Published in final edited form as:

Shock. 2009 September ; 32(3): 258–262. doi:10.1097/SHK.0b013e31819c3414.

THE NOVEL INOSINE ANALOGUE INO-2002 EXERTS AN ANTI-INFLAMMATORY EFFECT IN A MURINE MODEL OF ACUTE LUNG INJURY

Jon G. Mabley^{*}, Pal Pacher[†], Kanneganti G.K. Murthy[‡], William Williams[‡], Garry J. Southan[‡], Andrew L. Salzman[‡], and Csaba Szabo[§]

^{*}School of Pharmacy and Biomolecular Sciences, University of Brighton, Brighton, UK [†]Section on Oxidative Stress Tissue Injury, Laboratory of Physiological Studies, National Institutes of Health/ NIAAA, Bethesda, Maryland [‡]Inotek Pharmaceuticals Corporation, Beverly, Massachusetts

[§]Department of Anesthesiology, The University of Texas Medical Branch, Galveston, Texas

Abstract

Endogenous purines, including inosine, have been shown to exert immunomodulatory and anti-inflammatory effects in a variety of disease models. The dosage of inosine required for these effects has been shown to be between 200 and 600 mg kg⁻¹ because of the rapid metabolism of inosine *in vivo*. The aim of this study was to determine whether a metabolic resistant purine analog, INO-2002, exerts anti-inflammatory effects in an animal model of acute respiratory distress syndrome. Mice challenged with intratracheal LPS (50 µg) were treated with INO-2002 (30 or 100 mg kg⁻¹, i.p.) in divided doses at either 1 and 12 h or at 5 and 16 h. After 24 h, bronchoalveolar lavage fluid was obtained to measure leukocyte infiltration by myeloperoxidase levels, lung edema by protein levels, and proinflammatory chemokine (macrophage inflammatory protein 1α) and cytokine (TNF-α, IL-1, and IL-6) levels. INO-2002 (30 and 100 mg kg⁻¹) reduced the LPS-mediated infiltration of leukocytes and edema as evidenced by bronchoalveolar lavage fluid reduction in levels of myeloperoxidase and protein. INO-2002 also downregulated expression of the proinflammatory mediators macrophage inflammatory protein 1α, TNF-α, IL-1, and IL-6. Delaying the start of treatment by 5 h after LPS administration affected the potency of INO-2002 protective effects, with 100 but not 30 mg kg⁻¹ having anti-inflammatory effects. The inosine analog INO-2002 largely suppressed LPS-induced inflammation *in vivo* at doses lower than those needed for the naturally occurring purine inosine. These data support the proposal that purine analogs, resistant to metabolic breakdown, may represent a useful addition to the therapy of acute respiratory distress syndrome.

Keywords

Adenosine; cytokines; inflammation; inosine; lung; purine

INTRODUCTION

Acute respiratory distress syndrome (ARDS) is a major complication of conditions such as sepsis, trauma, and severe pneumonia. Currently, no satisfying therapy has emerged for ARDS

despite significant advances in our understanding of the underlying pathogenic mechanisms, and treatment remains largely supportive (1). Acute respiratory distress syndrome is characterized by an overwhelming lung inflammation involving the local recruitment and activation of polymorphonuclear neutrophils (2,3), the release of proinflammatory mediators such as chemokines and cytokines (4), and both reactive oxygen and nitrogen species (5,6). This inflammation cascade results in alveolocapillary damage, high-permeability pulmonary edema, altered lung mechanics, and severe gas exchange abnormalities (1). To investigate prospective therapeutic strategies to combat ARDS, several animal models have been developed, in particular, the *in vivo* administration of LPS, a component of the wall of gram-negative bacteria, has gained wide acceptance as a clinically relevant model of severe lung inflammation (7-9). We recently identified inosine as an effective suppressor of LPS-induced lung inflammation *in vivo* (10); however, the doses required (600 mg kg^{-1} administered over 24 h) preclude its use therapeutically.

Purine nucleosides such as adenosine and its primary metabolite inosine are low-molecular-weight molecules that are involved in a wide variety of intracellular biochemical processes (11). Additionally, nucleosides function as extracellular signaling molecules, and although both adenosine and inosine are present only at low levels in the extracellular space, metabolically stressful conditions such as I/R injury and inflammation dramatically increase their concentrations (11). Inosine has proved to be a powerful immunomodulatory agent both *in vitro* (12,13) and *in vivo* (10,12,14-19). Inosine treatment reduces the production of inflammatory cytokines by murine and human macrophages stimulated by LPS *in vitro* in addition to provided protective effects on both intestinal and vascular function in murine models of endotoxic shock *in vivo* (12,15). Inosine also attenuates the course of chronic autoimmune inflammatory diseases; these include type 1 diabetes (17), and colitis (16), the beneficial effects mediated by inosine reducing production of proinflammatory cytokines and chemokines. However, in all these models, the effective dosage of inosine approaches $600 \text{ mg kg}^{-1} \text{ d}^{-1}$ for acute inflammation and $200 \text{ mg kg}^{-1} \text{ d}^{-1}$ for chronic inflammatory conditions, dosages that would prove problematic in humans therapeutically.

The likely hypothesis explaining the high inosine requirement dose is the short half-life on inosine *in vivo*; this is due to its rapid metabolism by purine nucleoside phosphorylase to hypoxanthine, which is broken down further to xanthine and finally uric acid by xanthine oxidase (11). Attempts to overcome this by using phosphorylated inosine, inosine monophosphate as an immunomodulating agent have proved partially successful (20), but inosine monophosphate itself is rapidly broken down to inosine by 5'-nucleotidase. Therefore, development of a purine analog resistant to metabolism may overcome the dosage issues associated with inosine. INO-2002 is such an analog, a monosulfated modification of inosine that is resistant to breakdown by both purine nucleoside phosphorylase and 5'-nucleotidase (21). This study was undertaken to evaluate the effects of INO-2002 on acute lung inflammation induced by LPS *in vivo*.

METHODS

In vivo studies were performed in accordance with National Institutes of Health guidelines and with the approval of the local institutional animal care and use committee.

Intratracheal LPS administration

Male BALB/c mice 8 to 10 weeks old were anesthetized with a mixture of ketamine (80 mg kg^{-1} , i.p.) and xylazine (30 mg kg^{-1} , i.p.). A 1-cm midline cervical incision was made, exposing the trachea. Intratracheal administration of LPS (*Escherichia coli*; O127:B8) or vehicle (phosphate-buffered saline [PBS] pH 7.4) was performed with a bent 27-G tuberculin syringe (in a volume of $100 \mu\text{L}$) as described previously (10,22). The cervical incision was closed with

5.0 silk suture, and the mice were returned to their cage. The animals recovered rapidly after surgery.

Bronchoalveolar lavage

The mice were reanesthetized with ketamine/xylazine (i.p.) 24 h after the surgery. The animals were bled by transection of the inferior vena cava to reduce hemorrhage into the lungs. Bronchoalveolar lavage was performed by the intratracheal instillation of 1 mL PBS (pH 7.4) into the exposed lungs (maintained within the thoracic cavity). The lavage fluid was infused a total of three times into the lungs before final collection. The bronchoalveolar lavage fluid (BALF) was then centrifuged at 5,000 rpm for 10 min, and the cell-free supernatant was frozen at -70°C until further analysis. The volume of cell-free supernatant was measured for each animal.

Treatment groups

Animals challenged with intratracheal LPS were treated with INO-2002, 30 or 100 mg kg⁻¹ total dose given over 24 h (n = 12), or vehicle (isotonic saline; 0.2 mL; n = 12; control group), administered intraperitoneally BID, either at 1 and 12 h or delayed until 5 and 16 h after surgery. In addition, a group of n = 8 mice (sham group) was challenged with intratracheal PBS instead of LPS.

Measurements

Protein assay—The concentration of proteins in the BALF was determined using the Bradford assay. Proteins are expressed as micrograms of protein per milliliter of BALF (23).

Myeloperoxidase activity—The activity of myeloperoxidase (MPO), an indicator of neutrophil accumulation, was measured directly in the BALF (24). An aliquot of BALF (20 µL) was mixed with tetra-methyl-benzidine (1.6 mmol/L) and hydrogen peroxide (1 mmol/L). Myeloperoxidase activity was then measured as the change in absorbance at 650 nm at 37°C using a Spectramax microplate reader (Molecular Devices, Sunnyvale, Calif). Results are expressed as milliunits of MPO activity per milliliter of BALF.

Cytokines and chemokines—The concentration of the proinflammatory cytokines IL-1, IL-6, and TNF-α and chemokines macrophage inflammatory protein (MIP) 1α (a CC chemokine) were determined in the BALF using commercially available enzyme-linked immunosorbent assays (23), following the protocol provided by the manufacturer (R&D Systems, Minneapolis, Minn). For the measurement of proinflammatory cytokines and chemokines in mice challenged with LPS, BALF was diluted 1:2 to 1:5. Bronchoalveolar lavage fluid was assayed undiluted in sham animals.

Lung histology and immunohistochemistry—Histopathological changes induced by LPS were evaluated in five mice treated with INO-2002 treated with 100 mg kg⁻¹ BID at 1 and 12 h, and five mice were treated with vehicle. The animals were anesthetized 24 h after surgery, killed by exsanguination, and the lungs were inflated-fixed with 4% paraformaldehyde. Paraffin-embedded lungs were sectioned at 3 µm and stained with hematoxylin and eosin for morphological analysis (10,22). Photomicrographs were taken with a Zeiss Axiolab microscope equipped with a Fuji HC-300C digital camera.

Data analysis

Data are expressed as mean ± SEM. All the enzyme-linked immunosorbent assay and protein data were statistically evaluated using analysis of variance. When the relevant *F* values were

significant at the 5% level, pairwise comparisons were further made using Tukey post hoc test. Statistical significance was assigned to $P < 0.05$.

RESULTS

INO-2002 reduces lung leukocyte accumulation and formation of high-permeability edema after intratracheal instillation of LPS

The BALF of sham mice had a very low level of MPO activity associated with little or no leukocyte accumulation (Fig. 1A). Administration of LPS elicited a massive inflammation of the lung as demonstrated by a marked increase in MPO activity (Fig. 1A), indicating the presence of a significant proportion of polymorphonuclear cells. INO-2002 treatment, at both 30 and 100 mg kg⁻¹ treatment starting at 1 h after surgery, significantly suppressed the accumulation of leukocytes in the alveolar spaces, as indicated by a significant reduction BALF MPO activity from $1,059 \pm 116$ to 691 ± 121 and 385 ± 112 mU mL⁻¹, respectively (Fig. 1A). However, 30 mg kg⁻¹ INO-2002 failed to prevent the LPS-mediated increase in BALF MPO levels when the treatment start is delayed until 5 h after surgery (Fig. 1A); in contrast, the treatment start time had no effect on the effectiveness of 100 mg kg⁻¹ in reducing MPO levels (Fig. 1A).

A hallmark of ARDS is the development of high-permeability edema characterized by a high protein content of the edema fluid. Such abnormalities were present in the lungs of mice exposed to LPS, where the protein concentration in the BALF increased 3-fold from that in sham animals (Fig. 1B); however, the concentration of protein in the BALF was significantly reduced in LPS mice receiving INO-2002 at both 30 and 100 mg kg⁻¹ treatment starting 1 h after surgery (Fig. 1B). Similar to the MPO data, delaying the start of the INO-2002 treatment prevented 30 mg kg⁻¹ having a protective effect on edema, but the delay had no effect on the 100 mg kg⁻¹ dose (Fig. 1B).

INO-2002 downregulates the expression of proinflammatory chemokine MIP-1 α and cytokines TNF- α , IL-1, and IL-6 in acute lung inflammation

The BALF from sham animals had virtually undetectable levels of the inflammatory chemokine MIP-1 α and cytokines TNF- α , IL-1, and IL-6 (Table 1). Administration of LPS dramatically increased BALF levels of MIP-1 α , TNF- α , IL-1, and IL-6 to 540 ± 35 , $4,763 \pm 334$, 258 ± 18 , and 916 ± 97 pg mL⁻¹, respectively, effects which are attenuated by INO-2002 at both 30 mg kg⁻¹ (427 ± 39 , $3,392 \pm 433$, 240 ± 25 , and 652 ± 40 pg mL⁻¹) and 100 mg kg⁻¹ (281 ± 54 , $2,581 \pm 735$, 150 ± 17 , and 394 ± 62 pg mL⁻¹) when treatment is started 1 h after LPS administration (Table 1). The protective effects of 30 mg kg⁻¹ INO-2002 on chemokine and cytokine levels are reduced if treatment start is delayed until 5 h after LPS administration, with treatment only significantly reducing the LPS-mediated increase in TNF- α (Table 1). However, delaying the start of treatment with 100 mg kg⁻¹ INO-2002 has no effect on its protective action with significant reductions in MIP-1 α , TNF- α , IL-1, and IL-6 observed when treatment is started at 1 or 5 h after LPS administration (Table 1).

INO-2002 reduces morphologic damage in lungs exposed to LPS

As illustrated in Figure 2, the lungs of mice exposed to LPS showed marked inflammatory alterations characterized by a thickening of the alveolocapillary membrane, the presence of alveolar hemorrhage, and massive extravasation of both mono- and polymorphonuclear leukocytes into the alveolar space. In contrast, the histological damage was less pronounced in mice treated with INO-2002, where the number of both erythrocytes and leukocytes in the alveolar spaces was clearly reduced (Fig. 2).

DISCUSSION

The data presented in this study not only support the proposal that purines can have potent anti-inflammatory effects in lungs exposed to LPS but also that by using an inosine analog INO-2002, which is resistant to metabolic breakdown, the potency of purines can be greatly increased. INO-2002 down-regulated the expression of proinflammatory chemokines and cytokines, reduced the infiltration of activated polymorphonuclear neutrophils in the airways, decreased pulmonary edema, and improved lung morphology in a model of acute lung inflammation.

We have previously shown that inosine has anti-inflammatory effects in a wide range of inflammatory conditions (12,14,16,17), including lung inflammation (10), but at doses that preclude its use in the clinical setting, probably due to its short half-life. By producing an analog of inosine, INO-2002, resistant to metabolism by both purine nucleoside phosphorylase and 5'-nucleotidase (21), we have a purine likely to have a longer half-life *in vivo* and, hence, be more effective at lower doses. Indeed, the data reported here demonstrate INO-2002 is effective at 100 mg kg⁻¹ as compared to inosine, which exerted a protective effect in acute lung inflammation at 600 mg kg⁻¹ (10). INO-2002 also proved to be effective when the treatment start time was delayed attenuating lung inflammation when treatment started 1 h or 5 h after LPS exposure. These data are similar to that observed in animal models of type I diabetes, where INO-2002 proved to be more effective than inosine at reducing the incidence of diabetes at equivalent doses (21).

The presence of infiltrating leukocytes is the hallmark of pulmonary inflammation associated with acute lung injury (2,25) with chemokines (26) such as MIP-1 α regulating the recruitment to and activation of these leukocytes at sites of inflammation. LPS treatment *in vivo* increases lung MIP-1 α levels with subsequent leukocyte infiltration, an effect reduced by INO-2002 treatment. Although the molecular mechanisms underlying the effects of INO-2002 on chemokine expression cannot be inferred from our data, it is interesting to note that inosine has been shown to reduce MIP-1 α production in LPS-stimulated murine macrophages *in vitro* via activation of adenosine receptors (12). Therefore, a major effect of INO-2002 as with inosine may be to alter the development of chemotactic gradients, thereby acting as a powerful down-regulator of leukocyte trafficking in inflammatory conditions.

Acute respiratory distress syndrome is associated with the development of interconnected inflammatory cascades, with proinflammatory cytokines playing a central role in the initiation and propagation of the inflammatory response leading to lung injury. Of these cytokines, TNF- α , IL-1, and IL-6 are considered pivotal, with high levels of these cytokines observed in nonsurviving ARDS patients than in surviving patients (27-29). INO-2002 significantly reduced the LPS-mediated increase in all three of these inflammatory cytokines. These cytokines have also been implicated in the destruction of alveolar epithelium and leakage of capillaries leading to pulmonary edema, a hallmark of ARDS (30). The LPS-mediated increase in pulmonary edema was attenuated by INO-2002 treatment. Delaying the start of the treatment again affects the potency of INO-2002, with only the 100-mg kg⁻¹ dose effective at reducing proinflammatory cytokine levels. The reduction in leukocyte infiltration is an important mechanism underlying the beneficial effects on edema as neutrophils and the pro-inflammatory environment they produce are considered the primary effectors of alveolocapillary damage in ARDS (2,31).

The cellular and molecular mechanisms underlying the effects of purines such as inosine on inflammation are incompletely understood, but there have been three cellular mechanisms identified as possibly playing a role in the anti-inflammatory effect. Inosine has been shown to activate both A2a and A3 adenosine receptors to affect proinflammatory cytokine production

by macrophages and lymphocytes (12,32,33), and it is this mechanism that most likely explains INO-2002 protective effects. However, inosine, at millimolar concentrations (34) may also act partially by interfering with the activation of the nuclear enzyme poly (adenosine diphosphate–ribose) polymerase, whose activation has been shown to be a major mechanism of tissue injury in inflammation (35), including acute lung inflammation (22,36). The very high concentrations required for purine-mediated inhibition of poly (adenosine diphosphate–ribose) polymerase suggests that this may not account for the INO-2002–mediated protection in ARDS. Finally, inosine has also been shown to affect oxyradicals and peroxynitrite, both of which have been implicated in inflammation and resulting cell damage (37). Inosine can reduce radical formation both directly, by inhibiting superoxide production by human neutrophils (13), and indirectly, via its metabolite uric acid, a scavenger of oxyradicals and peroxynitrite (38). The lack of uric acid formation by INO-2002, which is incapable of being broken down through this purine metabolizing pathway, suggests that it is the INO-2002–mediated downregulation of inflammatory cytokines and the inhibition of leukocyte superoxide production that may reduce radical formation.

The data presented in this study indicate that a metabolic resistant analog of inosine, INO-2002, produces marked anti-inflammatory effects probably through adenosine receptor activation in a clinically relevant animal model of ARDS. Purines and their analogs have a very good toxicological profile and targeting an endogenous anti-inflammatory pathway, which have an enormous clinical potential in inflammatory disease. INO-2002 has also proved effective when the start of the treatment protocol is delayed, an important consideration in developing agents for clinical use. However, although the improvement of potency of INO-2002, as compared with inosine, as an anti-inflammatory agent strengthens the possibility that a purine analog may have therapeutic use in various inflammatory indications, the doses required remain high, and development of even more potent inosine analogs before their use in a clinical setting becomes possible.

Acknowledgments

This study was supported, in part, by the National Institutes of Health (grant no. RO1GM60915) to C.S.

References

1. Artigas A, Bernard GR, Carlet J, Dreyfuss D, Gattinoni L, Hudson L, Lamy M, Marini JJ, Matthay MA, Pinsky MR, et al. The American-European Consensus Conference on ARDS, part 2. Ventilatory, pharmacologic, supportive therapy, study design strategies and issues related to recovery and remodeling. *Intensive Care Med* 1998;24:378–398. [PubMed: 9609420]
2. Abraham E, Carmody A, Shenkar R, Arcaroli J. Neutrophils as early immunologic effectors in hemorrhage- or endotoxemia-induced acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L1137–L1145. [PubMed: 11076804]
3. Chignard M, Balloy V. Neutrophil recruitment and increased permeability during acute lung injury induced by lipopolysaccharide. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L1083–L1090. [PubMed: 11076798]
4. Martin TR. Cytokines and the acute respiratory distress syndrome (ARDS): a question of balance. *Nat Med* 1997;3:272–273. [PubMed: 9055847]
5. Matthay MA, Geiser T, Matalon S, Ischiropoulos H. Oxidant-mediated lung injury in the acute respiratory distress syndrome. *Crit Care Med* 1999;27:2028–2030. [PubMed: 10507639]
6. Peng X, Abdunnour RE, Sammani S, Ma SF, Han EJ, Hasan EJ, Tudor R, Garcia JG, Hassoun PM. Inducible nitric oxide synthase contributes to ventilator-induced lung injury. *Am J Respir Crit Care Med* 2005;172:470–479. [PubMed: 15937288]
7. Borron P, McIntosh JC, Korfhagen TR, Whitsett JA, Taylor J, Wright JR. Surfactant-associated protein A inhibits LPS-induced cytokine and nitric oxide production in vivo. *Am J Physiol Lung Cell Mol Physiol* 2000;278:L840–L847. [PubMed: 10749762]

8. Harrod KS, Mounday AD, Whitsett JA. Adenoviral E3-14.7K protein in LPS-induced lung inflammation. *Am J Physiol Lung Cell Mol Physiol* 2000;278:L631–L639. [PubMed: 10749739]
9. Kabir K, Gelinas JP, Chen M, Chen D, Zhang D, Luo X, Yang JH, Carter D, Rabinovici R. Characterization of a murine model of endotoxin-induced acute lung injury. *Shock* 2002;17:300–303. [PubMed: 11954830]
10. Liaudet L, Mabley JG, Pacher P, Virag L, Soriano FG, Marton A, Hasko G, Deitch EA, Szabo C. Inosine exerts a broad range of antiinflammatory effects in a murine model of acute lung injury. *Ann Surg* 2002;235:568–578. [PubMed: 11923614]
11. Hasko G, Sitkovsky MV, Szabo C. Immunomodulatory and neuroprotective effects of inosine. *Trends Pharmacol Sci* 2004;25:152–157. [PubMed: 15019271]
12. Hasko G, Kuhel DG, Nemeth ZH, Mabley JG, Stachlewitz RF, Virag L, Lohinai Z, Southan GJ, Salzman AL, Szabo C. Inosine inhibits inflammatory cytokine production by a posttranscriptional mechanism and protects against endotoxin-induced shock. *J Immunol* 2000;164:1013–1019. [PubMed: 10623851]
13. Marton A, Pacher P, Murthy KG, Nemeth ZH, Hasko G, Szabo C. Anti-inflammatory effects of inosine in human monocytes, neutrophils and epithelial cells in vitro. *Int J Mol Med* 2001;8:617–621. [PubMed: 11712075]
14. Liaudet L, Mabley JG, Soriano FG, Pacher P, Marton A, Hasko G, Szabo C. Inosine reduces systemic inflammation and improves survival in septic shock induced by cecal ligation and puncture. *Am J Respir Crit Care Med* 2001;164:1213–1220. [PubMed: 11673212]
15. Garcia Soriano F, Liaudet L, Marton A, Hasko G, Batista Lorigados C, Deitch EA, Szabo C. Inosine improves gut permeability and vascular reactivity in endotoxic shock. *Crit Care Med* 2001;29:703–708. [PubMed: 11373452]
16. Mabley JG, Pacher P, Liaudet L, Soriano FG, Hasko G, Marton A, Szabo C, Salzman AL. Inosine reduces inflammation and improves survival in a murine model of colitis. *Am J Physiol Gastrointest Liver Physiol* 2003;284:G138–G144. [PubMed: 12388199]
17. Mabley JG, Rabinovitch A, Suarez-Pinzon W, Hasko G, Pacher P, Power R, Southan G, Salzman A, Szabo C. Inosine protects against the development of diabetes in multiple-low-dose streptozotocin and nonobese diabetic mouse models of type 1 diabetes. *Mol Med* 2003;9:96–104. [PubMed: 12865945]
18. Darlington DN, Gann DS. Inosine infusion prevents mortality in endotoxic shock. *J Trauma* 2005;59:1432–1435. [PubMed: 16394918]discussion 1435
19. Szabo G, Stumpf N, Radovits T, Sonnenberg K, Gero D, Hagl S, Szabo C, Bahrle S. Effects of inosine on reperfusion injury after heart transplantation. *Eur J Cardiothorac Surg* 2006;30:96–102. [PubMed: 16730445]
20. Qiu FH, Wada K, Stahl GL, Serhan CN. IMP and AMP deaminase in reperfusion injury down-regulates neutrophil recruitment. *Proc Natl Acad Sci U S A* 2000;97:4267–4272. [PubMed: 10760293]
21. Mabley JG, Pacher P, Murthy KG, Williams W, Southan GJ, Salzman AL, Szabo C. The novel inosine analogue, INO-2002, protects against diabetes development in multiple low-dose streptozotocin and non-obese diabetic mouse models of type I diabetes. *J Endocrinol* 2008;198:581–589. [PubMed: 18562629]
22. Liaudet L, Pacher P, Mabley JG, Virag L, Soriano FG, Hasko G, Szabo C. Activation of poly(ADP-ribose) polymerase-1 is a central mechanism of lipopolysaccharide-induced acute lung inflammation. *Am J Respir Crit Care Med* 2002;165:372–377. [PubMed: 11818323]
23. Liaudet L, Szabo C, Evgenov OV, Murthy KG, Pacher P, Virag L, Mabley JG, Marton A, Soriano FG, Kirov MY, et al. Flagellin from Gram-negative bacteria is a potent mediator of acute pulmonary inflammation in sepsis. *Shock* 2003;19:131–137. [PubMed: 12578121]
24. Soriano FG, Liaudet L, Szabo E, Virag L, Mabley JG, Pacher P, Szabo C. Resistance to acute septic peritonitis in poly(ADP-ribose) polymerase-1-deficient mice. *Shock* 2002;17:286–292. [PubMed: 11954828]
25. Abraham E, Arcaroli J, Shenkar R. Activation of extracellular signal-regulated kinases, NF-kappa B, and cyclic adenosine 5'-monophosphate response element-binding protein in lung neutrophils occurs

- by differing mechanisms after hemorrhage or endotoxemia. *J Immunol* 2001;166:522–530. [PubMed: 11123332]
26. Strieter RM, Kunkel SL, Keane MP, Standiford TJ. Chemokines in lung injury: Thomas A. Neff Lecture. *Chest* 1999;116:103S–110S. [PubMed: 10424625]
 27. Meduri GU, Kohler G, Headley S, Tolley E, Stentz F, Postlethwaite A. Inflammatory cytokines in the BAL of patients with ARDS. Persistent elevation over time predicts poor outcome. *Chest* 1995;108:1303–1314. [PubMed: 7587434]
 28. Meduri GU, Headley S, Kohler G, Stentz F, Tolley E, Umberger R, Leeper K. Persistent elevation of inflammatory cytokines predicts a poor outcome in ARDS. Plasma IL-1 beta and IL-6 levels are consistent and efficient predictors of outcome over time. *Chest* 1995;107:1062–1073. [PubMed: 7705118]
 29. Takala A, Jousela I, Takkunen O, Kautiainen H, Jansson SE, Orpana A, Karonen SL, Repo H. A prospective study of inflammation markers in patients at risk of indirect acute lung injury. *Shock* 2002;17:252–257. [PubMed: 11954822]
 30. Ware LB. Pathophysiology of acute lung injury and the acute respiratory distress syndrome. *Semin Respir Crit Care Med* 2006;27:337–349. [PubMed: 16909368]
 31. Kollef MH, Schuster DP. The acute respiratory distress syndrome. *N Engl J Med* 1995;332:27–37. [PubMed: 7646623]
 32. Gomez G, Sitkovsky MV. Differential requirement for A2a and A3 adenosine receptors for the protective effect of inosine in vivo. *Blood* 2003;102:4472–4478. [PubMed: 12947007]
 33. Nemeth ZH, Bleich D, Csoka B, Pacher P, Mabley JG, Himer L, Vizi ES, Deitch EA, Szabo C, Cronstein BN, et al. Adenosine receptor activation ameliorates type 1 diabetes. *FASEB J* 2007;21:2379–2388. [PubMed: 17405852]
 34. Virag L, Szabo C. Purines inhibit poly(ADP-ribose) polymerase activation and modulate oxidant-induced cell death. *FASEB J* 2001;15:99–107. [PubMed: 11149897]
 35. Mabley JG, Jagtap P, Perretti M, Getting SJ, Salzman AL, Virag L, Szabo E, Soriano FG, Liaudet L, Abdelkarim GE, et al. Anti-inflammatory effects of a novel, potent inhibitor of poly (ADP-ribose) polymerase. *Inflamm Res* 2001;50:561–569. [PubMed: 11766996]
 36. Murakami K, Enkhbaatar P, Shimoda K, Cox RA, Burke AS, Hawkins HK, Traber LD, Schmalstieg FC, Salzman AL, Mabley JG, et al. Inhibition of poly (ADP-ribose) polymerase attenuates acute lung injury in an ovine model of sepsis. *Shock* 2004;21:126–133. [PubMed: 14752285]
 37. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev* 2007;87:315–424. [PubMed: 17237348]
 38. Becker BF, Reinholz N, Ozcelik T, Leipert B, Gerlach E. Uric acid as radical scavenger and antioxidant in the heart. *Pflugers Arch* 1989;415:127–135. [PubMed: 2556684]

Fig. 1. Protective effect of INO-2002 against leukocyte infiltration (A) and edema (B) in an acute model of lung inflammation

Male BALBc mice received an intratracheal instillation of LPS (from *E. coli* serotype 0127:B8; 50 μ g) and were treated with INO-2002 at 30 or 100 mg kg⁻¹ divided into two doses at 1 and 12 h after surgery or delayed until 5 and 16 h after surgery. Control mice are sham-treated receiving vehicle instead of LPS intratracheally. Myeloperoxidase activity and protein levels in the BALF were determined 24 h after the LPS administration. Data are expressed as mean $\pm$ SEM from 8 to 12 animals; * P < 0.05; $^{\dagger}P$ < 0.01 vs. control mice; $^{\ddagger}P$ < 0.05 vs. LPS-treated mice.

Fig. 2. Lung morphology in mice challenged with LPS

Representative histological sections of lungs harvested 24 h in sham mice (A, D), LPS-treated mice (B, E), and mice treated with LPS and INO-2002 100 mg kg⁻¹ (C, F) starting at 1 and 12 h. The morphologic alterations induced by LPS (infiltration of the alveolar spaces with mono- and polymorphonuclear cells, presence of alveolar hemorrhages and exudates, thickening of the alveolar septa) were reduced by INO-2002 treatment. Pictures are representative of n = 6 mice per treatment group, magnification shown at both 100× and 400×.

Table 1

Concentrations of proinflammatory chemokines and cytokines in the BALF from sham, LPS-treated, and LPS + INO-2002-treated mice

Treatment	Cytokine/chemokine (pg mL ⁻¹)			
	MIP-1 α	TNF- α	IL-1	IL-6
Sham	6 $\pm$ 2	169 $\pm$ 85	—	—
LPS	540 $\pm$ 36 [*]	4,763 $\pm$ 334 [*]	258 $\pm$ 18 [*]	916 $\pm$ 97 [*]
LPS + INO-2002, 30 mg kg ⁻¹	427 $\pm$ 39 ^{*†}	3,392 $\pm$ 433 ^{*†}	240 $\pm$ 25 [*]	652 $\pm$ 40 ^{*†}
LPS + INO-2002, 100 mg kg ⁻¹	281 $\pm$ 54 ^{*†}	2,581 $\pm$ 735 ^{*†}	150 $\pm$ 17 ^{*†}	394 $\pm$ 62 ^{*†}
LPS + INO-2002, 30 mg kg ⁻¹ delay	474 $\pm$ 83 [*]	2,778 $\pm$ 900 ^{*†}	264 $\pm$ 54 [*]	769 $\pm$ 146 [*]
LPS + INO-2002, 100 mg kg ⁻¹ delay	247 $\pm$ 43 ^{*†}	2,223 $\pm$ 423 ^{*†}	150 $\pm$ 18 ^{*†}	482 $\pm$ 70 ^{*†}

LPS (50 μ g) induced a massive increase in BALF levels of MIP-1 α , TNF- α , IL-1, and IL-6 24 h after administration that was significantly suppressed by INO-2002 at both 30 and 100 mg kg⁻¹ when given at 1 and 12 h after LPS treatment. Delaying the start of INO-2002 treatment 4 h (starting 5 and 16 h) after LPS administration reduced the protective effects seen at 30 but not at 100 mg kg⁻¹. Results are mean $\pm$ SEM from 8 to 12 animals.

* $P < 0.01$ vs. sham;

[†] $P < 0.05$ vs. LPS-treated mice.