

## **Rapid Interferon $\gamma$ -dependent Clearance of Influenza A Virus and Protection from Consolidating Pneumonitis in Nitric Oxide Synthase 2-deficient Mice**

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### **Summary**

Viral infection often activates the interferon (IFN)- $\gamma$ -inducible gene, *nitric oxide synthase 2* (*NOS2*). Expression of *NOS2* can limit viral growth but may also suppress the immune system and damage tissue. This study assessed each of these effects in genetically deficient *NOS2*<sup>-/-</sup> mice after infection with influenza A, a virus against which IFN- $\gamma$  has no known activity. At inocula sufficient to cause consolidating pneumonitis and death in wild-type control mice, *NOS2*<sup>-/-</sup> hosts survived with little histopathologic evidence of pneumonitis. Moreover, they cleared influenza A virus from their lungs by an IFN- $\gamma$ -dependent mechanism that was not evident in wild-type mice. Even when the IFN- $\gamma$ -mediated antiviral activity was blocked in *NOS2*<sup>-/-</sup> mice with anti-IFN- $\gamma$  mAb, such mice failed to succumb to disease. Further evidence that this protection was independent of viral load was provided by treating *NOS2*<sup>+/+</sup> mice with the NOS inhibitor, N<sup>ω</sup>-methyl-L-arginine (1-NMA). 1-NMA prevented mortality without affecting viral growth. Thus, host *NOS2* seems to contribute more significantly to the development of influenza pneumonitis in mice than the cytopathic effects of viral replication. Although *NOS2* mediates some antiviral effects of IFN- $\gamma$ , during influenza infection it can suppress another IFN- $\gamma$ -dependent antiviral mechanism. This mechanism was observed only in the complete absence of *NOS2* activity and appeared sufficient to control influenza A virus growth in the absence of changes in cytotoxic T lymphocyte activity.

**Key words:** nitric oxide synthase • interferon  $\gamma$  • virus infection • influenza A • cytotoxic T lymphocyte

The discovery that the IFN- $\gamma$ -inducible *nitric oxide synthase 2* (*NOS2*) gene could confer potent antiviral activity (1, 2) substantially extended its role as a critical component of the innate immune system (3). Yet although a number of viruses are susceptible to the nitric oxide (NO)-derived products of *NOS2*, not all are inhibited (3, 4, and references therein). Moreover, as a broadly reactive, cell-permeant radical, NO has the potential to injure uninfected cells near those harboring virus (3–7, and references therein). For this reason, *NOS2* has also been implicated in the pathology associated with several viral models (4, 5). That most of the studies mentioned above have relied on the use of NOS inhibitors of limited isoform specificity complicates interpretation of disparate results, since all three NOS isoforms can exhibit antiviral activity, and at least two of them (*NOS2* and *NOS1*) can damage cells (3, 4, 8, 9).

We have resorted to *NOS2* gene-targeted mice (10) in

an effort to assess the contribution made by this locus towards pathogenesis and immunoregulation during viral infection. Such mice have confirmed *NOS2*'s ability to protect the host during infection by Coxsackie B3 (11) and ectromelia viruses (11a). Here we studied infection by pneumotropic influenza A virus for two reasons. First, an earlier report with pharmacologic inhibitors implicated *NOS2* in host pathology during influenza pneumonitis (6). Second, although IFN- $\gamma$  has well-characterized antiviral activity and is important for *NOS2* induction in vivo (1–4), control of influenza A virus does not seem to require IFN- $\gamma$ , as evidenced by studies using anti-IFN- $\gamma$  antibodies (12, and G. Karupiah, unpublished observations) and IFN- $\gamma$ <sup>-/-</sup> mice (13, and G. Karupiah, unpublished observations). Surprisingly, treatment with neutralizing antibody revealed that IFN- $\gamma$  can control influenza A virus, but only when *NOS2* is absent.

## Materials and Methods

**Mice.** *NOS2*<sup>-/-</sup> mice were generated as described (10). *NOS2*<sup>-/-</sup> and *NOS2*<sup>+/+</sup> littermates (129/SvEv × C57BL/6 F<sub>2</sub>) were bred at the specific pathogen-free unit, John Curtin School of Medical Research. *NOS2* genotype was confirmed by PCR (14). Experiments were performed according to institutional guidelines for animal care and use.

**Virus Infection and Enumeration.** Influenza A virus (strain A/PR/8/34) was directly instilled in 30  $\mu$ l PBS into the nasal cavity under Avertin (1.5 ml/100 g) anesthesia, and titers were thereafter determined via serial dilutions of lung homogenates plated onto MDCK cell monolayers for detection of PFU (15). The limit of assay detection was 2.0 log<sub>10</sub> PFU.

**Lung Immunocyte Isolation.** Leukocytes were isolated as outlined (12) from HBSS-perfused and aseptically excised lungs from groups of influenza virus-infected mice (*n* = 5 per group). Populations consisted of ~40–50% T lymphocytes (CD3<sup>+</sup>), 20–30% B lymphocytes (CD45R/B220<sup>+</sup>), and 10–12% macrophages (F4/80<sup>+</sup>) as determined by flow cytometry.

**Bronchoalveolar Lavage Fluid NO<sub>x</sub> Assay.** Bronchoalveolar lavage fluid (BALF) consisted of washings from intratracheal instillation of 1.0 ml PBS. Nitrite was measured by a diazotization assay (14) with a sensitivity of 4  $\mu$ M after NO<sub>3</sub><sup>-</sup> was converted to NO<sub>2</sub><sup>-</sup> via nitrate reductase.

**Cytotoxicity Assays.** Influenza A virus-infected and uninfected EL-4 lymphoma cells were used as targets for measurement of anti-influenza A virus-specific, class I MHC-restricted CTL activity of lung parenchymal cells using standard <sup>51</sup>Cr-release assays (16, 17).

**IFN- $\gamma$  ELISA Analysis.** A sandwich ELISA (18) was used to measure IFN- $\gamma$  levels in BALF and from culture supernatants of lung immunocytes (5 × 10<sup>6</sup>; ~50% CD3<sup>+</sup>) of influenza A/PR/8/34 virus-infected mice coincubated for 48 h with influenza virus-infected, mitomycin C-treated (50  $\mu$ g/ml) genotype-matched lung parenchymal cells (5 × 10<sup>6</sup>). Recombinant MuIFN- $\gamma$  (Genzyme Corp., Cambridge, MA) served as the standard, with an assay detection limit of 3 U/ml. Con A was added at 4  $\mu$ g/ml (Sigma Chemical Co., St. Louis, MO).

**Histology.** Formalin-fixed, paraffin-embedded tissues were sectioned (5  $\mu$ m) and stained with hematoxylin and eosin as outlined (18).

**Neutralization of *NOS2* and IFN- $\gamma$  In Vivo.** *N*<sup>o</sup>-methyl-L-arginine (1-NMA; Sigma Chemical Co.) or its inactive d-enantiomer (d-NMA) was administered via daily intraperitoneal injection, and efficacy was verified by BALF nitrite plus nitrate (NO<sub>x</sub>) assay. Neutralization of IFN- $\gamma$  was achieved by injection of 1 mg i.p. anti-MuIFN- $\gamma$  mAb (clone XMG-6, rat IgG<sub>1</sub> [17, 18]) in 200  $\mu$ l PBS on days 0, 2, and 4 after infection. Control animals received an isotype-matched mAb against bacterial  $\beta$ -galactosidase (clone GL 113, rat IgG<sub>1</sub>).

**Statistical Analysis.** Unpaired Student's *t* tests at 95% confidence levels were performed using InStat software, Version 2.00 (GraphPad Software for Science, San Diego, CA).

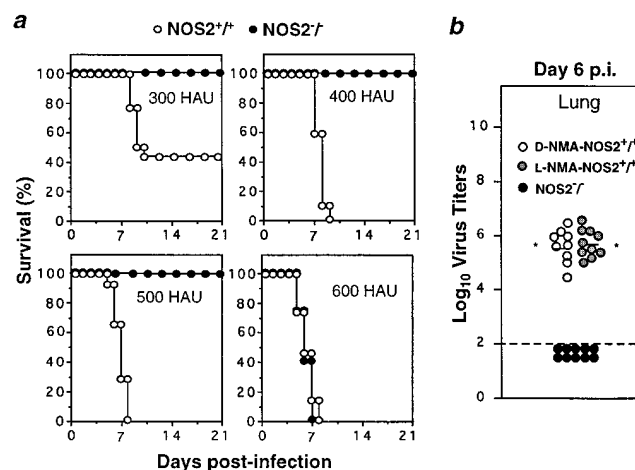
## Results and Discussion

**Augmented Virus Clearance and Enhanced Survival of *NOS2*<sup>-/-</sup> Mice Infected with Influenza A Virus.** In previous work, mice treated with anti-IFN- $\gamma$  antibodies (12, and G. Karupiah, unpublished observations) or lacking IFN- $\gamma$  (13, and G. Karupiah, unpublished observations) responded to influenza A virus similarly to control mice, even though

their capacity for *NOS2* expression is known to be impaired (19, 20). Our findings in *NOS2*<sup>-/-</sup> mice not only corroborated the implication that *NOS2* is dispensable for the control of influenza A virus in the lower respiratory tract, but also demonstrated that *NOS2* is detrimental in this setting. Thus, intranasal inoculation with the virulent A/PR/8/34 strain led to rapid pneumonitis and early mortality in wild-type mice given 300, 400, or 500 hemagglutinin units (HAU) of virus. In contrast, *NOS2*<sup>-/-</sup> mice started to succumb only at 600 HAU (Fig. 1 a).

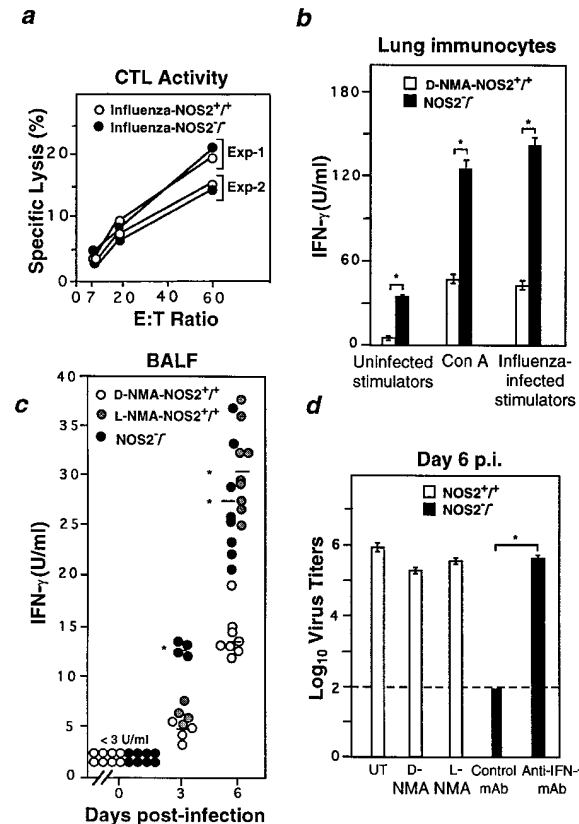
Influenza A virus titers in the lungs of d-NMA-treated controls given 400 HAU of virus reached 5.6 ± 0.2 log<sub>10</sub> PFU by day 6 after infection, whereas in *NOS2*<sup>-/-</sup> mice lung virus titers were below the limit of detection (<2.0 log<sub>10</sub> PFU; Fig. 1 b). Surprisingly, 1-NMA-treated wild-type mice harbored viral loads similar to their d-NMA-treated littermates (Fig. 1 b) but, unlike the latter, they showed no signs of morbidity and survived through the 21-d period of observation (data not shown). This outcome suggested (a) that *NOS2*<sup>-/-</sup> mice selectively expressed a potent antiinfluenza effector mechanism, and (b) the high output NO pathway may contribute to pathogenesis, since both 1-NMA- and d-NMA-treated wild-type mice failed to eradicate the virus but only those given the inactive d-enantiomer proved susceptible, dying between days 7 and 8 after infection, like their untreated counterparts (Fig. 1 a, and data not shown).

**An IFN- $\gamma$ -dependent Pathway Restricts Influenza Virus Replication in *NOS2*<sup>-/-</sup> Mice.** Experiments in  $\beta_2$ -microglobulin<sup>-/-</sup> mice (21) and Fas<sup>-/-</sup> × perforin<sup>-/-</sup> chimeras (22) demon-



**Figure 1.** Disruption of *NOS2* confers a protective effect during influenza A virus infection. (a) Mortality in male *NOS2*<sup>+/+</sup> or *NOS2*<sup>-/-</sup> mice (*n* = 20 per group at each dose) inoculated intranasally with 300, 400, 500, or 600 HAU influenza A (strain A/PR/8/34) and monitored for 21 d. Data represent four independent experiments. (b) Lung influenza A titers in d-NMA- or 1-NMA-treated *NOS2*<sup>+/+</sup> mice and *NOS2*<sup>-/-</sup> mice intranasally infected with 400 HAU of virus and determined at day 6 after infection. Dashed line, Assay detection limit. Data are derived from three experiments and expressed as log<sub>10</sub> virus titers/gram tissue ± SEM. \**P* < 0.01 versus *NOS2*<sup>-/-</sup> group, unpaired *t* test.

strated the importance of CD8<sup>+</sup> T lymphocytes for elimination of influenza A virus from the lung. To determine if this effector arm of the immune response contributed to rapid virus clearance in the NOS2<sup>-/-</sup> host, we examined influenza-specific CTL activity on day 6 after infection. Lung immunocytes lysed the influenza virus-infected targets equally well irrespective of NOS2 status (Fig. 2 a). However, measurement of local IFN- $\gamma$  production by these cells (Fig. 2 b) and in BALF (Fig. 2 c) revealed that



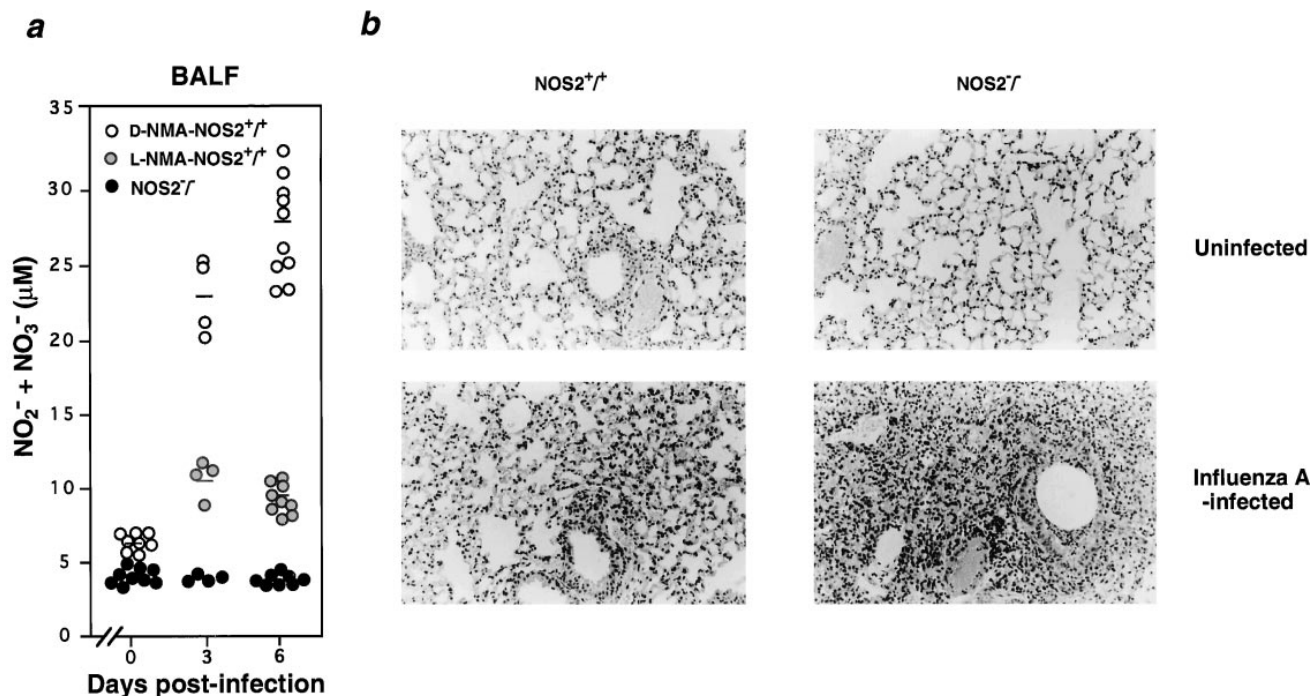
**Figure 2.** IFN- $\gamma$ -dependent antiinfluenza virus activity in NOS2<sup>-/-</sup> mice is not accompanied by changes in CTL response. (a) Ex vivo CTL activity of lung parenchymal cells (day 6 after infection) from d-NMA- or l-NMA-treated NOS2<sup>+/+</sup> mice and NOS2<sup>-/-</sup> mice intranasally infected with 400 HAU of virus. Shown are group means ( $n = 4$  for each of two independent experiments); SEMs fall within the symbols. (b) IFN- $\gamma$  production by explanted lung parenchymal cells from influenza A virus-infected NOS2<sup>+/+</sup> and NOS2<sup>-/-</sup> mice cultured in vitro with uninfected lung immunocytes, influenza-infected stimulators, or Con A for 48 h. Data are presented as means of triplicate cultures  $\pm$  SEM (from pooled lung immunocytes of five mice per group, and represent two independent experiments). (c) IFN- $\gamma$  levels in the BALF of d-NMA- or l-NMA-treated NOS2<sup>+/+</sup> mice and NOS2<sup>-/-</sup> mice intranasally infected with 400 HAU of virus determined at day 6 after infection. Individual samples were assayed in triplicate; horizontal bars, group means. \* $P < 0.01$  versus d-NMA-treated group, unpaired  $t$  test. (d) Influenza A virus titers in the lungs of NOS2<sup>-/-</sup> mice treated with anti-MuIFN- $\gamma$  ( $n = 14$ ) or control IgG<sub>1</sub> mAb ( $n = 14$ ); or in NOS2<sup>+/+</sup> mice given d-NMA ( $n = 10$ ) or l-NMA ( $n = 14$ ). Untreated NOS2<sup>+/+</sup> mice (UT) served as drug-free controls ( $n = 4$ ). Dashed line, Assay detection limit. Data shown represent three experiments and are expressed as log<sub>10</sub> virus titers/gram tissue  $\pm$  SEM. \* $P < 0.01$  versus control IgG<sub>1</sub> mAb-treated group, unpaired  $t$  test.

NOS2<sup>-/-</sup> mice exhibited 1.9–3.4-fold higher levels of IFN- $\gamma$  than wild-type hosts; this increase was independent of antigen load (Fig. 1 b).

Whether such an increase in IFN- $\gamma$  secretion could account for the reduced viral burden in mutant animals was assessed by intraperitoneal injection with mAb to IFN- $\gamma$  (18). Assay of virus infectivity levels 6 d after infection demonstrated that IFN- $\gamma$ -dependent anti-influenza A virus mechanisms were operative in mutant mice as depicted by the high titers comparable to those of l-NMA or d-NMA-treated wild-type controls (Fig. 2 d). In contrast, NOS2<sup>-/-</sup> animals receiving an IgG<sub>1</sub> isotype-matched control mAb still retained their capacity to restrict influenza virus replication within the lungs; titers were below the level of detection (Fig. 2 d).

*Elevated NO Production Is Associated with Pulmonary Pathology in Influenza Virus-infected Wild-type Hosts.* The demise of untreated or d-NMA-treated NOS2<sup>+/+</sup> mice was not due to uncontrolled viral growth, since l-NMA-treated wild-type or anti-IFN- $\gamma$ -treated NOS2<sup>-/-</sup> hosts also displayed overwhelming lung viral burdens yet showed no signs of morbidity throughout the 3-wk experimental period (Figs. 1 b, 2 d, and data not shown). Survival of the latter two groups implied that pulmonary injury in d-NMA-treated animals arose as a consequence of excessive NO production rather than by direct viral cytopathic effects. Indeed, NO<sub>x</sub> levels within the BALF of d-NMA-treated NOS2<sup>+/+</sup> mice rose appreciably ( $\sim 3.4$ – $4.0$ -fold) during the course of infection (Fig. 3 a), whereas this increase was absent in mutant mice and inhibited by 77–90% in l-NMA-treated controls (Fig. 3 a). Histopathologic examination revealed massive inflammatory foci and edema within the lungs of all d-NMA-treated wild-type mice as part of the consolidating pneumonitis observed 6 d after infection (Fig. 3 b). Neutrophils and alveolar macrophages were especially prominent. Both are capable of releasing cytotoxic oxidants besides NO (6, 23), such as the superoxide anion (O<sub>2</sub><sup>-</sup>), which can react with the latter to yield more damaging products like peroxynitrite (ONOO<sup>-</sup> [3]). Akaike et al. have demonstrated the local formation of both ONOO<sup>-</sup> and NO-hemoglobin in murine influenza infection using nitrotyrosine footprinting and electron spin resonance spectroscopy (6), while NO-thiol adducts also appear elevated in wild-type but not NOS2<sup>-/-</sup> mice during tuberculosis (14). Thus, it is conceivable that several NO-derived species acted in concert to cause influenza-associated pathology.

*Absence of Consolidating Pneumonitis in NOS2<sup>-/-</sup> Mice.* In marked contrast to the severe pneumonitis seen for all d-NMA-treated NOS2<sup>+/+</sup> mice, the majority of mutant animals ( $\sim 70\%$ ) lacked evidence of pathology. A mild degree of inflammation was observed in the remainder; an example of the latter is depicted in Fig. 3 b. These results are consistent with the idea that NOS2 promotes the development of viral pneumonia. Additional support for this concept comes from recent studies using NOS inhibitors during infection with HSV-1 (7), murine CMV (24), or the influenza A/Kumamoto/Y5/67 (H2N2) mouse



**Figure 3.** Absence of inducible NO production and consolidating pneumonitis in the lungs of infected NOS2<sup>-/-</sup> mice. (a) NO<sub>2</sub><sup>-</sup> + NO<sub>3</sub><sup>-</sup> (NO<sub>x</sub>) levels in the BALF of d-NMA- or l-NMA-treated NOS2<sup>+/+</sup> mice and NOS2<sup>-/-</sup> mice intranasally infected with 400 HAU of virus determined at day 6 after infection. Individual samples were assayed in triplicate after conversion to NO<sub>2</sub><sup>-</sup>; horizontal bars, group means. (b, top) Histological analyses of lungs from uninfected NOS2<sup>+/+</sup> and NOS2<sup>-/-</sup> hosts; original magnification: ×250. (Bottom) Consolidating pneumonitis in NOS2<sup>+/+</sup> mice which was absent in NOS2<sup>-/-</sup> mutant animals at day 6 after infection; original magnification: ×250. At least nine mice per group were examined.

adapted strain (6). Inhibition of NOS fully protected mice from HSV-1-dependent lung injury despite a 17-fold increase in viral titers (7). That NO was necessary to control HSV-1 in vivo substantiated earlier experiments in vitro (1, 2). In contrast, in the present work, the functional loss of NOS2 alleviated pneumonitis and greatly improved survival irrespective of whether influenza virus was cleared (NOS2<sup>-/-</sup> hosts) or not (NOS2<sup>+/+</sup> mice given l-NMA). Reduced pathology has also recently been noted in NOS2<sup>-/-</sup> mice infected with *Mycobacterium avium* (25) and *Toxoplasma gondii* (26).

IFN-γ has been implicated as the major cytokine responsible for NOS2 induction during influenza A-induced pneumonitis (6). Aside from IFN-γ's purported proinflammatory action in wild-type mice, we found that heightened IFN-γ release was both associated with and required for influenza A virus clearance in NOS2<sup>-/-</sup> animals, an effect unaccompanied by changes in antiviral CTL activity (Fig. 2, a-d). The effect was specific for mutant mice, since l-NMA-treated wild-type mice had comparable IFN-γ levels but failed to limit viral growth. IFN-γ thus appears to cooperate with immune components otherwise subject to suppression by NOS2 and not by NOS1 and/or NOS3. Complete inhibition of NOS2 by disruption of the gene encoding it, rather than the partial inhibition afforded by treatment of NOS2<sup>+/+</sup> mice with l-NMA, also appeared

necessary to permit expression of the IFN-γ-dependent antiinfluenza mechanism. Among pertinent candidates for components of this mechanism are MHC class II expression by pulmonary dendritic cells (27) and Th1 cell proliferation plus cytokine production (28, 29). Each could be compromised by NOS2, which can limit T cell responsiveness to IL-2 in the lungs of rats and mice (28) as well as inhibit the release of IL-12 and elevate the expression of TGF-β as shown in *Leishmania major*-infected NOS2<sup>-/-</sup> mice (30). In this manner, the high output NO pathway may regulate IFN-γ secretion via a feedback mechanism, such that once infection is cleared, autotoxic NO production is extinguished.

Beyond this, how NOS2 might influence the underlying molecular events leading to cytokine gene expression and action during antiviral immunity remains speculative. NO can activate or disable protein tyrosine kinases of the Janus kinase (JAK), extracellular signal-regulatory kinase (ERK), and src families, as well as the transcription factor nuclear factor (NF)-κB, and thus may affect cytokine signaling (31, and references therein). Indeed, NOS2<sup>-/-</sup> animals have altered levels of NF-κB and signal transducer and activator of transcription (STAT)3 activation in hemorrhage/resuscitation (32) and perturbed IFN-γ release in *L. major* (28, 29) and influenza A infections (Fig 2, b and d). Cytokine expression could also be regulated by NOS2 at the posttrans-

lational level. For example, S-nitrosylation of the active site cysteine residue in caspases leads to their inactivation (33). If this inhibition were to be relieved in NOS2<sup>-/-</sup> mice, abnormally active caspase-1 might more readily promote the release of IL-18 (34, 35), a powerful inducer of IFN- $\gamma$ .

During infection, NOS2 probably displays all three of its major effects to variable degrees—antimicrobial, inflammatory, and immunosuppressive. The results seen here with influenza A virus-infected mice appear to illustrate one extreme, in which the latter two effects dominate.

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