Role of Nitric Oxide in the Pathogenesis of Encephalomyocarditis Virus-Induced Diabetes in Mice

Young-Sun Lee¹, Na Li², Seungjin Shin², and Hee-Sook Jun¹*

Lee Gil Ya Cancer and Diabetes Institute, Gachon University of Medicine and Science, Yeonsu-ku, Incheon
Department of Pathology, Chicago Medical School, Rosalind Franklin University of Medicine, North Chicago, USA

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*Corresponding author: Hee-Sook Jun, PhD, Lee Gil Ya Cancer and Diabetes Institute, Gachon University of Medicine and Science, 7-45 Sondo-dong, Yeonsu-ku, Incheon 406-840 Korea; Tel: 11-82-32-899-6056; Fax: 11-82-32-899-6057; E-mail: hsjeon@gachon.ac.kr

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The D variant of encephalomyocarditis (EMC-D) virus causes diabetes in mice by destroying pancreatic β cells. In mice infected with a low dose of EMC-D virus, macrophages play an important role in β cell destruction by producing soluble mediators such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and nitric oxide (NO). To investigate the role of NO and inducible nitric oxide synthase (iNOS) in the development of diabetes in EMC-D virus-infected mice, we infected iNOS-deficient DBA/2 mice with EMC-D virus (2×10^2 PFU/mouse). Mean blood glucose levels of EMC-D-infected iNOS-deficient mice and wild-type mice were 205.5 and 466.7 mg/dl, respectively. Insulitis and macrophage infiltration were reduced in islets of iNOS-deficient mice compared with wild-type mice at 3 days after EMC-D infection. Apoptosis of β cells was decreased in iNOS-deficient mice, evidenced by reduced numbers of TUNEL-positive cells. mRNA expression for anti-apoptotic molecules, Bcl-2, Bcl-xL, Bcl-w, Mcl-1, cIAP-1 and cIAP-2, was not different between wild-type and iNOS-deficient mice, whereas expression of pro-apoptotic Bax and Bak mRNA was significantly decreased in iNOS-deficient mice. Expression of IL-1β and TNF-α mRNA was significantly decreased in both islets and macrophages of iNOS-deficient mice as compared with wild-type mice after EMC-D infection. Nuclear factor-κB was less activated in macrophages of iNOS-deficient mice after virus infection. We conclude that NO plays an important role in the activation of macrophages and apoptosis of pancreatic β cells in EMC-D virus-infected mice, and deficient iNOS gene expression inhibits macrophage activation and β cell apoptosis, contributing to prevention of EMC-D virus-induced diabetes.
INTRODUCTION

Type 1 diabetes results from absolute insulin deficiency caused by destruction of insulin-producing pancreatic β cells. The D variant of encephalomyocarditis (EMC-D) virus induces diabetes in genetically susceptible strains of mice by infecting and destroying β cells (13-18). In mice infected with a low dose ($1 \times 10^2$ PFU/mouse) of EMC-D virus, macrophages play a central role in the destruction of pancreatic β cells (4, 5, 13-15), evidenced by a significant increase in the incidence of diabetes if macrophages are activated prior to viral infection and complete prevention of EMC-D virus-induced diabetes if macrophages are inactivated prior to viral infection (4). Additional studies found that selective EMC-D viral infection of pancreatic β cells results in an initial recruitment of macrophages into the islets, followed by infiltration of other immunocytes including T cells, natural killer cells, and B cells (5).

EMC-D virus infects and activates macrophages without replication (13) and induces the production of soluble mediators such as interleukin (IL)-1β, tumour necrosis factor (TNF)-α, and inducible nitric oxide synthase (iNOS), which play important roles in the destruction of β cells (14). These infected macrophages express significantly more iNOS than either IL-1β or TNF-α (13). Treatment of EMC-D virus-infected mice with the tyrosine kinase inhibitor AG126, which inhibits nitric oxide (NO) production in EMC-D virus-infected macrophages, decreases the expression of IL-1β and TNF-α in the pancreatic islets and the incidence of diabetes and insulitis, as compared with vehicle-treated control mice (13). As well, treatment of EMC-D virus-infected mice with an iNOS inhibitor decreases the incidence of diabetes (14). These results suggest that iNOS and NO significantly contribute to the destruction of pancreatic β cells in mice infected with a low dose of EMC-D virus, although their roles are not fully understood.

To directly test whether iNOS and NO play a critical role in the pathogenesis of EMC-D
virus-induced diabetes in mice, we used iNOS knockout (KO) DBA/2 mice. We found that iNOS-deficient mice infected with EMC-D virus (2 × 10^2 PFU/mouse) showed a significantly lower incidence of diabetes. There was a reduced expression of IL-1β and TNF-α in macrophages and decreased infiltration of immunocytes in the islets of iNOS-deficient mice, resulting in reduced apoptosis of β cells as compared with EMC-D virus-infected wild-type mice. This study provides direct evidence of NO in the activation of macrophages by EMC-D viral infection and the pathogenesis of low dose (2 × 10^2 PFU/mouse) EMC-D virus-induced diabetes.
**MATERIALS AND METHODS**

**Virus.** EMC-D virus was prepared as described elsewhere (37, 38). Viral pools were prepared from L929 cells, and the virus titer was determined by plaque assay.

**Mice.** iNOS KO mice in C57BL/6J background (Jackson Laboratories, Bar Harbor, Maine) were backcrossed with DBA/2 mice, which are susceptible to EMC-D virus-induced diabetes. After 10-12 generations, heterozygous iNOS KO mice were crossed to produce homozygous iNOS KO mice. The wild iNOS (+/+) and KO iNOS (-/-) genotypes were screened by PCR. Animals were bred and maintained at animal facilities at the Rosalind Franklin University of Medicine and Science and the Lee Gil Ya Cancer and Diabetes Institute, Gachon University of Medicine and Science under a 12 h light:12 h dark photoperiod. All animal experiments were approved by the Institutional Animal Care and Use Committee at the Rosalind Franklin University of Medicine and Science and the Lee Gil Ya Cancer and Diabetes Institute.

**Infection of mice with EMC-D virus and measurement of blood glucose.** Six-week-old male mice were injected intraperitoneally with EMC-D virus (200 PFU/mouse). Blood glucose levels of unfed mice were determined at 6 days after EMC-D viral infection using a One-Touch Basic glucometer (Lifescan, Burnaby, BC, Canada). Blood glucose levels greater than 250 mg/dl were considered to be diabetic.

**Peritoneal macrophage preparation.** Peritoneal macrophages were harvested from iNOS KO and wild-type mice at 3 days after EMC-D virus infection, and cells were plated on 100-mm dishes. Cells were incubated for 1 h at 37°C in 5% CO₂ and then washed three times to remove...
nonadherent cells. Adherent cells were used for RT-PCR analysis of gene expression and electrophoretic mobility shift assay for nuclear factor (NF)-κB.

**Islet isolation.** Mice were infected with EMC-D virus. Three days later, food was removed for 4 h. The peritoneal cavity was exposed, and the pancreas was distended by intraductal injection of collagenase solution (Worthington Biochemical, Lakewood, NJ; 470 U/ml Hank’s Balanced Salt Solution [HBSS]). The distended pancreas was excised, transferred to a 15 ml glass tube, and incubated for 15-20 min at 37°C. After incubation, the digestion was stopped by addition of cold HBSS, and islets were dislodged by mild pipetting. More HBSS was added, and the tube was centrifuged for 5 min at 200 g. Islet separation was done by centrifugation on a Ficoll gradient (1900 g, 10 min, 4°C). To increase the purity of the preparation, islets were handpicked and counted under an inverted microscope (17, 35).

**Quantitative real-time RT-PCR analysis.** Total RNA was isolated from the islets and peritoneal macrophages. cDNA was synthesized using Superscript™ III Reverse Transcriptase Kit (Invitrogen, Carlsbad, Calif.). PCR was carried out in a LightCyclerTM (Roche Applied Science, Indianapolis, Indiana) at 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, 55°C for 20 s, and 72°C for 35 s. The following primer sequences were used: for insulin, sense 5’-TCAGAGACCACATGCAAGCAG-3’ and antisense 5’-GTCTGAAGGTCCCCGGGGCT-3’; for Bcl-2, sense 5’-ATACCTGGGCCACAAGTGAG-3’ and antisense 5’-TGCCTGAATGTGTGTGTGT-3’; for pro-apoptotic Bax, sense 5’-AAACAGGGCAGGAGAGAAG-3’ and antisense 5’-CAGCCTTGGGAAAATACAAGC-3’; for TNF-α, sense 5’-GACCCTCACACTCAGCAAGCAG-3’ and antisense 5’-CGCTGGCTCAGCACTCC-3’.
-3'; and for IL-1β, sense 5'-CAGGACAGGTATAGATTCTTTTCTTTT-3' and antisense 5'-ATGGCAACTGTTCCTGAACTCAACT-3'. Expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was analyzed as an internal control using the following primers: sense 5'-AGTGCCAGCCTCGTCCGTA-3' and antisense 5'-TGAGCCCTTCCACAATGCAA-3'. The relative copy number was calculated using the threshold crossing point (Ct) as calculated by the LightCycler software combined with the delta delta CT calculations.

For additional anti- or pro-apoptosis genes, cDNA was synthesized using PrimeScript™ 1st strand cDNA Synthesis kit (TaKaRa. Bio, Inc., Otsu, Japan). PCR was carried out in a 7900HT Fast Real-Time PCR Systems (Applied BioSystems Inc., Foster City, CA) at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. The following primer sequences were used: for Bak, sense 5'-CTGCTTTTTGCTGATATCATACTG-3' and antisense 5'-CACCCAACCGGCTCTCTGT-3'; for Bcl-xL, sense 5'-GCTGGGACACTTTTGTGGATCT-3' and antisense 5'-AAGCGCTCGGCTTTC-3'; for Bcl-w, sense 5'-TGAGAGCAGGACAGCAGACTTTTC-3' and antisense 5'-AAGCGCTCGGCTTTC-3'; for Mcl-1, sense 5'-GGATGGGTTTGTGGAGTTCTTC-3' and antisense 5'-CAAAAGCCAGCAGCACATTTC-3'; for cIAP-1, sense 5'-AGCCAGTCTGCGAGTCTCCATCT-3' and antisense 5'-CTGCTTTTTGCTGATATCATACTG-3'; for cIAP-2, sense 5'-GCTGAGGTGCTGGGAATCTG-3' and antisense 5'-GTACTCACACCTTGGAAACCACTTG-3'. Expression of cyclophillin mRNA was analyzed as an internal control in these genes; for cyclophillin, sense 5'-TGAGAGCAGGACAGCAGACTTTTC-3' and antisense 5'-TGCCGGTGCAGCAGACTTTTC-3'.
The relative copy number was calculated using the threshold crossing point (Ct) as calculated by
the 7900HT Fast Real-Time PCR Systems software combined with the delta delta CT calculations.

Immunohistochemical and histological staining of pancreatic sections. Mice were killed at 3
days after EMC-D virus infection, and pancreata were removed, fixed in 10% formalin, and
embedded in paraffin. Serial sections were incubated with guinea pig anti-swine insulin antibody
(Dako, Carpenteria, Calif.) or rabbit anti-Bax antibody (Calbiochem, La Jolla, Calif.) followed by
Cy3-conjugated anti-guinea pig IgG or Cy2-conjugated goat anti-rabbit IgG (1:200, Jackson
ImmunoResearch, West Grove, Penn.), respectively. To detect macrophage infiltration in the
pancreas, serial sections were blocked with 5% normal donkey serum and incubated with purified
anti-F4/80 (BM8; eBioscience, San Diego, Calif.) primary antibody. Sections were then incubated
in biotinylated donkey anti-rat antibody (Jackson ImmunoResearch) followed by
Alexa448-conjugated streptavidin (Molecular Probes, Eugene, Oregon). Images were analyzed
with light microscope (Nikon, Hicksville, NY) and laser scanning confocal microscope (Olympus
Fluoview 300, Center Valley, Penn.). Adobe Photoshop version 7.0 was used to process the
images. To detect histological changes in the pancreatic islets, paraffin-embedded sections were
stained with hematoxylin and eosin. Histological changes in the pancreatic islets were classified as
“peri-islet infiltration”, “mild to moderate insulitis,” “severe insulitis,” and “atrophied
morphology” as previously described (13).

TUNEL staining. Mice were sacrificed at 3 days after EMC-D virus infection, and pancreata were
removed, fixed in 10% formalin, and embedded in paraffin. The DeadEnd colorimetric kit for
TUNEL (Promega Corp., Madison, Wisconsin) was used per the manufacturer’s instructions.
Briefly, paraffin sections were deparaffinized, permeabilized with proteinase K, and incubated
with a mixture of biotinylated nucleotides and rTdT enzyme for 60 min at 37°C. Incorporated nucleotides were detected by incubation with streptavidin-horseradish peroxidase-conjugate followed by diaminobenzidine and counterstained with methyl green (Sigma, St. Louis, Missouri).

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) was performed using double-stranded oligonucleotides (Santa Cruz Biotechnology) for the consensus binding site of the nuclear factor (NF)-κB nucleotide (5'-AGTTGAGGGGACTTTCCCAGGC-3'). Oligonucleotides were labeled in the following reaction: 2 µl oligonucleotide (1.4 pmol/µl), 5 µl 10× kinase buffer, 1 µl T4 polynucleotide kinase (10 units/µl), and 2 µl [r-32P]ATP (3,000 Ci/mmol at 10 mCi/ml) incubated at 37°C for 30 min. To separate the labeled probe from unbound ATP, the reaction mixture was eluted in a Sephadex G-25 column following the manufacturer’s instructions. Two micrograms of crude nuclear protein were incubated for 10 min on ice in binding buffer (10 mmol/l Tris-HCl, pH 7.5, 50 mmol/l NaCl, 1 mmol/l DTT, 1 mmol/l EDTA, 2% glycerol, and 60 µg/ml poly(dI-dC), in a final volume of 15 µl. Labeled probe (50-100 Kcpm) was added, and the reaction was incubated for 30 min at room temperature. NF-κB p65 antibody was added 1 h before incubation with the labeled probe at 4°C. The competitor oligonucleotide was added before the labeled probe and incubated for 10 min at room temperature. DNA-protein complexes were resolved by electrophoresis on 5% polyacrylamide gels in TBE buffer 45 mmol/l Tris borate, 1 mmol/l EDTA). The gels were subsequently dried and autoradiographed with intensifying screens at -80°C.

Measurement of viral titers. The pancreas was removed from each mouse at 1, 3, and 5 days after EMC-D viral infection, and islets were isolated at 3 days after infection. EMC-D viral titer in the pancreas and isolated islets was determined by plaque assay on L929 cells as described previously.


Statistical Analyses. Data are presented as mean ± SD. The statistical significance of differences was analyzed by unpaired Student’s $t$ test. $P < 0.05$ was accepted as significant.
RESULTS

Prevention of EMC-D virus-induced diabetes in iNOS-deficient mice. We infected iNOS-deficient DBA/2 mice with $2 \times 10^2$ PFU/mouse of EMC-D virus and examined blood glucose levels and incidence of diabetes. Mean blood glucose levels of iNOS-deficient DBA/2 mice infected with EMC-D virus were significantly lower (205.5 mg/dl) than infected wild-type mice (466.7 mg/dl) at 6 days after virus infection (Fig. 1A) compared with wild-type DBA/2 mice. The cumulative incidence of diabetes in iNOS-deficient mice infected with EMC-D virus was 42.3 ±19.9%, whereas 100% of wild-type mice developed diabetes at 6 days after infection. When we measured insulin mRNA expression by real-time RT-PCR in islets isolated at 3 days after virus infection, insulin mRNA expression was significantly higher in iNOS-deficient mice than in wild-type mice (Fig. 1B), suggesting that insulin-producing \( \beta \) cells are preserved in iNOS-deficient mice after EMC-D viral infection.

As large numbers of macrophages are known to infiltrate the islets of EMC-D virus-infected mice (4, 13, 16), we examined the presence of macrophages in the islets at 3 days after viral infection by immunohistochemical staining of pancreatic sections. When sections were double-stained with F4/80, a macrophage-specific antibody, and anti-insulin antibody, few macrophages and many insulin-positive cells were found in the islets of iNOS-deficient mice (Fig. 2A), whereas many macrophages and few insulin-positive were found in the islets of wild-type mice (Fig. 2B). Histological analysis of the islets revealed that the majority (61.9%) of the examined islets from iNOS-deficient mice showed mild to moderate insulitis with peri-islet infiltration, 32.1% showed severe insulitis, and 6.0% showed atrophied morphology. In contrast, 41.0% of the examined islets from wild-type mice showed mild to moderate insulitis, 51.2% showed severe insulitis, and 7.8% showed an atrophied morphology (Table 1). These results
indicate that NO may play a role in the destruction of β cells in EMC-D virus-infected mice, and the absence of iNOS prevents the destruction of β cells, resulting in the prevention of diabetes.

Decrease of apoptosis in islets of iNOS-deficient DBA/2 mice infected with EMC-D virus. To investigate whether apoptosis of β cells is decreased in iNOS-deficient mice infected with EMC-D virus, pancreatic sections were prepared at 3 days after viral infection and DNA fragmentation was examined by TUNEL staining. Fewer TUNEL-positive cells were seen in the islets in pancreatic sections from iNOS-deficient mice as compared with wild-type mice (Fig. 3A), and the number of TUNEL-positive cells was significantly decreased in the islets of iNOS-deficient mice (5.95 ± 4.37%) as compared with wild-type mice (14.78 ± 6.05%) (Fig. 3B). We examined the mRNA expression of pro-apoptotic molecules, Bax and Bak, and anti-apoptotic molecules, Bcl-2, Bcl-xL, Bcl-w, Mcl-1, cIAP-1, and cIAP-2, by real-time RT-PCR at 3 days after EMC-D viral infection. We found that EMC-D viral infection induced Bax and Bak mRNA expression, however expression of Bax and Bak mRNA was significantly lower in the islets of iNOS-deficient mice as compared with wild-type DBA/2 mice (Fig. 4A, B). In contrast, the expression of Bcl-2, Bcl-xL, Bcl-w, Mcl-1, cIAP-1, and cIAP-2 mRNA was not significantly different between iNOS-deficient and wild-type DBA/2 mice (Fig. 4C-H). When we double-stained pancreatic sections with anti-insulin and anti-Bax antibody, Bax-positive cells were rare in the islets of iNOS-deficient mice, but present in the islets of wild-type mice (Fig. 5).

Decreased expression of IL-1β and TNF-α mRNA in macrophages and islets in iNOS-deficient DBA/2 mice infected with EMC-D virus. To examine whether the expression of IL-1β and TNF-α is affected by iNOS gene knockout, we examined these mRNAs by real-time RT-PCR in peritoneal macrophages and islets at 3 days after EMC-D viral infection. The
expression of IL-1β and TNF-α mRNA was significantly decreased in peritoneal macrophages (Fig. 6A) and islets (Fig. 6B) of iNOS-deficient mice as compared with wild-type DBA/2 mice infected with EMC-D virus.

**Decreased NF-κB activation in macrophages of iNOS-deficient DBA/2 mice infected with EMC-D virus.** Activation of NF-κB is involved mainly in the expression of inflammatory responsive genes such as IL-1β and TNF-α (6, 20). To test whether the decreased expression of IL-1β and TNF-α mRNA in iNOS-deficient mice infected with EMC-D virus is mediated by reduction of NF-κB activation, we evaluated the activation of NF-κB in macrophages by EMSA at 3 days after virus infection. EMC-D virus infection increased DNA binding activity of NF-κB in nuclear extracts of macrophages in wild-type DBA/2 mice. Addition of excess unlabeled NF-κB oligonucleotide abolished the binding activity and addition of antibody against the p65 subunit of NF-κB resulted in super-shift, indicating the specificity of the binding activity (Fig. 7A). We found that NF-κB binding activity was inhibited in macrophages of iNOS-deficient mice infected with EMC-D virus (Fig. 7B).

**Viral replication in iNOS-deficient DBA/2 mice infected with EMC-D virus.** As it is known that NO is involved in the inhibition of viral replication (24), we determined whether the absence of iNOS affects EMC-D viral replication in the pancreas by measuring infectious viral titers in pancreatic tissue at 1, 3, and 5 days after infection. Viral titers peaked in the pancreas at 3 days after EMC-D viral infection, but there was no significant difference in titers between iNOS-deficient and wild-type mice. (Fig. 8A). In addition, we measured viral titers in isolated islets at 3 days after EMC-D virus infection and found the titer was not significantly different between iNOS-deficient and wild-type mice (p value was 0.084) (Fig. 8B).
A high titer ($5 \times 10^5$ PFU/mouse) of EMC-D viral infection results in the development of diabetes within 3 days in susceptible strains of mice, primarily as a result of rapid destruction of pancreatic $\beta$ cells by viral replication within the cells (36). However, in mice infected with a low titer (50-200 PFU/mouse) of EMC-D virus, macrophages play an important role in the destruction of pancreatic $\beta$ cells (4, 16). Macrophages are the predominant islet-infiltrating cell type in the early stages after EMC-D viral infection, followed by T cells, natural killer cells, and B cells (5). Depletion of macrophages prevented the development of diabetes, whereas the incidence of diabetes increase when macrophages are activated before viral infection in mice infected with 100 PFU/mouse (4). Further studies found that activated macrophages produce soluble mediators such as IL-1$\beta$, TNF-$\alpha$, and iNOS, which play an important role in the destruction of $\beta$ cells in the infected mice (14). In addition, infection of peritoneal macrophages with EMC-D virus in vitro induces the expression of iNOS mRNA and NO production (13), and treatment of EMC-D virus-infected mice with an iNOS inhibitor reduces the incidence of diabetes (14). However, the precise mechanisms by which iNOS and NO destroy pancreatic $\beta$ cells in EMC-D virus-infected mice are not fully understood.

NO produced by cytokine-induced iNOS plays an important role in $\beta$ cell damage and dysfunction (2, 10, 21, 23, 32). Treatment with the NOS inhibitor, N$^G$-nitro-L-arginine methyl ester, significantly reduces the incidence of diabetes in BioBreeding rats, an animal model of type 1 diabetes (22), and islets from iNOS-deficient mice are more resistant to $\beta$ cell destruction in an allogenic transplantation model (8). Transgenic mice in which iNOS is specifically expressed in $\beta$ cells show markedly reduced $\beta$ cell mass and develop diabetes. Treating these mice with an iNOS inhibitor prevents or delays the development of diabetes (33).
In the present study, we found that infection of iNOS-deficient DBA/2 mice with EMC-D virus significantly decreased the incidence of diabetes and reduced immunocyte infiltration into the islets. NO is known to induce apoptosis in isolated islets and in a β cell line (3, 29), and inhibition of NOS also inhibits DNA cleavage (19), an indicator of apoptosis. In addition, NOS inhibitor prevents IL-1β-induced Fas expression, whereas NO donors such as sodium nitroprusside and NO-releasing compound-18 induce Fas expression and pancreatic β cell destruction (31). Therefore, we examined apoptotic cell death in the islets of iNOS-deficient mice infected with EMC-D virus. The significant reduction of TUNEL-positive cells in islets of iNOS-deficient mice indicates that iNOS and NO play an important role in the destruction of β cells by apoptosis.

Cellular apoptosis is regulated by the balance between pro-apoptotic proteins, such as Bax, and anti-apoptotic proteins, such as Bcl-2, which are members of the Bcl-2 protein family (1, 30, 34). We found that EMC-D viral infection significantly induced Bax and Bak mRNA and Bax protein expression in the islets of wild-type mice, and this expression was significantly reduced in the islets of virus-infected iNOS-deficient mice as compared with wild type mice. In contrast, expression of anti-apoptotic molecules including Bcl-2, Bcl-xL, Bcl-w, Mcl-1, cIAP-1, cIAP-2 in the islets was not different between virus-infected wild-type and iNOS-deficient mice. There are two major apoptotic pathways: the death receptor-mediated pathway and the mitochondria-mediated pathway (26). Bax and Bak are known to be required for mitochondria-dependent apoptosis (11), and the expression of Bax increases in NO-induced apoptosis (28). In addition, loss of Bax or Bak partially inhibits death receptor-mediated islet cell death (25), suggesting that Bax and Bak are also involved in death receptor-mediated apoptosis. Thus, the increase Bax and Bak expression in islets of EMC-D virus-infected mice may contribute to β cell apoptosis, whereas reduced Bax and Bak expression in iNOS-deficient mice may
It was previously found that EMC-D viral infection activates macrophages in vitro and induces the expression of proinflammatory cytokines (13). NO is known to regulate immune responses (7), and NO plays an immunoregulatory role in Kilham rat virus-induced diabetes by upregulating macrophage-derived proinflammatory cytokines and Th1 immune responses (27). We found that mRNA expression of the inflammatory cytokines IL-1β and TNF-α was significantly downregulated in peritoneal macrophages of iNOS-deficient mice infected with EMC-D virus, as compared with virus-infected wild-type mice. In addition, activation of NF-κB, a transcription factor that regulates a number of genes involved in inflammation (6), was also significantly downregulated in iNOS-deficient mice infected with EMC-D virus, as compared with virus-infected wild-type mice. Thus, it is possible that iNOS deficiency inhibits the activation of macrophages by EMC-D virus, resulting in the decreased expression of IL-1β and TNF-α.

However, when macrophages were infected with EMC-D virus in vitro, there were no differences in expression of IL-1β and TNF-α between iNOS KO and wild-type mice (data not shown). Therefore, iNOS-dependent NO arising from other cells or immunocytes may contribute to the production of inflammatory cytokines in macrophages by amplifying cytokine cascade in vivo. IL-1β and TNF-α mRNA expression after EMC-D viral infection was also decreased in the islets of iNOS-deficient mice as compared with wild-type mice. Thus, it is possible that the suppression of NO production may result in reduced destruction of β cells and subsequently reduced recruitment of macrophages. In accord with this, it was found that iNOS KO mice produce less TNF-α, IL-6, and Th1 cytokines after mycobacterial infection and endotoxin challenge, which protects against liver injury (12).

It is known that iNOS and NO inhibit viral replication and play a role in host defense against viruses (9, 24, 39). However, we found no significant difference in viral titers of both
pancreas and islets between infected wild-type and iNOS-deficient mice, indicating that NO does not affect EMC-D viral replication in our animal model.

In conclusion, our studies show that the absence of iNOS inhibits the activation of macrophages by EMC-D virus, evidenced by decreased IL-1β and TNF-α expression and reduced activation of NF-κB, and inhibits recruitment of macrophages to the islets. In addition, iNOS deficiency results in decreased apoptotic death of β cells, thus preventing the development of diabetes in EMC-D virus-infected DBA/2 mice.
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FIGURE LEGENDS

FIG. 1. Prevention of EMC-D virus-induced diabetes in iNOS-deficient mice. iNOS KO (-/-) and wild-type (+/+ ) mice were infected with EMC-D virus (200 PFU/mouse). (A) Blood glucose levels were measured at 6 days after EMC-D viral infection. Each point represents an individual animal; dotted line indicates the mean. (B) At 3 days after EMC-D viral infection, islets were isolated and insulin mRNA was analyzed by real-time quantitative PCR and normalized by GAPDH expression. The fold change was calculated as the ratio of the mean expression level in iNOS KO mice. *P < 0.05.

FIG. 2. Reduction of macrophage infiltration in iNOS-deficient mice infected with EMC-D virus. Serial sections of pancreas from iNOS KO (-/-) and wild-type mice (+/+ ) were prepared at 3 days after EMC-D viral infection (200 PFU/mouse) and stained with hematoxylin and eosin (H&E) or anti-F4/80 and anti-insulin antibodies.

FIG. 3. Decrease of apoptotic cells in islets of iNOS-deficient mice infected with EMC-D virus. Pancreatic sections were prepared from iNOS KO (-/-) and wild-type (+/+ ) mice at 3 days after EMC-D viral infection and stained with TUNEL. (A) Representative islets are shown (× 200). (B) The number of TUNEL-positive cells were calculated as percentage of the total number of cells (38-40 islets/group). *P < 0.0001.

FIG. 4. Decreased expression of Bax and Bak in islets of iNOS-deficient mice infected with EMC-D virus. iNOS KO (-/-) and wild-type mice (+/+ ) were infected with EMC-D virus (200 PFU/mouse). At 3 days after EMC-D viral infection, islets were isolated and the expression of (A)
Bax, (B) Bak, (C) Bcl-2, (D) Bcl-xL, (E) Bcl-w, (F) Mcl-1, (G) cIAP-1, and (H) cIAP-2 mRNA were analyzed by real-time quantitative PCR and normalized by GAPDH or cyclophilin expression. The mRNA expression level was calculated as the ratio of the mean expression in islets of iNOS KO mice (n=3-7 per group). *P < 0.01.

FIG. 5. Decreased expression of Bax in islets of iNOS-deficient mice infected with EMC-D virus. Pancreatic sections were prepared from iNOS KO (-/-) and wild-type (+/+) mice at 3 days after EMC-D viral infection and stained with hematoxylin and eosin or anti-Bax and anti-insulin antibodies.

FIG. 6. Decreased expression of IL-1β and TNF-α mRNA in both macrophages and islets in iNOS-deficient mice infected with EMC-D virus. iNOS KO (-/-) and wild-type (+/+) mice were infected with EMC-D virus (200 PFU/mouse). At 3 days after infection, (A) macrophages and (B) islets were isolated and the expression of IL-1β and TNF-α mRNA was analyzed by real-time quantitative PCR and normalized by GAPDH expression. The mRNA expression level was calculated as a ratio of the mean expression level in iNOS KO mice. *P < 0.05.

FIG. 7. Reduced NF-κB activation in macrophages of iNOS-deficient mice infected with EMC-D virus. Peritoneal macrophages were isolated from iNOS KO (-/-) and wild-type (+/+) mice at 3 days after EMC-D virus infection, nuclear protein extracts (NE) were prepared, and EMSA was performed with 32P-labeled NF-κB consensus sequence. (A) Autoradiogram of EMSA after competition with a molar excess of unlabeled probe (Competitor) and supershift analysis performed by incubating nuclear extracts of wild-type mice with an antibody against p65 NF-κB. Arrows indicate the p65 NF-κB band (left) and the supershifted band by anti-p65 antibody (right).
FIG. 8. Viral replication in the pancreas and islets of iNOS-deficient mice infected with EMC-D virus. iNOS KO (-/-) and wild-type (+/+) mice were infected with EMC-D virus. (A) Pancreata were removed at 1, 3 and 5 days and (B) isolated islets at 3 days after EMC-D virus infection, and the viral titer was determined by plaque assay on L929 cells.
TABLE 1. Histological analysis of pancreatic islets in iNOS-deficient mice infected with EMC-D virus.

<table>
<thead>
<tr>
<th>Mice</th>
<th>No. of islets examined</th>
<th>Peri-islet infiltration</th>
<th>Mild to moderate insulitis</th>
<th>Severe insulitis</th>
<th>Atrophied morphology</th>
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<td>iNOS (-/-) (n = 9)</td>
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1 Mice were sacrificed at 3 days after EMC-D viral infection. Pancreata were sectioned and stained with hematoxylin and eosin, and the degree of insulitis was scored.
Fig. 1

A

Blood glucose (mg/dl)

iNOS (-/-) (n=12)
iNOS (+/+) (n=11)

B

Insulin mRNA

iNOS (-/-) (n=4)
iNOS (+/+) (n=3)

*
Fig. 2

H&E
F4/80/Insulin
iNOS (+/+)  iNOS (-/-)

40 μm
Fig. 3

A

B

<table>
<thead>
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<th>TUNEL-positive cells (%)</th>
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- iNOS (+/+)
- iNOS (-/-)

(n=4)

iNOS (-/-)

iNOS (+/+)

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Fig. 5

H&E  Bax  Insulin  Bax/Insulin

INOS (-/-)  INOS (+/+)
**Fig. 6**

**A**
- Macrophages: IL-1β mRNA
  - iNOS (-/-) (n=3)
  - iNOS (+/+) (n=3)
- Macrophages: TNF-α mRNA
  - iNOS (-/-) (n=6)
  - iNOS (+/+) (n=3)

**B**
- Islets: IL-1β mRNA
  - iNOS (-/-) (n=4)
  - iNOS (+/+) (n=3)
- Islets: TNF-α mRNA
  - iNOS (-/-) (n=3)
  - iNOS (+/+) (n=4)
Fig. 4

A. Bax mRNA
B. Bak mRNA
C. Bcl-2 mRNA
D. Bcl-xL mRNA
E. Bcl-w mRNA
F. Mcl-1 mRNA
G. cIAP-1 mRNA
H. cIAP-2 mRNA