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Role of Nitric Oxide Synthase Type 2 in Acute Infection with Murine Cytomegalovirus

Satoshi Noda,* Kazuo Tanaka,* Sada-aki Sawamura,* Masafumi Sasaki, Takako Matsumoto,* Katsunaka Mikami,* Yuji Aiba,* Hideaki Hasegawa,† Noboru Kawabe,† and Yasuhiro Koga*

Whether or not NO plays a critical role in murine CMV (MCMV) infection has yet to be elucidated. In this study, we examined the role of NO in acute infection with MCMV using NO synthase type 2 (NOS2)-deficient mice. NOS2−/− mice were more susceptible to lethal infection with MCMV than NOS2+/+ mice and generated a much higher peak virus titer in the salivary gland after acute infection. A moderate increase in the MCMV titer was also observed in other organs of NOS2−/− mice such as the spleen, lung, and liver. The immune responses to MCMV infection including NK cell cytotoxicity and CTL response in NOS2−/− mice were comparable with those of NOS2+/+ mice. Moreover, the ability to produce IFN-γ is not impaired in NOS2−/− mice after MCMV infection. The peritoneal macrophages from NOS2−/− mice, however, exhibited a lower antiviral activity than those from NOS2+/+ mice, resulting in an enhanced viral replication in macrophages themselves. Treatment of these cells from NOS2+/+ mice with a selective NOS2 inhibitor decreased the antiviral activity to a level below that obtained with NOS2−/− mice. In addition, the absence of NOS2 and NOS2-mediated antiviral activity of macrophages resulted in not only an enhanced MCMV replication and a high mortality but also a consequent risk of the latency. It was thus concluded that the NOS2-mediated antiviral activity of macrophages via NO plays a protective role against MCMV infection at an early and late stage of the infection. The Journal of Immunology, 2001, 166: 3533–3541.

Cytomegaloviruses classified as β-herpesviruses are ubiquitous microbes that commonly infect many hosts, including humans and mice. CMV causes no apparent clinical manifestations in immunocompetent individuals; however, it does cause severe diseases such as encephalitis, pneumonitis, retinitis, hepatitis, gastritis, and colitis, especially in immunocompromised hosts including fetuses, transplant recipients, and AIDS patients. Murine CMV (MCMV) infection in mice resembles its human counterpart in many ways with respect to the establishment of acute, persistent, and latent infections and host-virus interaction (1). Indeed, MCMV has a considerable homology to human CMV on molecular and biological levels (2). Like all herpesviruses, persistent CMV infection occurs in multiple organs after acute infection, resulting in latency and a consequent risk of reactivation (1).

The immune responses to acute viral infection can be classified into two categories: the innate response in the early phase; and specific responses in the late phase. In innate immunity to MCMV infection, NK cells (3, 4), macrophages (5, 6), and cytokines collaboratively act as the first line of antivirus defense. In contrast, the specific immune responses by CD4+ and CD8+ T cells help remove the virus late in infection, thus resulting in a protective effect from the virus-associated pathogenicities (7, 8). Although the complete clearance of the virus may require specific T cell immunity, the innate immunity in the early phase plays a critical role in controlling the overall extent of viral replication and spread, thereby reducing the overall mortality.

Proinflammatory cytokines, such as IFN-γ and TNF-α, are also involved in the elimination of the virus early or late in infection (9–11). Among them, Th1-associated cytokine IFN-γ produced by NK cells or T cells have been shown to play an important role in the clearance of MCMV (12–14). Moreover, this factor also plays an important role in augmenting the early NK cell response (15) and late protective T cell response (16) to MCMV infection.

When comparing the protective role of NK cells and various cytokines, the role of macrophages in innate immunity against CMV infection had yet to be elucidated. Macrophages use NO as an antiviral effector (17, 18). NO is produced by the NO synthase (NOS) isoforms, which convert arginine and oxygen to citrulline and NO (19, 20). One of the isoforms, NOS type 2 (NOS2), is expressed by macrophages after stimulation by IFN-γ (21, 22). The expression of NOS2 leads to a high output production of NO from these cells in viral infection (18). NOS2-derived NO appears to inhibit the early steps of microbial replication by modulating their DNA synthesis (23, 24), resulting in the microbial clearance. Indeed, NOS2-derived NO is reported to be important in the elimination of such viruses as the vaccinia virus (25), HSV-1 (26, 27), and EBV (28), and the RNA viruses such as JEV (29), CVB3 (30), and vesicular stomatitis virus (31).

Recent works also indicated that NO inhibits CMV replication in vitro (32, 33). Moreover, Tay et al. (34) showed the importance of NOS2 activity for the clearance of CMV in vivo. However, these prior studies on the NO function in viral infections have been
performed by pharmacological assays using common NOS inhibitors. The use of NOS inhibitors to investigate the role of NOS2 in CMV infection is considered to be inconclusive, because these inhibitors have non-isozyme-selective nature and variation in the bioavailability. We therefore examined the in vivo role of NO in CMV infection using NOS2-deficient mice. Using these mice, the relationship between the viral clearance by innate immunity and the resultant viral latency was also examined.

Materials and Methods

Mice

Breeding pairs of 129/SvEv × C57BL/6 mice with a disrupted NOS2 gene (NOS2−/−) were purchased from Dr. D. Nathan (San Francisco, CA) and kindly provided by Drs. J. Mudgett, J. MacMicking, and C. Nathan (35) and kindly provided by Dr. Y. Abe (Fujita Health University, Toyoake, Japan). C57BL/10, C57BL/6, BALB/c, and C57BL/6J mice were purchased from Clea Japan (Tokyo, Japan) and Biological Research Laboratories (Füllinsdorf, Switzerland), respectively, and their F1 generation was used as wild-type controls (NOS2+/-). These mice were bred in specific pathogen-free conditions in our animal facility and then used at 6 wk. The mice in the all experiments were sacrificed after anesthetization with Nembutal. A disruption of the NOS2 in NOS2−/− mice was confirmed by RT-PCR, showing no expression of NOS2 mRNA in the peritoneal exudate cells of these mice, which had received 400 µL PBS 6 h before sacrifice (data not shown). Both RNA extraction and RNA-PCR were performed as described below.

Virus and virus titration

The Smith strain of MCMV (VR194) was obtained from the American Tissue Culture Collection (Manassas, VA). The stock solution was prepared from salivary glands from MCMV-infected BALB/c mice as described previously (36). The virus concentration of this MCMV stock suspension was 2.0 × 106 PFU/ml. This concentration and the virus titers in the organs of NOS2−/− and NOS2+−/− mice were determined by a plaque assay using subconfluent 3T3/Swiss albino cells as previously reported (37).

Light microscopy and transmission electron microscopy

The organs, including the salivary gland, lung, and liver, were removed after exsanguination and then fixed in 3.7% formaldehyde at room temperature for 18 h. The sections were stained with hematoxylin-eosin (38). Thin sections of the salivary gland were prepared for a morphological analysis by electron microscopy (JEM-2000EX; JEOL, Tokyo, Japan) according to the method described elsewhere (37).

NK cell cytotoxicity

The NK cell cytotoxicity was determined as described previously (15). In brief, spleen cells were prepared from both NOS2−/− and NOS2+−/− mice infected with 2 × 106 PFU MCMV on day 0. A single-cell suspension of these cells was obtained on days 1, 2, 3, and 4 after the infection and then layered on Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) and centrifuged at 400 × g for 30 min at 18°C. The lymphocytes were then harvested and washed twice with RPMI 1640 and used as effector cells. For the preparation of target cells, YAC-1 cells were labeled with 3.7 MBq 51Cr (per ml) (ICN Biomedicals, Costa Mesa, CA). The target cells and effector cells were mixed in 0.2 ml RPMI 1640 on a round-bottom microplate and then incubated for 4 h at 37°C. After centrifugation, the amount of radioactivity in the supernatant was measured. The NK cell activity of the effector cells was expressed as follows:

\[
\% \text{ specific lysis} = \frac{(\text{maximal } {51\text{Cr}} \text{ release} - \text{spontaneous } {51\text{Cr}} \text{ release}) - (\text{experimental } {51\text{Cr}} \text{ release} - \text{spontaneous } {51\text{Cr}} \text{ release})}{\text{maximal } {51\text{Cr}} \text{ release} - \text{spontaneous } {51\text{Cr}} \text{ release}} \times 100.
\]

Maximum release was obtained by counting acid-lysed target cells. Spontaneous release was obtained by incubating target cells in the absence of effector cells.

Cytolytic effector cell assay

Peritoneal exudate cells (PECs) from both MCMV-infected NOS2−/− and NOS2+−/− mice were harvested 2 days after the i.p. injection of 1.0 ml 10% protease peptone by peritoneal lavage. The organs of NOS2−/− mice were harvested and washed twice with RPMI 1640 and used as effector cells. For the preparation of target cells, MCMV-infected primary mouse embryonic fibroblasts (MEFs) were prepared from C57BL/6 as described previously (39), and then labeled with 3.7 MBq 51Cr (per ml) (40). The target cells and nonadherent PECs were mixed at various ratios in 0.2 ml RPMI 1640 on a V-bottom 96-well microplate and then incubated for 4 h at 37°C. After centrifugation, the amount of radioactivity in the supernatant was measured. The cytolytic activity was expressed by the same formula as that used in the NK cell cytotoxicity assay. Maximum and spontaneous releases were obtained by the same methods used in the NK cell cytotoxicity assay.

Cell preparations

To enrich NK cells and CD4+ T cells, B cells and CD8+ T cells were depleted from the spleen cells by immunomagnetic negative selection (41). In brief, spleen cells from NOS2−/− or NOS2+−/− mice were incubated at 4°C with magnetic particles bound to goat anti-mouse IgG (PerSeptive Biosystems, Framingham, MA). The magnetic particles attached to cells were then removed using a magnet (PerSeptive Biosystems), while leaving behind any surface IgG-negative cells. Such B cell-depleted spleen cells were further depleted of CD8+ T cells by incubation with magnetic particles bound to anti-CD8 (Lyt2) (Dynal, Oslo, Norway). The depletion of B cells and CD8+ T cells was confirmed by flow cytometry using the Abs FITC-conjugated anti-CD45R/B220 (RA3-6B2; Pharmingen, San Diego) and anti-CD8α (5-6.7; Pharmingen) (data not shown).

Cytokine measurement

A cytokine production assay was performed as reported previously (42). The spleen cells devoid of both B cells and CD8+ T cells were resuspended at a final concentration of 2.5 × 106/ml and then were cultured in 1.0 ml of media containing 10 µg/ml Con A in a 24-well plate. The supernatants were harvested after 48 h, and the cytokine level was quantified using the sandwich ELISA technique. Briefly, a 96-well ELISA plate was coated with anti-cytokine Ab. After washing and blocking, the well was incubated with samples or standards, and then biotinylated anti-cytokine Ab was added. Next, streptavidin-peroxidase and a peroxidase substrate were added to induce a colorimetric reaction. To assay the cytokines, the following coating Abs, biotinylated Abs, and standards were used: IFN-γ, R4-6A2 (PharMingen, San Diego, CA); XMG1.2 (PharMingen); IFN-γ (Life Technologies, Gaithersburg, MD); IL-4, BVD4-1D11 (PharMingen); BVD4-24G2 (PharMingen); and rIL-4 (Life Technologies).

Collection of protease peptone-induced peritoneal macrophages

Protease peptone-induced peritoneal macrophages were prepared as follows. The mice were injected i.p. with 10% protease peptone. At 2 days after the injection, PECs were harvested in HBSS. After centrifugation, these cells were suspended in RPMI 1640 supplemented with 10% FCS and cultured in a 24-well plate for 12 h at 37°C in a 5% CO2 atmosphere. Next, adherent cells were gently washed with PBS after removing any nonadherent cells and were used as protease peptone-induced peritoneal macrophages.

RNA-PCR for NOS2

Both RNA extraction and RNA-PCR were conducted as previously reported (40). In brief, RNA from the peritoneal macrophages of MCMV-infected mice was extracted using ISOGEN (Nippon Gene, Toayama, Japan) according to the manufacturer’s manual. RNA-PCR was performed with the mRNA-PCR kit (Takara Shuzo, Shiga, Japan). The primers for a disrupted region of NOS2 were selected from the published sequence data (38). In brief, RNA from the peritoneal macrophages of MCMV-infected mice were incubated at 37°C in a 5% CO2 atmosphere. Next, adherent cells were gently washed with PBS after removing any nonadherent cells and were used as protease peptone-induced peritoneal macrophages.

Measurement of NO from peritoneal macrophages

Protease peptone-induced peritoneal macrophages from infected NOS2−/− or NOS2+−/− mice were prepared as mentioned above. These cells after a new in vitro infection (multiplicity of infection (MOI), 0.005) were cultured in a 24-well plate at 1 × 105/well for 24 h at 37°C in a 5% CO2 atmosphere, and then the culture supernatants were harvested. The measurement of NO (NO2−/NO3−) in the supernatants was performed using the assay kit (NO/NO3 assay kit-Fluometric; Dojindo, Kumamoto, Japan) according to the manufacturer’s manual. The base sequences were as follows: NOS2−/−-primer CCGTGCCTCAGGTGTTTCTT; NOS2+−/−-primer GTTCCGTGTTTGTTC (680–707). RNA-PCR for β-actin was performed as previously reported (38), using commercially available primers (Clontech, Palo Alto, CA).

Measurement of NO from peritoneal macrophages

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ELISA reader (Fluoroscan II, excitation 365 nm, emission 450 nm; Dainippon Seiyaku, Osaka, Japan). The standard curve was obtained by the control solution of the kit.

**In vitro analysis of anti-MCMV activity of peritoneal macrophages**

Proteose peptone-induced peritoneal macrophages were prepared as described above. These cells were plated in a 96-well microplate at 1 × 10^6/well, and then the cells were infected with MCMV (MOI, 0.005). At various times after the new in vitro infection, the supernatant was harvested. The productive virus in the supernatant was determined by a plaque assay as described above. The intrinsic antiviral activity was determined by measuring the amount of productive virus in the supernatant. In some experiments, MEG ((2-mercaptoethyl)guanidine; Cayman Chemical, Ann Arbor, MI) was used to inhibit the NOS2 activity of peritoneal macrophages in this assay.

**Phagocytic activity of peritoneal macrophages**

The peritoneal macrophages were prepared as described above. These cells were pulsed with fluorescent FluoSpheres beads (Molecular probes, Eugene, OR) in a 24-well plate and then incubated at 37°C. At selected time points, the cells were harvested, and the bead accumulation was analyzed in a FACScan.

**Detection of MCMV DNA in organs**

DNA extraction from the organs and DNA-PCR for detection of MCMV DNA were performed as described previously (36). In short, the MCMV-DNA of the immediate early (IE) region was enhanced by PCR using two oligonucleotide primers (primer 1, 1701–1730; primer 2, 2371–2400) as reported previously (43). This primer pair amplified the 700-bp segment. PCR was performed in an automated thermal cycler (Program Temperature Control System PC-700; Astec, Fukuoka, Japan) after the addition of Taq polymerase (Ex taq; Takara Shuzo, Otsu, Shiga, Japan). The samples were then electrophoresed on 2% (w/v) agarose gel containing 1 μg/ml ethidium bromide in Tris-acetate buffer, and visualized by UV fluorescence (44).

**Results**

**Absence of NOS2 exerts a lethal effect on MCMV infection**

At first, the susceptibility of NOS2−/− mice against MCMV was examined by changing the inoculation doses of MCMV (Fig. 1). The inoculation of MCMV led to early mortality in NOS2−/− mice when given only 3.7 × 10^4 PFU of the virus. In contrast, the NOS2+/+ mice were still resistant to such a viral load; however, they began to succumb at 3.3 × 10^5 PFU, which was about a 10 times larger number than that for NOS2−/− mice. NOS2−/− mice thus appear to be highly susceptible to infection with MCMV in comparison with NOS2+/+ mice.

**Virus titer is markedly elevated in NOS2−/− mice infected with MCMV**

The absence of NOS2 led to a high mortality in MCMV infection (Fig. 1). We next investigated the role of NOS2 in viral replication. NOS2+/+ and NOS2−/− mice were sublethally infected with 2.0 × 10^4 PFU MCMV, and the infectious virus number in the organs was determined by a plaque assay (Fig. 2). The salivary gland, the optimal organ for MCMV replication, generated a much higher virus titer in NOS2−/− mice than in NOS2+/+ mice (Fig. 2A). The titer in NOS2−/− mice reached a peak 4 wk after infection (log_{10} PFU/organ = 10.5), whereas that in NOS2+/+ mice peaked 2 wk postinfection (log_{10} PFU/organ = 6.3). Consistent with these observations, an electron microscopic analysis showed a large amount of MCMV in the salivary gland of NOS2−/− mice, whereas only a few virus particles were noted in the organ of NOS2+/+ mice (Fig. 3). The productive virus was transiently detected in the spleen, lung, and liver of NOS2−/− mice, whereby it
was not detected in these organs of NOS2<sup>+/+ </sup>mice (Fig. 2B). It is thus demonstrated that the lack of NOS2 activity led to a marked increase of the virus number in the organs after MCMV infection. Whereas a large number of the virus were generated in the salivary gland, and to a lesser extent in the other organs including the lung and liver of NOS2<sup>2/2</sup> mice, histological examinations of these organs showed no apparent tissue injury or abnormality at this sublethal dose (Fig. 4, A and B, and data not shown). In contrast, the lung and liver of NOS2<sup>2/2</sup> mice exhibited a marked tissue inflammation and injury with a higher lethal dose, 1.1×10<sup>5</sup> PFU of the virus (Fig. 4C), resulting in virus-induced diseases including pneumonitis and hepatitis with necrosis and bleeding (45), resulting in death (Fig. 1).

**NK cell cytotoxicity and CTL response were not impaired in MCMV-infected NOS2<sup>2/2</sup> mice**

The early nonspecific responses by NK cells have been clearly shown to provide resistance in acute stage of MCMV infection (3, 4), and the CTL is critical in the late stage of the infection (8). We therefore examined how a high mortality accompanied with a high virus titer in NOS2<sup>2/2</sup> mice is concerned with the immune responses to MCMV in these mice. As shown in Fig. 5, the MCMV-induced NK cell cytotoxicity in NOS2<sup>2/2</sup> mice was comparable to that from NOS2<sup>+/+</sup> mice. Moreover, the specific CTL response to this virus was not impaired in MCMV-infected NOS2<sup>2/2</sup> mice (Fig. 6). As a result, the cell-mediated immunity to MCMV infection was intact in NOS2<sup>2/2</sup> mice, thus suggesting that the susceptibility to this virus infection is due to the absence of NOS2.

**NOS2<sup>2/2</sup> mice showed a successive production of IFN-γ after acute infection**

Th1-associated cytokine IFN-γ, especially produced by NK cells (13) or CD4<sup>+</sup> T cells (9), is also important for MCMV clearance early or late in infection. Moreover, the T cell-dependent IFN-γ production has been found to play a critical role in the augmentation of NK cell activity (15). We therefore measured the ability of IFN-γ production in NOS2<sup>2/2</sup> mice to examine whether a failure in such cytokine production is involved in the susceptibility to MCMV infection. As shown in Fig. 7A, CD4<sup>+</sup> T cell-enriched spleen cells from MCMV-infected NOS2<sup>2/2</sup> mice successively produced a large amount of IFN-γ lasting up to day 14 after infection when stimulated with Con A, whereas production by those from NOS2<sup>+/+</sup> mice was transient with a peak on day 7. In contrast, the production of Th2-associated cytokine, IL-4, was undetectable in NOS2<sup>2/2</sup> mice on days 7 and 14 after MCMV infection (Fig. 7B). As a result, the unimpaired IFN-γ productivity and the skewing to Th1 response in immunity were observed in NOS2<sup>2/2</sup> mice. These results indicated that the high susceptibility to MCMV infection in NOS2<sup>2/2</sup> mice is not due to the defect of the IFN-γ productivity.
Macrophage-mediated antiviral activity in NOS2<sup>+/+</sup> and NOS2<sup>−/−</sup> mice

NOS2<sup>−/−</sup> mice showed a high susceptibility to MCMV infection (Figs. 1–3) despite the normal immune responses (Figs. 5–7). We next examined whether macrophages, a dominant source of NOS2-derived NO, contribute to the control of MCMV infection in the acute phase. The enhanced expression of NOS2 mRNA in peritoneal macrophages of NOS2<sup>+/+</sup> mice was observed on day 3 after infection, lasting up to day 7, whereas no NOS2 mRNA was expressed in NOS2<sup>−/−</sup> mice (Fig. 8A). This result implied that NOS2-associated antiviral activity of macrophages play a role in inhibiting the viral replication and eliminating the virus in vivo. It has been demonstrated that the proficiency of MCMV replication in the macrophages themselves determines the virulence and the virus-induced pathogenesis in vivo (6, 45, 46). The efficiency of inhibiting viral replication in macrophages themselves has been defined as the intrinsic antiviral activity of these cells (45). We therefore examined the intrinsic antiviral activity of the macrophages from NOS2<sup>+/+</sup> and NOS2<sup>−/−</sup> mice. The intrinsic antiviral activity of naive macrophages from NOS2<sup>−/−</sup> mice was comparable with that from NOS2<sup>+/+</sup> mice on day 3 after in vitro MCMV infection (the productive virus titer in the culture supernatant was 213 ± 83.3 PFU/ml in NOS2<sup>−/−</sup> mice and 200 ± 40.0 PFU/ml in NOS2<sup>+/+</sup> mice). However, the activity of these cells from NOS2<sup>−/−</sup> mice was lower than that from NOS2<sup>+/+</sup> mice on day 9 after the in vitro infection (the productive virus titer in the supernatant was 1800 ± 174 PFU/ml in NOS2<sup>−/−</sup> mice and 653 ± 92.4 PFU/ml in NOS2<sup>+/+</sup> mice) (Fig. 8B). The treatment of these cells from NOS2<sup>−/−</sup> mice with MEG, a selective NOS2 inhibitor, decreased the antiviral activity to a level below that obtained in the NOS2<sup>−/−</sup> mice (Fig. 8B). The phagocytic activity in the macrophages of the both mice was intact (data not shown). Consistent with this finding, the peritoneal macrophages from NOS2<sup>−/−</sup> mice on day 3 of infection, in which a high expression of NOS2 mRNA (Fig. 8A) was observed, produced a higher amount of NO in the culture supernatant on in vitro reinfection (NO<sub>2</sub> /NO<sub>3</sub> on days 1 and 2 after the reinfection were 12.9 ± 0.8 and 18.6 ± 2.3 μM in NOS2<sup>−/−</sup> mice and 3.6 ± 0.9 and 10.7 ± 0.8 μM in NOS2<sup>+/+</sup> mice, respectively), thus leading to a significantly higher intrinsic antiviral activity in these cells (Fig. 8C). However, the productive virus titer increased after the cells from NOS2<sup>−/−</sup> mice were treated with MEG, thus resulting in a marked reduction of the antiviral activity (Fig. 8C). As a result, NOS2-derived NO produced by macrophages and/or the NOS2-associated antiviral function may greatly help control MCMV infection at an early stage of infection.

Role of NOS2 in establishing latent MCMV infection

MCMV latently infects its host for a long time after acute infection. A latent MCMV infection, which is determined as the presence of viral DNA without any infectious particles, has been observed in a wide variety of organs, including the lung, spleen, kidney, and salivary gland (1). We, therefore, investigated the role of NOS2 in latent MCMV infection using a PCR analysis (47). No infectious virus was observed in the lung of NOS2<sup>−/−</sup> or NOS2<sup>+/+</sup> mice 4 wk after infection (Fig. 2B). In contrast, MCMV-DNA was definitely detected in the lung of NOS2<sup>−/−</sup> mice up to 32 wk after infection (Table I; see the boxed data). Furthermore, the NOS2<sup>−/−</sup> mice exhibited a delayed clearance of MCMV-DNA in the salivary gland, spleen, and kidney. It is thus indicated that NOS2 plays a significant role not only in the initial clearance but also in its latency after acute MCMV infection.

**FIGURE 4.** Histological examination of the organs from NOS2<sup>+/+</sup> and NOS2<sup>−/−</sup> mice infected with MCMV. The salivary gland (A) and lung (B) of these mice inoculated i.p. with a sublethal dose, 2 × 10<sup>4</sup> PFU of the virus 2 wk earlier. The lung and liver (C) of both mice inoculated i.p. with a higher lethal dose (1.1 × 10<sup>5</sup> PFU MCMV) 5 days before. Hematoxylin-eosin staining; original magnifications, ×44 (A, B), ×50 (C; lung), and ×25 (C; liver).
Discussion
The expression of NOS2 has been demonstrated to play an important role in microbial infection (48). Moreover, the NOS2-dependent pathways also contribute to a defense in a variety of viral infections (17, 18). In this study, we investigated the role of NOS2 in acute infection with MCMV using NOS2-deficient mice. Our data demonstrated that the absence of NOS2 results in 1) a high mortality in MCMV infection, 2) an enhancement of the viral replication in the organs, 3) a reduced anti-MCMV activity of macrophages, and 4) a prolongation of MCMV-DNA latency. These observations thus provide conclusive evidence regarding crucial role of NOS2 and macrophages in CMV infection.

There are three well-characterized isoforms of NOS including NOS1 (neuronal NOS), NOS2, and NOS3 (endothelial NOS) (17). In particular, NOS2-derived NO has appeared to be important in the elimination of many viruses including CMV in vitro and in vivo (32–34). However, the role of NOS2-derived NO in CMV infection has been unclear, especially in vivo, because most of these studies used common NOS inhibitors that exhibit nonspecific effects. We resolved this issue using an in vivo mouse model.

Immune responses are engaged in both the clearance of the virus from infected cells and the inhibition of virus dissemination among the cells, which thus results in a reduced viral burden in the host. The importance of NK cells or T cells in controlling MCMV infection has been definitely established (49). In the present study, however, the susceptibility to MCMV infection in NOS2−/− mice (Figs. 1–3) is not due to a failure of such immune responses as NK cell activity, CTL response, and IFN-γ productivity, because these responses to the virus are intact even in NOS2−/− mice after infection (Figs. 5–7). These findings also suggested that the NOS2-dependent pathway plays a critical role in controlling MCMV infection.

As for NK cell cytotoxicity in microbial infections, Diefenbach et al. (50) recently showed that the activity to Leishmania major infection is significantly reduced in NOS2−/− mice, and NOS2 is required for the induction of this activity. This impairment of NK activity may be due to a defect in the NO-mediated JAK/STAT signaling pathway via IL-12 and IFN-αβ in NK cells (51). In the current study, however, the NK activity was not impaired even in NOS2−/− mice after MCMV infection (Fig. 5). Consistent with our findings, Bartholdy et al. (52) reported that the NK activity to lymphocytic choriomeningitis virus or vesicular stomatitis virus...
infection remains intact in NOS2−/− mice. The discrepancy between the results may be attributable to cytokine release (i.e., IL-18) after infection, because the IL-18-dependent NK activity, which is independent of the JAK/STAT signaling pathway (53), can be induced during MCMV infection (54). In contrast to the situation in microbial infection, NOS2-derived NO appears not to be involved in the enhancement of the NK activity after virus infections.

Specific immune responses including Th and CTL are required to remove the virus (49). These responses are strictly regulated by the Th1/Th2 balance. This balance is therefore critical for obtaining effective immunity in microbial infection (55). Our current study shows a Th1 bias (predominant IFN-γ production) in NOS2−/− mice after MCMV infection that appears to be induced by the lack of NOS2 activity (Fig. 7). Indeed, the data from many laboratories have shown that NO affects the Th1/Th2 immuno-regulatory balance and specifically down-regulates the Th1-type cytokine IL-2 and IFN-γ and/or increases the Th2-associated cytokine IL-4 (56). In addition, increased Th1 responses in the absence of NO have been reported in the mouse in vivo model of leishmaniasis (57), toxic shock syndrome (35), bacterial septic arthritis (58), vaccination with attenuated Schistosoma mansoni (59), HSV-1 infection (60), and influenza virus infection (61). These findings strongly suggested that Th1 bias in some microbial infections is due to the absence of NOS2-derived NO. Th1 bias, however, does not seem to be detrimental to NOS2−/− mice infected with MCMV, because IFN-γ, a potent antiviral cytokine, is produced much after infection in these mice (Fig. 7A). Moreover, IFN-γ and/or other Th1-related cytokines seem to play an essential role in establishing late cell-mediated protective function including T or B cell response (16, 62). In fact, CTL activity is not impaired in NOS2−/− mice (Fig. 6). Such effectors as CTL and IFN-γ may contribute to the final clearance of MCMV in the late phase of infection even in the NOS2−/− mice.

Macrophages appear to have dual, even paradoxical, roles: one as a host for CMV replication; and another as an effector cell for eliminating the virus. Hanson (46) recently reported that the proficiency of MCMV replication in macrophages themselves positively correlates with virulence in vivo. Indeed, macrophages, a dominant source of NOS2-derived NO, play a protective role against MCMV infection by the expression of the intrinsic antiviral activity (6, 45). These findings show the possibility that the absence of NOS2-mediated antiviral activity of macrophages may enhance the viral replication in these cells, hence severe virus-induced pathogenicity. In line with these findings, our data show that NOS2−/− mice exhibit a high susceptibility to MCMV infection such as a high virus titer and increased mortality (Figs. 1–3). The immune response to MCMV including NK cells and CTL is

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**FIGURE 8.** NOS2-associated antiviral activity of macrophages in MCMV infection. A, RT-PCR analysis for the expression of NOS2 mRNA in peritoneal macrophages after acute infection. NOS2+/+ and NOS2−/− mice were inoculated i.p. with 2 × 10⁶ PFU of MCMV on day 0. Peritoneal macrophages were obtained from each mouse on each day after infection as indicated in the figure. Both RNA extraction and RT-PCR were performed as described in Materials and Methods. B, In vitro analysis of the intrinsic antiviral activity of naive peritoneal macrophages. The naive peritoneal macrophages were obtained from both mice as described in Materials and Methods. These cells were infected with MCMV (MOI = 0.005), and then were treated with MEG (100 μM) in vitro. The culture supernatants were harvested on day 3 or 9 after the in vitro infection, and then the productive virus titer was determined by a plaque assay. Data represent the means ± SD of triplicate cultures. C, In vitro analysis of the intrinsic antiviral activity of macrophages after acute infection. The peritoneal macrophages on day 3 of infection were obtained as described in Materials and Methods. These cells were reinfected with MCMV and then were treated with MEG as described above. The culture supernatants were harvested, and then the productive virus titer was determined as shown in B. The titer was expressed as follows: PFU/ml = (experimental production of the virus in the supernatant − spontaneous production of the virus in the supernatant). The spontaneous virus titer on each day was below 110 PFU/ml (Ex. 1) and 140 PFU/ml (Ex. 2) in the both mice.
shown), also suggesting that macrophages of NOS2
2
macrophages at an early stage of infection (Fig. 8). In addition, a
exhibit a reduced NOS2-dependent intrinsic antiviral activity of
themselves are good reservoirs of MCMV in vivo because of the
lower intrinsic antiviral activity. These results strongly demon-
strated that NOS2 is required for the induction of antiviral activity of macrophages and that NOS2-derived NO from these cells plays
a crucial role in controlling MCMV infection in the acute phase.

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Table I. Detection of MCMV-DNA in latently infected NOS2+/+ or NOS2+/- mice

a To detect MCMV latency, DNA-PCR was used to amplify the MCMV-specific DNA sequences (IE1) from various organs of the mice. The clearance of the MCMV-DNA in each tissue is underlined. For explanation of boxed material, see the text.

b Number of positive mice/total.

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References


