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# Defective Nitric Oxide Production by Alveolar Macrophages during *Pneumocystis* Pneumonia

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**Running title:** Nitric oxide and *Pneumocystis* pneumonia

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**Non-standard abbreviations**: *Pneumocystis*, Pc; PCP, *Pneumocystis* pneumonia; L-NIO, L-$N^5$-(1-iminoethyl)ornithine; Spm NONOate, (Z)-1-[N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino]diazen-1-ium-1,2-diolate; DEA NONOate, diethylammonium (Z)-1-(N,N-diethylamino)diazen-1-ium-1,2-diolate; Tmp/Smx, trimethoprim/sulfamethoxazole; BH4, tetrahydrobiopterin; PMSF, phenylmethanesulphonylfluoride.

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## Abstract

The toxicity of nitric oxide (NO) on *Pneumocystis* (Pc) organisms, the role of NO in the defense against Pc infection, and NO production by alveolar macrophages (AMs) during *Pneumocystis* pneumonia (PCP) were investigated. Results indicated that NO was toxic to Pc organisms and inhibited their proliferation in culture. When NO production was inhibited by intraperitoneal injection of rats with the nitric oxide synthase inhibitor L-$N^5$-(1-iminoethyl) ornithine (L-NIO), progression of Pc infection in immunocompetent rats was enhanced. NO levels in bronchoalveolar lavage (BAL) fluids from immunosuppressed, Pc-infected rats and mice were greatly reduced as compared to those from uninfected animals, and AMs from these animals were defective in NO production. However, inducible nitric oxide synthase (iNOS) mRNA and

protein levels were high in AMs from Pc-infected rats and mice. Immunoblot analysis showed that iNOS in AMs from Pc-infected rats existed primarily as a monomer, but homo-dimerization of iNOS monomers was required for NO production. When iNOS dimerization cofactors, including calmodulin, were added to macrophage lysates, iNOS dimerization increased, whereas incubation of the same lysates with all cofactors except calmodulin did not rescue iNOS dimer formation. These data suggest that NO is important in the defense against Pc infection, but production of NO in AMs during PCP is defective due to reduced dimerization of iNOS.

**Keywords:** *Pneumocystis,* nitric oxide, iNOS, alveolar macrophage, AIDS-related opportunistic infection, innate immunity, calmodulin

## Introduction

Complex interactions between host and pathogen occur during PCP, triggering both the innate and adaptive immune responses. Studies have shown that the alveolar macrophage (AM) is an important cell for the clearance of *Pneumocystis* organisms from the lungs during PCP (1, 2). Clearance of organisms by alveolar macrophages (AMs) is mediated by direct phagocytosis (2-4), production of toxic metabolites (5, 6), and modulation of inflammatory cytokines (2-4). Some of these functional abilities are defective (7, 8), and the number of AMs (7, 9) is decreased during PCP. The reasons for reduced function of AMs are largely unknown.

AMs are an important source of nitric oxide (NO) in the lung during infection (10). NO is a short-lived, freely diffusible molecule with many functions in normal physiology (11) and in response to cytokines (12), infectious agents, or bacterial products (13). It has an antimicrobial effect on organisms such as *Toxoplasma gondii* (14), *Leishmania donovani* (15), and *Cryptococcus neoformans* (16). NO also causes redistribution of the actin pool in some cells (17). This reorganization is mediated by calmodulin, as inhibition of calmodulin activity suppresses the redistribution despite high NO levels (17). Calmodulin is recently shown to be an important molecule for survival of AMs, but its level in AMs is decreased during PCP (18).

Pc organisms have been shown to stimulate AMs from humans and rats to produce NO (19), and both chemically produced (20) and IFN-$\gamma$-stimulated NO (21) are toxic to Pc organisms. However, elevated levels of iNOS mRNA and protein in AMs in Pc-infected, $CD4^+$ cell-depleted mice were found not to reduce the organism burden or slow disease progression (22, 23). In this study, we further investigated this problem and found that the inability of the iNOS protein to form dimers was a cause of this defect.

## Materials and Methods

**Reagents.** Spm NONOate [(Z)-1-[N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino]diazen-1-ium-1,2-diolate], DEA NONOate [diethylammonium (Z)-1-(N,N-diethylamino)diazen-1-ium-1,2-diolate], and L-NIO [L-$N^5$-(1-iminoethyl)ornithine] were purchased

from Cayman Chemical (Ann Arbor, MI). Tetrahydrobiopterin (BH4) was purchased from Alexis Biochemicals (Lausen, Switzerland). Heme was obtained from MP Biochemicals (Irvine, CA), and L-arginine hydrochloride was purchased from Fisher Bioreagents (Pittsburgh, PA). Anti-rat and mouse iNOS antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-GAPDH antibody was obtained from US Biological (Swampscott, MA). Calmodulin protein was purchased from ANIARA (Manson, OH). All other reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) unless stated otherwise.

**Short-term culture of Pc organisms.** Pc organisms were grown on monolayers of human embryonic lung (HEL 299) cells (CCL-137; ATCC, Manassas, VA) on 24-well culture dishes in 0.5 ml minimum essential medium containing 2 mM L-glutamine, 17.9 mM sodium bicarbonate, 0.1 mM nonessential amino acids, 10% fetal calf serum (FCS), 100 units/ml penicillin, and 0.1 mg/ml streptomycin at 37°C and 5% $O_2$, 10% $CO_2$, 85% nitrogen. *P. carinii* innoculum was isolated and added to the cultures as previously described (24).

**Rodent models of PCP.** Immunosuppression of rats with dexamethasone (7) and mice with anti-CD4 antibody (1, 25), transtracheal inoculation of animals with Pc organisms (7), bronchoalveolar lavage (26), and isolation of AMs from BAL fluids (7) were performed as previously described.

**Infection of mice with *Histoplasma capsulatum.*** *H. capsulatum* (IU-CT) yeasts were grown in HMM broth as described previously (27). Six-week old C57/BL6 mice (Jackson laboratories, Bar Harbor, Maine) were anesthetized with 5% isoflurane and then intranasally instilled with 50 μl inoculum containing 1 x $10^6$ yeasts of *H. capsulatum*. At days 7 and 14 post infection, BAL fluids and AMs were obtained from these mice by bronchoalveolar lavage as described above.

**Assessment of nitrate/nitrite levels.** The nitrite assay was performed using the NO Quantitation Kit (Active Motif, Carlsbad, CA) according to manufacturer's instructions.

**RNA isolation and real-time RT-PCR**. Total RNA was isolated from AMs using the TRIzol reagent according to manufacturer's instructions (Invitrogen). Real-time RT-PCR for rat or mouse iNOS was performed using the Assays-on-Demand™ gene expression kits (Applied Biosystems, Foster City, CA) on a Smartcycler (Cepheid, Sunnyvale, CA). Ribosomal protein S8 (RPS8) mRNA was assayed in an identical manner as a control as described (28). Organism viability was estimated based on the rRNA levels of Pc mitochondrial large subunit ribosomal RNA gene as described (29).

**Immunoblot blot analysis of iNOS protein.** Determination of protein concentration and Western blotting were performed as described (30). For determination of iNOS dimer formation, electrophoresis was performed under partially denatured conditions to allow the iNOS dimer to remain intact as previously described (31). Immunoblot

autoradiographs were analyzed using the ImageJ software package (32).

**Statistics.** Comparisons between the mean values of the treatment and control groups and data of effects of NO on Pc proliferation over time were analyzed by the Mann-Whiteney *U* test. A $p < 0.05$ was considered significant.

## Results

**NO production by chemical generators in culture media and effect of NO on Pc viability.** Spm NONOate and DEA NONOate were incubated in complete medium, and release of NO from these chemicals was induced by decreasing the pH of the medium from 8.0 to 7.4 with 1 N HCl. At pH 7.4 and 37˚C, the half-life of DEA NONOate is 2.1 min and that of Spm NONOate is 39 min (Product information, Cayman Chemical). The pH of medium in a well of each condition was checked daily and maintained at 7.0 - 7.4. Both chemicals produced NO in a dose-dependent manner. NO production (measured as nitrites) by DEA NONOate (0.1 mM) peaked (20.2 ± 2.0 µM) at 15 min after lowering the pH; little NO was produced after this time point. Production of NO by Spm NONOate (0.1 mM) was still increasing 60 min after pH change (23.7 ± 1.8 µM at 60 min). Lower concentrations (0.01 mM) of Spm NONOate and DEA NONOate generated roughly 30% as much NO as the higher concentrations (6.9 ± 1.2 µM for Spm NONOate and 6.5 ± 2.0 µM for DEA NONOate). The times for peak production were the same as those for the higher concentrations of each compound.

To test the toxicity of these compounds, one half-million *P. carinii* organisms were incubated with carrier (0.01 mM NaOH) or NO generators and then assessed for viability by measuring Pc mitochondrial large subunit rRNA levels at 2 or 4 h (Fig. 1). Compared to carrier-treated control levels at 4 h, 0.01 mM Spm NONOate decreased Pc viability by 17%, while 0.1 mM Spm NONOate reduced it by 33% at 2 h and 43% at 4 h (Fig. 1). Pc organisms incubated with 0.01 mM or 0.1 mM DEA NONOate did not show significant decreases in viability at either time point, probably due to the shorter duration of NO production by DEA NONOate.

**Suppression of Pc proliferation in short-term culture by NO.** Pc was cultured on HEL cell monolayers. High (0.1 mM) and low (0.01 mM) concentrations of NO generators were included in the culture media and replenished every 24 h. Separate cultures treated with carrier (0.1% DMSO in saline) or trimethoprim/sulfamethoxazole (Tmp/Smx, 50/250 µg/ml) were used as negative and positive controls, respectively. The cultures were examined under an inverted microscope every other day to assess the health of the monolayer. Any culture showing signs of contamination by other microorganisms (bacteria or fungi) was discarded. Organisms were sampled and counted every other day as described before (24). Untreated cultures showed a significant increase in organism numbers after seven days of culture (21.3 ± 4.1 organisms per 1000x microscopic field, Fig. 2). Tmp/Smx inhibited propagation of the organisms in culture by 89% (2.4 ± 0.4

organisms per 1000x microscopic field, $p<0.0001$ vs. untreated control) at the same time point (7 days). DEA NONOate at either concentration and Spm NONOate at 0.01 mM had minimal effects (Fig. 2), whereas Spm NONOate at 0.1 mM suppressed 97% of Pc growth at seven days (0.58 ± 0.1 organisms per 1000x field). These results indicate that the NO generated is inhibitory to Pc replication in short-term culture.

To control for the toxicity of NO to HEL cells, the health of the monolayer was monitored in each condition. Without Pc, HEL cell culture alone had only 5.4 ± 1.0% cell death, but 54.6 ± 6.6% of Pc-infected cells died ($p<0.05$ vs. control) after seven days of culture. Spm NONOate at 0.1 mM inhibited 97% of Pc growth as mentioned above, but caused only 10.7 ± 2.3% cell death by Day 7, indicating that the suppression of Pc growth in cultures by NO generators was not due to damage of the cell monolayer by NO.

**Increased Pc proliferation *in vivo* by suppressing NO production.** To determine if NO participates in the control of Pc proliferation *in vivo*, uninfected (Normal) and Pc-inoculated Normal (Normal-Pc) rats were injected (i.p.) daily with L-NIO at 2 mg/kg to inhibit NO production and then assessed for organism burden and NO levels in AMs and BAL fluids. NO is quickly converted to nitrites and nitrates in vivo (33-35). Nitrates can be converted to nitrites through the action of nitrate reductase. Therefore, NO levels in AMs and BAL fluids were determined by measuring nitrites after conversion of nitrates to nitrites using the nitrate reductase in the NO quantitation kit. AMs were isolated, incubated for 1 h in saline, lysed by sonication, and assayed for nitrite levels.

AMs from rats treated with L-NIO for one week produced 1.5 ± 0.2 μM (normalized to 1 mg of AM protein) of nitrites; this was 62% less than that (3.9 ± 0.5 μM) produced by AMs from untreated rats ($p<0.05$) (Fig. 3). The ability to produce NO by AMs was further impaired with longer treatment of L-NIO. After two weeks of L-NIO injection, nitrite production in AMs was decreased by 73% (1.0 ± 0.1 μM vs. 3.7 ± 0.2 μM for untreated controls, $p<0.05$) (Fig. 3).

The response of AMs to stimulation by IFN-γ and *E. coli* organisms to produce NO was then assessed. One hour incubation with 10 ng/ml IFN-γ and $5 \times 10^6$ *E. coli* organisms increased nitrite production in AMs from Normal rats by approximately 3 fold (from 3.9 ± 0.5 to 11.6 ± 2.2 μM for those treated with carrier for one week and from 3.7 ± 0.2 to 10.2 ± 1.1 μM for those treated with carrier for two weeks). In contrast, AMs from L-NIO-treated rats increased nitrite production by only 1.4 and 1.2 fold, respectively, from 1.5 ± 0.2 to 2.1 ± 0.4 μM for those treated for one week and from 1.0 ± 0.1 to 1.2 ± 0.2 μM for those treated for two weeks (Fig. 3). AMs from Normal-Pc rats which had been inoculated with Pc for one week or two weeks produced significantly more nitrites (14.7 ± 2.0 μM and 15.3 ± 1.8 μM, respectively, $p<0.05$ vs. controls at the same time point). IFN-γ and *E. coli* stimulation did not increase the levels of nitrites produced by AMs at either time point.

In contrast to the increase in nitrite production in Normal rats inoculated with Pc, AMs from L-NIO treated Normal-Pc rats did not show an

increase in nitrite production in response to Pc inoculation (3.2 ± 0.4 μM after one week and 2.3 ± 0.2 μM after two weeks of L-NIO treatment, $p<0.05$ for each versus Normal-Pc rats). IFN-γ and *E. coli* also failed to further stimulate nitrite production in these cells (3.0 ± 0.3 μM for 1 week and 2.1 ± 0.3 μM for two weeks) (Fig. 3).

Organism burdens in Normal-Pc rats with or without L-NIO treatment were also determined. After one week of infection, impression smears of lungs from Normal-Pc rats showed an average of 0.6 ± 0.1 trophic forms and 0.1 ± 0.1 cysts per 1000x microscopic field (Fig. 3). Rats treated with L-NIO showed higher burdens with an average of 1.9 ± 0.2 trophic forms and 0.2 ± 0.1 cysts at the same time point. These represent 3.1 fold and 2 fold increases in trophic forms and cysts, respectively, and both increases were statistically significant ($p<0.05$ vs. Normal-Pc). At two weeks, the difference in organism burden between untreated and L-NIO-treated rats was even larger. Normal-Pc rats had begun to clear the organisms, and both trophic form (0.5 ± 0.1) and cyst (0.1 ± 0.1) numbers were reduced or unchanged. In contrast, organism burdens in L-NIO treated rats were increased (from 1.9 ± 0.2 to 3.6 ± 0.2 trophic forms/field and from 0.2 ± 0.1 to 0.6 ± 0.1 cysts/field, $p<0.0001$ vs. Normal-Pc and Normal-Pc + L-NIO at one week) (Fig. 3). These results demonstrate that AMs do produce NO in response to Pc infection, and that inhibition of NO production hampers the clearance of Pc from the lungs of immunocompetent rats. Since L-NIO treated rats were not immunosuppressed, they all cleared the infection and survived.

**Low nitrite levels in BAL fluids of Pc-infected rats and mice.** To investigate NO production in the lung during PCP, the first 5 ml of the BAL fluids from uninfected and Pc-infected immunosuppressed rats were assayed for nitrite levels. Results showed that BAL fluids from Dex-Pc rats had 35% less nitrites than those from either Normal or Dex rats ($p<0.05$, Table 1). BAL fluids from Normal-Pc rats had 3 fold more nitrites than those from Normal rats that were not inoculated with Pc and 4.5 fold more than those from Dex-Pc rats ($p<0.05$, Table 1).

Previous results indicate that CD4-depleted-Pc mice respond differently from rats in the timing of AM number decreases (9); therefore, BAL fluid (first 1 ml) nitrites were assessed at both four and seven weeks of infection and were found to have 41% (1.3 ± 0.1 μM) and 55% (1.0 ± 0.2 μM), respectively less nitrites than those from CD4-depleted mice ($p<0.05$, Table 1). These data suggest that the decrease in nitrites in BAL fluids during PCP is not species-specific and is not related to method of immunosuppression.

**Decreased nitrite levels in AMs during PCP in rats and mice.** To assess NO production by AMs from rats and mice with PCP, AM-produced nitrites were measured after 1 h of culture. Nitrite levels were first assayed in AM samples treated with saline alone to determine background values of nitrites for the assay. AMs from Dex-Pc rats (n=15) were found to produce only 20% as much NO ($p<0.05$ vs. Dex control, Table 1) as those from Normal or Dex rats (n=12 each). IFN-γ and *E. coli* stimulation of these cells did not significantly increase nitrite levels.

Likewise, CD4-depleted-Pc mice from early (4 weeks) and late (7 weeks) infections had significantly lower nitrite levels ($p<0.05$, Table 1) ($n=15$-20). These results indicate that AMs from PCP rats and mice are defective in NO production.

**NO production in AMs from *H. capsulatum*-infected mice.** To determine whether the defect in NO production by AMs is specific to Pc infection, mice were infected with the yeast form of *H. capsulatum* by intranasal instillation. At 7 (mid stage) and 14 (late stage) days after infection, nitrite levels in AMs and BAL fluids were measured. Results showed that nitrite levels in both AMs and BAL fluids from mice infected for 7 days had no significant difference as compared to uninfected controls. However, a 5.56 fold and 4.62 fold increase in nitrite levels in AMs and BAL fluids, respectively were observed at 14 days after infection (Table 2). These results suggest that in contrast to Pc infection, NO production in AMs is increased during *H. capsulatum* infection.

**iNOS transcription in PCP rats and mice.** Real-time RT-PCR for iNOS mRNA levels was then performed to determine if the defect in NO production was due to reduced transcription of the iNOS gene. RNAs from two million AMs from Normal, Dex, and Dex-Pc rats, or one million AMs from Normal, CD4-depleted, or CD4-depleted–Pc mice were converted to cDNAs and then used for the real-time RT-PCR. As shown in Fig. 4, iNOS transcripts, normalized to RPS8, were 14.6 fold higher in AMs from Dex-Pc rats than in those from Dex rats ($p<0.05$). This is in direct contrast to the low nitrite levels in AMs from Dex-Pc rats (Table 1). iNOS mRNA levels in AMs from Normal and Dex rats were very similar, indicating that immunosuppression by dexamethasone did not significantly affect iNOS transcription in AMs. AMs from Normal-Pc rats had increased iNOS transcripts as compared to those from Normal rats (14.7 fold, $p<0.05$). As with rats, real-time RT-PCR results indicated that AMs from CD4-depleted-Pc mice had higher levels (13.6 fold) of iNOS mRNA than those from CD4-depleted mice (Fig. 4). These results indicate that up-regulation of iNOS transcription in AMs is a normal immune response to Pc.

**iNOS protein levels in rats and mice with PCP.** To determine whether the iNOS mRNA detected in AMs from rats was translated, Western immunoblotting was performed. Equal amounts of AM soluble protein from Normal, Dex, and Dex-Pc rats were electrophoresed on a polyacrylamide gel and then transferred to a PVDF membrane. The membrane was reacted with antibody against the iNOS protein, with GAPDH as a loading control. The iNOS protein was detected in all samples (Fig. 5A), and AMs from Dex-Pc rats were found to produce significantly more iNOS protein than those from Normal or Dex rats.

The CD4-depleted mouse model of PCP was also assessed for iNOS protein expression. As shown in Fig. 5B, the amount of iNOS protein was low in AMs from Normal and CD4-depleted mice, but AMs from those infected with *P. murina* for either four or seven weeks had significantly increased levels of iNOS protein.

**iNOS dimerization in AMs during PCP.** In order to determine if the decreased NO production by AMs was due to reduced iNOS dimerization, partially denaturing immunoblotting was performed as described previously (31) to identify both the iNOS dimmer (260 kDa) and monomer (130 kDa) in AM lysates. As shown in Fig. 6A, pooled AMs from three Normal or Dex rats had approximately equal levels of iNOS monomer and dimer. This ratio was shifted in AMs from Dex-Pc rats with high levels of the iNOS monomer and low levels of the iNOS dimer (Fig. 6A). Image analysis of the immunoblots showed that Normal and Dex animals dimerized 46-58% of their AM iNOS (Fig. 6B). In contrast, AMs from Dex-Pc rats dimerized only 15% of the iNOS protein (Fig. 6B).

**Effects of cofactors on iNOS dimerization.** To determine whether the low iNOS dimer levels were due to reduced availability of cofactors necessary for iNOS dimerization, AM lysates from Normal, Dex, or Dex-Pc rats were incubated for 30 min with iNOS dimerization cofactors in sterile saline containing 1 mM phenylmethylsulphonylfluoride (PMSF) before they were analyzed for iNOS dimers. These cofactors included heme, tetrahydrobiopterin (BH4), FAD, FMN, NADPH, arginine, and in some conditions, calmodulin. The concentrations of cofactors used were similat to those of previously published (36, 37).

Normal or Dex AM lysates supplemented with all cofactors showed similar iNOS dimer levels (Fig. 7A). For Dex-Pc AM lysates, levels of iNOS monomers were high and those of iNOS dimers were low without the presence of cofactors; addition of all cofactors significantly increased the levels of iNOS dimers, as the levels of monomers were decreased (Fig. 7A). However, when calmodulin was not included in the cofactors, Dex-Pc AM lysates had very low iNOS dimer levels (Fig. 7A). Image analysis of three independent immunoblots (Fig 7B) showed that 17.9 ± 1.9% of the iNOS proteins were dimerized without addition of any cofactors and that 18.4 ± 2.1% of the iNOS proteins dimerized when the cell lysate was incubated with all cofactors except calmodulin. In contrast, 62.0 ± 9.8% of the iNOS proteins were dimerized when the cofactors included 5 µM calmodulin (Fig. 7B).

## Discussion

In order to survive in the lung, Pc must evade the actions of AMs. We have previously shown that Pc infection renders AMs defective in phagocytosis (8) and induces AM apoptosis (1, 7, 9). In this study, we found that AM defense is further weakened during Pc infection due to decreased NO production. We first demonstrated that Pc is susceptible to NO produced *in vitro* by NO generators, Spm NONOate or DEA NONOate. Spm NONOate at the concentration of 0.1 mM also inhibited the proliferation of Pc in culture on HEL monolayers (Fig. 2). This inhibition was due to effects of NO on the organism rather than on the feeder HEL cells. These data agree with previous results on the toxicity of NO to Pc (20, 21) and confirm that its action is directly against the organism.

NO production by AMs is mediated by iNOS, and regulation of iNOS expression takes place primarily at the transcriptional level. Therefore, Shellito

et al. determined iNOS mRNA and protein levels in AMs from uninfected and Pc-infected CD4 cell-depleted mice and SCID mice to investigate the role of NO in host defense against Pc infection (23). Since organism burden remained high despite elevated levels of iNOS mRNA and protein in AMs of these mice, they concluded that iNOS is an unlikely participant in host defense against Pc and speculated that NO is not important in this regard, but they did not measure NO or nitrite levels (23). In the present study, we also found that the levels of both iNOS mRNA and protein were increased in AMs from Pc-infected animals (Figs. 4 and 5), but NO production by these cells was defective (Table 1). This finding explains the reason why organism burden was high even though iNOS levels were high. It is possible that iNOS detected in the study of Shellito et al. (23) was not functional; therefore, insufficient NO was produced to kill the organisms. The defect in NO production was not observed in AMs from *H. capsulatum*-infected mice (Table 2). Since *H. capsulatum* can infect immunocompetent animals, these *H. capsulatum*-infected mice were not immunosuppressed. Therefore, whether host immunosuppression or Pc infection itself is responsible for decreased NO production in AMs remains to be confirmed.

We found that AMs from immunocompetent rats challenged with Pc responded to the organism by producing large amounts of NO, and the levels remained high for two weeks while the animals were clearing the organisms. These data suggest that NO production is a normal host response to Pc infection in immunocompetent animals. This possibility is supported by the observation that lower NO levels in L-NIO–treated Normal-Pc rats were accompanied by increased organism burdens (Fig. 3). The increase in organism burden was more pronounced after two weeks of treatment with L-NIO, suggesting that NO plays a role in the control of Pc proliferation during the normal immune response.

When AMs from normal rats were stimulated with IFN-γ and *E. coli*, NO production was increased approximately three fold (Table 1). Surprisingly, no additional NO production was observed when AMs from normal rats challenged with Pc were treated with IFN-γ and *E. coli*. This lack of response to IFN-γ and *E. coli* stimulation in NO production was also seen in AMs from Pc-infected rats (Table 1). These results suggest that Pc infection can render NO production in AMs defective. This is confirmed by the finding that AMs from Pc-infected rats produced very low levels of NO and that these AMs were also unresponsive to IFN-γ and *E. coli* stimulation (Table 1). It is possible that expression of iNOS in AMs is maximally induced during Pc infection; therefore, additional response does not occur when the cells are stimulated with IFN-γ or *E. coli*.

The decrease in NO production in Dex-Pc rats was not due to dexamethasone, since antibody-mediated immunosuppression of mice by depletion of CD4+ cells showed the same phenomena. Previous studies indicate that these mice have a transient increase in AM number prior to a profound decrease by seven weeks of infection (9). The fact that

nitrite levels are low in mouse lung at four weeks of infection when AM number is maximal suggests that NO production in AMs is suppressed even at the early stage of infection.

In addition to transcription regulation, production of iNOS can also be regulated at the translational or post-translational level. TGF-β can promote degradation of the iNOS protein and decrease the translation of the transcript (36). Imidazole-based anti-fungal agents (37) and carbon monoxide (38) can inhibit dimerization of iNOS or activity of the dimer. Cofactor availability and binding also modulate the stability of iNOS as suppression of BH4 by cytokines results in reduced iNOS dimerization (39). In this study, we showed that iNOS protein synthesis was increased during PCP (Fig. 5), indicating that the reduction in NO production during PCP was not due to defects in the production of the iNOS protein.

iNOS must homodimerize to form a functional enzyme and requires the cofactors BH4, heme, arginine, NADPH, FAD+, FMN, and calmodulin for this action (40, 41). iNOS converts it substrate arginine to citrulline and NO (40, 42). If arginine levels are low, the iNOS protein will not dimerize (41). Dimerization of iNOS is initiated by binding of calmodulin, FAD+, and FMN to the monomer, followed by addition of a heme molecule near the N-terminus of the monomer. This stimulates addition of another iNOS monomer, which is stabilized by arginine and BH4 (41). The results of the present study showed that dimerization of the iNOS monomers takes place normally in AMs from uninfected and Pc-challenged immunocompetent animals, but was reduced in AMs from Dex-Pc rats (Figs. 6 and 7).

The observation that addition of calmodulin and other cofactors, but not the other cofactors alone rescued iNOS dimer formation (Fig. 7) suggested that available calmodulin was insufficient in AMs during PCP. This conclusion is supported by our recent results indicating that calmodulin mRNA and protein levels are significantly decreased in AMs during PCP (18). Calmodulin is a ubiquitous calcium-sensing protein. Binding of calcium to calmodulin results in an altered conformation and permits it to bind other proteins. iNOS dimerization requires apocalmodulin (calcium-free calmodulin) as a cofactor (40, 43).

Results of the present study suggest that NO production is an important host response to Pc and that the defect in iNOS dimerization confers a survival advantage to the organism. The AM attempts to increase NO production by up-regulating iNOS expression, but fails to achieve its goal due to decreased calmodulin availability for dimerization. Since calmodulin down-regulation also contributes to AM defects in survival and GM-CSF signaling (18), additional study is required to define mechanisms which prevent calmodulin down-regulation and to restore iNOS dimerization. Although NO inhalation in conjunction with Tmp/Smx and prednisolone has been used to treat PCP (44, 45), the roles of iNOS and NO in the defense against Pc infections in humans are not known. This animal study will serve as a model for further study of the involvement of NO in human PCP.

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## Figure Legends

**Figure 1.** Effect of NO on *Pneumocystis* organisms. *P. carinii* organisms were incubated with 0.01 or 0.1 mM Spm NONOate or DEA NONOates for 2 or 4 h and then assayed for viability by real-time RT-PCR for *P. carinii* mitochondrial large subunit rRNA. * $p<0.05$ vs. carrier (0.01 mM NaOH) controls at the same time point. $^{\$}$ $p<0.05$ vs. same condition at 2 h.

**Figure 2.** Inhibitory effect of NO on Pc proliferation in short-term culture. *P. carinii* organisms were inoculated onto HEL cell monolayers in 24-well plates, and numbers of organisms in the cultures were counted every other day. Some wells contained Tmp/Smx (50/250 µg/ml) or the nitric oxide generators Spm NONOate or DEA NONOate at 0.1 mM or 0.01 mM, and the media were adjusted to pH 7.4 daily. Results are averages ± SD of quadruplicate wells of each condition in five independent experiments.

**Figure 3**. Effect of L-NIO on AM nitrite levels and *Pneumocystis* burden. (A). Uninfected (Normal) and Pc-challenged (Normal-Pc) immunocompetent rats were injected (i.p.) daily with 2 mg/kg of L-NIO, starting two days after Pc inoculation. AMs were isolated from these rats one or two weeks after initiation of L-NIO treatment and assessed for nitrite levels with or without stimulation of the AMs with 10 ng/ml IFN-$\gamma$ and 5 x $10^6$ *E. coli*. Nitrite levels are expressed in µM relative to 1 mg AM protein in cell lysate. (B). The numbers of *Pneumocystis* organisms (both trophic and cyst forms) in Normal-Pc rats after L-NIO treatment were determined by examining Giemsa-stained lung impression smears. Numbers of organisms were average counts of ten 1,000X microscopic fields. Asterisk (*) denotes $p<0.05$ as compared to untreated group.

**Figure 4**. iNOS mRNA levels in AMs during PCP. Levels of iNOS mRNA relative to those of the control gene RPS8 in AMs from *Pneumocystis*-infected rats (A) and mice (B) were determined by real-time RT-PCR. Dex-Pc AMs were from rats that had been infected with *P. carinii* for 6 weeks. CD4-depl-Pc AMs were from mice infected with *P. murinii* for 4 weeks. Normal-Pc AMs were isolated from normal rats 17 days after inoculation with *P. carinii*. Results represent mean fold change ± SD relative to normal control from 4 to 7 animals in each group. Asterisk (*) denotes $p<0.05$ vs. immunosuppressed group (Dex or CD4-depl), and the pound sign (#) indicates $p<0.05$ vs. Normal group.

**Figure 5.** iNOS protein levels in AMs from PCP rats and mice. (A) Total soluble protein from AMs of Normal, Dex, and Dex-Pc rats was separated by SDS-PAGE and then reacted with an anti-iNOS antibody. (B) AM proteins from Normal, CD4+ lymphocyte depleted (CD4-depl), and CD4-depleted Pc-infected (CD4-depl-Pc) mice were treated as described for rat AMs in Panel A. CD4-depl-Pc mice were infected with *P. murina* for four or seven weeks prior to sacrifice. The iNOS protein shown in the figure is the130 kDa monomer. Results are representative of four independent experiments.

**Figure 6.** iNOS dimer formation in rat AMs during PCP. (A) Total soluble protein from rat AMs was separated by SDS-PAGE under partially denaturing conditions as previously described (31) and then subjected to Western blotting with an anti-iNOS antibody. Pooled AMs from three Normal and Dex rats and five Dex-Pc rats were used. The molecular weight of iNOS monomer and dimer are 130 and 260 kDa, respectively. Image analysis of four independent Western blots was performed by normalizing iNOS monomer and dimer signals to that of the control protein GAPDH. Average iNOS dimer levels ± SD are plotted as a percent of iNOS monomer levels. Asterisk (*) denotes $p<0.05$ versus Dex control samples.

**Figure 7.** iNOS dimer formation in AM lysates after incubation with cofactors and calmodulin. (A) AMs from Normal (3 rats), Dex (3 rats), and Dex-Pc (5 rats) rats were pooled and sonicated on ice. AM lysates were incubated with iNOS dimerization cofactors including heme (10 µM), BH4 (10 µM), FAD (4 µM), FMN (4 µM), NADPH (2 mM), and arginine (2 mM) with or without calmodulin (5 µM) in sterile saline containing 1 mM PMSF. After 30 min of incubation, cell lysates were subjected to partially denaturing Western blot analysis as described in Fig. 6 legend. The histogram (B) represents average iNOS dimerization levels ± SD from three independent trials. Asterisks (*) denotes $p<0.05$ versus Dex controls.

Table 1. Nitrite levels in BAL fluids and AMs from rats and mice with PCP[1]

| Sample | Animal | Normal | Suppressed[2] | 4 wk PCP | 7 wk PCP | IFN-γ/*E. coli*[3] | Normal-Pc[4] |
|---|---|---|---|---|---|---|---|
| BALF | Rat | 3.2 ± 0.2 | 3.4 ± 0.6 | NA[6] | 2.1 ± 0.3* | NA | 9.6 ± 1.3† |
| | Mouse | 2.3 ± 0.4 | 2.2 ± 0.2 | 1.3 ± 0.1* | 1.0 ± 0.1* | NA | NA |
| AMs | Rat | 4.2 ± 0.3 | 4.5 ± 0.4 | NA | 0.8 ± 0.1* | 1.0 ± 0.1* | 15.6 ± 1.2† |
| | Mouse | 4.8 ± 0.4 | 4.3 ± 0.3 | 2.0 ± 0.2* | 1.7 ± 0.2* | 2.1 ± 0.3* | NA |

[1]BALF (BAL fluid) and AM lysates were treated with nitrate reductase to convert nitrates to nitrites prior to determination of nitrite levels. Nitrite levels in the first 5 ml rat BALF or the first 1 ml mouse BALF are expressed in μM, and those in AMs (1 x $10^6$ from rats and 5 x $10^5$ from mice) are expressed in μM relative to 1 mg of protein in the cell lysates. [2]Rats were immunosuppressed with dexamethasone, and mice were immunosuppressed with anti-CD4 antibody. [3]Preincubated with 10 ng/ml IFN-γ and 5 x $10^6$ *E. coli* for 1 h prior to assay. [4]Normal rats challenged with *P. carinii*. NA, not assessed. *$p<0.05$ vs. immunosuppressed control. †$p<0.05$ vs. Normal control.

Table 2. Nitrite levels in BAL fluids and AMs from *H. capsulatum*-infected mice*.

| | Uninfected | *H. capsulatum*-infected | |
|---|---|---|---|
| | | Day 7 | Day 14 |
| BALF | 0.29 ± 0.35 | 0.26 ± 0.41 | 1.34 ± 0.34* |
| AMs | 0.18 ± 0.20 | 0.10 ± 0.32 | 1.00 ± 0.12* |

*BALF (BAL fluid) and AM lysates were treated with nitrate reductase to convert nitrates to nitrites prior to determination of nitrite levels. Nitrite levels in the first 1 ml BALF are expressed in µM, and those in AMs ($5 \times 10^5$) are expressed in µM relative to 1 mg of protein in the cell lysates. *$p<0.05$ vs. uninfected control.

Figure 1

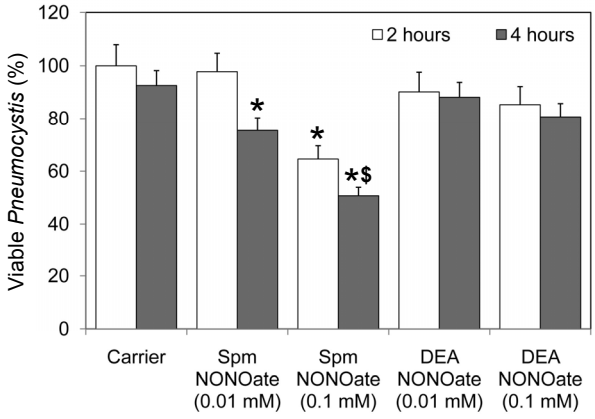

120
100
80
60
40
20
0
Viable *Pneumocystis* (%)
2 hours
4 hours
*
*
*$
Carrier
Spm NONOate (0.01 mM)
Spm NONOate (0.1 mM)
DEA NONOate (0.01 mM)
DEA NONOate (0.1 mM)

Figure 2

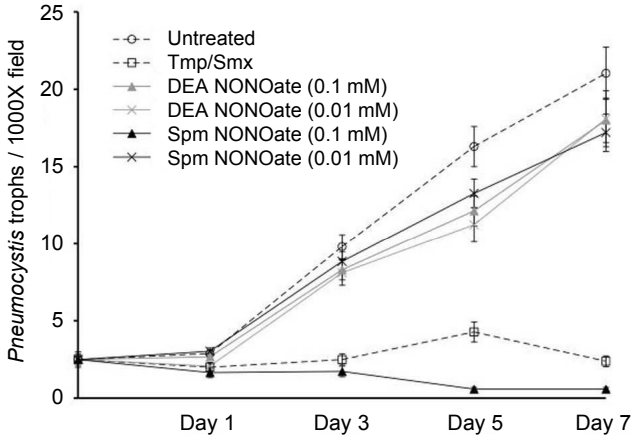

25
20
15
10
5
0
*Pneumocystis* trophs / 1000X field
Untreated
Tmp/Smx
DEA NONOate (0.1 mM)
DEA NONOate (0.01 mM)
Spm NONOate (0.1 mM)
Spm NONOate (0.01 mM)
Day 1
Day 3
Day 5
Day 7

Figure 3

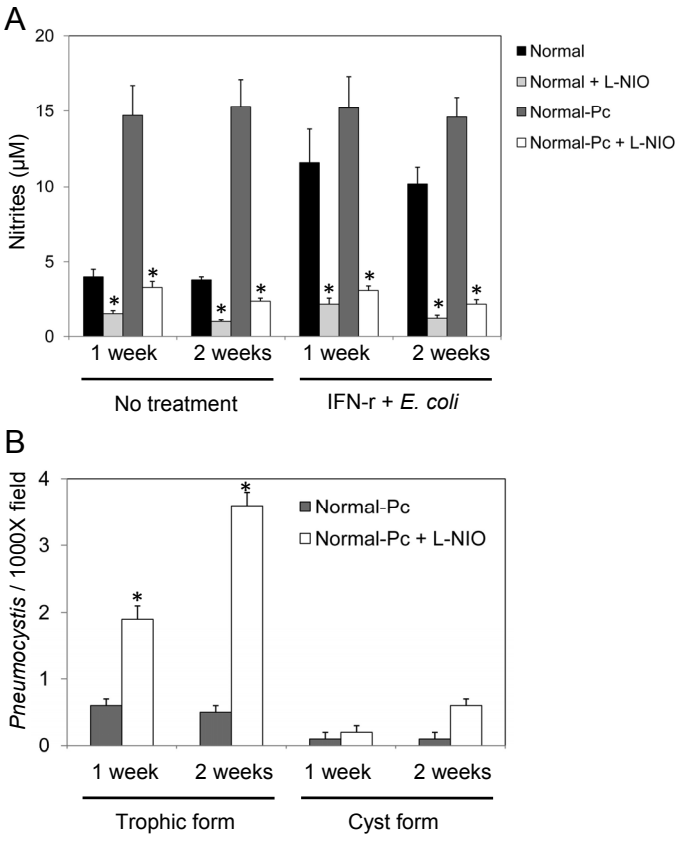

A
Nitrites (µM)
20
15
10
5
0
Normal
Normal + L-NIO
Normal-Pc
Normal-Pc + L-NIO
1 week
2 weeks
1 week
2 weeks
No treatment
IFN-r + E. coli
B
Pneumocystis / 1000X field
4
3
2
1
0
Normal-Pc
Normal-Pc + L-NIO
1 week
2 weeks
1 week
2 weeks
Trophic form
Cyst form

Figure 4

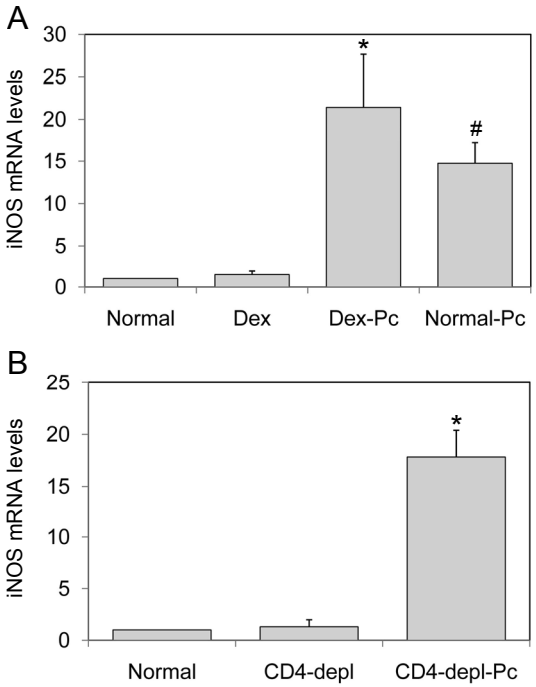

A
iNOS mRNA levels
30
25
20
15
10
5
0
*
#
Normal
Dex
Dex-Pc
Normal-Pc
B
iNOS mRNA levels
25
20
15
10
5
0
*
Normal
CD4-depl
CD4-depl-Pc

Figure 5

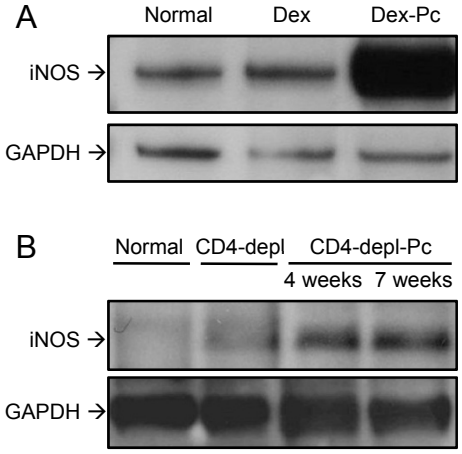

A
Normal
Dex
Dex-Pc
iNOS
GAPDH
B
Normal
CD4-depl
CD4-depl-Pc
4 weeks
7 weeks
iNOS
GAPDH

Figure 6

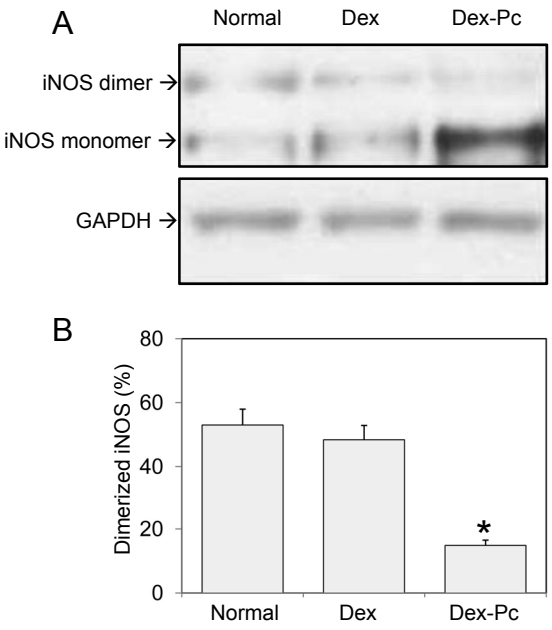
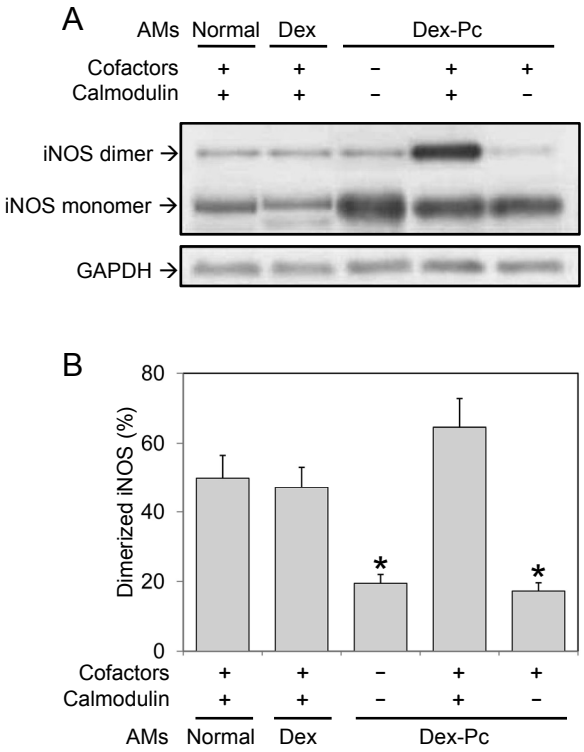

A
Normal
Dex
Dex-Pc
iNOS dimer
iNOS monomer
GAPDH
B
Dimerized iNOS (%)
80
60
40
20
0
*
Normal
Dex
Dex-Pc

Figure 7


A
AMs
Normal
Dex
Dex-Pc
Cofactors
Calmodulin
iNOS dimer
iNOS monomer
GAPDH
B
Dimerized iNOS (%)
80
60
40
20
0
*
*
Cofactors
Calmodulin
AMs
Normal
Dex
Dex-Pc