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Characterization of NO and Cytokine Production in Immune-Activated Microglia and Peritoneal Macrophages Derived from a Mouse Model Expressing the Human NOS2 Gene on a Mouse NOS2 Knockout Background

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ABSTRACT

Significant differences exist in the production and release of nitric oxide (NO) from human macrophages versus macrophages of mouse origin. Human macrophages have been shown to respond poorly to stimuli that provoke strong inflammatory reactions from mouse macrophages. To address the differences in macrophage function in an animal model, a transgenic mouse was created that contained the entire human NOS2 gene, including the human promoter and all of its exons and introns. The huNOS2 transgenic mouse was then mated to mice lacking a functional NOS2 gene (muNOS2$^{-/-}$ or NOS2 knockout mice) to generate a double transgenic mouse (huNOS2$^{+/0}$/muNOS2$^{-/-}$) that expresses a functional human NOS2 gene in place of the mouse NOS2 gene. These double transgenic mice were found to express only human NOS2 mRNA and human iNOS proteins in response to immune stimulation. The production and release of nitric oxide from isolated macrophages from the doubly transgenic mouse also more closely paralleled human responses rather than mouse. Peritoneal macrophages from double transgenic mice generated nanomolar levels of nitrite in response to inflammatory stimuli, while peritoneal macrophages from wild-type mice generated micromolar levels of nitrite in response to the same inflammatory stimuli. Similarly, microglia from the huNOS2$^{+/0}$/muNOS2$^{-/-}$ mice accumulated nanomolar levels of nitrite following inflammatory stimulation. Reduced nitrite release persisted in spite of normal responsiveness to inflammatory stimulation as measured by tumor necrosis factor alpha and interleukin-6 production and release. These data suggest that the human-specific release of nanomolar levels of nitrite may largely result from differences between the human and mouse NOS2 genes, which may program different degrees of nitric oxide responses to inflammatory signals in humans than in mice. *Antioxid. Redox Signal.* 8, 893–901.

INTRODUCTION

THE ACTION OF NITRIC OXIDE in biological tissues depends on multiple factors starting with the temporally and spatially integrated level of NO in that tissue. The levels of NO depend, in turn, on the specific isoforms of NOS that are activated to produce NO, the target molecules whose biochemical integrity and/or function is altered by NO, such as redox reactants, structural proteins, enzymes, lipids, and/or DNA and the proximity of the reactants to the source of NO production (11, 41, 49, 55). Specific isoforms of nitric oxide synthase (NOS) generate distinct levels of NO and these levels have been associated with defined actions of NO. For example, the transient production of nanomolar levels of NO by constitu-

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tive calcium-dependent forms of NOS (NOS1; NOS3) is involved in physiological regulation of synaptic transmission, in regulation of blood vessel tone, in platelet adherence, and in intracellular signaling (12, 24, 35, 38, 39, 49). Induction of NOS2 expression typically lead to high levels of NO production that have been associated with deleterious actions of NO, including induction of apoptosis, killing of bacteria and viruses, and inactivation of critical proteins such as ion channels and mitochondrial proteins (8, 14, 23, 26). The latter actions of NO and its secondary oxidant metabolites, known collectively as reactive oxygen–nitrogen species (RNOS) (55), have been adapted by organisms to protect the cell from microbial invaders (3, 25). Thus, a primary component of the innate immune response, the body's first line of defense, is activation of a calcium-independent isoform of NOS and the sustained release of high levels of NO by this enzyme (23, 38).

The role of NO and RNOS in innate immunity has been well characterized and is the subject of multiple reviews (6, 23, 44, 53). The NOS2 gene and its protein product, inducible NOS (iNOS), have been localized to polymorphonuclear and mononuclear blood phagocytes and their tissue counterparts, tissue macrophages that include microglia (brain macrophages) and Kupffer cells (liver macrophages). It is important to realize, however, that other cell types, including CNS astrocytes and neurons, also express iNOS under specific conditions (42, 45, 51).

Production of NO by iNOS is primarily regulated by transcriptional processes (29). The binding of pattern-associated molecular pathogens (PAMPs) to membrane receptors results in signal cascades that lead to iNOS transcription (5, 9). The series of events leading to induction of the NOS2 gene, translation of iNOS, and the subsequent production of NO has been established using primary macrophages or neoplastic cell lines derived from mice or rats. For rodent cells, immune induction of macrophage NOS2 and iNOS protein *in vitro* by factors such as lipopolysaccharide (LPS) and interferon-γ (IFNγ) typically leads to high μ*M* amounts of NO (>1 μ*M*/μg protein/24 h). However, the induction of NOS2 and translation of iNOS in immune activated human macrophages *in vitro* demonstrate a different time course and result in significantly lower overall levels of NO production and release (7, 18, 21, 46, 53, 54). Nevertheless, the increased expression of iNOS protein within specific disease settings such as HIV encephalopathy or malaria, clearly indicates a role for NO in the innate immune response in humans (2, 13, 31, 53). Because differences in expression of NOS2 and in the overall levels of NO generated by mouse and human macrophages may be critical to understanding the tissue changes in disease states that involve innate immune responses, we have generated a mouse model that expresses the entire human NOS2 gene on a mouse knockout background. Using *in vitro* assays, we have characterized the response of macrophages from this "humanized iNOS" mouse to immune stimulators.

METHODS

Generation of transgenic mice

Transgenic mice expressing the human NOS2 gene and iNOS protein were generated by microinjection of a fragment of PAC 24ixx (Genome Systems Inc., St Louis, MO) containing the entire human NOS2 gene. The PAC DNA containing the human NOS2 gene was digested to completion with NotI, and a 45 Kbp fragment containing the NOS2 gene's promoter and all of its exons and introns (as defined by hybridization to oligonucleotide probes and PCR amplification) was purified from low melting agarose gels. Purified DNA was injected into blastocyst stage embryos, which were then implanted into pseudopregnant mice. Of the resulting litters, three mice were found to contain the human NOS2 gene by PCR analysis of genomic DNA isolated from tail snips. Of these three founders, one passed the human NOS2 gene through the germline to its progeny. The human NOS2-gene transgenic mouse was then crossbred to NOS2$^{-/-}$ mice (B6.129P2-*Nos2*^{tm1Lau}/J; Jackson Labs, Bar Harbor, ME) (33) to create doubly transgenic mice that express only human iNOS proteins (human NOS2$^{+/0}$/mouse NOS2$^{-/-}$). These animals were bred under standard conditions and each offspring was genotyped to confirm the presence of the human NOS2 gene and the absence of an intact mouse NOS2 gene. Animals were kept under 12 h light/12 h dark cycle and fed normal chow and water *ad libitum*.

Cell cultures

Microglia. Microglia were isolated from postnatal day 1 mouse pups using standard techniques (17). Briefly, pups were euthanized, and cortices removed and placed into sterile PBS. Under a dissecting scope, meninges were removed and the cortical tissue was minced into tissue digestion medium (Papain Dissociation System, Worthington Biochemicals, Lakewood, NJ). Tissue was dissociated according to the manufacturer's instructions and a mixed glial culture was prepared from each mouse brain by plating the dissociated cells into 75 cm^2 flasks in growth media (high glucose DMEM containing 10% fetal bovine serum, 2 m*M* glutamine, and 100 U/ml Penstrep). After incubation in a humidified 5% CO_2, 95% air atmosphere for 4–5 days, media containing FBS was replaced by media containing 10% horse serum to promote the proliferation of microglia. Microglia were then detached from the surface of the mixed culture by gentle shaking for 2 h. Microglia in the cell supernatants were pelleted, resuspended in serum free DMEM, and replated into tissue culture plates for the assay procedures.

Peritoneal macrophages. Peritoneal macrophages from 20–25-week-old adult human NOS2$^{+/0}$/mNOS2$^{-/-}$mice and NOS+/+ mice were elicited by i.p. injection of 10% thioglycollate diluted into PBS. After 72 h, the macrophages were harvested using peritoneal lavage (PBS, 37°C). Cells in the lavage fluid were extracted from the peritoneum and the fluid containing cells from 2 to 3 mice of the same genotype were pooled. In this manner, a sufficient number of macrophages were obtained for the experimental assays. The cells were pelleted by centrifugation and then resuspended into media (high glucose DMEM containing 5% fetal calf serum and 5% horse serum, 2 m*M* glutamine, and 50 μg/ml gentamycin). Cells were counted, plated directly into 96-well plates, and cultured for 2–3 days in a humidified 5% CO_2, 95% air atmosphere. During this time, the macrophages attached to the plastic of the culture plate and spread, resembling typical tissue macrophages.

Nitric oxide assay

Nitric oxide production by resting and immune-stimulated macrophages was determined by measuring the supernatant levels of nitrite, the stable aqueous oxidation product of NO. Cells were plated into 96-well dishes in serum-free high glucose DMEM and allowed to equilibrate overnight. Cells were then immune activated for 24 or 72 h using polyinosinic-polycytidylic acid (PIC), a double stranded RNA, lipopolysaccharide (LPS), a bacterial coat component, and/or recombinant murine interferon-γ (rmIFNγ). Nitrite values for each well were determined using a Sievers 280 Nitric Oxide Analyzer (Boulder, CO) with sodium nitrite standards. Nitrite values were normalized to μg protein using the Pierce BCA assay with bovine serum albumin standards. Data are presented as the average nitrite (nmoles/μg protein) ± SEM. A minimum of four wells were analyzed per experimental condition for a minimum of four different culture groups. Significance was determined using an unpaired Student's *t* test.

NOS activity

NOS activity was determined from the conversion of [H^3] L-arginine to [H^3] L-citrulline, essentially as described by Weinberg (54). Microglia or peritoneal macrophages were plated into six-well dishes and cultured in serum-free media for 24 or 72 h. The media was then removed and the cells were washed two times with ice-cold Ca^{2+}, Mg^{2+}-free phosphate buffered saline containing 1 X protease inhibitor cocktail (Sigma Chemical Co, St. Louis, MO). Cells were suspended using trypsin-versene, transferred to a prechilled conical tube, pelleted by centrifugation at 1,200 RPM for 2 min, and the supernatant discarded. One ml of lysis buffer containing 1 x protease inhibitor cocktail was added to each tube and the resulting cell lysate was sonicated. Protein levels were determined using the BioRad protein assay (BioRad Life Sciences, Hercules, CA). Solubilized protein (10–50 μg) was added to the NOS activity reaction mixture (50 m*M* Tris-HCl, pH 7.5, 1.0 m*M* NADPH, 10 μ*M* FAD, 10 μ*M* tetrahydrobiopterin (BH4), 0.75 m*M* $CaCl_2$, 100 μ*M* arginine, 10 μ*M* FMN, and 25 μl ^{3}H-L-arginine (~1 Ci/ml; New England Nuclear, Boston, MA). The reaction mixture was incubated at 37ºC for 60 min and the reaction terminated by addition of three volumes of ice-cold stop buffer (0.1 *M* HEPES, 1 m*M* L-citrulline, 5 m*M* EDTA; pH 5.5). The eluate was loaded onto a prewashed Dowex 50 × 8–200 H+ resin converted to the Na+ form to separate L-[^{3}H]-arginine from L-[^{3}H]-citrulline. The eluate containing citrulline was then collected in scintillation vials containing 5 ml scintillation fluid and was counted using a Packard Tri-Carb 2300TR Liquid Scintillation Analyzer (Packard, Downer's Grove, IL). To determine specificity for iNOS, the assay was also performed in the presence of a specific iNOS inhibitor, 1400W. Specific iNOS activity is determined by subtracting the background level determined in the presence of 1400W from the total activity and data were normalized to mg protein/min.

Reverse transcriptase–polymerase chain reaction (RT–PCR)

Total RNA from treated and untreated microglia or peritoneal macrophages was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA). For this process, 1 μg of total RNA was treated with RQ1 DNase (Promega Biosciences Inc., San Luis Obispo, CA) to remove DNA contamination and then reverse-transcribed using AMV transcriptase (Promega) and random primers. The reverse transcription reactions were incubated at room temperature for 10 min, and then at 42°C for 40 min. The transcriptase was subsequently inactivated by heating at 99°C for 5 min and cooling to 4°C for 5 min. Samples lacking reverse transcriptase were also used to ensure that genomic DNA was not amplified in the PCR reaction. The resulting cDNA templates were then mixed with Taqman Universal PCR Master Mix and primer/probe sets from Assay-on-Demand Gene Expression Assays from Applied Biosystems Incorporated (Foster City, CA) for murine arginase I (AG1), for a common sequence of both isoforms of the cationic amino acid transporter 2 gene (murine *CAT2A* and *B;* also known as *SLC7A2*), for human NOS2, and for murine TNFα. The PCR amplification were performed on the ABI 7000HT Sequence Detection System and the conditions were programmed as heating at 95°C for 10 min, followed by 40 cycles of denaturing at 95°C for 15 s, and annealing/extending at 60°C for 1 min. The threshold cycle (C_T, the cycle number at which the amount of amplified target gene reaches a fixed threshold) was subsequently determined. Relative quantitation of mRNA expression is calculated by the comparative C_T method with the amount of target = $2^{-\Delta\Delta CT}$ (36). Data are presented as the fold change where the value of target is normalized to that of the endogenous control (18S) and relative to a calibrator. The calibrator in this case is untreated cells. Triplicate samples of lysates were run for each gene examined for at least three different culture groups and an average fold change determined. In some cases, the PCR products were analyzed by electrophoresis, stained with ethidium bromide and imaged with a Kodak imager (Eastman Kodak Co., Rochester, NY).

Cytokine production

Cytokine levels in culture supernatants were determined using the Mouse Cytokine Ten-Plex antibody bead kit (Biosource International, Inc., Camarillo, CA). Cytokines measured included IL-2, IL-4, IL-5, IL-10, IL-1β, GM–CSF, rmIFNγ, IL-12, IL-6, and TNFα. Assays were performed on the same supernatants taken from immune activated or untreated microglia as described above for NO production. After mixing with pre-prepared antibody-coated beads as described by the manufacturer, supernatant cytokine levels were determined using a BioPlex Array Reader (BioRad). The absolute values of cytokine levels (pg/ml) in the experimental and control samples are determined by extrapolation to a standard curve generated using a series of standards containing known concentrations of each cytokine in the kit. A minimum of two wells was analyzed per experimental condition for a minimum of four different culture groups. Significance was determined using an unpaired Student's *t* test.

RESULTS

Double transgenic HuNOS2$^{+/0}$/mouse NOS2$^{-/-}$mice were viable and fertile with no observable abnormal gross pheno-

type. To determine the presence of the human NOS2 transgene, peritoneal macrophages and microglia were assayed for the presence of human NOS2 mRNA. Untreated values for NOS2 mRNA in both cell types were at the minimal level of detection as predicted for an inducible gene (Figs. 1A and B). Stimulation with either LPS (100 ng/ml) + rmIFNγ (10 U/ml) or PIC (50 μg/ml) + rmIFNγ (10 U/ml) resulted in a significant increase in NOS2 mRNA expression in both macrophage types (Figs. 1A and B). Human NOS2 mRNA increased 228-fold for LPS + rmIFNγ treatment and 44-fold for PIC + rmIFNγ treatment. Similarly treated microglia derived from mice expressing wild-type murine NOS2 demonstrated a much larger increase (11,400-fold) when stimulated with LPS + rmIFNγ (Fig. 1B). Protein expression was also detected using Western blot and an antibody specific for human iNOS in immune activated peritoneal macrophages (Fig. 1C).

NO production was examined in peritoneal macrophages and/or microglia in response to stimulation with IFNγ alone, PIC alone, PIC + rmIFNγ, LPS alone, LPS + rmIFNγ and IL-1b + rmIFNγ. Peritoneal macrophages were treated for 72 h and microglia were treated for 24 h in serum-free media. In both macrophage types, supernatant nitrite levels were increased in immune stimulated compared to untreated cells (Fig. 2). The combination of rmIFNγ with either PIC or LPS produced higher levels of NO compared to either PIC or LPS alone. This synergistic effect of IFNγ has been well described for both human and murine NOS2 induction (1, 9, 19, 53). As also shown in Fig. 2, LPS + rmIFNγ was the most effective immune activator in peritoneal macrophages from HuNOS2$^{+/0}$/mNOS2$^{-/-}$mice. For HuNOS2$^{+/0}$/mNOS2$^{-/-}$ microglia, however, LPS alone or in combination with rmIFNγ failed to induce NO production after 24 h of exposure (Fig. 2B). In contrast, a significant increase in NO production was observed in microglia stimulated with PIC + rmIFNγ. Independent of macrophagic cells isolated from the periphery (peritoneal macrophages) or from the brain (microglia), mice expressing the wild-type *NOS2* gene (*NOS2*$^{+/+}$) responded to both PIC + rmIFNγ and LPS + rmIFNγ. Compared to similarly treated peritoneal macrophages from HuNOS2$^{+/0}$/mouse NOS2$^{-/-}$mice, the overall level of NO production in peritoneal macrophages from wild-type NOS2 mice was approximately 10x greater (Fig. 2C).

NOS-mediated NO production by peritoneal macrophages from the HuNOS2$^{+/0}$/mNOS2$^{-/-}$mice was confirmed by examining the activity of iNOS in cell lysates using the L-[H^3] arginine to L-[H^3] citrulline conversion assay. Peritoneal macrophages were stimulated for 24 or 72 h with 10 U/ml rmIFNγ and iNOS activity determined at each time point. NOS activity values significantly increased from 46 ± 0.4 pmoles/mg protein/min at 24 h poststimulation to 68 ± 0.8 pmoles/mg protein/min at 72 h poststimulation ($p < 0.001$). These values are within previously published value ranges for immune activated human macrophages or human mixed glia cultures (7–70 pmoles/mg protein/min) (22, 37) and are notably less than previously published values for wild-type NOS2 peritoneal macrophages (950 pmole/mg protein/min) (33).

The integrity of the innate immune response in the NOS2$^{+/0}$/mNOS2$^{-/-}$ mouse was examined by determining the expression of other known genes involved in immune activation. Both CAT2 and TNFα mRNA are expressed in immune

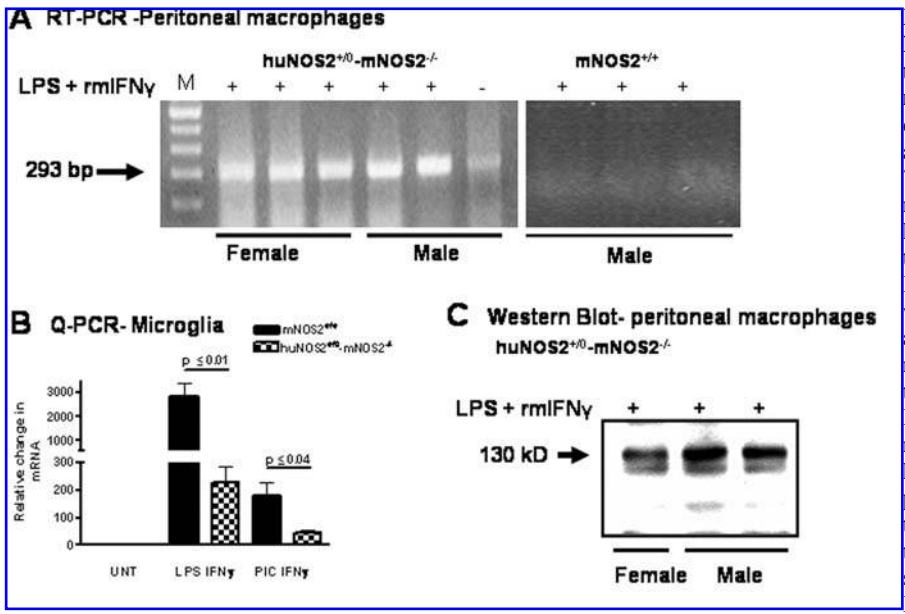

FIG. 1. Expression of human NOS2 gene products in double transgenic huNOS2$^{+/0}$/mNOS2$^{-/-}$ mice. (**A**) Peritoneal macrophages derived from individual adult male and female mice were activated for 72 h with 100 ng/ml LPS + 10 U/ml rmIFNγ, and the expression of human NOS2 mRNA from each culture lysate determined using RT–PCR. Human NOS2 expression was observed in individual female and male huNOS2$^{+/0}$/mNOS2$^{-/-}$ mice, but was not observed in wild-type (murine NOS2$^{+/+}$) mice. (**B**) Relative change in NOS2 mRNA in neonatal microglia derived from huNOS2$^{+/0}$/mNOS2$^{-/-}$ mice compared to microglia expressing wild-type NOS2 (mNOS2$^{+/+}$) under untreated conditions and after immune stimulation for 5 h with 100 ng/ml LPS + 10 U/ml rmIFNγ or 50 μg/ml PIC + 10 U/ml rmIFNγ. Using quantitative RT–PCR, relative mRNA expression is calculated by the comparative C_T method with the amount of target = $2^{-\Delta\Delta CT}$. Data are presented as the average fold change where the value of target is normalized to that of the endogenous control RNA (beta-actin or 18S) and relative to a calibrator RNA. The calibrator in this case is untreated wild-type cells. Significance was determined using an unpaired Student's *t* test; $n = 2$ wells analyzed per experimental condition for six different mouse pups of each genotype. (**C**) Western blot for iNOS protein in cell lysates of LPS- rmIFNγ stimulated peritoneal macrophages from NOS2$^{+/0}$/mNOS2$^{-/-}$ mice.

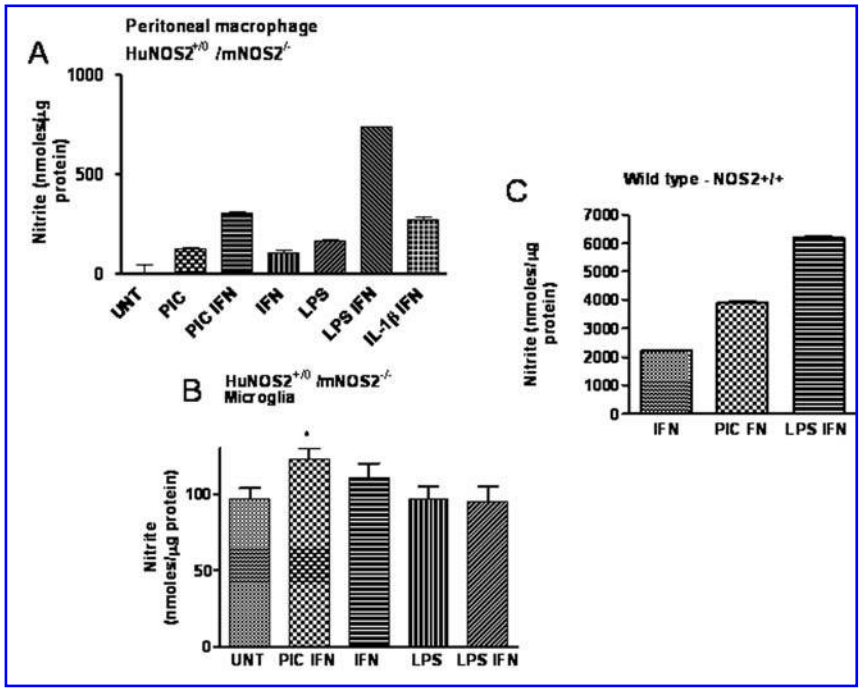

FIG. 2. Differential release of nitric oxide from microglia and macrophages of double transgenic huNOS2$^{+/0}$/ mNOS2$^{-/-}$ mice versus wild-type mNOS2$^{+/+}$ mice. Supernatant nitrite levels from (**A**) peritoneal macrophage and (**B**) neonatal microglial cultures derived from huNOS2$^{+/0}$/mNOS2$^{-/-}$ mice, and from (**C**) wild-type (murine NOS2$^{+/+}$) peritoneal macrophage cultures. Cells either remained untreated or were immune activated with PIC (50 µg/ml), PIC (50 µg/ml) + rmIFNγ (10 U/ml), rmIFNγ (10 U/ml), LPS (100 ng/ml), LPS (100 ng/ml) + rmIFNγ (10 U/ml), or recombinant murine IL-1b (0.2 ng/ml) + rmIFNγ (10 U/ml). Please note the difference in Y-axis scales. Values represent the average nitrite (nmoles/µg protein) ± SEM. *$p < 0.01$ compared to untreated; n = 6–19 wells analyzed for each experimental condition.

activated macrophages during the innate immune response (5, 16, 28, 44). As shown in Fig. 3, mRNA expression levels for the cationic amino acid transporter 2 (CAT2) and TNFα were increased in microglia stimulated for 24 h with either LPS + rmIFNγ or PIC + rmIFNγ. Arginase I mRNA levels were not observed to significantly change with stimulation at this time point.

We also examined cytokine production profiles for microglia derived from the HuNOS2$^{+/0}$/mNOS2$^{-/-}$mice using a multiplex cytokine bead assay to determine cytokine levels in the same experimental samples as assayed for nitrite. Of the 10 cytokines assayed, significant changes were observed for TNFα, IL-6, and IL-12 (Fig. 4). The remaining cytokines (IL-2, IL-4, IL-5, IL-10, IL-1β, GM-CSF, and rmIFNγ) were below detection levels of the assay in both the untreated and treated conditions at the time point studied. A significant increase in TNFα, IL-6, and IL-12 levels was observed in HuNOS2$^{+/0}$/mouse NOS2$^{-/-}$ microglia treated for 24 h with

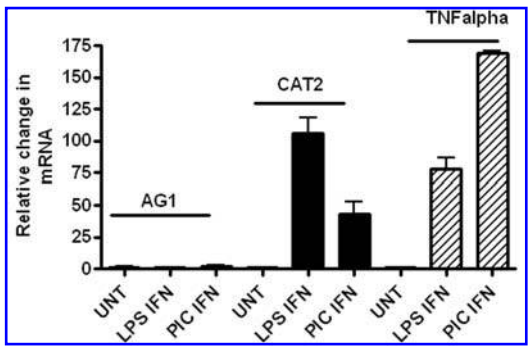

FIG. 3. Relative changes in mRNA expression of arginase I, cationic amino acid transporter 2, and TNFα in treated and untreated huNOS2$^{+/0}$/mNOS2$^{-/-}$ neonatal microglia. mRNA levels were determined using quantitative RT–PCR. Microglia were treated with PIC (50 µg/ml) + rmIFNγ (10 U/ml), or LPS (100 ng/ml) + rmIFNγ (10 U/ml). Data are presented as the average fold change where the value of target is normalized to that of the endogenous control RNA (beta-actin or 18S) and relative to a calibrator RNA. The calibrator in this case is untreated cells. n = 2 wells analyzed per experimental condition for 6 different huNOS2$^{+/0}$/mNOS2$^{-/-}$ mice pups.

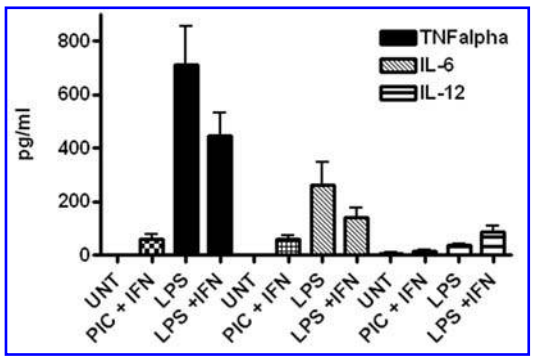

FIG. 4. Production of proinflammatory cytokines in PIC + rmIFNγ or LPS + rmIFNg treated and untreated huNOS2$^{+/0}$/mNOS2$^{-/-}$ neonatal microglia. Data values represent the average supernatant level of cytokines (pg/ml) ± SEM determined simultaneously in the same sample using a cytokine multiplex bead–based analysis system. n = at least duplicate wells analyzed from individual cultures derived from 6 huNOS2$^{+/0}$/mNOS2$^{-/-}$ mice. All data points for immune stimulated conditions were significant at the $p < 0.01$ level compared to untreated cells.

LPS, rmIFNγ alone, or in combination with PIC compared to untreated values. These values were compared with LPS-rmIFNγ –stimulated microglia derived from mice expressing wild-type NOS2 (mNOS2$^{+/+}$). No significant difference was observed in the levels of TNFα (25.5 ± 8.3 pg/ml/μg protein, nine wells assayed) or IL-6 (33 ± 18 pg/ml/μg protein, six wells assayed) in mNOS2$^{+/+}$ microglia compared to similarly activated microglia derived from mice expressing the huNOS2 gene (TNFα = 50 ± 10 pg/ml/μg protein, 10 wells assayed and IL-6 = 16 ± 4.2 pg/ml/μg protein, 10 wells assayed).

DISCUSSION

Macrophages derived from the NOS2$^{+/0}$/mNOS2$^{-/-}$ mouse demonstrate a characteristic *in vitro* response to well-known immune activators such as interferon-γ, a proinflammatory cytokine; PIC, a double stranded RNA that serves as a viral mimetic; and LPS, a bacterial coat component. Human NOS2 mRNA, iNOS protein, iNOS activity, and NO production were significantly increased in neonatal microglia and/or in adult peritoneal macrophages over background levels in response to these immune activators. In addition, the combination of rmIFNγ with either PIC or LPS produced a significantly larger response than either agent alone. Synergism of immune activators is characteristic of both immune activated murine and human macrophages (1, 9, 19, 29). Overall, these data strongly suggest that the human NOS2 transgene is functional in the NOS2$^{+/0}$/mNOS2$^{-/-}$ mouse.

NOS2 induction and NO production are only single elements of the multigene innate immune response. Coordinate gene activity is initiated by PAMP signaling and regulates a large range of macrophage functions. For example, intracellular arginine levels are maintained during the immune response despite the increased demand for arginine from increased iNOS and arginase enzymatic activity. Recent studies by Kakuda *et al*. (28) demonstrate that increased expression of arginine-specific transporters termed cationic amino acid transporters (CATs) are critical for intracellular arginine supplies (10, 16, 28). Increased expression of CAT2 transporter mRNA is observed in immune activated microglia from NOS2$^{+/0}$/mNOS2$^{-/-}$ mice and demonstrates the integrity of the innate immune response in this mouse.

In addition, genes encoding proinflammatory cytokines like TNFα, IL-6, and IL-12 are induced by the same immune signals that induce NOS2 (5). Once produced and released, these cytokines can act as paracrine regulators of NOS2 via activation of NFkB or other transcription factors (4, 15, 27, 29, 34). Our data from HuNOS2 macrophages demonstrate a typical increased expression of TNFα mRNA and increased TNFα secretion in immune activated cells. In addition to TNFα, IL-6 and IL-12 are also produced and at comparable levels to cells from mice expressing wild-type mNOS2. These data suggest that coordinate activity is retained and is unaffected by the exchange of the mouse NOS2 gene for the human NOS2 gene. Thus, the reduced level of NOS2 mRNA expression and reduced NO production in the huNOS2 mouse cannot be explained on this basis.

The presence of the human NOS2 gene in place of the mouse NOS2 gene dictates a human-specific pattern for NO production in our mouse model. This is best exemplified by the difference in the level of NOS2 mRNA expression, iNOS activity and NO production. Our *in vitro* data demonstrate a significantly lower level of NOS2 mRNA expression, iNOS activity, and NO production in immune activated microglia and/or peritoneal macrophages compared to similarly activated macrophages expressing the normal (wild-type) murine NOS2 gene. For example, the level of NO produced in the NOS2$^{+/0}$/mNOS2$^{-/-}$ macrophages is approximately 1/10 the value observed in NOS2$^{+/+}$ macrophages under comparable stimulation conditions. The level of induction of NOS2 mRNA and the activity of iNOS are also significantly lower than mice expressing the normal murine NOS2 gene and are comparable to those values observed in human macrophages under similar conditions. Low levels of NOS2 expression, iNOS activity, and NO levels have been well documented for human cells that express NOS2 and iNOS (7, 18, 21, 22, 46, 53, 54). In addition to reduced levels of NO, the time course of iNOS induction/activation and the pattern of PAMP stimulation required for activation are different in human versus rodent cells. An extensive literature review by Weinberg *et al*. (53) provides a comprehensive view of the conditions under which human macrophages can be induced to produce NO.

Interestingly, PIC is a more reliable and effective induction agent for NO production than LPS in NOS2$^{+/0}$/mNOS2$^{-/-}$ brain macrophages (microglia). In rodent, LPS is a predominant and commonly used activator for macrophages, including microglia (17, 20). Lack of response to LPS is also observed in human microglia (18), whereas double stranded RNA and its signaling pathways play a critical role in human macrophage activation (32, 46, 50, 52).

The difference in PAMP activation patterns between human and rodent macrophages underscores the species-specific regulatory differences in expression of the NOS2 gene. A number of studies have defined the transcription factor binding sites relevant for immune-mediated induction of NOS2. Overall, it is clear that only about a 1,000 base pair (bp) region close to the transcriptional start site is required for full induction of murine NOS2 by cytoactive factors (29). This is not true for the human NOS2 gene. In human cells expressing NOS2 and iNOS, multiple enhancer and silencer sites upstream from the 5′ flanking sequence are required for induction (15, 27, 29, 47, 48). Thus, although human gene regulatory sites such as the NFκB binding sites are homologous to rodent sites, the presence of additional functional elements in different gene locations is likely to dictate a human specific pattern of induction and ultimately, NO production. Thus, we predict that the differences in the promoter are a major factor in the reduced levels of NO observed in activated human macrophages compared to activated rodent macrophages. Our humanized mouse model expresses the entire human NOS2 promoter in place of the mouse promoter. Thus, it is likely that immune-mediated signaling pathways triggered in the mouse macrophage interact with the human gene to generate the observed specific response pattern. We cannot rule out, however, that the human NOS2 promoter does not fully respond to mouse transcription factors or that posttranscriptional factors such as mRNA stabil-

ity and translational or posttranslational regulation (6, 30, 38, 53) influence iNOS expression and NO production in $NOS2^{+/0}$/$mNOS2^{-/-}$ macrophages.

The double transgenic $NOS2^{+/0}$/$mNOS2^{-/-}$ mice provide an important model system in which to more fully examine the differences between human and rodent NOS2 expression, activity, and NO levels. Importantly, these mice also allow us to examine the impact of differences in "human" NOS2 activation compared to rodent NOS2 activation on inflammatory-mediated disease processes. Redox-specific outcomes such as oxidation or nitrosation are critical to host resistance and repair after damage (2, 3, 25, 44). There is little doubt that NO participates in altering the redox environment at the wound site in both humans and rodents although the role of NO during acute or chronic inflammation is clearly multifactorial. Both damaging and protective roles for NO have been proposed (23, 26, 40). The use of mouse NOS2 and the subsequent potential for differences in levels of NO has the potential to shift the redox changes in a manner that is not representative of human disease processes. Consequently, interventions that are used to "normalize" the tissue redox balance in mouse models of inflammatory disease may not faithfully reflect human outcomes. Using the $NOS2^{+/0}$/$mNOS2^{-/-}$ mice as a basis for disease expression may, thus, provide a better understanding of human inflammatory disease process and a better evaluation of therapeutic approaches to these diseases.

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ABBREVIATIONS

CAT, cationic amino acid transporter; IFNγ, interferon γ; iNOS, inducible NOS; LPS, lipopolysaccharide; NO, nitric oxide; NOS, nitric oxide synthase; PAMP, pattern-associated molecular pathogens; PIC, polyinosine–polycytidylic acid; RNOS, reactive nitrogen–oxygen species; TNFα; tumor necrosis factor alpha.

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