of iNOS to GAPDH.

Protocol 1: AM Depletion

AM depletion in iNOS+/- and iNOS-/- mice was accomplished through the intratracheal instillation of liposomes filled with dichloromethyleneephosphonic acid (Cl₂MDP), as previously described (54). In brief, phosphatidylcholine (100 mg/ml) and cholesterol (0.8 mg/ml) in chloroform were gently mixed in an 11-to-1 ratio. The chloroform phase of this solution was evaporated under low vacuum rotation in a 500-ml round-bottom flask. The white phospholipid film on the wall of the flask was dispersed in 0.6 M Cl₂MDP by gentle rotation at room temperature for 10 min. Excess nonencapsulated Cl₂MDP was removed by centrifugation (10,000 g; 30 min), and the liposomes were washed twice (21,000 g for 30 min) and resuspended in PBS.

Under halothane anesthesia, the anterior neck was incised and a tracheostomy was performed for bronchoalveolar lavage. The lungs were inflated with saline to 30 cmH₂O pressure, and lavage was performed through the tracheostomy tube. The return volume was aspirated three times, and the return volume was pooled. Sixty-six hours after prior AM depletion, 1.5 million iNOS+/- or iNOS-/- donor AM were harvested by BAL fluid from iNOS+/- or iNOS-/- donor mice. Three 1-ml aliquots of 0.5 mM EDTA-PBS were each instilled and aspirated three times, and the return volume was pooled.

Bacterial ALI was induced from several donor mice were pooled, washed twice with sterile PBS, and resuspended in PBS at a density of 3 × 10⁷ AM/ml.

Sixty-six hours after prior AM depletion, 1.5 million iNOS+/- vs. iNOS-/- AM (50 μl suspension) were instilled into AM-depleted iNOS+/- and iNOS-/- mice via a tracheostomy. On the basis of the iNOS genotype of donor AM and recipient mice, two reciprocal
iNOS chimeric groups of AM-reconstituted mice were created and studied: the + to − group, in which iNOS +/+ AM were reconstituted in AM-depleted iNOS −/− mice, and the − to + group, in which iNOS −/− AM were reconstituted in AM-depleted iNOS +/+ mice. In addition, an important control group was also created and studied, + to +, in which AM-depleted iNOS +/+ mice were reconstituted with iNOS +/+ AM. Although these mice are genetically identical to naïve wild-type mice in that all cells, including AM, are iNOS +/+, these + to + mice control for the anesthesia and surgical AM-depletion/reconstitution interventions and potential effects of these interventions on subsequent CLP-induced sepsis and ALI.

To ensure AM retained their NO-producing capability after harvest and were also not activated by the harvesting procedures, a pilot study was conducted. iNOS +/+ and iNOS −/− AM were harvested and instilled into AM-depleted mice. AM were then harvested from these animals and either incubated with PBS or stimulated in vitro with 10 μg/ml lipopolysaccharide (LPS) and 10 U/ml IFN-γ for 24 h. NO production was assessed by measuring nitrite levels by chemiluminescence.

To assess the pulmonary distribution of intratracheally instilled AM, isolated AM were fluorescently labeled by incubation with 100 μg/ml bisbenzamide (15 min at 37°C). AM were then washed twice with PBS, resuspended in 50 μl PBS, and then intratracheally instilled via a tracheostomy. Two hours after instillation of fluorescently labeled AM, mice were killed and the lungs were harvested and flash frozen in OCT under inflation at 25 cmH2O. Frozen lung tissue was sectioned and visualized by fluorescence microscopy (DMR Leica upright fluorescent microscope, with a charge-coupled device camera connected to a Macintosh computer running Openlab 4.0.2 software).

**Degree of Peritonitis After AM Depletion**

Peritoneal lavage was done in a subset of animals to determine the total cellularity and protein concentration in the peritoneal cavity after AM depletion and sepsis. We counted cell numbers with a hemacytometer and determined protein concentrations using the Bradford protein assay.

**Statistical Analysis**

Data are expressed as means ± SE. Differences between groups were assessed by one-way ANOVA. Post hoc tests were done with a Student-Newman-Keuls t-test or Tukey’s test where appropriate. Statistical significance was accepted when *P* < 0.05.

**RESULTS**

**Protocol 1: AM Depletion**

Treatment of naïve mice with Cl2MDP-filled liposomes effectively depleted AM with a nadir of ~90% depletion observed at 72 h (Fig. 1A). Thus, for all subsequent studies, Cl2MDP-liposome treatment was administered 68 h before induction of sepsis by CLP, such that when septic ALI was assessed at 4 h after CLP, this time point always coincided with the nadir in BAL AM counts. Indeed, in such AM-depleted mice subsequently subjected to CLP, we confirmed persistent, effective AM depletion at 4 h (Fig. 1B).

**Effect of AM depletion on septic ALI.** As previously shown (57), induction of sepsis was associated with a significant increase in EB-albumin leak in iNOS +/+ mice (Fig. 2A). In contrast, sepsis was not associated with an increase in EB albumin leak in iNOS −/− mice [0.4 ± 0.1 vs. 0.2 ± 0.1 μg EB·g lung−1·min−1 in sham; *P* = not significant (NS)]. The septic increase in pulmonary EB-albumin leak in iNOS +/+ mice was abolished after AM depletion, with the level of leak being reduced to that in sham mice (Fig. 2A).

**Protocol 2: AM Reconstitution**

We first assessed whether AM harvest and reconstitution procedures activated these AM, especially with regard to basal and stimulated iNOS expression, as reflected by NO production in vitro. As such, iNOS +/+ mice were AM depleted (time 0) and reconstituted (at 66 h) with 1.5 million iNOS +/+ AM.
These + to + AM-reconstituted mice were killed 2 h later (at 68 h), and the instilled AM were harvested again by BAL. In the absence of stimulation, these instilled/harvested AM had low levels of NO production ($3.38 \pm 0.35 \mu mol$ nitrates/10^6 AM) similar to unstimulated freshly harvested iNOS+/+ AM not used for AM reconstitution. Moreover, after stimulation with LPS and IFN-γ, NO production was increased to a similar degree in both instilled/harvested AM (34.1 $\pm$ 19.7 μmol nitrates; $P < 0.05$) and in freshly harvested, noninstilled AM (38.5 $\pm$ 9.2 μmol nitrates; $P < 0.05$).

Fluorescent imaging after AM reconstitution showed that the bisbenzamide-labeled donor macrophages were successfully delivered into the alveoli (Fig. 3).

**Effect of AM reconstitution on septic ALI.** To confirm that AM-depletion/reconstitution procedures, before induction of sepsis, did not generate any ALI, pulmonary EB-albumin leak and MPO were assessed in sham + to + AM-reconstituted mice. The rate of pulmonary microvascular leak in sham + to + AM-reconstituted mice was not significantly different vs. that shown in naïve iNOS+/+ mice ($0.39 \pm 0.10$ vs. $0.33 \pm 0.13$ μg EB/g lung^{-1}.min, respectively; $P = NS$). Pulmonary MPO activity was also similar in sham + to + AM-reconstituted mice vs. naïve iNOS+/+ mice (9.1 $\pm$ 1.9 vs. 7.0 $\pm$ 0.9 U/g lung, respectively; $P = NS$). Thus the AM-depletion/reconstitution procedures themselves had no significant effects on markers of ALI.

Induction of sepsis in + to + AM-reconstituted mice was associated with a significant increase in EB-albumin leak (Fig. 4). Indeed, septic pulmonary EB-albumin leak, which had been abolished in AM-depleted iNOS+/+ mice, was completely restored by AM reconstitution with the use of iNOS+/+ donor AM. In stark contrast, iNOS−/− AM reconstitution of AM-depleted iNOS+/+ mice (− to + group; all cells iNOS+/+, except AM) was not associated with any significant septic increase in pulmonary EB-albumin leak. Thus septic pulmonary EB-albumin leak, which had been abolished in AM-depleted iNOS+/+ mice, was not restored after AM reconstitution using iNOS−/− donor AM. More strikingly, reconstitution of AM-depleted iNOS−/− mice with iNOS+/+ donor AM (+ to − group; only AM iNOS+/+) was associated with a similar increase in septic pulmonary EB-albumin leak vs. + to + AM-reconstituted mice.

After induction of sepsis, there was a similar increase in pulmonary neutrophil infiltration, as reflected by pulmonary MPO activity, in all AM-depleted/reconstituted groups (Fig. 5). Moreover, the sepsis-induced increase in pulmonary MPO activity was not significantly different in AM-depleted/reconstituted mice vs. non-AM-depleted septic iNOS+/+ mice.

**Effect of AM-Depletion/Reconstitution on Endogenous NOS System**

Sepsis was associated with significant systemic iNOS activation, as reflected by increased plasma nitrite/nitrate concen-
trations in iNOS+/+ vs. sham mice (51 ± 9 vs. 10 ± 2 μM, respectively; P < 0.05). This increase in plasma nitrite/nitrate levels was slightly, but not significantly, reduced in septic AM-depleted mice (35 ± 5 μM; P = NS vs. septic non-AM-depleted iNOS+/+ mice). As our group previously reported (57), there was no significant increase in plasma nitrite/nitrate levels in septic iNOS−/− mice (9 ± 2 vs. 5 ± 1 μM in sham; P = NS).

The AM-depletion/reconstitution procedures had no significant effect on basal plasma nitrites/nitrates, which were similar in sham + to + AM-reconstituted mice vs. naïve iNOS+/+ mice (14 ± 5 vs. 10 ± 2 μM, respectively; P = NS). After induction of sepsis, plasma nitrites/nitrates also increased significantly in + to + mice (Fig. 6). Moreover, in septic − to + mice (all cells iNOS+/+, except AM), the septic increase in plasma nitrites/nitrates was preserved and was similar to that seen in septic + to + mice. Most strikingly, sepsis in + to − mice (only AM iNOS+/+; all other cells iNOS−/−) was not associated with any increase in plasma nitrites/nitrates. The data in the reciprocal AM-iNOS chimeric mice suggest a trivial contribution of AM iNOS to systemic NO production, as reflected by plasma nitrites/nitrates.

Sepsis was also associated with significant pulmonary iNOS mRNA expression, which was increased in septic vs. naïve iNOS+/+ mice (0.41 ± 0.02 vs. 0.09 ± 0.01 iNOS/GAPDH band density, respectively; P < 0.05). Prior AM depletion in iNOS+/+ mice had no effect on the septic increase in pulmonary iNOS mRNA expression (0.40 ± 0.12 iNOS/GAPDH band density; P = NS), which was similar to that in septic non-AM-depleted iNOS+/+ mice. Pulmonary iNOS mRNA expression was not evident in either septic or naïve iNOS−/− mice (Fig. 7).

In AM-depleted iNOS+/+ mice in which AM were subsequently reconstituted, there was a significant and similar sep-
sis-induced increase in iNOS mRNA expression, regardless of iNOS+/+ donor AM (+ to + AM depleted/reconstituted group) or iNOS−/− donor AM (− to + group; all cells iNOS+/+, except AM; Fig. 7). Thus AM iNOS contributed negligibly to total pulmonary iNOS expression. Moreover, sepsis in + to − mice (only AM iNOS+/+) was only associated with a slight increase in pulmonary iNOS mRNA expression, compared with the complete absence of obvious iNOS mRNA expression in septic iNOS−/− animals.

Potential Mechanism of AM iNOS-mediated ALI

We assessed the effects of sepsis on two additional markers of ALI: BAL concentrations of multiple cytokines of relevance to ALI and BAL nitrite/nitrate levels. In BAL fluid, the early inflammatory cytokines TNF-α and IL-1β were undetectable in iNOS+/+ and iNOS−/− mice under both sham and septic conditions (Table 1). In contrast, there were significant increases in BAL levels of two key cytokines, MIP-2 and IL-6, after sepsis. However, the sepsis-induced increases in these cytokines were not significantly different between iNOS+/+ and iNOS−/− mice (Table 1). Therefore, given the lack of any significant effect of total iNOS on these cytokines, further assessment of these markers of ALI in our AM-chimera mice to define a specific effect of AM iNOS was not warranted.

BAL nitrite/nitrate levels were significantly higher in iNOS+/+ vs. iNOS−/− mice under both basal and septic conditions (P < 0.05 for each). However, there were no significant increases in BAL nitrites/nitrates after the induction of sepsis in either iNOS+/+ (4.1 ± 0.4 vs. 3.4 ± 1.1 μM in sham, n = 6/group) or iNOS−/− (1.9 ± 0.1 vs. 1.5 ± 0.5 μM in sham, n = 6/group) mice. Given the lack of any significant sepsis-induced signal and the small differences between iNOS+/+ and iNOS−/− mice, these experiments were not repeated in our AM-chimera mice.

DISCUSSION

Effective AM depletion of iNOS+/+ mice before induction of sepsis completely abrogated the septic increase in pulmonary microvascular protein leak and significantly reduced pulmonary neutrophil influx. This attenuated sepsis-induced pulmonary microvascular protein leak in iNOS+/+ mice after AM depletion was restored in + to + AM-reconstituted mice (iNOS+/+ donor AM) but not in − to + AM-reconstituted mice (iNOS−/− donor AM). Although sepsis in iNOS−/− mice was not associated with any increase in pulmonary protein leak, sepsis-induced pulmonary microvascular protein leak could be induced in iNOS−/− mice by AM depletion and subsequent AM reconstitution with iNOS+/+ donor AM (+ to − AM chimeric mice). In all AM-depleted mice, AM reconstitution with either iNOS+/+ or iNOS−/− AM restored septic pulmonary neutrophil influx. In addition, AM iNOS appears to contribute minimally to the sepsis-induced increases in both pulmonary iNOS expression and systemic NO production.

Septic ALI is characterized by a significant increase in the permeability of the pulmonary microvascular endothelial cell (EC) barrier, resulting in the leak of protein-rich plasma fluid and circulating inflammatory cells into the pulmonary interstitium and air spaces (10, 57). A variety of cells, including inflammatory cells (e.g., neutrophils, AM) and pulmonary parenchymal cells (e.g., epithelial cells, EC) participate in the inflammatory process during septic ALI (12, 25, 36, 52, 58).

AM are the predominant resident immune cells in the pulmonary air spaces and may contribute importantly to ALI. In the present study, effective AM depletion completely eliminated the septic increase in pulmonary microvascular protein leak. Similarly, AM depletion was associated with reduced lung vascular permeability after unilateral lung ischemia-reperfusion in rats (33). AM after intratracheal IgG-immune complex is also AM dependent because AM depletion attenuated the severity of ALI, as measured by lung vascular permeability, NF-κB activation, and intercellular adhesion molecule-1 expression, as well as BAL levels of TNF-α and MIP-2 (27). In contrast, AM depletion in mice did not attenuate alveolar albumin leak after intratracheal MCP-1/LPS treatment (29). Differences between studies may be due to interspecies differences and to the model of ALI employed, as AM may be more important in some forms of ALI.

AM may contribute to ALI through production and release of several mediators, including reactive oxygen species, NO, and cytotoxic or proinflammatory cytokines, including chemokine receptors (chemokines) (25, 33). AM express iNOS and are important sources of endogenous pulmonary NO production in inflammatory states, such as septic ALI (9, 56, 57). A role for iNOS in septic ALI, and specifically in septic pulmonary microvascular protein leak, has been suggested previously by our group and others (24, 42, 57). Moreover, using reciprocally bone marrow-transplanted iNOS chimeric mice, we have previously shown that iNOS specifically in inflammatory cells, including neutrophils and AM, was responsible for septic ALI, e.g., pulmonary microvascular protein leak, oxidant, and nitrosative stress (42, 57). In the present study, the selective presence of iNOS in AM (in + to − AM-depleted/reconstituted mice) was absolutely required for phenotypic expression of septic pulmonary microvascular protein leak. Our findings are consistent with the role suggested for AM iNOS in pancreatitis-associated early ALI in rats (53). Moreover, in this previously published study, the cytotoxicity of activated AM against human umbilical vein EC was shown to be NO dependent because injury was reduced after preincubation of AM with L-NMMA, an NO inhibitor. AM iNOS may also be clinically relevant: enhanced AM iNOS expression has been observed in AM isolated by BAL in patients with acute respiratory distress syndrome (ARDS) (21). It is noteworthy that AM iNOS contributed negligibly to total pulmonary iNOS activity and total systemic NO production, as reflected by plasma nitrates/nitrites, in septic mice. However, this is consistent with our concept of cell source-specific

Table 1. Effects of sepsis on the levels (pg/ml) of selected key cytokines in the bronchoalveolar lavage fluid of iNOS+/+ and iNOS−/− mice

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>iNOS+/+ Naïve</th>
<th>iNOS+/+ Septic</th>
<th>iNOS−/− Naïve</th>
<th>iNOS−/− Septic</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1.2 ± 0.4</td>
<td>47.2 ± 2.9*</td>
<td>1.1 ± 0.1</td>
<td>42.1 ± 3.7*</td>
</tr>
<tr>
<td>MIP-2</td>
<td>10.2 ± 0.2</td>
<td>2560 ± 975*</td>
<td>10.8 ± 0.7</td>
<td>2071 ± 668*</td>
</tr>
<tr>
<td>IL-10</td>
<td>17.8 ± 10.2</td>
<td>14 ± 4.9</td>
<td>7.6 ± 3.2</td>
<td>9.4 ± 5.9</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6/group. MIP, macrophage inflammatory protein. *P < 0.05 vs. naïve.
effects of iNOS, such that selective expression of low levels of iNOS in AM, relative to other cells in the lungs, may still be pathophysiologically important in ALI.

One of the major roles of AM is to facilitate the recruitment of circulating neutrophils to the lung, through production and release of neutrophil chemokines (25, 31). Indeed, AM depletion in the present study also significantly attenuated septic pulmonary neutrophil influx (6, 22, 33). Consistent with this important role of AM, septic pulmonary neutrophil infiltration was restored to the levels observed in septic wild-type mice after AM reconstitution of AM-depleted mice. However, AM iNOS did not appear to modulate septic pulmonary neutrophil infiltration, which was restored in AM-depleted mice after reconstitution with either donor iNOS+/+ or iNOS−/− AM. This is in stark contrast to the absolute role of AM iNOS in septic pulmonary microvascular protein leak. Thus the mechanism of AM iNOS mediation of septic pulmonary microvascular protein leak was not through AM-mediated modulation of pulmonary neutrophil infiltration. It should be noted that AM iNOS-dependent ALI still required pulmonary neutrophil infiltration, which was present in all AM iNOS chimeric mice. Indeed, neutrophils are key circulating inflammatory cells that contribute to the microvascular and tissue injury of ALI (8, 28, 30, 42, 46, 55).

The mechanism of AM iNOS-mediated septic pulmonary protein leak remains uncertain. In the setting of inflammation, such as in ALI, many of the effects of NO are thought to be mediated through its rapid reaction with superoxide, leading to generation of extremely potent oxidants, e.g., peroxynitrite, hydroxyl radical (4, 11, 23). Peroxynitrite is associated with covalent modification of proteins through nitrosation of sulfhydryl groups and nitration of tyrosine residues in proteins (2, 47, 50, 63). Tyrosine nitration generates 3-nitrotyrosine residues in proteins, an important posttranslational modification that can regulate the function of proteins, which may play a role in ALI, such as surfactant-associated proteins, actin, and NF-κB (2, 5, 37, 63). The presence of 3-nitrotyrosine is considered a “footprint” of peroxynitrite action and has been identified in proteins isolated from the BAL fluid of patients with ARDS (47, 63). Indeed, activated rat AM were shown to nitrate a tyrosine analog, which was inhibited by NOS inhibition (19).

Alternatively, AM iNOS may also regulate AM cytokine and chemokine production, which could modulate neutrophil activation and function (48, 62). However, in the present study, the levels of key cytokines in the BAL, e.g., IL-6 and MIP-2, were similar in septic iNOS+/+ or iNOS−/− mice, suggesting minimal contribution of iNOS to pulmonary levels of these key cytokines in septic AM. Moreover, these data suggest that AM iNOS-dependent septic lung injury is not mediated through iNOS-dependent changes in pulmonary cytokine expression. In other studies, NO increased expression of proinflammatory cytokines such as IL-1, TNF-α, and MIP-2 (35, 41, 48). NO may also have an anti-inflammatory effect through inhibition of NF-κB activation (38, 62). Given these conflicting data, further studies will be required to define the possible role of cell source-specific iNOS and NO on pulmonary cytokine expression.

We recognize limitations of our study. Although the murine CLP model of sepsis has been recognized as representative of sepsis in humans, the degree of ALI is quite mild (43, 44), such that no mechanical ventilation or supplemental oxygenation is required. However, both ventilation and oxygen therapy can exacerbate ALI, which can confound assessment of the role of iNOS. Moreover, our findings may be of greater relevance to the early, initiating events in human ALI. Although intrapulmonary instillation of clodronate liposomes has been widely used and is highly selective for AM, which avidly take up liposomes (20, 54), clodronate may cause cytoplasmic edema in alveolar epithelial cells (6). However, in the present study, pulmonary microvascular leak and neutrophil influx were not increased in sham mice after AM depletion, indicating the clodronate liposomes did not cause any apparent ALI. In addition, AM depletion had no effect on the degree of peritonia after the CLP procedure, indicating that the effects of the clodronate liposomes were localized to the lung.

In summary, AM contribute significantly to septic pulmonary protein leak and pulmonary neutrophil influx. In addition, we provide data in support of our concept of cell source-specific effects of iNOS-derived NO and clearly define a critical role of AM iNOS in mediating septic pulmonary microvascular leak, independent of iNOS expression in other cells. Targeting macrophage iNOS may be a therapeutic option in human ALI/ARDS, as opposed to nonspecific systemic NO inhibition, which can worsen cardiac, hepatic, and renal injury and does not improve survival (56, 60).

GRANTS

This work was supported by the Ontario Thoracic Society, Lawson Health Research Institute, University of Western Ontario, and London Health Sciences Center Department of Medicine.

REFERENCES

4. Beckman JS, Beckman TW, Chen J, Marshall PA, and Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 87: 1620–1624, 1990.


