Enhanced antiviral antibody secretion and attenuated immunopathology during influenza virus infection in nitric oxide synthase-2-deficient mice

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INTRODUCTION

Nitric oxide (NO) is a pleiotropic free radical that is involved in numerous constitutive physiological functions, such as the control of vascular tone (Palmer et al., 1987), platelet aggregation (Mellion et al., 1981) and neurological activity (Garthwaite, 1991). NO synthase (NOS)-2, the enzyme responsible for much of the high-output, inducible expression of NO, mediates many of the immune-regulatory properties of NO. The role of NO in protection against viral infections both in vitro and in vivo has been well documented. In a number of studies, viral infection of mice treated with drugs that selectively block NOS2 or that have mutations in the NOS2 gene resulted in increased replication and spread of the virus (Karupiah et al., 1993; Noda et al., 2001; Zaragoza et al., 1998). The antiviral activity of NO is thought to be mediated at least in part through its direct action on viral proteins critical for virus replication (Saura et al., 1999).

Whilst playing an important role in restricting and clearing virus in some models of infection, NO has also been shown to intensify immune-mediated pathology and contribute to lethal outcomes in other viral infections (Adler et al., 1997; Akaike et al., 1996). During infection with Influenza A virus (IAV), lung pathology and morbidity are associated with the formation of peroxynitrite (Akaike et al., 1996). More recently, mice are less susceptible to IAV infection when treated with NOS2-inhibiting compounds (Akaike et al., 1996) or when deficient in NOS2 gene expression (Akaike et al., 2003; Karupiah et al., 1998a). In an earlier report, highly virulent IAV was cleared from the lungs of NOS2-deficient (NOS2−/−) mice much earlier than in control (NOS2+/+) mice. The mechanism of virus clearance in the absence of NOS2 activity was shown to be dependent on gamma interferon (IFN-γ) (Karupiah et al., 1998a).

Previous studies have shown that, during IAV infection, both innate and adaptive elements of the immune response work synergistically to limit virus replication. Virus clearance is perturbed in the absence of natural killer (NK) cells (Stein-Streilein & Guffee, 1986), T cells (Bender et al., 1992; Topham et al., 1997) or antibody (Iwasaki & Nozima, 1977; Mozdzanowska et al., 1997). We undertook this study to examine the nature of the IFN-γ-dependent antiviral response in NOS2−/− mice. It is well known that NO can shift the Th1/Th2 balance in vitro. Th1 cells are
thought to be more sensitive to the cytotoxic effects of NO (Taylor-Robinson, 1997). Therefore, we hypothesized that cytotoxic T cells, primed by more robust Th1-cell activity in the absence of high NO output, mediate much of the enhanced antiviral activity in NOS2−/− mice. Surprisingly, our data suggest that an increased virus-specific antibody response, rather than an enhanced cytotoxic T-cell response, may account for the reduced susceptibility of NOS2−/− mice to IAV infection.

**METHODS**

**Mice and virus.** Male NOS2−/− mice (129/SvEv × C57BL/6 F2) and control NOS2+/+ littermates (129/SvEv × C57BL/6 F2) (Karupiah et al., 1998b; MacMicking et al., 1997) were bred at the specific-pathogen-free unit at the ICSMR. Mice between 7 and 12 weeks of age were used in experiments approved by the Animal Ethics Committees of the University of Sydney and the Australian National University. For IAV infection studies, mice were anaesthetized by intraperitoneal injection of 2,2,2-tribromoethanol at a dose of 0.3 mg (g body weight)−1 and inoculated intranasally with 500 p.f.u. IAV in a 20 µl volume.

The A/PR/8/34 (A/PR/8) strain of influenza virus was grown for 2 days in the allantoic cavity of 10-day-old embryonated hen eggs. For some assays, virus stock was further purified by centrifugation at 85 000 g for 35 min and the pelleted virus was resuspended in PBS. Virus was titrated on Madin–Darby canine kidney cell monolayers to obtain p.f.u. values or incubated with chicken red blood cells to determine haemagglutinin units (h.a.u.). Aliquots of virus stored at −70 °C were used in experiments.

**Tissues for assays and histology.** Lung-cell suspensions were prepared according to a modified procedure (Baumgarth & Kelso, 1996). Briefly, perfused lung lobes were digested with 1 mg collagenase ml−1 (Roche) for 60 min at 37 °C. Red blood cells were lysed and the remaining cells were used in assays. Single-cell suspensions of mediastinal lymph nodes and spleens were obtained by passing the tissues through nylon mesh. Red blood cells were lysed unless indicated otherwise. For determination of microscopic pulmonary histopathology, formalin-fixed tissues were embedded in paraffin and 5 µm sections were stained with haematoxylin and eosin. Bronchoalveolar lavage (BAL) was performed by using 1 ml PBS containing protease inhibitors (Roche). BAL fluid was centrifuged to remove cellular debris and stored at −70 °C. Cytokines were measured by using OptEIA sets (PharMingen). IFN-γ was measured by using an in-house ELISA.

**Flow-cytometric staining and analysis.** For analysis of cells by flow cytometry, samples were incubated for 30 min with anti-IFR antibody (clone 2.4G2). Cells were stained with mAbs Ly6G+, CD3− and NK1.1−fluorescein isothiocyanate; CD138−, CD4−, CD19−, and CD11b−phycoerythrin; B220−, CD8− and CD45−allophycocyanin (PharMingen). Stained cells were analysed by flow cytometry.

**Cytotoxicity assays.** For the determination of cytotoxic T-lymphocyte (CTL) activity in lung parenchyma samples, RMA target cells infected with A/PR/8 virus (10 p.f.u. per cell) were labelled with 100 µCi (3.7 MBq) Na125I (ICN) for 45 min. Effector cells were added to labelled target cells at a 27:1 effector:target ratio and cultured for 4 h. To ascertain the level of NK-cell cytotoxic activity in various tissue samples, 1 × 105 YAC-1 target cells were labelled with Na125I for 45 min. Effector and target cells were cultured at a ratio of 100:1 for 4 h. Counts in supernatants were read on a TopCount machine (Packard). Percentage specific lysis was determined by the formula: (experimental release−medium release)/(maximum release−medium release) × 100.

**Measurement of virus-specific antibody titres.** Virus-specific antibody titres were ascertained by ELISA (Jackson et al., 1986). Serial dilutions of serum were added to microtitre plates coated with 1000 h.a.u. purified A/PR/8 ml−1 and incubated overnight at 4 °C. Isotype- or IgG subtype-specific peroxidase-conjugated secondary antibodies (ICN) were added and incubated for 2 h at room temperature. Plates were developed with ABTS solution (Moss Inc.) and A405 was read.

The virus haemagglutination–inhibition (HI) titre of serum samples was measured according to standard procedures. Briefly, an equal volume of virus containing h.a.u. was added to serial dilutions of serum and the suspension was incubated at room temperature for 30 min. Subsequently, an equal volume of 1% chicken red blood cells in PBS was added to the wells. The HI titre was recorded as the reciprocal of the highest dilution at which haemagglutination was inhibited.

**B-cell stimulation assays.** For the measurement of IFN-γ produced by mitogen-stimulated splenocytes, 106 cells were added to individual wells of 96-well flat-bottom plates. One hundred microlitres of serially diluted, purified goat anti-mouse Ig antibody (SouthernBiotech) was added to each well in triplicate and cultures were incubated in a 5% CO2 incubator for 16 h. Supernatants were analysed for the presence of IFN-γ by ELISA.

**Carboxyfluorescein diacetate succinimidyl ester (CFSE) labelling and mitogen stimulation.** CFSE labelling was performed by using the method of Lyons & Parish (1994). Briefly, cells were suspended at 5·0 × 107 ml−1 and incubated for 10 min at 37 °C with 5 μM CFSE (Molecular Probes). CFSE-labelled splenocytes were suspended at 106 cells ml−1 in RPMI medium supplemented with 10% fetal calf serum. Cells were cultured with 20 μg lipopolysaccharide (LPS) ml−1, 10 μg affinity-purified goat anti-mouse anti-IgM F(ab′)2 (Jackson Immunoresearch) or anti-IgG and 10 μg rat anti-CD40 ml−1 for 4 days at 37 °C in 5% CO2, then analysed by flow cytometry.

**Clinical illness observations.** To meet ethical guidelines, individual mice were observed daily for signs of morbidity. Detailed laboratory records of these observations were kept. To determine the relationship between clinical disease and lung pathology, a series of scoring parameters was devised. Clinical illness scores were assigned based on the appearance of cachexia and ruffled fur (score of 2) or cachexia and ruffled fur associated with severely decreased locomotor activity (score of 3). Infected mice with no visible clinical symptoms were given a score of 1. Mice were killed by cervical dislocation and lungs were removed from the thoracic cavity for visual inspection. The degree of pleural haemorrhage was recorded as none (score of 1), mild (score of 5), medium (score of 10) and severe (score of 15).

**Statistics.** Data were analysed by using the statistics software program InStat (GraphPad Software). t-test analysis was performed on most data and means ± SEM are provided unless otherwise indicated (*P < 0.05, **P < 0.01, ***P < 0.001). Mann–Whitney tests were performed on data containing individual data points. Data with P values of <0.05 were regarded as significant.

**RESULTS**

NOS2−/− mice have decreased mortality and viral titres (Karupiah et al., 1998a; data not shown) compared with NOS2+/+ mice infected with IAV. Previous studies have
demonstrated that IAV replication is sensitive to CD8\textsuperscript{+} T cell-mediated cytolysis (Bender et al., 1992; Topham et al., 1997), NK-cell effector activity (Stein-Streilein & Guffee, 1986) and the presence of virus-specific antibody (Iwasaki & Nozima, 1977; Mozdzanowska et al., 1997). We therefore examined each of these components of the immune response during infection of NOS2\textsuperscript{−/−} mice with a sublethal dose of virulent IAV.

**T-cell and NK-cell cytotoxic responses do not contribute to increased virus clearance in NOS2\textsuperscript{−/−} mice**

To ascertain whether CD8\textsuperscript{+} T-cell cytolysis was enhanced in NOS2\textsuperscript{−/−} mice, we measured cytolytic activity in the lungs of IAV-infected NOS2\textsuperscript{+/+} and NOS2\textsuperscript{−/−} mice. As shown in Fig. 1(a), the majority of NOS2\textsuperscript{−/−} lung parenchyma samples had lower virus-specific cytolytic activity than those of NOS2\textsuperscript{+/+} mice, but the difference between the two strains was not statistically significant. Therefore, it is clear that, as a whole, IAV-specific CD8\textsuperscript{+} T-cell cytolytic activity is similar in NOS2\textsuperscript{−/−} and NOS2\textsuperscript{+/+} mice.

NK cells are an important part of the early antiviral innate immune response. During IAV infection, there is an early increase in the number and activity of these cells in the lung. As virus replication is reduced early in IAV-infected NOS2\textsuperscript{−/−} mice, we studied the NK-cell response in the lungs of these mice by cytotoxicity assays. As shown in Fig. 1(b), NK-cell cytolytic activities in the lungs of NOS2\textsuperscript{−/−} mice were comparable with those of NOS2\textsuperscript{+/+} mice.

Thus, CD8\textsuperscript{+} T-cell and NK-cell cytolytic activity in the lungs of NOS2\textsuperscript{−/−} mice cannot explain the enhanced clearance of IAV in these mice.

**Elevated levels of virus-specific IgG2a in NOS2\textsuperscript{−/−} mice**

Antibody-mediated immunity to IAV plays a major role in limiting virus replication during primary IAV infection. To measure the humoral response to IAV infection in NOS2\textsuperscript{−/−} mice, sera obtained from infected mice were used in virus-specific ELISA. As shown in Fig. 2(a), levels of IAV-specific IgM and IgG were similar in NOS2\textsuperscript{−/−} and NOS2\textsuperscript{+/+} mice on day 7 of infection. IgA levels were also comparable in NOS2\textsuperscript{−/−} and NOS2\textsuperscript{+/+} mice (data not shown). Characterization of IgG subtypes (Fig. 2b) demonstrated that both strains produced similar levels of IAV-specific IgG1, IgG2b and IgG3. Strikingly, however, IAV-specific IgG2a antibody titres were significantly higher in the serum of NOS2\textsuperscript{−/−} mice.

To determine whether there was an increased ability of NOS2\textsuperscript{−/−} serum to block the haemagglutinin (HA) of IAV and thus neutralize the virus in vitro, the level of HI activity in whole serum from individual mice was also measured. HI levels were comparable in both strains of mice (Fig. 2c), indicating that HI titres do not correspond directly with levels of IgG2a. Nevertheless, the possibility that the elevated levels of IgG2a in NOS2\textsuperscript{−/−} mice may contribute to IAV neutralization and/or complement fixation and lysis of infected cells in vivo remains.

To determine whether the difference between the levels of IgG2a in these mice was consistent at other time points, we measured IgG2a at days 4, 7 and 9 post-infection. As can be seen from the kinetics of this isotype response (Fig. 3), IgG2a levels in NOS2\textsuperscript{−/−} mice showed clearly increasing titres over this period. At day 4, there was no significant difference between NOS2\textsuperscript{−/−} and NOS2\textsuperscript{+/+} mice. However, titres increased significantly in NOS2\textsuperscript{−/−} mice compared with levels in NOS2\textsuperscript{+/+} mice by day 7 of infection (49-fold, P<0.0001). The difference between the two strains was reduced, yet still significant, on day 9 (19-fold, P=0.0306).

**B cells in NOS2\textsuperscript{−/−} splenocyte cultures have increased viability and proliferation in response to B-cell mitogens**

Antigens can activate B cells either through T cell-dependent (TD) or T cell-independent (TI) interactions. IAV shares important biochemical and physical properties of typical TI type-2 (TI-2) antigens: (i) they possess a regular, repetitive structure of surface glycoproteins (Bachmann & Zinkernagel, 1996), (ii) they activate serum complement deposition (Beebe et al., 1983) and (iii) they are capable of generating an antibody response in the absence of T cells (Sullivan et al., 1976).

Given that IAV has characteristics similar to those of TI antigens and that the magnitude of the IgG2a response to IAV was greater in NOS2\textsuperscript{−/−} mice, we postulated that B cells in these mice might elicit a stronger response to stimulation with TI mitogens. Therefore, we measured the ability of
NOS2−/− B cells to proliferate in response to B-cell mitogen stimulation. Splenocytes from NOS2+/+ and NOS2−/− mice were labelled with CFSE and stimulated with B-cell mitogens in vitro. Four days later, the degree of B-cell proliferation was measured by flow cytometry. As shown by 7AAD and Syndecan-1 (CD138) labelling (Fig. 4), viable B cells from both strains of mice proliferated and became plasmablasts when stimulated with mitogens (Fig. 4b–d). Significantly, at the end of the 4 day period, there were 3- to 4-fold more viable B cells in the NOS2−/− cultures stimulated with a T1 mitogen, anti-IgM (Fig. 4c), and with anti-IgM and anti-CD40 (Fig. 4d). In both cultures, the increased B-cell viability resulted in a higher total number of plasmablasts. CFSE dilution showed that 5- to 16-fold more NOS2−/− B cells underwent at least one cycle of division compared with NOS2+/+ B cells.

As IFN-γ is responsible for IgM class-switching to IgG2a, we ascertained the level of IFN-γ production in NOS2−/− splenocyte cultures when B cells were stimulated with a commonly used B-cell mitogen, anti-mouse Ig antibody. As shown in Fig. 5, IFN-γ production was detected at all concentrations of mitogen used in the splenocyte cultures of both NOS2+/+ and NOS2−/− mice. Significantly higher levels of IFN-γ (P<0.05) were secreted by NOS2−/− splenocytes in each case.

**Attenuated clinical illness in influenza virus-infected NOS2−/− mice**

To study the pathogenesis of viral pneumonia in NOS2−/− mice, clinical illness was measured in IAV-infected NOS2−/− and NOS2+/+ mice (Fig. 6). On the indicated days, each mouse was given a score between 1 and 3 for clinical disease symptomatology, extracted from animal ethical protocol observation records, as described in Methods. NOS2−/− mice demonstrated a delay in manifestation of clinical illness and developed a statistically lower clinical illness score from day 7 of infection compared with NOS2+/+ mice (P<0.0001).

To ascertain whether there was a correlation between clinical disease and lung pathology, we measured the extent of externally visible macroscopic lung lesions. There was a significant attenuation in the extent of lung lesions in NOS2−/− mice compared with NOS2+/+ mice on days 7 and 10 of infection (data not shown). A comparison of clinical illness and severity of lung lesions demonstrated that the greatest difference between the two strains occurred on day 7 of infection.

**Reduced levels of leukocytes and proinflammatory cytokines in the lungs of NOS2−/− mice**

IAV pneumonia is characterized by pulmonary consolidation, consisting of large numbers of leukocytes, and the presence of high levels of proinflammatory cytokines. To determine whether there were differences in the composition of cells and cytokines in the lungs of mice that may have contributed to the clinical and macroscopic observations between the two strains, these factors were examined. Histological analysis revealed areas of extensive pulmonary leukocytic consolidation in both NOS2+/+ and NOS2−/− mice on day 7 of infection (Fig. 7a). These leukocytes consisted of macrophages, neutrophils and lymphocytes. As
can be seen from Fig. 7(a), there were no obvious qualitative or quantitative histological differences between these two strains observed microscopically in areas of lung consolidation.

Quantification and detailed phenotypic analysis of lung parenchymal cells by flow cytometry (Table 1) showed that by day 7 there was a significantly greater increase in leukocytes in the lungs of NOS2$^{+/+}$ mice compared with NOS2$^{-/-}$ mice (4.9-fold vs 3.1-fold, $P=0.0099$). Although the change in leukocyte cell numbers was significantly different between NOS2$^{+/+}$ and NOS2$^{-/-}$ mice, the difference in absolute cell numbers on day 7 was not significant ($P=0.3988$). This was due to the presence of significantly higher baseline numbers of leukocytes in the lungs of NOS2$^{-/-}$ mice ($P=0.0080$). The reason for this initial difference in absolute cell numbers is not clear, but NOS2 can affect the development of some subpopulations of leukocytes (J. P. Jayasekera, unpublished observations). As changes in pathology and clinical illness are reflected over the course of infection and uninfected mice are healthy, the change in cell numbers over baseline levels provided a more representative depiction of cellular changes in infected lungs. Analysis of the kinetics of inflammatory leukocyte migration into the lungs of infected mice demonstrated that there was a characteristic early rise in neutrophils by day 4 of infection, which subsided rapidly and returned to baseline levels by day 9. Whereas the increase in neutrophils was not statistically different between NOS2$^{+/+}$ and NOS2$^{-/-}$ mice throughout the course of infection, the increase in macrophages was significantly higher in NOS2$^{+/+}$ mice compared with NOS2$^{-/-}$ on day 7 (4.4-fold vs 2.7-fold, $P=0.0052$) and day 9 (3.0-fold vs 1.4-fold, $P=0.0254$).

Previous studies have shown a direct relationship between elevated levels of proinflammatory cytokines and the
development of clinical illness during viral pneumonia. To determine whether proinflammatory cytokines were diminished in NOS2⁻/⁻ mice, tumour necrosis factor (TNF) and interleukin (IL)-6 proteins were measured in BAL fluid throughout the course of infection. Levels of both cytokines were elevated in NOS2⁺/⁺ (TNF, 65±12 pg ml⁻¹; IL-6, 1074±143 pg ml⁻¹) and NOS2⁻/⁻ (TNF, 49±14 pg ml⁻¹; IL-6, 591±290 pg ml⁻¹) mice on day 4 of infection (Fig. 7b). However, by day 7, there was a marked reduction in TNF (5±4 pg ml⁻¹) and IL-6 (53±53 pg ml⁻¹) in the BAL fluid of NOS2⁻/⁻ mice, whilst these cytokines remained elevated in the lungs of NOS2⁺/⁺ mice (TNF, 87±5 pg ml⁻¹; IL-6, 1239±68 pg ml⁻¹). These equated to levels that were higher by 17-fold for TNF and 23-fold for IL-6 in the NOS2⁺/⁺ mice over and above the levels in NOS2⁻/⁻ mice at day 7.

Thus, inflammatory cell migration and proinflammatory cytokine production are attenuated significantly in the lungs of IAV-infected NOS2⁻/⁻ mice. Importantly, this correlates directly with lower clinical illness (Fig. 6) and lung lesions observed in NOS2⁻/⁻ mice, suggesting that NOS2

**Fig. 5.** IFN-γ production by splenocytes stimulated with a Tl mitogen. Splenocytes obtained from NOS2⁺/⁺ (filled bars) and NOS2⁻/⁻ (empty bars) mice were cultured in the presence of anti-mouse Ig antibody at the indicated dilutions. Supernatants were assayed for the presence of IFN-γ. Bars represent the means±SEM from triplicate cultures. Results are representative of two independent experiments.

**Fig. 6.** Clinical illness in NOS2⁻/⁻ mice is attenuated. Groups of NOS2⁺/⁺ (■) and NOS2⁻/⁻ (○) mice were infected with IAV. On the days indicated, signs of clinical illness were recorded. Results from multiple experiments consisting of four or five mice were combined and are shown as means±SEM.

**Fig. 7.** Transient expression of proinflammatory cytokines in the lungs of NOS2⁻/⁻ mice. (a) Representative photomicrographs of haematoxylin and eosin-stained lung tissue from mice infected with IAV (bars, 250 μm). (b, c) BAL fluid was obtained at the indicated times and analysed for the presence of cytokines. Results are representative of at least two similar experiments consisting of three or four mice per group and are shown as means±SEM.
Table 1. Leukocyte subsets in the lungs of IAV-infected mice

<table>
<thead>
<tr>
<th>Cell subset</th>
<th>NOS2 mice</th>
<th>Day 0</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 9</th>
</tr>
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<tbody>
<tr>
<td>Leukocytes</td>
<td>+/+</td>
<td>3.30 (1.86)</td>
<td>8.75 (2.64)</td>
<td>16.1 (3.06)</td>
<td>14.6 (1.93)</td>
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<tr>
<td></td>
<td>+/-</td>
<td>6.13 (1.76)</td>
<td>10.3 (4.05)</td>
<td>19.0 (8.65)</td>
<td>12.9 (8.92)</td>
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<tr>
<td>CD4^+ T cells</td>
<td>+/+</td>
<td>0.39 (0.20)</td>
<td>0.40 (0.11)</td>
<td>0.74 (0.35)</td>
<td>1.61 (0.23)</td>
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<tr>
<td></td>
<td>+/-</td>
<td>0.77 (0.28)</td>
<td>1.00 (0.64)</td>
<td>1.22 (0.67)</td>
<td>1.25 (0.82)</td>
</tr>
<tr>
<td>CD8^+ T cells</td>
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<td>0.25 (0.06)</td>
<td>0.98 (0.59)</td>
<td>2.86 (0.43)</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>0.29 (0.13)</td>
<td>0.41 (0.19)</td>
<td>1.17 (0.80)</td>
<td>2.13 (2.10)</td>
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<td>Macrophages</td>
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<td>1.34 (1.22)</td>
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<td>Neutrophils</td>
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<td>0.69 (0.18)</td>
<td>0.65 (0.22)</td>
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<td></td>
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<td>1.07 (0.73)</td>
<td>0.73 (0.22)</td>
<td>0.61 (0.52)</td>
</tr>
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</table>

exacerbates viral pneumonia by contributing to the recruitment of inflammatory leukocytes and to the prolonged expression of proinflammatory cytokines.

**Attenuated local T-cell response to influenza virus infection in the lungs of NOS2^{−/−} mice**

T-cell effector activity during IAV infection has previously been shown to contribute both to the resolution of inflammation and to the exacerbation of immunopathology (Cerwenka et al., 1999). Characterization of the T-cell response by flow cytometry showed that CD4^+ T-cell levels increased by day 7 of infection (1.9-fold) in the lungs of NOS2^{+/+} mice, reaching peak levels by day 9 (4.2-fold) (Table 1). Levels did not increase on subsequent days (data not shown). In contrast, CD4^+ T-cell levels in infected NOS2^{−/−} mice did not change significantly from baseline levels by day 9 of infection (1.6-fold). CD8^+ T cells were elevated to the same extent in the lungs of NOS2^{+/+} and NOS2^{−/−} mice on both day 7 (4.1-fold and 4.0-fold, respectively) and day 9 (12.1- and 7.3-fold, respectively).

To evaluate the level of soluble T-cell effector activity, T-cell cytokines in BAL fluid were measured by ELISA. IFN-γ and IL-10 proteins were found to reach peak levels in NOS2^{+/+} mice on day 7 of infection (Fig. 7c). In NOS2^{−/−} mice, IFN-γ levels peaked on day 7, but were nevertheless significantly lower (P<0.0001) than levels in NOS2^{+/+} mice. Interestingly, IL-10 protein was not detected in the lungs of NOS2^{−/−} mice to any significant degree (Fig. 7c), suggesting that immune regulation was not being mediated by this molecule in these mice. Remarkably, also, IL-2 levels were higher in NOS2^{−/−} mice on day 5 of infection and equivalent in both strains on day 7 of infection (Fig. 7c), indicating that T cells in the lungs of NOS2^{−/−} mice were functional, but had a different pattern of cytokine expression. The level of IL-2 protein was significantly higher (P=0.0164) in NOS2^{+/+} mice on day 9.

**DISCUSSION**

It has been proposed that Th1 responses are sensitive to NO (Taylor-Robinson, 1997) and there are data to suggest that Th1-mediated immunity is enhanced in NOS2^{−/−} mice (James et al., 1998). As Th1 responses are particularly effective at controlling virus infections, we expected that NOS2^{−/−} mice would exhibit an increased level of cellular immunity. However, we found that NOS2^{−/−} mice exhibited a low to moderate antiviral CTL response and NK-cell cytotoxic activity that were not significantly different from those of NOS2^{+/+} mice.

Antibody plays a prominent role in clearing IAV infection, particularly in the absence of an effective CTL response (Eichelderger et al., 1991). Viral infections elicit a predominant IgG2a antibody response in mice (Coutelier et al., 1987). This is known to be due to the production of IFN-γ that typically accompanies a viral infection, as IFN-γ promotes class-switching from IgM to IgG2a in B cells. Previous studies have shown that, in the absence of IFN-γ, virus-specific IgG2a levels are reduced significantly in IAV-infected mice (Baumgarth & Kelso, 1996; Graham et al., 1993). Similarly, in β-2 microglobulin-deficient mice, which lack the usual class-I major histocompatibility complex-restricted CTL clearance mechanisms, virus clearance is delayed when mice are treated with a neutralizing anti-IFN-γ antibody (Sarawar et al., 1994). Importantly, IgG2a has a number of unique functions that make it ideally suited for mediating viral clearance *in vivo*. These include antibody-dependent, cell-mediated cytotoxicity (Kipps et al., 1985), binding Fc receptors on phagocytes (Unkeless & Eisen, 1975) and a strong capacity for initiating complement fixation (Neuberger & Rajewsky, 1981). Indeed, Palladino et al. (1995) have demonstrated that virus-specific IgG2a antibodies are preferentially able to cure IAV infection in immunocompromised mice. Our results are consistent with these studies in that a heightened IgG2a antibody response early during IAV infection in NOS2^{−/−} mice was associated...
with early IFN-γ-dependent virus clearance in these mice. The elevated IgG2a levels did not correspond with the ability of NOS2−/− serum to inhibit haemagglutination by IAV in vitro. Nevertheless, there are numerous other ways for antibody to neutralize virus in vivo. Indeed, Feng et al. (2002) have shown that IAV-specific antibodies with relatively low HI activity, as might be expected to be produced during an early primary immune response, can exhibit strong virus-neutralization activity in the presence of other serum factors.

Thus, it is likely that virus-specific IgG2a exerts more profound effects in vivo and may be responsible, in part, for the reduced virus levels in NOS2−/− mice. Additionally, the contribution of other IFN-γ-dependent antiviral mechanisms (Shtrichman & Samuel, 2001) in mediating virus clearance in NOS2−/− mice cannot be discounted. Importantly, IFN-γ levels were lower in the lungs of NOS2−/− mice than in those of wild-type mice, which suggests that the quantitative beneficial effects of IFN-γ may be systemic rather than local.

Cross-linking B-cell surface Ig with an anti-Ig antibody resulted in IFN-γ secretion in both NOS2+/+ and NOS2−/− splenocyte cultures; however, NOS2−/− splenocytes produced 2- to 4-fold higher levels of IFN-γ. These results are consistent with previous studies that have similarly demonstrated heightened production of IFN-γ by NOS2−/− and NOS2+/+ splenocyte cultures treated with NOS2 inhibitors (Karupiah et al., 1998b). How are IgG2a and IFN-γ levels elevated in IAV-infected NOS2−/− mice when the T-cell response in these mice is reduced? In addition to T cells, accessory cells such as NK cells, macrophages and dendritic cells are capable of providing ‘help’ to activated B cells (Szomolanyi-Tsuda & Welsh, 1998). Studies performed on the interaction between NK cells and B cells have shown that activated NK cells can promote purified B cells to secrete Ig and amplify the B-cell response to TI antigens (Snapper et al., 1994). Additionally, B cells activated by TI mitogens acquire the ability to stimulate IFN-γ production by NK cells (Michael et al., 1989). IAV has many properties of a TI antigen and thus could partly elicit a T cell-independent response during infection. Our findings that elevated levels of IgG2a in the serum of NOS2−/− mice are not accompanied by a corresponding increase in HI activity suggest that at least part of the heightened IgG2a response may be independent of germinal centre-mediated affinity maturation. Thus, increased levels of IgG2a may be the product of a T cell-independent response elicited by TI properties of IAV.

In addition to stimulating B cells to produce IgG2a, IFN-γ also acts as a survival factor. When purified B cells are stimulated with mitogens, many undergo cell death; however, the addition of exogenous IFN-γ causes more B cells to survive and proliferate in culture (Hasbold et al., 1999). Furthermore, IFN-γ has been shown to be the main inducer of BAFF (B-cell activation factor from the TNF family) secretion by macrophages, dendritic cells and neutrophils (Moore et al., 1999; Nardelli et al., 2001) and BAFF is a key promoter of B-cell (Batten et al., 2000) and plasmablast (Balázs et al., 2002) survival. We observed increased survival and total numbers of plasmablasts in NOS2−/− splenocyte cultures stimulated with anti-IgM alone as well as in conjunction with anti-CD40. When purified B cells were treated under similar conditions, proliferation was comparable between NOS2+/+ and NOS2−/− B cells (J. P. Jayasekera & C. G. Vinuesa, unpublished observations). These results suggest that the increased stimulatory effect of B-cell mitogens on NOS2−/− B-cell proliferation is due to a B cell-extrinsic mechanism, such as IFN-γ secretion by accessory cells.

NO has also been shown to modulate lymphocyte activity directly. A number of reports have sought to ascertain the role of NO in regulating normal B cells. During graft-versus-host disease (GVHD), B cells have a greatly diminished ability to proliferate and secrete antibody in response to LPS stimulation. Inhibition of NO in cultures of splenocytes from mice undergoing acute GVHD restored the sensitivity of B cells to LPS-induced proliferation (Wall et al., 1988).

Similarly, the ability of IL-6-activated macrophages to reduce both antibody production and the viability of B cells has been shown to be mediated by NO (Takagi et al., 1994). Nitrite alone can reduce DNA synthesis and antibody production by LPS-activated B cells (Takagi et al., 1992). Clearly, the absence of high levels of NO production in mitogen-activated splenocyte cultures may account for the increased viability and enhanced proliferation of B cells from NOS2−/− mice in the present study.

Our results indicate that NO not only regulates the antibody response during IAV infection, but also contributes to exacerbating clinical illness significantly in NOS2+/+ mice. Elevated clinical illness was associated directly with increased immunopathology, characterized by higher overall accumulation of inflammatory leukocytes in lungs, elevated and sustained expression of proinflammatory cytokines and increased T-cell cytokines. Proinflammatory cytokines, such as TNF, IL-1 and IL-6, are associated with the clinical symptoms of IAV infection in humans (Fritz et al., 1999; Hayden et al., 1998) and contribute significantly to morbidity and mortality in mice (Kozak et al., 1997; Peper & Van Campen, 1995; Swiergel et al., 1997). We found significantly lower levels of TNF and IL-6 in the lungs of NOS2−/− mice at the time when clinical illness peaked in NOS2+/+ mice.

Viral load is an important determining factor in promoting localized cytokine expression in viral pneumonia. Mice infected with a lethal dose of IAV produce higher levels of TNF, IL-1 and IL-6 than mice infected with a lower dose of virus (Conn et al., 1995). We have shown previously that virus levels are similar in NOS2+/+ and NOS2−/− mice early during infection (Karupiah et al., 1998a), thus suggesting strongly that IAV replication in lung epithelial cells of both strains is similar. However, as virus levels are reduced significantly in NOS2−/− mice by day 7 of infection,
whereas IAV persists in the lungs of NOS2⁺/⁺ mice up to day 10 (unpublished observation), the increased presence of virus in NOS2⁺/⁺ mice may stimulate a sustained production of proinflammatory cytokines. Indeed, proinflammatory cytokines and chemokines were elevated significantly in the lungs of mice infected with a virulent IAV expressing the HA derived from the 1918 Spanish influenza virus (Kobasa et al., 2004). Similarly, elevated and prolonged production of these factors was associated with delayed viral clearance and elevated immunopathology in the lungs of infected mice.

Whilst NO has been shown to have beneficial antiviral effects both in vitro and in vivo, high levels of NO can also be detrimental to the host. This study reveals an important physiological role for NOS2 in regulating the antibody response and in exacerbating immunopathology during a viral infection. A wide body of evidence exists supporting the concept that an inappropriately vigorous cellular immune response during primary IAV infection is detrimental to the host. Thus, it is particularly important to consider the environment in which such a response takes place. Strong inflammatory reactions that affect particularly sensitive organs such as the lungs, brain and heart can be deleterious to the host. Therefore, in attempting to reduce morbidity during viral pneumonia, strategies aimed at limiting the cellular response whilst stimulating the humoral response may prove beneficial in the management of illness (Sato et al., 1998; Schiltknecht & Ada, 1985).

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