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Intestinal Myofibroblasts Produce Nitric Oxide in Response to Combinatorial Cytokine Stimulation

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Abstract

Inflammatory bowel disease (IBD) patients display elevated levels of intraluminal nitric oxide (NO). NO can react with other molecules to form toxic compounds, which has led to the idea that NO may be an important mediator of IBD. However, the cellular source of NO and how its production is regulated in the intestine are unclear. In this study we aimed to determine if intestinal myofibroblasts produce NO in response to the IBD-associated cytokines IL-1 β , TNF α , and IFN γ . Intestinal myofibroblasts were isolated from mice and found to express inducible nitric oxide synthase (iNOS) mRNA, but not endothelial NOS or neuronal NOS. Individual treatment of myofibroblasts with IL-1 β , TNF α , or IFN γ had no effect on NO production, but stimulation with combinations of these cytokines synergistically increased iNOS mRNA and protein expression. Treatment with TNF α or IFN γ increased cell surface expression of IFN γ RI or TNFRII, respectively, suggesting that these cytokines act in concert to prime NO production by myofibroblasts. Impairment of NF- κ B activity with a small molecule inhibitor was sufficient to prevent increased expression of IFN γ RI or TNFRII, and inhibition of Akt, JAK/STAT, or NF- κ B blocked nearly all NO production induced by combinatorial cytokine treatment. These data indicate that intestinal myofibroblasts require stimulation by multiple cytokines to produce NO and that these cytokines act through a novel pathway involving reciprocal cytokine receptor regulation and signaling by Akt, JAK/STAT, and NF- κ B.

Intestinal myofibroblasts (IMF) participate in a variety of biological processes, including wound healing, inflammation, and tumorigenesis (Powell et al., 1999; Pucilowska et al., 2000). These cells are situated in the lamina propria adjacent to intestinal epithelia and smooth muscle cells, and form a syncytium that extends throughout the gastrointestinal tract (Joyce et al., 1987). IMF cultured in vitro secrete large amounts of extracellular matrix and

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elevated numbers of IMF are found within and around intestinal strictures (McKaig et al., 2002, 2003), suggesting they participate in the development of intestinal fibrosis. They also produce a number of factors important for the development and resolution of inflammation that include IL-1, IL-6, IL-8, IL-10, IL-33, MCP-1, TNF α , and PGE₂ (Pang et al., 1994; Shao et al., 2006; Kobori et al., 2010). IMF regulation of fibrosis and cytokine production, and their close proximity to several other intestinal cell types, indicates these cells play an essential role in inflammatory bowel disease (IBD).

Nitric oxide (NO) is a highly reactive gas that is reported to have both protective and inflammatory properties in the intestine. Nitric oxide synthases (NOSs) produce NO from L-arginine, NADPH and oxygen, and three isoforms have been identified (Knowles and Moncada, 1994). Endothelial NOS (eNOS) or neuronal NOS (nNOS) are constitutively expressed by some cell types, while inducible NOS (iNOS) is expressed after exposure to cytokines and microbial products. NO consumption by guanylate cyclase in the formation of cGMP has been suggested to mediate intestinal homeostasis (Kolios et al., 2004). Reduction of NO bioavailability with the NO-scavenging compound NG-nitro-L-arginine methyl ester (L-NAME) results in decreased levels of cGMP, and this reduction correlates with increased interepithelial junctions, decreased mucous production, and a leaky mucosal barrier (Brown et al., 1993; Alican and Kubes, 1996). Other studies suggest a pro-inflammatory role for NO in murine models of colitis. Inhibition of NO with L-NAME attenuates disease in the TNBS colitis model (Miller et al., 1993), and genetic deletion of eNOS, nNOS, or iNOS decreases disease susceptibility in the DSS and TNBS models (Zingarelli et al., 1999; Beck et al., 2004). While NO clearly modulates disease in experimental colitis models in mice, many of these results are conflicting and a precise role for NO in murine colitis has yet to emerge.

In humans a strong correlation exists between NOS activity and IBD. Elevated NO levels in the colons of IBD patients have been reported by several groups and citrulline, a bi-product of NOS reactions, is more abundant in rectal biopsy samples from patients with active ulcerative colitis (Boughton-Smith et al., 1993; Middleton et al., 1993; Lundberg et al., 1994). Moreover, expression of iNOS is substantially increased in intestinal samples of IBD patients compared to samples from healthy donors (Singer et al., 1996; Kolios et al., 1998). Staining of intestinal samples from IBD patients with iNOS-specific antibodies has shown that iNOS is expressed by epithelial cells, but punctate staining of non-epithelial cells is also observed (Kolios et al., 1998; Ljung et al., 2006). Monocytes in patients with active IBD and crypt-associated macrophages also express elevated levels of iNOS and NO (Dijkstra et al., 2002; Chin et al., 2008). Furthermore, cytokines associated with autoimmune diseases and also found in the intestines of IBD patients (e.g., IL-1 β , TNF α , and IFN γ), are reported to stimulate iNOS expression in intestinal epithelial cells and macrophages (Kolios et al., 1995; Fonseca et al., 2003). While a strong link exists between iNOS expression in intestinal epithelial cells and cells of monocyte/macrophage lineage during intestinal inflammation, it is unclear if additional cell types, such as IMF, also contribute to NO production in the pathogenesis of IBD.

The aim of this study was to determine if IMF produce NO after exposure to IBD-associated cytokines, such as IL-1 β , TNF α , and IFN γ . We found that IMF express iNOS, but not eNOS or nNOS, and that iNOS expression is dramatically increased after stimulation with

combinations of IL-1 β , TNF α , and IFN γ . NO production was undetectable in un-stimulated IMF or in IMF stimulated with single treatments of IL-1 β , TNF α , or IFN γ . However, when IL-1 β , TNF α , or IFN γ were administered as combinations, we observed a substantial production of NO that was similar to amounts made by activated macrophages. We also found that synergistic effects seen with combinatorial cytokine treatments results from regulation of reciprocal receptor expression and a signaling pathway involving Akt, JAK/STAT, and NF- κ B.

Materials and Methods

Intestinal myofibroblast isolation and culture

Primary intestinal myofibroblasts (IMF) were isolated from the small intestines of 3–4 week old C57BL/6 mice as previously described with slight modification (Fruchtman et al., 2005; Theiss et al., 2005). IMF were isolated from 3- to 4-week-old mice because this is the age where we obtained the greatest number of viable cells per isolation. Mice were housed within an environmentally controlled specific pathogen-free animal facility at the University of California, San Francisco and all experiments were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco. Briefly, intestines were collected, flushed with HBSS (with Ca²⁺ and Mg²⁺, without phenol red, UCSF Cell Culture Facility, San Francisco, CA), minced into 1 mm segments, and digested with 4 U/ml Dispase (Worthington Biochemicals, Lakewood, NJ) and 300 U/ml collagenase I (Worthington Biochemicals) in HBSS on a rotary shaker (60–80 rpm) at room temperature for 25 min. Digested tissue was pipetted up and down, placed on ice for 3 min, and centrifuged at 800–1000 rpm for 10 min. The supernatant was aspirated and the tissue pellet was washed once with culture media before being plated into culture flasks. Cells were cultured in DMEM (UCSF Cell Culture Facility, San Francisco, CA) supplemented with 10% heat inactivated Certified Fetal Bovine Serum (Invitrogen, Grand Island, NY) and 1 \times penicillin/streptomycin (UCSF Cell Culture Facility) in a 37°C humidified 5% CO₂ incubator and washed with fresh media twice a week. These culture conditions allow IMF to proliferate while other intestinal cell types perish during extended culture. By passage 3, isolated cells were highly enriched for IMFs as demonstrated by immunohistochemical staining for the IMF markers smooth muscle actin, desmin, and vimentin. Enriched IMF were routinely at least 84% positive for smooth muscle actin, 71% positive for desmin, and 99% positive for vimentin and used for experiments at passage 3–5. The Raw 264.7 murine macrophage cell line was obtained from ATCC (TIB-71) and cultured under identical conditions.

Cytokine stimulation and treatment with small molecule inhibitors

IMF were seeded in 10- or 15-cm dishes (Corning, Tewksbury, MA) and cultured to 80–90% confluence, or seeded in Black Clear Bottom, TC surface, 96-well plates (BD Falcon, San Jose, CA) at 8,000–10,000 cells per well. Passaged cells were placed in complete DMEM and allowed to recover for 20–24 h in a 37°C humidified 5% CO₂ incubator before experiments. For cytokine and inhibitor treatments, culture medium was replaced with fresh medium that consisted of DMEM supplemented with 5% heat-inactivated Certified Fetal Bovine Serum, 0.5 \times penicillin/streptomycin, and one or more of the following: IL-1 α ,

IL-1 β , TNF α , IFN γ (all from R&D Systems, Minneapolis, MN), lipopolysaccharide (LPS; Sigma), IKK Inhibitor X, AG 490, and WHI-P154 (all from Calbiochem, San Diego, CA).

RNA Isolation and RT-PCR

RNA was isolated from cultured cells or mouse brain tissue using an RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Valencia, CA). RT-PCR was conducted on a GeneAmp PCR System 9700 (Foster City, CA) with an OneStep RT-PCR Kit (Qiagen). Primers were designed with Primer3 software (developed by HHMI and NIH) using mRNA sequences obtained from GenBank. The primer sequences were as follows: mouse nNOS, forward, 5'-ACC AGC TCT TCC CTC TAG CC-3', reverse, 5'-ATG GGT ACT TCC AGC ACC AG-3'; mouse iNOS, forward, 5'-CCT TGT TCA GCT ACG CCT TC-3', reverse, 5'-GGC TGG ACT TTT CAC TCT GC-3'; mouse eNOS, forward, 5'-TCT TCG TTC AGC CAT CAC AG-3', reverse, 5'-CAC AGG GAT GAG GTT GTC CT-3', and GAPDH, forward, 5'-CGA CAA GGG AGC TAA AAC CA-3', reverse, 5'-GCC TTG CAG AAA CTT TGG AG-3'. One step RT-PCR was performed with the following conditions: Reverse transcription for 30 min at 50°C; Initial PCR activation for 15 min at 95°C; 3-step cycling for 45 sec at 94°C, 1 min at 59°C for eNOS and iNOS, at 55°C nNOS and GAPDH, and 1 min at 72°C for a total of 35 cycles; final extension for 10 min at 72°C. DNA from RT-PCR reactions was separated on a 1.5% agarose (Sigma, St. Louis, MO) gel containing ethidium bromide and images of gels exposed to UV light were acquired.

Western blot

To measure expression of iNOS and phosphorylation of signal transduction proteins, cells were plated in 10-cm dishes. The culture medium was replaced with fresh serum-free DMEM for measurement of signal transduction proteins or with DMEM supplemented with 5% heat-inactivated Certified Fetal Bovine Serum, 0.5 \times penicillin/streptomycin for measurement of iNOS protein expression. Cells were then treated with the indicated cytokine and inhibitor conditions. Cells were then placed in a lysis buffer containing 1 \times RIPA buffer with 1 \times protease inhibitor cocktail and 1 \times phosphatase inhibitors (Pierce, Rockford, IL), and snap-frozen on dry-ice. Cell lysates were melted on ice and re-frozen on dry-ice three times to ensure complete lysis. Lysates were centrifuged at 15,400g for 15 min at 4°C and the supernatant was collected. Samples (15 μ g/lane) were separated by 4–20% Ready Gel Tris–HCl gel (Bio-Rad, Hercules, CA), transferred to an Immobilon-P PVDF membrane (Bio-Rad), and blocked for 30 min in Tris-buffered saline plus 0.1% Tween 20 (TBST) containing 1.5% skim milk. Membranes were incubated for overnight at 4°C with antibodies to iNOS (BD Pharmingen, San Jose, CA), β -actin (Abcam, Cambridge, MA), p-NF- κ B p65 (Ser536), NF- κ B p65 (Cell Signaling, Danvers, MA), p-Akt (Ser473), Akt (Cell Signaling), p-STAT1 (Tyr701), and STAT1 (Santa Cruz Biotechnology, Santa Cruz, CA). After washing with TBST, membranes were incubated with a peroxidase-conjugated secondary antibody (anti-rabbit IgG, HRP-linked; Cell Signaling) overnight at 4°C, washed again, and developed with ECL Plus reagent (Pierce).

Flow cytometry

Cells were washed with PBS and then stained with PE-conjugated rat anti-mouse IL-1R (CD121a, BD Biosciences, San Jose, CA), hamster anti-mouse TNFR1 (CD120a, BD Biosciences), PE-conjugated goat anti-hamster IgG (H + L, CalTag, Burlingame, CA), biotinylated hamster anti-mouse TNFR2 (CD120b, BD Biosciences), or biotinylated hamster anti-mouse IFN γ RI (CD 119, BD Biosciences). Streptavidin-PE (eBioscience, San Diego, CA) was used to detect cells labeled with α -TNFR2 and α -IFN γ RI, and a PE conjugated hamster IgG antibody (BD Biosciences) was used as an isotype control. Flow cytometry data analysis was performed with Flowjo software (Treestar, Ashland, OR) and mean fluorescence intensity (MFI) of the staining was used to measure expression of the receptors. MFI values were corrected for non-specific labeling by subtracting the MFI of cells stained with secondary antibodies alone or with isotope control antibodies. The relative fold increase of MFI was calculated by dividing the MFI of myofibroblasts treated with cytokines or cytokine-inhibitor combinations by the MFI of myofibroblasts treated with medium alone.

Measurement of NOS activity

IMFs and Raw 246.7 cells were cultured as described above in black 96-well plates and nitric oxide synthase (NOS) activity was assayed with the Fluorimetric Cell-Associate Nitric Oxide Synthase Detection System (Sigma) according to the manufacturer's instructions. Relative fluorescence units (RFU) was measured with a fluorimeter (Synergy HT, Bio-Tek Instruments, Winooski, VT) set to an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Relative fold increase in RFU was used to represent changes in NOS activity between different conditions and was calculated by dividing the RFU of treated cells by the RFU of non-treated cells.

Statistical analysis

To determine the statistical significance of differences between groups, GraphPad Prism (GraphPad Software) was used to perform a 2-tailed Student's *t*-test or one-way analysis of variance (ANOVA). Differences with a *P*-value of less than 0.05 were considered statistically significant.

Results

IMF produce NO by iNOS after combinatorial cytokine stimulation

To determine if primary mouse intestinal myofibroblasts (IMFs) express enzymes that synthesize NO, we analyzed these cells for expression of the three known NOS genes (Knowles and Moncada, 1994). RT-PCR with primary mouse IMFs revealed the presence of inducible NOS (iNOS) mRNA, but minimal to undetectable amounts of endothelial NOS (eNOS) or neuronal NOS (nNOS; Fig. 1A). We next determined if iNOS in these cells was regulated by pro-inflammatory cytokines implicated in IBD. Interestingly, individual treatment with IL-1 β , TNF α , or IFN γ had little effect on iNOS expression in IMF, but combinations of IL-1 β and IFN γ or TNF α and IFN γ considerably increased iNOS mRNA expression (Fig. 1A). We then used the above cytokines and IL-1 α to examine whether

iNOS protein expression was regulated in a similar fashion. Individual stimulation with IL-1 α , IL-1 β , TNF α , or IFN γ had little effect on iNOS protein levels, but combinations of IL-1 α , IL-1 β , and TNF α with IFN γ potently stimulated expression of iNOS (Fig. 1B). Our laboratory previously reported that C-type natriuretic peptide (CNP) induces IMF relaxation through cGMP-associated reductions in MLC phosphorylation (Chitapanarux et al., 2004). However, no change in iNOS expression in IMF was observed after CNP treatment (Fig. 1B). Together these data suggest that cytokines implicated in IBD synergize to trigger expression of iNOS in IMFs.

To determine if combinatorial cytokine stimulation of iNOS expression results in enhanced NO production in IMF, we measured NO production with a fluorimetric cell-associated NOS assay. This assay measures the conversion of the non-fluorescent compound 4,5-diaminofluorescein to triazolofluorescein, a fluorescent compound generated in the presence of NO (Kojima et al., 1998). IMF loaded with 4,5-diaminofluorescein had minimal changes in fluorescence after treatment with TNF α or IFN γ , but combinations of TNF α and IFN γ dose-dependently stimulated a 6- to 10-fold increase in fluorescence (Fig. 1C,D). This result indicates that combinations of TNF α and IFN γ synergize to induce production of NO through expression of iNOS in primary IMFs.

IBD cytokines induce reciprocal receptor expression in IMF

We postulated that IMFs were sensitized by one cytokine to respond to another cytokine by increasing the number of the corresponding receptors at the cell surface. Surface expression of receptors for IL-1, TNF- α , and IFN γ was measured in response to treatment with IL-1 α , IL-1 β , TNF α , or IFN γ . Primary IMF expressed relatively low levels of IL-1R and TNFR1, but higher levels of TNFR2 and IFN γ RI (Fig. 2A). Treatment with TNF α , but not IL-1 α or IL-1 β , significantly increased expression of IFN γ RI on the surface of IMF (Fig. 2B). Likewise, treatment with IFN γ , but not IL-1 α or IL-1 β , significantly increased TNFR2 (Fig. 2C). This result suggests that regulation of iNOS and NO production by combinatorial cytokine treatment occurs from distinct signaling events triggered by each cytokine, which primes IMFs to respond to additional cytokines.

We investigated the signaling mechanism responsible for iNOS regulation by sequentially treating IMF with IL-1 β , TNF α , or IFN γ and measuring iNOS expression and NO production. Individual treatment with IL-1 β , TNF α , or IFN γ for 24 h had minimal effects on iNOS expression (Fig. 2D). However, sequential treatment with IL-1 β , TNF α , or IFN γ for 20 h, followed by removal of the cytokine and subsequent treatment for 24 h with IL-1 β , TNF α , or IFN γ resulted in a similar synergistic regulation of iNOS as seen with combinatorial treatment (Fig. 2D). We observed multiple bands on the iNOS Western blot and we believe these to be iNOS splice variants as previously reported (Bloch et al., 1995; Xu et al., 1995). We also compared the effects of sequential and combinatorial cytokine treatment on NO production by IMF. Treatment of IMF with IL-1 β , TNF α , or IFN γ for 20 h, followed by removal of the cytokine and subsequent treatment for 24 h with IL-1 β , TNF α , or IFN γ resulted in synergistic regulation of NO production (Fig. 2E). Taken together, these data suggest that TNF α and IFN γ prime IMF to synthesize iNOS and produce NO in a synergistic fashion by stimulating expression of IFN γ RI and TNFR2.

NO production by IMF is mediated through Akt, JAK/STAT, and NF- κ B signaling

To determine how iNOS expression is regulated in IMF after combinatorial treatment with TNF α , or IFN γ , respectively, we examined signaling cascades that are typically associated with these cytokines. Stimulation with TNF α for 15 min had no detectable effect on phosphorylation of Akt, NF- κ B, and STAT1 (Fig. 3A). However, treatment with IFN γ for 15 min induced phosphorylation of Akt, NF- κ B, and STAT1 (Fig. 3A). Combinatorial treatment with TNF α and IFN γ for 15 min further increased phosphorylation of NF- κ B and STAT1, but had no effect on phosphorylation of Akt (Fig. 3A). Phosphorylation of NF- κ B and STAT1 was maximally enhanced after pretreatment of IMF with TNF α for 20 h followed by 15 min of stimulation with IFN γ (Fig. 3A). We employed small molecule inhibitors to determine the signaling proteins downstream of TNF α or IFN γ stimulation. Addition of IFN γ for 15 min after 20 h of pretreatment with TNF α and AG490, a protein tyrosine kinase inhibitor with selectivity for members of the Janus-associated kinase (JAK) family, prevented phosphorylation of NF- κ B and significantly reduced phosphorylation of Akt and STAT1 (Fig. 3A). IKK inhibitor X, an inhibitor of NF- κ B phosphorylation, and WHI-P154, a specific JAK3 inhibitor, also reduced phosphorylation of NF- κ B, Akt, and STAT1, but were less potent than AG490 under similar conditions. Pretreatment with IFN γ for 20 h and treatment with TNF α for 15 min also induced phosphorylation of NF- κ B, Akt, and STAT1. However, this condition resulted in less phosphorylation of NF- κ B, Akt, and STAT1 when compared to treatment with TNF α for 20 h and treatment with IFN γ for 15 min. The inclusion of AG490 during IFN γ pretreatment for 20 h impaired phosphorylation of NF- κ B, Akt, and STAT1 after TNF α stimulation, but inclusion of IKK inhibitor X or WHI-P154 only blocked phosphorylation of STAT1 (Fig. 3A). Stimulation with IL-1 β for 20 h and treatment with TNF α or IFN γ for 15 min both increased phosphorylation of Akt and NF- κ B, but only IFN γ provoked phosphorylation of STAT1. These data suggest that Akt, JAK/STAT, and NF- κ B drive the synthesis of iNOS and production of NO by IMF in response to TNF α and IFN γ .

Inhibitors of Akt, JAK/STAT, and NF- κ B signaling were also used to examine the role of this pathway on iNOS expression and NO production by IMF in response to TNF α and IFN γ . AG490, IKK inhibitor X, and WHI-P154 all reduced iNOS expression, but with varying degrees of potency, in response to IL-1 β and IFN γ or TNF α and IFN γ combinatorial treatment (Fig. 3B). While these inhibitors had varying effects on iNOS expression, they prevented nearly all NO produced upon TNF α and IFN γ sequential stimulation (Fig. 3C,D). These data indicate that Akt, JAK/STAT, and NF- κ B potentially control NO production by IMF in response to TNF α and IFN γ .

We determined if small molecule inhibitors of Akt, JAK/STAT, and NF- κ B signaling in IMF were directly regulating expression of iNOS or were working through an indirect pathway by preventing induction of IFN γ RI and TNFRII expression. IMF were stimulated with TNF α or IFN γ in the presence of IKK inhibitor X or AG490. As previously shown in an earlier experiment from this study (Fig. 2A), IFN γ RI expression on IMF was increased upon treatment with TNF α , and TNFRII expression was increased upon treatment with IFN γ (Fig. 4A,B). IMF treated with TNF α or IFN γ and IKK inhibitor X, but not AG490, significantly blocked expression of IFN γ RI and TNFRII, respectively (Fig. 4A,B). These

data suggest that increased expression of IFN γ RI and TNFRII upon stimulation with TNF α or IFN γ is regulated by NF- κ B. Furthermore, these data indicate that distinct signaling pathways are engaged after single or combinatorial treatment with TNF α or IFN γ .

To determine if a similar signaling pathway regulates NO production in other cell types, we examined Raw 264.7 cells, a well-characterized murine macrophage cell line that has been used extensively for studying the regulation of iNOS (Walker et al., 1997; Ricote et al., 1998). Untreated Raw 264.7 cells expressed high levels of phosphorylated NF- κ B and phosphorylated STAT1 when compared to untreated IMF (Fig. 5A). Phosphorylation of NF- κ B and STAT1 in Raw 264.7 cells was further increased by treatment with LPS or IFN γ , but TNF α had no effect (Fig. 5A). In contrast, IMF required treatment with a combination of IFN γ and IL-1 β to generate comparable levels of phosphorylated STAT1, but NF- κ B phosphorylation never reached similar levels as Raw 264.7 cells at any condition tested (Fig. 5A). Phosphorylated Akt was undetectable in Raw 264.7 cells, even after stimulation with TNF α , IFN γ , or LPS (Fig. 5B). Similar to IMF, stimulation of Raw 264.7 cells with IFN γ alone induced expression of iNOS that was further potentiated with combinatorial treatment with IFN γ and TNF α (Fig. 5C). AG490 inhibited stimulation of iNOS expression with IFN γ treatment alone, but had a negligible effect when IFN γ and TNF α were administered in combination. Also, in contrast to iNOS regulation in IMF, IKK inhibitor X had no effect on stimulation of iNOS expression by IFN γ and TNF α (Fig. 5C). Finally, we performed a side-by-side comparison of NO production in IMF and Raw 264.7 cells after treatment with cytokines and JAK/STAT or NF- κ B inhibitors. Counter to iNOS regulation in IMF, individual treatment with IFN γ was sufficient to induce production of NO in Raw 264.7 cells (Fig. 5E). This induction of NO synthesis was partially inhibited with AG490, but not nearly as dramatic as the near-complete inhibition with AG490 seen in IMFs after IFN γ and IL-1 β or TNF α combinatorial treatment (Fig. 5E). Also in contrast to NO regulation in IMF, IKK inhibitor X did not block NO production by Raw 264.7 cells in response to IFN γ or LPS (Fig. 5E). These data indicate that regulation of iNOS and NO production in Raw 264.7 cells is primarily mediated by signaling pathways distinct from those in IMF.

Discussion

Excessive intestinal NO production is thought to contribute to the pathogenesis of IBD, but the cellular sources of NO and regulation of NO synthesis in the intestine is unclear. In this study we show that production of NO is undetectable in primary IMF cultured under basal conditions and dramatically increased upon combinatorial cytokine stimulation. Individual treatment with IFN γ , TNF α , or IL-1 β resulted in modest effects on iNOS expression and undetectable changes in NO production, whereas combinations of IFN γ and TNF α or IL-1 β at the same doses triggered a large increase in iNOS expression and production of NO. These findings suggest IMF may promote intestinal inflammation through production of NO when other inflammation-associated cytokines are present.

Based on the data reported here, we propose a model for regulation of iNOS expression and NO production by IMF (Fig. 6). We found that IMF responded to stimulation with IFN γ or TNF α by increasing the expression of TNFRII or IFN γ RI, respectively, through a signaling pathway mediated by NF- κ B (Fig. 6A). Thus, our data suggests a mechanism of iNOS

regulation in IMF where stimulation with one cytokine induces the expression of a receptor for a second cytokine, thereby sensitizing the cell to respond when the second cytokine is detected. We have also identified a signaling pathway in IMF that triggers iNOS expression after cell sensitization. While TNF α -sensitized IMF responded to IFN γ by expressing iNOS through pathways involving both JAK-mediated phosphorylation of STAT1 at Tyrosine701 and Akt phosphorylation at Serine473, NF- κ B p65 phosphorylation at Serine536, IFN γ -sensitized IMF responded to TNF α by expressing iNOS through Akt phosphorylation at Serine473 and NF- κ B p65 phosphorylation at Serine536 (Fig. 6B). Treatment with small molecule inhibitors of JAK family members or NF- κ B reduced iNOS expression and completely blocked NO production after stimulation with IFN γ or TNF α , which suggests that this pathway is required for NO production by IMF. These findings are in line with a previous report showing that iNOS expression in STAT1-deficient mouse embryonic fibroblasts is not induced upon treatment with IFN γ in combination with LPS, TNF- α or IL-1 (Samardzic et al., 2001).

Additional lines of evidence from this study support our model of sensitization and response to inflammatory cytokines in the regulation of NO production by IMF. We observed that stimulation with IFN γ , TNF α , or IL-1 β for 20 h, followed by removal of the cytokine and stimulation with a different cytokine (i.e., IFN γ or TNF α) for 24 h resulted in nearly the same increase in iNOS expression and NO production as simultaneous administration of cytokines. Secondary stimulation with IL-1 β elicited a similar, but less potent, stimulation of iNOS expression and NO synthesis. While pretreatment with IL-1 β did not significantly enhance expression of TNFR2 or IFN γ RI, it did sensitize IMF to respond to IFN γ . This result suggests that IL-1 β sensitizes IMF to respond to IFN γ through modulation of IFN γ intracellular signaling pathways. Together these findings suggest that primary exposure of IMF to IFN γ , TNF α , or IL-1 β induces transcriptional changes independently of the other cytokines.

We also saw that AG490 was able to inhibit iNOS expression and block NO production without affecting expression of TNFR2 or IFN γ RI, suggesting that the sensitization and responses phases are regulated through distinct signaling pathways. However, NF- κ B inhibition prevented both increased expression of TNFR2 or IFN γ RI and blocked iNOS expression and NO production. Since NF- κ B is activated by a variety of stimuli in addition to cytokines, including oxidized LDL, free radicals, UV radiation and microbial antigens, it is not entirely surprising that different signaling pathways would converge on this transcription factor (Piette et al., 1997; Ballard, 2001). We speculate that NF- κ B may partner with different co-activators for the sensitization and response phases of NO synthesis in IMF, but this question remains for future work.

This study identifies IMF as producers of NO and sheds light on a signaling pathway that results in iNOS expression. Intestinal barrier function is known to be regulated by IFN γ -mediated stimulation of TNFR2 expression in epithelial cells (Wang et al., 2006). However, to the best of our knowledge, the present study is the first to describe a requirement for combinations of cytokines in the expression of iNOS and NO production in a cell type of the myofibroblast lineage. Since multiple intestinal cell types express NOS isoforms, either constitutively or in response to inflammatory stimuli, we believe that future work should

focus on delineating the relative contribution of NO production by intestinal cells in homeostatic and disease states. Further characterization of the pathways controlling NO production by IMF and other intestinal cell types may reveal new targets for the treatment of IBDs.

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Literature Cited

- Alican I, Kubes P. A critical role for nitric oxide in intestinal barrier function and dysfunction. *Am J Physiol*. 1996; 270:G225–G237. [PubMed: 8779963]
- Ballard DW. Molecular mechanisms in lymphocyte activation and growth. *Immunol Res*. 2001; 23:157–166. [PubMed: 11444381]
- Beck PL, Xavier R, Wong J, Ezedi I, Mashimo H, Mizoguchi A, Mizoguchi E, Bhan AK, Podolsky DK. Paradoxical roles of different nitric oxide synthase isoforms in colonic injury. *Am J Physiol Gastrointest Liver Physiol*. 2004; 286:G137–G147. [PubMed: 14665440]
- Bloch KD, Wolfram JR, Brown DM, Roberts JD Jr, Zapol DG, Lepore JJ, Filippov G, Thomas JE, Jacob HJ, Bloch DB. Three members of the nitric oxide synthase II gene family (NOS2A, NOS2B, and NOS2C) colocalize to human chromosome 17. *Genomics*. 1995; 27:526–530. [PubMed: 7558036]
- Boughton-Smith NK, Evans SM, Hawkey CJ, Cole AT, Balsitis M, Whittle BJ, Moncada S. Nitric oxide synthase activity in ulcerative colitis and Crohn's disease. *Lancet*. 1993; 342:338–340. [PubMed: 7687730]
- Brown JF, Keates AC, Hanson PJ, Whittle BJ. Nitric oxide generators and cGMP stimulate mucus secretion by rat gastric mucosal cells. *Am J Physiol*. 1993; 265:G418–G422. [PubMed: 8214062]
- Chin MP, Schauer DB, Deen WM. Prediction of nitric oxide concentrations in colonic crypts during inflammation. *Nitric Oxide*. 2008; 19:266–275. [PubMed: 18501201]
- Chitapanarux T, Chen SL, Lee H, Melton AC, Yee HF Jr. C-type natriuretic peptide induces human colonic myofibroblast relaxation. *Am J Physiol Gastrointest Liver Physiol*. 2004; 286:G31–G36. [PubMed: 12958024]
- Dijkstra G, Zandvoort AJ, Kobold AC, de Jager-Krikken A, Heeringa P, van Goor H, van Dullemen HM, Tervaert JW, van de Loosdrecht A, Moshage H, Jansen PL. Increased expression of inducible nitric oxide synthase in circulating monocytes from patients with active inflammatory bowel disease. *Scand J Gastroenterol*. 2002; 37:546–554. [PubMed: 12059056]
- Fonseca SG, Romao PR, Figueiredo F, Morais RH, Lima HC, Ferreira SH, Cunha FQ. TNF-alpha mediates the induction of nitric oxide synthase in macrophages but not in neutrophils in experimental cutaneous leishmaniasis. *Eur J Immunol*. 2003; 33:2297–2306. [PubMed: 12884305]
- Fruchtman S, Simmons JG, Michaylira CZ, Miller ME, Greenhalgh CJ, Ney DM, Lund PK. Suppressor of cytokine signaling-2 modulates the fibrogenic actions of GH and IGF-I in intestinal mesenchymal cells. *Am J Physiol Gastrointest Liver Physiol*. 2005; 289:G342–G350. [PubMed: 15831713]
- Joyce NC, Haire MF, Palade GE. Morphologic and biochemical evidence for a contractile cell network within the rat intestinal mucosa. *Gastroenterology*. 1987; 92:68–81. [PubMed: 3536654]
- Knowles RG, Moncada S. Nitric oxide synthases in mammals. *Biochem J*. 1994; 298:249–258. [PubMed: 7510950]
- Kobori A, Yagi Y, Imaeda H, Ban H, Bamba S, Tsujikawa T, Saito Y, Fujiyama Y, Andoh A. Interleukin-33 expression is specifically enhanced in inflamed mucosa of ulcerative colitis. *J Gastroenterol*. 2010; 45:999–1007. [PubMed: 20405148]

- Kojima H, Sakurai K, Kikuchi K, Kawahara S, Kirino Y, Nagoshi H, Hirata Y, Nagano T. Development of a fluorescent indicator for nitric oxide based on the fluorescein chromophore. *Chem Pharm Bull (Tokyo)*. 1998; 46:373–375. [PubMed: 9501473]
- Kolios G, Brown Z, Robson RL, Robertson DA, Westwick J. Inducible nitric oxide synthase activity and expression in a human colonic epithelial cell line, HT-29. *Br J Pharmacol*. 1995; 116:2866–2872. [PubMed: 8680718]
- Kolios G, Rooney N, Murphy CT, Robertson DA, Westwick J. Expression of inducible nitric oxide synthase activity in human colon epithelial cells: Modulation by T lymphocyte derived cytokines. *Gut*. 1998; 43:56–63. [PubMed: 9771406]
- Kolios G, Valatas V, Ward SG. Nitric oxide in inflammatory bowel disease: A universal messenger in an unsolved puzzle. *Immunology*. 2004; 113:427–437. [PubMed: 15554920]
- Ljung T, Lundberg S, Varsanyi M, Johansson C, Schmidt PT, Herulf M, Lundberg JO, Hellstrom PM. Rectal nitric oxide as biomarker in the treatment of inflammatory bowel disease: Responders versus nonresponders. *World J Gastroenterol*. 2006; 12:3386–3392. [PubMed: 16733856]
- Lundberg JO, Hellstrom PM, Lundberg JM, Alving K. Greatly increased luminal nitric oxide in ulcerative colitis. *Lancet*. 1994; 344:1673–1674. [PubMed: 7996962]
- McKaig BC, Hughes K, Tighe PJ, Mahida YR. Differential expression of TGF-beta isoforms by normal and inflammatory bowel disease intestinal myofibroblasts. *Am J Physiol Cell Physiol*. 2002; 282:C172–C182. [PubMed: 11742810]
- McKaig BC, McWilliams D, Watson SA, Mahida YR. Expression and regulation of tissue inhibitor of metalloproteinase-1 and matrix metalloproteinases by intestinal myofibroblasts in inflammatory bowel disease. *Am J Pathol*. 2003; 162:1355–1360. [PubMed: 12651627]
- Middleton SJ, Shorthouse M, Hunter JO. Increased nitric oxide synthesis in ulcerative colitis. *Lancet*. 1993; 341:465–466. [PubMed: 8094492]
- Miller MJ, Sadowska-Krowicka H, Chotinaruemol S, Kakkis JL, Clark DA. Amelioration of chronic ileitis by nitric oxide synthase inhibition. *J Pharmacol Exp Ther*. 1993; 264:11–16. [PubMed: 7678645]
- Pang G, Couch L, Batey R, Clancy R, Cripps A. GM-CSF, IL-1 alpha, IL-1 beta, IL-6, IL-8, IL-10, ICAM-1 and VCAM-1 gene expression and cytokine production in human duodenal fibroblasts stimulated with lipopolysaccharide, IL-1 alpha and TNF-alpha. *Clin Exp Immunol*. 1994; 96:437–443. [PubMed: 8004813]
- Piette J, Piret B, Bonizzi G, Schoonbroodt S, Merville MP, Legrand-Poels S, Bours V. Multiple redox regulation in NF-kappaB transcription factor activation. *Biol Chem*. 1997; 378:1237–1245. [PubMed: 9426183]
- Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI, West AB. Myofibroblasts. II. Intestinal subepithelial myofibroblasts. *Am J Physiol*. 1999; 277:C183–C201. [PubMed: 10444394]
- Pucilowska JB, Williams KL, Lund PK. Fibrogenesis. IV. Fibrosis and inflammatory bowel disease: Cellular mediators and animal models. *Am J Physiol Gastrointest Liver Physiol*. 2000; 279:G653–G659. [PubMed: 11005750]
- Ricote M, Li AC, Willson TM, Kelly CJ, Glass CK. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature*. 1998; 391:79–82. [PubMed: 9422508]
- Samardzic T, Jankovic V, Stosic-Grujicic S, Trajkovic V. STAT1 is required for iNOS activation, but not IL-6 production in murine fibroblasts. *Cytokine*. 2001; 13:179–182. [PubMed: 11161462]
- Shao J, Sheng GG, Mifflin RC, Powell DW, Sheng H. Roles of myofibroblasts in prostaglandin E2-stimulated intestinal epithelial proliferation and angiogenesis. *Cancer Res*. 2006; 66:846–855. [PubMed: 16424017]
- Singer II, Kawka DW, Scott S, Weidner JR, Mumford RA, Riehl TE, Stenson WF. Expression of inducible nitric oxide synthase and nitrotyrosine in colonic epithelium in inflammatory bowel disease. *Gastroenterology*. 1996; 111:871–885. [PubMed: 8831582]
- Theiss AL, Simmons JG, Jobin C, Lund PK. Tumor necrosis factor (TNF) alpha increases collagen accumulation and proliferation in intestinal myofibroblasts via TNF receptor 2. *J Biol Chem*. 2005; 280:36099–36109. [PubMed: 16141211]

- Walker G, Pfeilschifter J, Kunz D. Mechanisms of suppression of inducible nitric-oxide synthase (iNOS) expression in interferon (IFN)-gamma-stimulated RAW 264.7 cells by dexamethasone. Evidence for glucocorticoid-induced degradation of iNOS protein by calpain as a key step in post-transcriptional regulation. *J Biol Chem.* 1997; 272:16679–16687. [PubMed: 9195984]
- Wang F, Schwarz BT, Graham WV, Wang Y, Su L, Clayburgh DR, Abraham C, Turner JR. IFN-gamma-induced TNFR2 expression is required for TNF-dependent intestinal epithelial barrier dysfunction. *Gastroenterology.* 2006; 131:1153–1163. [PubMed: 17030185]
- Xu W, Charles IG, Liu L, Koni PA, Moncada S, Emson P. Molecular genetic analysis of the duplication of human inducible nitric oxide synthase (NOS2) sequences. *Biochem Biophys Res Commun.* 1995; 212:466–472. [PubMed: 7542877]
- Zingarelli B, Szabo C, Salzman AL. Reduced oxidative and nitrosative damage in murine experimental colitis in the absence of inducible nitric oxide synthase. *Gut.* 1999; 45:199–209. [PubMed: 10403731]

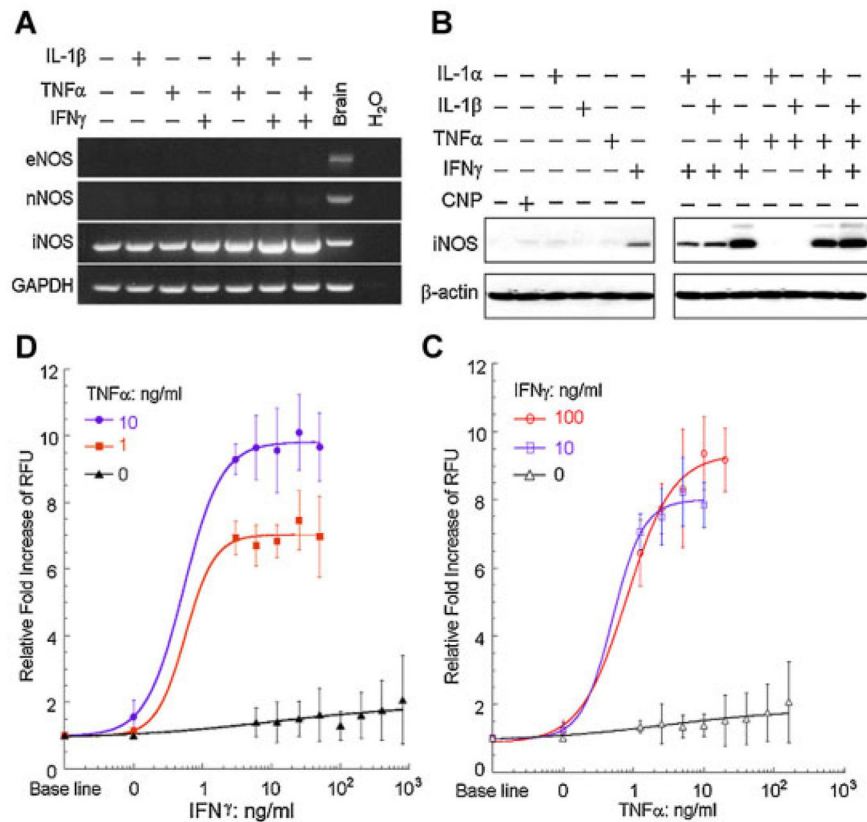


Fig. 1. Treatment with combinations of cytokines stimulates the expression of iNOS and synthesis of NO by IMF. A: RT-PCR performed on RNA isolated from IMF treated alone or with combinations of 5 ng/ml of IL-1 β , 10 ng/ml of TNF α or 100 ng/ml of IFN γ for 16 h. Reactions were performed with primers specific for eNOS, nNOS, iNOS, and GAPDH and separated by gel electrophoresis. RNA isolated from murine brain and reactions with no RNA input (H₂O) were included as controls. B: Western blots with antibodies specific to iNOS or β -actin using IMF treated alone or with combinations of 5 ng/ml IL-1 α , 5 ng/ml IL-1 β , 10 ng/ml TNF α , 100 ng/ml IFN γ , or 2 μ M CNP for 24 h. C,D: Fluorescence measurements of IMF loaded with 4,5-diaminofluorescein after treatment with 0, 10, or 100 ng/ml IFN γ and increasing doses of TNF α (C) or with 0, 1, or 10 ng/ml TNF α and increasing doses of IFN γ (D) for 24 h. Data are presented as the fold increase of relative fluorescence units (RFU) above baseline RFU values.

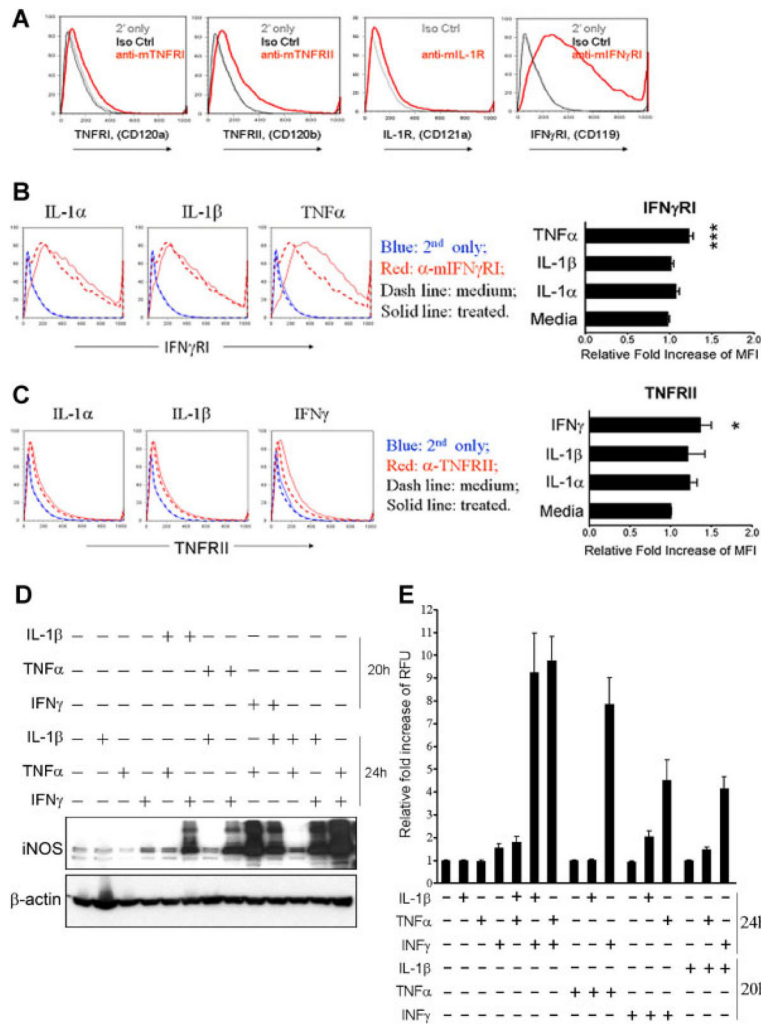


Fig. 2. Stimulation with TNF α or IFN γ sensitizes IMF to produce NO by increasing expression of IFN γ RI or TNFRII. **A:** Measurement of TNFR1, TNFR2, IL-1R, and IFN γ RI expression by flow cytometry on the surface of IMF. Gray and black lines indicate staining controls with secondary antibody alone and isotype control antibodies, except for IL-1R where the secondary alone condition was omitted because a fluorophore-conjugated antibody was used. **B:** Measurement of IFN γ RI expression by flow cytometry on the surface of IMF before (dashed red line) and after (solid red line) treatment for 24 h with 5 ng/ml IL-1 α , 5 ng/ml IL-1 β , or 10 ng/ml TNF α . Histogram plots show number of cells positively labeled on Y-axis and fluorescence intensity of staining on X-axis. IFN γ RI antibody was omitted to control for non-specific labeling from samples before (dashed blue line) and after (solid blue line) treatment. $***P < 0.001$. Results are reported as mean \pm S.D. **C:** Measurement of TNFR2 expression by flow cytometry on the surface of IMF before (dashed red line) and after (solid red line) treatment for 24 h with 5 ng/ml IL-1 α , 5 ng/ml IL-1 β , or 100 ng/ml IFN γ . $*P < 0.05$. **D:** Western blots with antibodies specific to iNOS or β -actin using IMF treated alone, in combination, or sequentially with 5 ng/ml IL-1 β , 10 ng/ml TNF α , 100 ng/ml IFN γ . For sequential cytokine treatment, cells were stimulated for 20 h with one

cytokine, washed with PBS, and the media replaced with a different cytokine for 24 h. E: Fluorescence measurements of IMF loaded with 4,5-diaminofluorescein after treatment alone, in combination, or sequentially with the indicated cytokines as in (D).

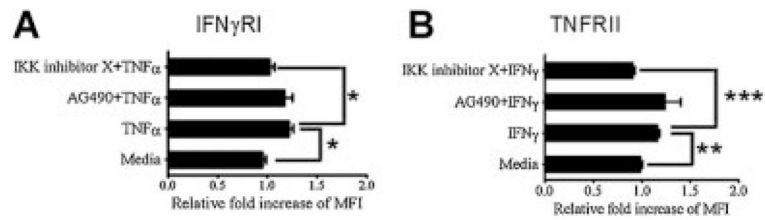


Fig. 4.

Increased expression of IFN γ RI or TNFRII on IMF after sensitization with TNF α or IFN γ , respectively, is regulated by NF- κ B. Flow cytometry measurements of IFN γ RI (A) or TNFRII (B) expression on the surface of IMF before and after treatment for 24 h with 10 ng/ml TNF α (A) or 100 ng/ml IFN γ (B) and 50 μ M AG490 or 15 μ M IKK inhibitor X. Data are expressed as the relative fold increase of mean fluorescence intensity (MFI) in IMF after treatment of cells with TNF α or IFN γ . One-way ANOVA analysis was used to determine significant differences between the means of multiple groups, and a post compression test (Tukey's multiple comparison test) was used to confirm where differences occurred between groups. * P 0.05; ** P 0.01, and *** P 0.001. Columns are mean \pm SD.

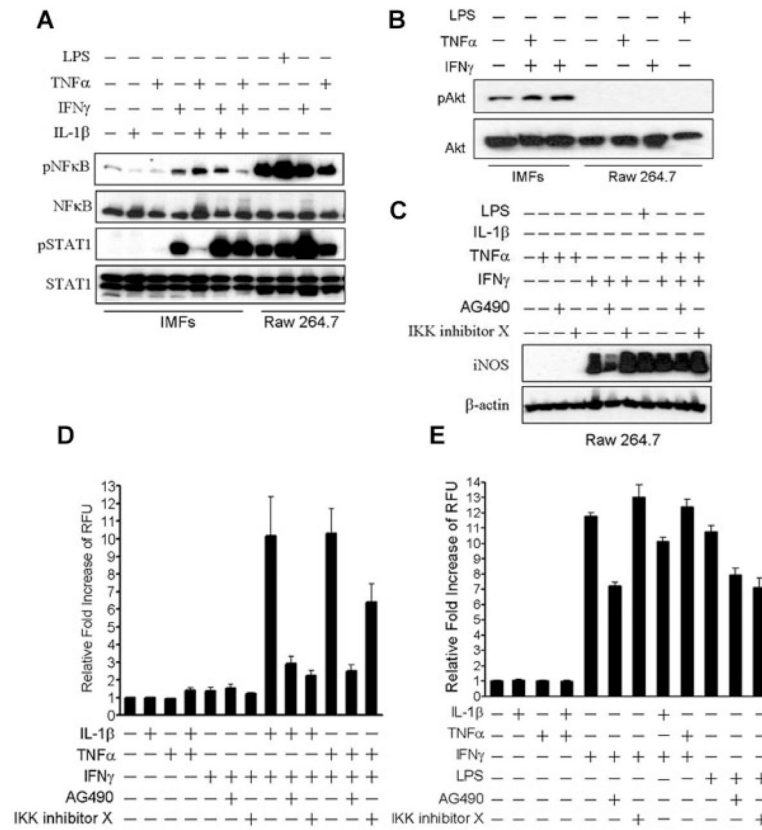
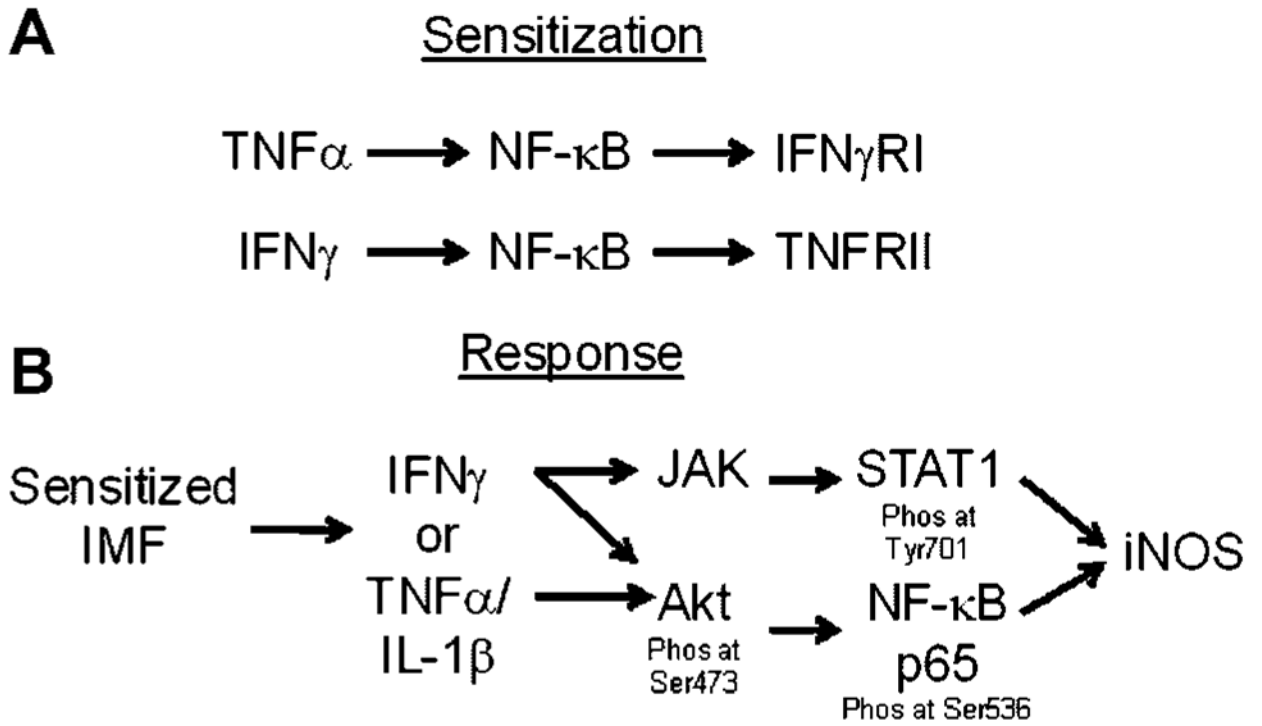


Fig. 5. NO production mediated by increased iNOS expression in IMF is regulated through a pathway distinct from macrophages. **A:** Western blots with antibodies specific to phosphorylated Ser536 on p65 NF- κ B, total NF- κ B, phosphorylated Tyr701 on STAT1, and total STAT1 using IMF and Raw 264.7 murine macrophages stimulated for 15 min with 100 ng/ml LPS, 5 ng/ml IL-1 β , 100 ng/ml IFN γ , or 10 ng/ml TNF α . **B:** Western blots with antibodies specific to phosphorylated Ser473 on Akt or total Akt using IMF and Raw 264.7 cells stimulated for 20 h with 100 ng/ml LPS or the indicated cytokines as above. **C:** Western blots with antibodies specific to iNOS or β -actin using IMF and Raw 264.7 cells treated with the indicated inhibitors and cytokines for 20 h. Fluorescence measurements of IMF (**D**) and Raw 264.7 cells (**E**) loaded with 4,5-diaminofluorescein after treatment with the indicated inhibitors and cytokines for 24 h.

**Fig. 6.**

Model of combinatorial cytokine regulation of iNOS expression in IMF. Stimulation of IMF with $TNF\alpha$ increases expression of $IFN\gamma RI$ and thereby sensitizes the cell to respond to $IFN\gamma$ (A). Subsequent exposure to $IFN\gamma$ triggers activation of JAK/STAT and Akt/NF- κB pathways that lead to increased expression of iNOS (B). Alternatively, stimulation of IMF with $IFN\gamma$ increases the expression of $TNFR II$ and thereby sensitizes the cell to respond to $TNF\alpha$ (A). Subsequent exposure to $TNF\alpha$ alone or in combination with $IL-1\alpha/\beta$ triggers activation of an Akt/NF- κB pathway that leads to increased expression of iNOS (B).