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# Epigenetic silencing of the human *NOS2* gene: Rethinking the role of nitric oxide in human macrophage inflammatory responses[1]

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## Abstract

Macrophages, including alveolar macrophages, are primary phagocytic cells of the innate immune system. Many studies of macrophages and inflammation have been done in mouse models, where inducible nitric oxide synthase (NOS2) and nitric oxide (NO) are important components of the inflammatory response. Human macrophages, in contrast to mouse macrophages, express little detectable NOS2 and generate little NO in response to potent inflammatory stimuli. The human *NOS2* gene is highly methylated around the *NOS2* transcription start site. In contrast, mouse macrophages contain unmethylated cytosine-phosphate-guanine dinucleotides (CpGs) proximal to the *NOS2* transcription start site. Further analysis of chromatin accessibility and histone modifications demonstrated a closed conformation at the human *NOS2* locus and an open conformation at the murine *NOS2* locus. In examining the potential for CpG demethylation at the *NOS2* locus, we found that the human *NOS2* gene was resistant to the effects of demethylation agents both *in vitro* and *in vivo*. Our data demonstrates that epigenetic modifications in human macrophages are associated with CpG methylation, chromatin compaction and histone modifications that effectively silence the NOS2 gene. Taken together, our findings suggest there are significant and under-appreciated differences in how murine and human macrophages respond to inflammatory stimuli.

### Keywords

Gene regulation; macrophages; inflammation

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## Introduction[4]

Alveolar macrophages are primary phagocytic cells of the innate immune system (1, 2). They are responsible, among other functions, for clearing infectious, toxic and allergic particles from the respiratory tract. In the case of murine macrophages, the response to inflammatory stimuli is characterized by the production of large amounts of NO via NOS2 (3–5). NO produced by murine alveolar macrophages is a critical component in the removal of bacteria and foreign particles (6). It has been shown to be crucial to murine alveolar macrophage function and host survival (6–8).

The murine alveolar macrophage has been frequently used as a model for human alveolar macrophage physiology. NO production by murine macrophages has been inferred to explain human inflammatory pathophysiology. However, there is scant evidence describing NO production in human alveolar macrophages to support such an extrapolation (9, 10). What data there is consistently shows that, even when stimulated, healthy human macrophages make little to no NO (9, 11–13). In murine models, inhibiting NO production impairs clearance of intracellular pathogens. Similar treatment of human alveolar macrophages has no effect (14). Further complicating the current landscape is data showing that human alveolar macrophages recovered from patients with chronic inflammatory diseases are capable of making measurable, but small, amounts of NO (15–18). Thus, it remains unclear whether NO production is relevant to human macrophage physiology or macrophage-mediated pathophysiology. Given this apparent contrast with the central role NO plays in murine macrophage inflammation, we consider it essential to clarify whether these animal findings can be appropriately extrapolated to the human arena.

Epigenetic programming is one of the mechanisms that controls cell type specific transcription of genes. Epigenetic control involves multiple regulatory mechanisms that include untranslated RNA, histone acetylation, and methylation of histones and DNA CpG dinucleotide sequences (19–22). DNA methylation at the C-5 positions of cytosine (5mC) in the CpG dinucleotide is a well-characterized epigenetic modification controlling gene expression (23, 24). Methylation of CpG motifs in the promoter region has been repeatedly shown to influence DNA transcription (primarily decreasing transcription) and is an important step in both normal and malignant cell function (25–28). CpG methylation, coupled with subsequent histone deacetylation, condenses chromatin, and leads to gene silencing (29–31). This mechanism relies on methyl-CpG-binding domain proteins that recognize methylated cytosines in the CpG dinucleotides. Altering gene regulation through the reversal of DNA methylation and histone acetylation is a recent addition to clinical therapeutics. In some clinical trials, cancer chemotherapeutic agents are used specifically to promote gene transcription via CpG demethylation and histone deacetylation (32–34).

In this study, we examined the effect of classic *NOS2* activating stimuli on *NOS2* expression and NO production in human macrophages. We found that human macrophages failed to produce NO even after stimulation with potent inflammatory signals. Furthermore, we found that in contrast to mouse macrophages, human macrophages did not express detectable levels of NOS protein coded by the *NOS2* gene. We found that CpG motifs proximal to the

[4]The abbreviations used are: 5-aza-dC, 5-aza-2′deoxycytidine; BMDMs, bone marrow-derived macrophages; ChIP, Chromatin immunoprecipitation; CpG, cytosine-phosphate-guanine dinucleotides; FBS, fetal bovine serum; FEV1, Forced expiratory volume in 1 second; FVC, forced vital capacity; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H3K27me3, trimethyl-histone H3 (Lys27); H3K4me3, trimethyl-histone H3 (Lys4); HBB, β-globin gene; HbF, hemoglobin F; HDAC, histone deacetylase; ICAM1, intercellular adhesion molecule 1; IFNγ, interferon gamma; LPS, lipopolysaccharide; MHS, murine alveolar macrophage cell line; NOS2, inducible nitric oxide synthase; NO, nitric oxide; PBS, phosphate buffered saline; RAW 264.7, murine peritoneal macrophage cell line; RPL30, 60S ribosomal protein L30; THP-1, human monocytic leukemia cell line; TNFα, tumor necrosis factor α; TSS, transcription start site.

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transcription start site (TSS) of the human *NOS2* promoter region are fully methylated, consistent with an epigenetically repressed gene. In contrast, CpG motifs proximal to the TSS in mouse macrophage *NOS2* were not methylated. Assays for chromatin accessibility and histone regulatory marks were consistent with the hypothesis that the human macrophage *NOS2* gene is a silenced gene. These data describe previously unrecognized differences between human and mouse macrophages and suggests that murine data be used with caution when inferring links to human disease.

## Materials and Methods

### Cell lines

Human alveolar macrophages, human blood monocytes, a human monocytic leukemia cell line (THP-1), murine alveolar macrophage cell line (MHS) and murine peritoneal macrophage cell line (RAW 264.7) were used. THP-1 and MHS cells were cultured in RPMI supplemented with 5ml L-glutamine, 5ml 1M Hepes, 5ml 100mM sodium pyruvate, 5ml of 250mg/ml glucose, 5% fetal bovine serum (FBS), 5ml Pen/Strep per liter. Human monocytes were cultured in RPMI-1640 supplemented with gentamicin 40μg/ml and 5% FBS and human and murine alveolar macrophages were cultured in RPMI-1640 supplemented with gentamicin 40μg/ml.

### Bronchoalveolar lavage

After informed consent was obtained under a Carver College of Medicine IRB-approved protocol, nonsmoking volunteer research subjects performed spirometry with exclusion criteria of an FEV1 or FVC < 80%. Next, they underwent standard flexible fiberoptic bronchoscopy. Premedication with intramuscular morphine 10mg injection was followed by local anesthesia with lidocaine instillation into the upper airway. Standard bronchoalveolar lavage was performed by serially instilling and suction retrieving 20 ml aliquots of normal saline five times in three different lung segments. The first collection was discarded to avoid possible contamination with upper airway secretions or lidocaine. The remaining lavage was filtered through sterile gauze and centrifuged at 200g for 5 minutes to pellet cellular material. The resulting pellet was suspended in phosphate buffered saline (PBS) and centrifuged at 200g for 5 minutes. A sample of the cells were labeled with Wright stain and microscopically examined to determine the proportion of the cells that were macrophages. Aliquots of $5\times10^6$ cells were stored at minus 80°C until RNA and DNA isolation procedure was performed. The procedure generated a relatively pure population of alveolar macrophages with fewer than 5% neutrophils or lymphocytes.

### Human blood monocyte isolation

As part of the bronchoscopy protocol, some subjects underwent venipuncture with 180cc of blood drawn. Mononuclear cells were isolated from the gradient interface after centrifugation with Histopaque (Sigma-Aldrich, St. Louis, MO, USA). Monocyte isolation was performed with BigEasy Easy Sep system (StemCell, Vancouver, Canada) according to the manufacturer's protocol and as described previously (35).

### Mouse bronchoalveolar lavage

Mice were anesthetized, and the chest cavity was opened. The trachea was exposed, an incision made, and tubing inserted. The lungs were flushed with 3 × 1 ml PBS. Cells were pelleted. Slides were then made from the pelleted cells and determined to be greater than 95% macrophages by Wright stain.

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### Murine bone marrow derived macrophage isolation

To generate bone marrow derived macrophages, C57/Blk6 mice were euthanized and the tibias and femurs were harvested. Inside a sterile hood, bones were dipped in ethanol and submerged in DMEM supplemented with 10% fetal calf serum, L-glutamine and penicillin/streptomycin (DMEM). The ends of the bones were cut off with a sharp scissors and the bone marrow flushed out with DMEM using a syringe and 25G needle (approximately 3 mls per fibula and 2mls per tibia). Cells were centrifuged and resuspended in DMEM (6 ml/mouse) with an added 7 mls of DMEM and 2 mls of L cell conditioned medium (LCCM, obtained by culture of L929 cells in DMEM for 5 days). Cells were incubated for 6 days at 37°C and then removed from plates using .05% Trypsin/EDTA (Life Technologies). Cells were resuspended in DMEM at 1 million per ml and seeded onto the appropriate tissue culture dishes for the proposed experiments.

### DNA and RNA isolation

DNA was isolated from macrophages using QIAgen DNAeasy Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA was isolated using the mirVana miRNA Isolation kit (Applied Biosystems, Austin, TX, USA). The quantity and quality of the RNA samples was assessed using an Experion Automated Electrophoresis Station (Bio-Rad, Hercules, CA, USA). The RNA quality indicator was above 8 for all samples where values of greater than 8 indicate primarily intact RNA on a scale of 1–10. After preparation, RNA samples were stored at −80°C until use.

### DNA methylation analysis

Determination of genome-wide methylation values was conducted under contract by the University of Minnesota BioMedical Genomics Center (Minneapolis, MN, USA) using the Illumina Infinium 450K Human Methylation array, which contains 485,000 probes that interrogate CpG residues in 99% of RefSeq annotated genes (NCBI, Bethesda, MD, USA). The resulting microarray data were inspected for complete bisulfite conversion of the DNA. Average β-values (i.e., average methylation) for each CpG residue were determined using the GenomeStudio V2009.2, methylation module version 1.5.5 version 3.2 (Illumina, San Diego, CA, USA). Comparison of β-values (i.e., methylation) between cases and controls was conducted using Student's *t*-test, whereas comparisons of the relationship between overall values between individual arrays were conducted using Pearson's correlation coefficient.

### Primer design

We used Genome Browser (http://genome.ucsc.edu/index.html) to obtain the sequence of the *NOS2* gene promoter region from −2000 base pairs upstream of TSS to the second exon. We used the publicly available online program Meth Primer (http://www.urogene.org/methprimer/index1.html) to obtain predicted bisulfite-converted sequence. Primers were designed based on this sequence (Supplemental Table 1).

### Bisulfite sequencing

DNA was bisulfite modified then amplified using EZ DNA Methylation Kit (Zymo Research) according to the manufacturer's instructions. The DNA samples were amplified using a touchdown nested PCR protocol (Supplemental Table 1) with primers designed for the human and murine *NOS2* promoter regions (Supplemental Table 2). The PCR product was gel purified and extracted using QIAquick Gel Extraction Kit according to the manufacturer's instructions. PCR products were then sequenced at the University of Iowa DNA Facility.

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### Western analysis

Whole cell protein was obtained by lysing the cells on ice for 20 minutes in 200 μl of lysis buffer (0.05 M Tris pH 7.4, 0.15 M NaCl, 1% NP-40) with added protease and phosphatase inhibitors: 1 protease minitab (Roche Biochemicals)/10 ml and 100 ul 100X phosphatase inhibitor cocktail (Calbiochem)/10 ml. The lysates were sonicated for 20 seconds, kept at 4°C for 30 minutes, spun at 15,000 g f.or 10 minutes and the supernatant saved. Protein determinations were made using the Bradford Protein assay from Bio-Rad. Cell lysates were stored at −70° until use.

Western analysis was performed on whole cell proteins. 30 μg of protein was mixed 1:1 with 2x sample buffer (20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.05% bromophenol blue and 1.25 M Tris pH 6.8, all chemicals from Sigma Chemical Co.) heated to 95o for 5 minutes and loaded onto a 10% SDSPAGE gel and run at 100 V for 90 minutes. Cell proteins were transferred to PVDF membranes (Bio-Rad Hercules, CA) by semi-dry transfer (BioRad). Equal loading of the protein groups on the blots was evaluated using Ponceaus S, a staining solution designed for protein quantification or by stripping and reprobing with antibodies to β-actin or GAPDH. The PVDF was dried and then incubated with the primary antibody overnight in 5% milk. The blots were washed four times with TTBS and incubated for 1 hour with horseradish-peroxidase conjugated anti-rabbit or mouse IgG antibody as a control. Immunoreactive bands were developed using a chemiluminescent substrate (ECL Plus, Amersham, Arlington Heights, IL). An autoradiograph was obtained, with exposure times of 10 seconds to 2 minutes.

### Griess assay of nitric oxide production

Measurement of NO production was performed using the Griess reaction as described previously (13, 36) using Griess Reagent Kit for Nitrite Determination (Invitrogen, NY, USA) according to the manufacturer's protocol.

### Chromatin accessibility assay

Cells were cultured with or without stimulation with IFNγ and LPS, harvested and pelleted by centrifugation. Cells were washed with ice cold PBS and then lysed with lysis buffer (cold RSB + 0.10% NP-40). Nuclei were isolated by centrifugation. Nuclear DNA was digested with increasing concentrations of DNAse (0U, 2.5U, 5U, 10U) for 10 minutes at 37o. DNA was isolated with Qiagen DNAEasy Kit as described above. DNA was then purified and used for quantitative PCR. We designed primers that cover genomic sequences proximal to the TSS for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), a housekeeping gene; 60S ribosomal protein L30 (*RPL30),* a transcriptionally active gene; intercellular adhesion molecule 1 (ICAM1), an inflammatory response gene; β-globin gene (HBB) a known transcriptionally silent gene (silent in nonerythroid cells); tumor necrosis factor α (TNFα), an inflammatory response gene and NOS2 (Supplemental Table 2).

### Chromatin immunoprecipitation (ChIP) assay

ChIP Assay was performed using a ChIP assay kit from Cell Signaling Technology (Beverly, MA, USA). Briefly, macrophage proteins and DNA were cross-linked with formaldehyde. Cells were lysed in SDS-lysis buffer and then sonicated. For sonication, the amplitude setting was 20%. The time setting was as follows: 1″ sonicate, 1″ off for a total of 10 each or 20″ total, rest 30″ on ice then repeat for a total of 3 times. Sonication was performed using a Sonics Vibra Cell Model CV18, Sonics and Materials, Inc. 50 ul of sheared chromatin was used to analyze chromatin digestion, with most of DNA sample found to be in the appropriate 500–600 bp size range. 10 ul of purified chromatin was stored as "input sample". The remaining chromatin was diluted with provided ChIP buffer and then

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immunoprecipitation performed using anti-trimethyl-histone H3 (Lys27) (H3K27me3) polyclonal rabbit IgG (Cell Signaling, Beverly, MA), anti-trimethyl-histone H3 (Lys4) (H3K4me3) polyclonal rabbit IgG (Millipore, Temeluca, CA), or a negative control antibody (Normal Rabbit IgG #2729). ChIP-Grade Protein G Magnetic Beads were added to the incubation mix. After bead separation with a magnetic separation rack, chromatin was eluted from the antibody/Protein G beads with ChIP Elution Buffer. DNA was purified and used for quantitative PCR.

### Demethylation and histone deacetylation treatment of therapy-resistant melanoma patients

DNA samples were obtained from conventional treatment resistant metastatic melanoma patients enrolled in an experimental protocol at the University of Iowa Hospitals and Clinics. The research treatment protocol included 5-azacytidine (a DNA methyltransferase inhibitor), a histone deacetylase inhibitor and an oral alkylating agent. Patients were consented and blood drawn at designated time points (day 0 and 2 to 3 weeks following the second treatment cycle) (Figure 7A). Demethylation of DNA was monitored by measuring serum hemoglobin F levels with high power liquid chromatography as described previously (38). For an analysis of DNA methylation at the *NOS2* gene, DNA was isolated from subject blood mononuclear cells and bisulfite converted. CpG methylation was determined at the *NOS2* locus by sequencing the region proximal to the TSS (primers are described in the Supplemental Files).

## Results

### Human macrophages do not increase NO production or NOS2 expression in response to inflammatory stimuli

We studied primary human alveolar macrophages, the human macrophage cell line THP-1, bone marrow-derived murine macrophages, and the murine macrophage cell line MHS. Cells were stimulated with interferon gamma (IFNγ) and lipopolysaccharide (LPS) for twenty-four hours. Nitrite levels were measured in the cell culture supernatants. In human macrophages only minimal nitrite levels were released at baseline, with no sign of a response to the stimuli (Fig. 1A). In stark contrast, murine macrophages made large amounts of NO in response to stimulation (Fig. 1A). Next, we measured NOS2 protein levels in the cell lysates of human alveolar macrophages, human THP-1 cells, murine MHS macrophages and mouse bone marrow-derived macrophages after stimulation with IFNγ and LPS. NOS2 protein amounts were in line with our findings with NO showing a robust increase with stimulation in murine macrophages and no detectable NOS2 protein in human macrophages (Fig. 1B). NO measurements also correlated with *NOS2* mRNA levels determined by qRT-PCR. There was an impressive increase in NOS2 mRNA with stimulation in bone marrow-derived mouse macrophages (> 500 fold) and in the murine MHS cell line (>10,000 fold). There were barely detectable levels of *NOS2* mRNA present in human alveolar macrophages at baseline, with almost no increase following stimulation (Fig. 1C).

### Human alveolar macrophages have an intact LPS/ IFNγ inflammatory pathway when stimulated

Having found that human alveolar macrophages lacked NOS2 expression, we next asked if the signaling cascade downstream of the LPS and IFNγ receptors was intact. To evaluate signaling events downstream of the LPS (TLR4) and IFNγ receptors, we evaluated STAT1 activation. STAT1 is a transcription factor that is activated via phosphorylation synergistically induced by the combination of LPS and IFNγ (37). Figure 2A demonstrates substantial activation of STAT1 by LPS/IFNγ reflected in the rapid increase in phosphorylated STAT1. To examine whether proteins other than NOS2 known to be

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induced by LPS/IFNγ were up-regulated in stimulated human alveolar macrophages, we examined intracellular adhesion molecule-1 (ICAM1). ICAM1 is a transmembrane protein that is expressed upon cytokine stimulation and facilitates macrophage migration past the endothelium (38). Stimulation of human alveolar macrophages with LPS/IFNγ led to significant upregulation of *ICAM1* mRNA (Figure 2B); the murine macrophage cell line MHS showed a similar response. Stimulation of human alveolar macrophages also led to significant increase in ICAM1 protein (Figure 2C). These data demonstrate that there is not a global defect in LPS and IFNγ signaling in human macrophages and that the signaling cascade downstream of the LPS/IFNγ receptors in human alveolar macrophages is intact and upregulates inflammatory genes other than *NOS2*.

## CpG motifs in the NOS2 promoter region in human alveolar macrophages and monocytes are uniformly methylated

There is convincing evidence indicating that CpG methylation at the promoter region and first exon is an important epigenetic mechanism regulating the activation of gene transcription (39). Because we had found that intracellular signaling and downstream expression of other inflammatory genes was intact in stimulated human macrophages, we hypothesized that DNA methylation might be responsible for the lack of transcription of the *NOS2* gene in human alveolar macrophages.

First, we assessed methylation levels at the *NOS2* gene promoter region in data from an Illumina 450K array. The Illumina array allows for a broad analysis of methylation levels in more than 450,000 probes across the genome. The Illumina array includes a number of probes upstream of and leading into the *NOS2* gene. In examining beta values (average methylation of 7 probes covering −1500bp upstream of TSS to the end of 1$^{st}$ exon) from an Illumina array (alveolar macrophages from 5 normal subjects), we found high levels of methylation at probes that clustered around the TSS of the *NOS2* gene (Figure 3A). This was in stark contrast with the low methylation levels found at probes around the TSS of the LPS/IFNγ inducible gene, *ICAM1* (Fig 3 B).

Given the results from the Illumina methylation array, we next examined in greater detail the CpG motifs around the *NOS2* TSS. To do this, we compared bisulfite sequencing of macrophage DNA from human (alveolar macrophages, blood monocytes, monocytic cell line THP-1) and murine sources (C5BL6 alveolar and bone marrow-derived macrophages and MHS macrophage cell line). The DNA was bisulfite converted and DNA sequencing of the *NOS2* promoter region performed. Primers designed for the sodium bisulfite-treated DNA were used to sequence the region from approximately 1000 bp upstream of the TSS to the end first exon in the human and murine DNA. We found that across all the human macrophages, CpG motifs in that region were uniformly methylated (Fig 3C). For the murine cells, the data was distinctly different. By contrast, we found that CpGs in the region 400 bp upstream of the TSS and in exon 1 contained many unmethylated CpG motifs (Figure 3 D).

Based on previous data (40), we asked whether stimulation with cytokines could reverse CpG methylation. We stimulated THP-1 cells with a combination of cytokines and found unchanged methylation of CpG motifs in the promoter region after stimulation (data not shown).

## Chromatin Immunoprecipitation Assay (ChIP) reveals silencing histone modification at the NOS2 gene in human alveolar macrophages

A ChIP assay generates data on whether a particular gene region is associated with repressive or transcriptionally active histone marks; this, in part, determines whether a given

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promoter is “open” or “closed” to transcription factors. Methylation of lysines on histones can result in either promotion or inhibition of transcription, based on the level of methylation and position of the lysine. It has been previously shown that, near gene transcription start sites, tri-methylation of lysine H3K4 is linked to active transcription while tri-methylation of lysine H3K27 is linked to transcriptional silencing. (41).

First, we established the efficacy of our immunoprecipitation using antibodies to H3K4me3 and H3K27me3 in lysates of THP-1cells (Fig 4A). Next, a ChIP assay was performed on formaldehyde fixed THP-1 cells and human alveolar macrophages.. We used antibodies to H3K4me3 and H3K27me3 to immunoprecipitate histone H3 protein. We then isolated DNA from the precipitated protein and performed qPCR using primers for *NOS2*, *ICAM1*, housekeeping gene *GAPDH*, *RPL30*, *TNFα* and a known silent gene, *HBB*. In both human alveolar macrophages and THP-1 cells, there was association of actively transcribed genes (*GAPDH, ICAM1, RPL30, and TNFα*) with the activating histone mark H3K4me3 and association of *NOS2* and *HBB* with the silencing H3K27me3 modification (Fig. 4A & B). When these same studies were performed using mouse bone marrow-derived macrophages and the murine MHS cell line, *NOS2* DNA was found associated with the activating histone modification H3K4me3 as would be expected of an accessible gene (Fig 4C & D). Thus, *NOS2* has characteristics of a silenced gene in human macrophages and looks more like an open gene in mouse cells.

## Association of the human NOS2 gene with H3K27me3 is resistant to inflammatory stimuli

In mice, macrophage NO expression increases dramatically with inflammatory stimuli. To determine if the association of the human *NOS2* gene with the gene silencing histone modification, H3K27me3, was changed by inflammatory stimuli, human macrophages were stimulated with IFNγ and LPS. A ChIP assay was performed on control and stimulated cells. In both types of human macrophages, stimulation increased H3K4me3 association in the active genes (*ICAM1*, *RPL30, and TNFα*; Fig 5A). Stimulation did not increase activating histone marks associated with the known silenced gene HBB or *NOS2* (Fig 5B); the activating/silencing mark ratio remained negative after stimulation. In comparison, mouse macrophages (MHS) stimulated with IFNγ and LPS demonstrated an increase in the association of the *NOS2* gene with H3K4me3 (Fig 5C). Thus, *NOS2* behaves like a “closed” gene that cannot be modulated by acute inflammatory signaling in human macrophages.

## Chromatin accessibility assay indicates a closed chromatin conformation at the NOS2 locus in human alveolar macrophages

Because the ChIP assay suggested closed chromatin at the human *NOS2* gene, we went on to directly measure chromatin accessibility. Chromatin accessibility assays provide data regarding the “closed” or “open” state of chromatin proximal to a particular gene. The assay tests the ability of DNAse to cleave and degrade the relevant DNA. Data is outlined as an Accessibility Index (accessibility index = 2^((Ct DNAse treated) - (Ct Uncut)). Thus, a higher number implies more degradation by DNase and, therefore, a more accessible gene. Previous research has shown strong inverse correlation between DNA methylation and chromatin accessibility (42). qPCR performed on DNAse treated human THP-1 cell nuclei revealed striking differences in chromatin accessibility between known actively transcribed genes (*GAPDH, RPL30, ICAM1*) and a known transcriptionally silent gene, HBB. In a series of 3 separate experiments, the *NOS2* gene index was similar to the silent *HBB* gene (Fig. 6A). To ask if stimulation would change chromatin accessibility at the *NOS2* locus, we stimulated human alveolar macrophages with IFNγ and LPS, followed by a chromatin accessibility assay. qPCR performed for *ICAM1* and *TNFα* revealed even higher indices of accessibility after stimulation, while the *NOS2* gene remained inaccessible regardless of stimulation (Fig. 6B). To ask if the murine *NOS2* gene was more accessible than the human

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gene, we performed a chromatin accessibility assay on treated mouse MHS macrophages. In contrast with the human *NOS2* gene, murine *NOS2* gene segregated with known actively transcribed genes *GAPDH* and *ICAM1* (Fig. 6C).

These data collectively demonstrate that the difference between the mouse and human macrophage NO response to inflammatory signaling is due to epigenetic silencing of the gene in human cells.

### 5-aza-2′-deoxycytidine (5-aza-dC) and Trichostatin A (TSA) induce changes in NOS2 promoter methylation profile in murine MHS cells

We tested the ability of the DNA methyltransferase inhibitor 5-aza-dC and the histone deacetylase inhibitor TSA to alter CpG methylation at the human *NOS2* locus *in vitro*. The *NOS2* TSS in THP-1 cell was fully methylated (except for two CpGs at +117 and +165) and we were unable to change the methylation profile with drug exposure (Fig 7A); this data was consistent with previous findings that showed an inability to demethylate the heavily methylated *NOS2* promoter in human endothelial cells (43). This is consistent with our hypothesis that genes that are heavily methylated and associated with histone silencing marks are resistant to the effects of demethylating agents.

To ask if an already open locus (mouse *NOS2*) was sensitive to DNA methyltransferase inhibition, we treated the murine macrophage cell line, MHS, with 5-aza-dC and TSA. At baseline, sequencing of MHS DNA revealed a string of demethylated CpGs at the beginning of *NOS2* promoter and upstream of it. Subjecting these cells to a protocol of 5-aza-dC and TSA treatment resulted in further demethylation of CpG targets upstream and downstream of the *NOS2* promoter region (Figure 7A). This, again, supports our observations that the *NOS2* gene is open for regulation in the mouse, while it is closed to manipulation in human macrophages.

### 5-aza-dC and TSA do not induce demethylation changes in NOS2 promoter methylation profile in human blood DNA during in vivo treatment

We next asked if *in vivo* exposure to a demethylating agent and inhibitor combination would alter CpG methylation at the *NOS2* locus. 5-aza-dC and histone deacetylase inhibitors (HDAC) are being used in clinical trials for some malignancies, including melanoma. The hope is that the combined drug therapy will reverse methylation of tumor suppressor genes and slow the progression of the cancer. In a trial here at the University of Iowa, patients with advanced melanoma are treated with a protocol that includes 5-aza-dC and a histone deacetylase inhibitor (Figure 7A). We have collected blood before and after treatment and examined DNA methylation of the *NOS2* gene. The clinical effect of the demethylating agents was confirmed by examining Hemoglobin F levels (HbF). During fetal development, HbF is the dominant hemoglobin isoform. From approximately 6 months post-partum through adulthood it is gradually replaced by Hemoglobin A. This process of “hemoglobin switching” is controlled epigenetically by methylation of the HbF (γ-globin) gene promoter in bone marrow erythroblasts (44). We followed four patients enrolled in the current trial. Two of these had a rise in HbF while the other two had unchanged levels of HbF during the course of treatment (Figure 7 B). We analyzed bisulfate-converted DNA for methylation of the *NOS2* promoter in all 4 patients prior to treatment and then later post-treatment as outlined in the “Methods” section. We found two CpGs (+3 and +165) changed in one patient who showed a HbF response after treatment (Figure 7C). All other CpGs remained methylated. The second patient who responded to treatment with increase in HbF, along with 2 “non-responders” showed no changes in methylation pattern. This data suggests that the human *NOS2* gene is resistant to the effects of a demethylating agent and HDAC inhibitor *in vivo* as well as *in vitro*.

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## Discussion

The major conclusion of our study is that human macrophages do not produce NO in response to acute inflammatory stimuli as a result of epigenetic regulation of the *NOS2* gene (Figure 8). This conclusion is based on several complementary lines of experimental evidence. First, we demonstrate that despite an otherwise intact inflammatory response, human alveolar macrophages do not make NO or increase expression of *NOS2* when stimulated. Next, we demonstrate uniform methylation of CpGs in the *NOS2* promoter region in human macrophages. Consistent with the CpG methylation data, ChIP assays demonstrate that the human macrophage *NOS2* gene is associated with a silencing histone mark and has tightly compacted chromatin rendering it inaccessible to transcription factors. Stimulation of human macrophages with LPS and IFNγ does not alter the *NOS2* the ChIP assay results or chromatin accessibility. Finally, we show that this epigenetic silencing of the NOS2 region in human macrophages is resistant to the effects of demethylating agents both in circulating leukocytes *in vivo* and in a human macrophage cell line *in vitro*. Collectively, this experimental evidence strongly supports the overall concept that human macrophages, including primary alveolar macrophages, do not produce NO in response to inflammatory stimuli because of epigenetic silencing of the *NOS2* gene.

Our human findings stand in stark contrast to the physiology of normal mouse macrophages. We find that in mouse macrophages the CpG motifs proximal to the *NOS2* TSS are unmethylated. Furthermore, murine macrophage *NOS2* segregates with “open” genes on chromatin accessibility assay and ChIP analysis reveals murine *NOS2* is associated with H3K4me3, a histone mark that correlates with active transcription. Thus, it appears that the regulation of NO as an inducible inflammatory response in the mouse mononuclear phagocyte does not extend to human cells.

Our studies are consistent with a larger body of work on the overall differences in inflammation-related gene expression between mice and men (45). The literature is full of conflicting reports on the role of NO in human inflammation. Schneeman et al performed a comprehensive analysis of human monocytes in 1993 (11). They found that human mononuclear phagocytes exposed to an array of potent stimuli did not produce nitrite, consume L-arginine, produce L-citrulline or display NOS activity. Weinberg et al, found minimal immunoreactive NOS2 protein or *NOS2* mRNA in human macrophages stimulated for 3 days in culture; secreted NO was not detected (46). This is similar to our studies demonstrating neither induction of *NOS2* mRNA or protein with inflammatory stimuli. Any subtle discrepancies likely reflect differences in intensity of the stimulation used and/or purity of cell populations studied.

The actual role of NO in human lung disease remains incompletely defined. Exhaled NO is a marker of active inflammation in asthma, sarcoidosis and ARDS (18, 47–50). Whether NO is beneficial or harmful is unclear and the cells of origin not certain (51–55). There is evidence showing NO production in more chronic human lung diseases, such as granulomatous infections and pulmonary fibrosis (15–18). Immunoreactive NOS2 has been demonstrated in chronic granulomas, though absent in nodal or tissue mononuclear phagocytes (75). This suggests that in the appropriate chronic inflammatory context, epithelioid granulomas may uniquely acquire the ability to express functional NO. This does not, however, appear to be an important aspect of normal human macrophage physiology. Despite this defect in acute NO production, human alveolar macrophages have other mechanisms for effective pathogen killing (56, 57). This seems to stand in contrast with the purported central importance of high-level macrophage NO production in mouse lung defense (58, 59). Furthermore, there are conflicting data regarding the consequences of blocking NO production in murine models of acute lung injury and macrophage-dependent

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pathogen infection (5, 7). What these findings demonstrate is that we do not yet fully understand the role of NO in lung cell physiology across species. Identifying the molecular basis for this variant inflammatory response may yield important cross-species insights (9, 11, 60, 61).

This is the first study to explore epigenetic silencing of the *NOS2* gene in human macrophages and to compare it with mouse macrophages. Our conclusion that differential CpG methylation contributes to decreased NO response in human macrophages is supported by a study in endothelial cells where methylation of the *NOS2* core promoter areas contributed to decreased induction of the *NOS2* gene in human endothelial cells (43). Extending this observation, we find that differential methylation upstream of the *NOS2* TSS correlates with concurrent differences in chromatin accessibility and histone modifications that predict gene silencing. Human macrophages do not produce NO constitutively or in response to acute inflammatory signals because the *NOS2* gene is epigenetically switched off.

Methylation of CpG motifs is one of several universal epigenetic mechanisms that control gene expression in both plants and animals (62–64). The preponderance of data linking promoter area CpG methylation and gene expression comes from studies on genes with CpG islands (an area longer than 500 bp with an observed CpG/expected CpG ratio ≥ 0.65) at the promoter region (65). While there is no CpG island in the *NOS2* promoter, we have identified a number of CpGs proximal to the TSS that are differentially methylated between mouse and human macrophages. Recent literature supports the importance of these TSS-proximal, non-island, CpGs in regulation of gene expression (66–70). These particular studies identified the role of CpG methylation (proximal to the TSS without being in an island) in transcription of the IL-2, IFNγ, MMP13, IL1β and TNFα genes.

DNA methylation depends on an array of methyltransferases, enzymes capable of de novo methylation and demethylation of genomic DNA, along with maintenance of methylation in daughter cells (71, 72). Human and mouse macrophages express DNA methyltransferases DNMT1, 3A, and 3B that can be inhibited with 5-azacytidine with resultant reduced methylation in replicating daughter cells. Our efforts to demethylate the *NOS2* TSS met with only modest success. In murine cells that already had many demethylated CpG motifs at baseline, *in vitro* inhibition of DNA methyltransferase was successful. Human cells, both *in vivo* and *in vitro*, proved more resistant to the demethylation agent. In some melanoma patients who received a DNA methylation inhibitor on an experimental treatment protocol a rise in blood HbF levels signaled demethylation at the bone marrow erythroblast level. The dose or duration of chemotherapeutic agents may not have been inadequate to demethylate the NOS locus in circulating leukocytes or the *NOS2* gene may be more resistant to such manipulation. Toxicity of the DNA methyltransferase inhibitors can impair cell viability *in vitro* and demethylation agents work best on rapidly dividing cells. Our work is consistent with previous reports in which 5-aza-dC and TSA failed to alter DNA structure in tissue culture (73–75).

In this study we demonstrate silencing at the NOS2 gene at a number of levels: 1. CpG motifs proximal to the human TSS are methylated. 2. Histone modifications consistent with gene silencing are found at the NOS2 gene (H3K27me3). 3. Histone modifications consistent with active gene activity (H3K4me3) are in low abundance at the NOS2 promoter region. 4. The NOS2 locus is relatively protected from DNAse treatment suggesting closed chromatin. Our conclusion from these studies is that the NOS2 gene in human macrophages is silenced. Alternatively, production from this locus may be below the sensitivity of present measurement techniques or there may be transcription of a very unstable transcript. At this time, we can’t definitively rule out these possibilities, but consider them unlikely.

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There are a number of potential weaknesses in this study. One of these is the lack of data showing that with demethylation of the NOS2 proximal CpGs, NOS2 is transcribed. Another weakness is the limited number of human cell types that are analyzed. It would be nice to know if the silencing is true for macrophages alone or is also true in other cell types such as airway epithelial cells. For the patient studies, this is only preliminary data and future trials with more patients and higher does of the DNMT and HDAC inhibitors may be more fruitful.

In future studies, we will be examining more cell types for silencing of the NOS2 locus. We will be asking whether this locus is dynamically regulated and further testing whether demethylation with a DNMT inhibitor is possible. This project was started because of our interest in human inflammation and its links to both chronic and acute diseases. There are some papers suggesting that mycobacterial infection generates nitric oxide from human macrophages (76, 77). There are also suggestions that nitric oxide plays an important role in COPD, ARDS, sarcoidosis, asthma and viral infections (78–82). In light of the present work, it will be important to determine the conditions under which macrophage NOS2 expression might change and how that might affect disease outcome.

Our findings demonstrate that the susceptibility to epigenetic modification is gene specific and may be predictable from methylation sequencing analysis. Future clinical trials utilizing treatments aimed at demethylation and/or histone modifications should consider the susceptibility of the target gene(s) of interest. In this study, we show that human macrophages, including primary alveolar macrophages, do not make NO at baseline or in response to acute inflammatory stimuli. The lack of *NOS2* expression and subsequent NO secretion is explained by epigenetic changes that are consistent with silencing of the *NOS2* transcription locus. We believe this study points out the pitfalls of cross species extrapolation in understanding inflammatory mechanisms. Future studies exploring how human macrophages kill pathogens and respond to inflammation should focus on alternative pathways to *NOS2* and NO. To do otherwise is to risk having the best laid schemes of mice and men go awry (with apologies to Robert Burns).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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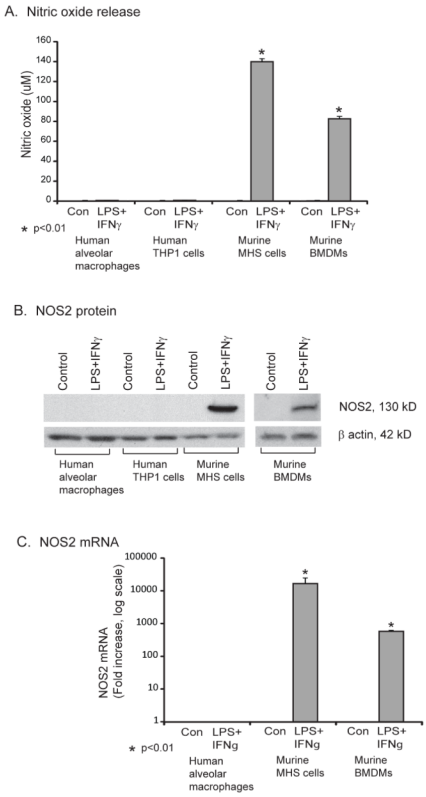


**Figure 1. Murine, but not human, macrophages increase NO release and *NOS2* expression after stimulation**

**A.** Human (alveolar macrophages obtained by bronchoscopy or THP-1 monocytic cell line) and murine cells (bone marrow-derived macrophages (BMDMs) or the mouse alveolar macrophage cell line, MHS) were put in culture at 1 million cells per ml and treated with and without LPS (100 ng/ml) and IFNγ (100 ng/ml) for 18 hours. Cells were cultured for 18 hours and supernatants collected for NO measurement (Greiss reaction). Data represents mean +/− standard error for three separate experiments. Significant differences from unstimulated control were evaluated using a Student's t-test. **B.** The same human and mouse cells were cultured with and without LPS (100 ng/ml) and IFNγ (100 ng/ml) for 18 hours. Total cell protein was isolated and Western analysis for *NOS2* performed. Shown is a representative blot from four replicate experiments. C. Human alveolar macrophages, MHS cells and murine bone marrow-derived macrophages were cultured with and without LPS (100 ng/ml) and IFNγ (100 ng/ml) for 4 hours. RNA was isolated and *NOS2* and ICAM1 relative levels were analyzed using qRT-PCR). Data represent mean +/− standard error (SE) from three separate experiments. Significance was evaluated using Student's t-test.

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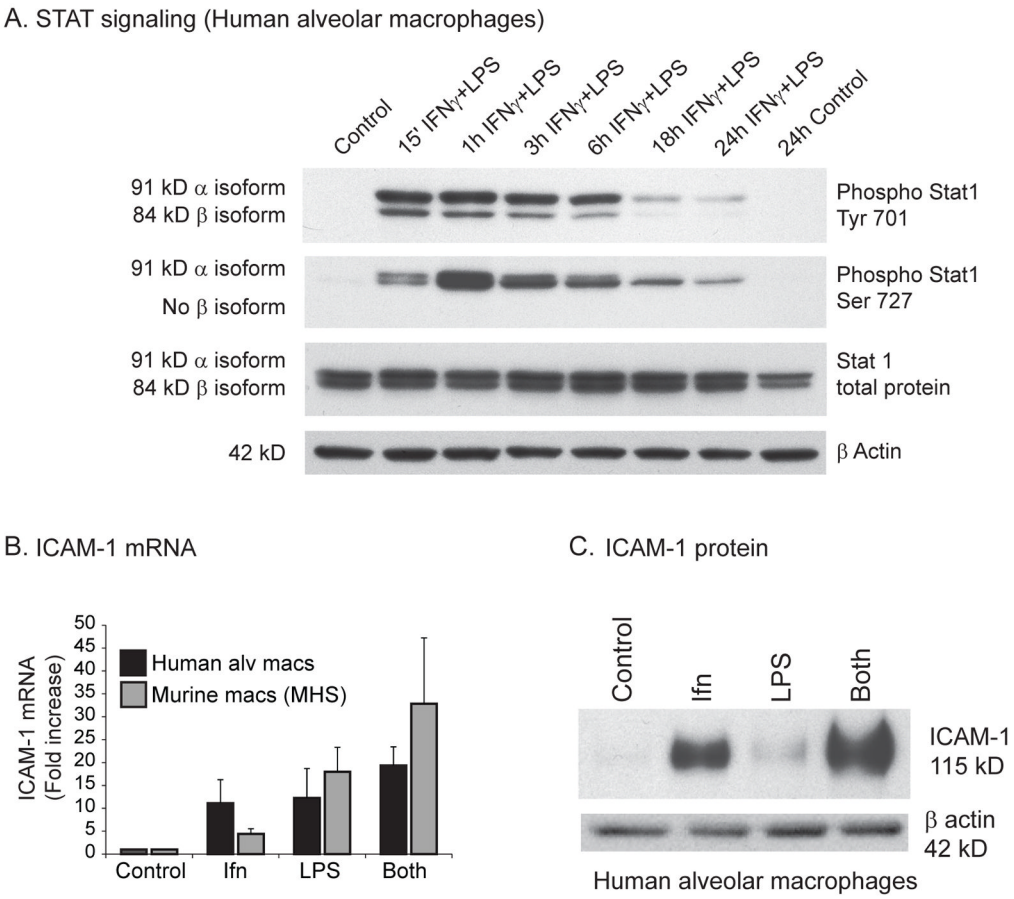


**Figure 2. LPS and IFNγ signaling is intact in human alveolar macrophages**

**A.** Human alveolar macrophages were stimulated with and without LPS (100 ng/ml) and IFNγ (100 ng/ml) for varying time points. Whole cell protein was isolated and Western analysis performed using antibodies specific for phosphorylated STAT1 (tyrosine 701 and serine 727) and total STAT1. Shown is a representative Western from three separate experiments. **B.** Human alveolar macrophages and MHS cells were stimulated with and without LPS (100 ng/ml) and IFNγ (100 ng/ml) for 4 hours. RNA was isolated and ICAM1 levels determined with qRT-PCR. **C.** Human alveolar macrophages were stimulated with and without LPS (100 ng/ml) and IFNγ (100 ng/ml) for 18 hours. Whole cell protein was isolated and Western analysis performed for ICAM1. Shown is representative Western from three separate experiments.

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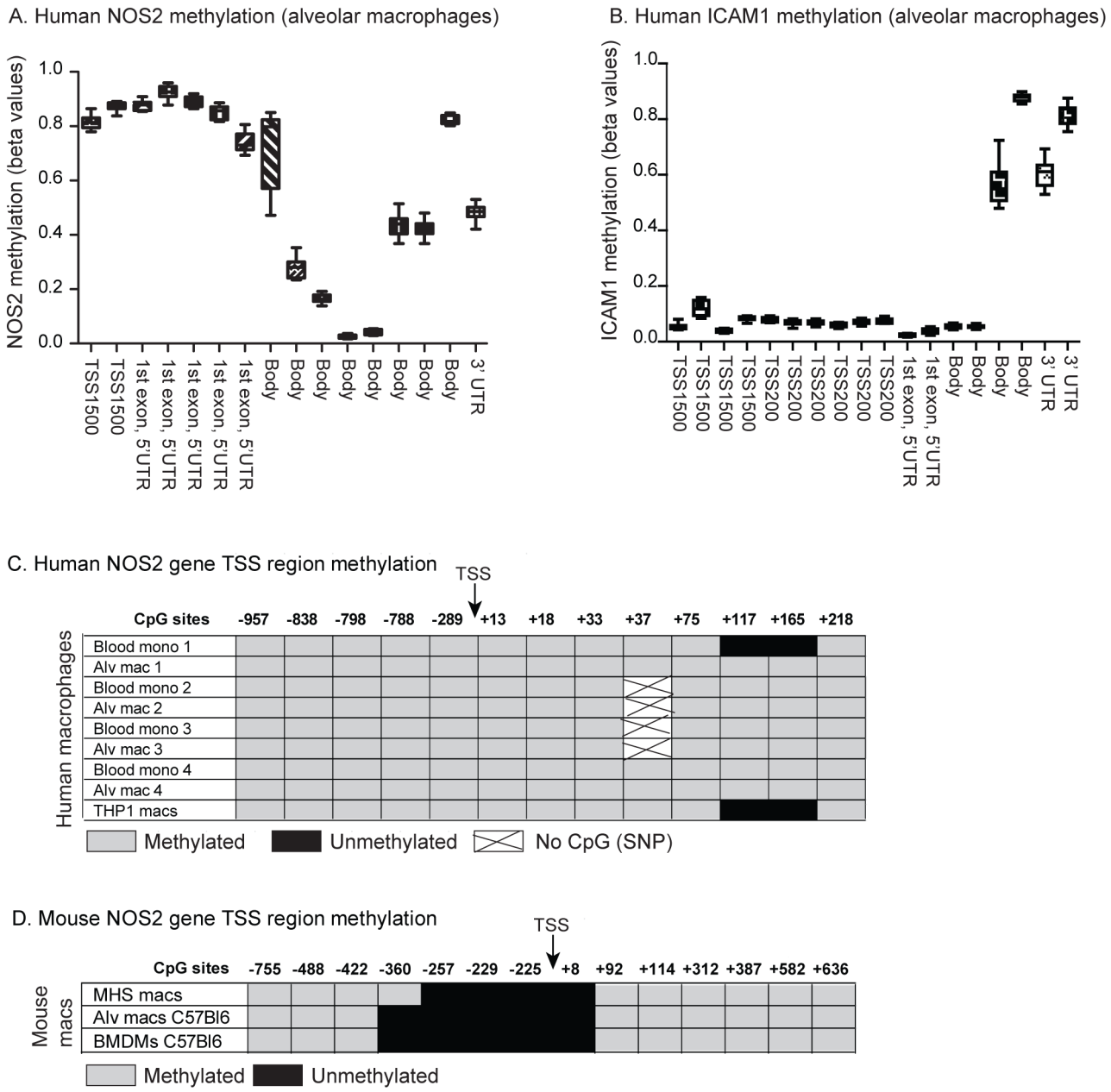


**Figure 3. The human *NOS2* gene is methylated at the promoter region**

**A+B.** Human alveolar macrophages were obtained from normal volunteers. DNA was isolated and CpG methylation was determined using bisulfite conversion and the Illumina Infinium 450K Human Methylation array. Shown are representative beta values from 10 individuals for the *NOS2* gene (A) and the ICAM1 gene (B). Probe sites from the Illumina array are listed in the Supplemental files. **C.** Individual bisulfite sequencing of CpG sites proximal to the *NOS2* promoter was determined in paired samples of human blood monocytes and alveolar macrophages from 4 donors and THP-1 cells. DNA was isolated, bisulfite conversion performed and sequencing performed. Sites that sequenced as CG were considered methylated, while those that sequenced as TG were considered unmethylated. All CpGs from −957 (relative to TSS) to +218 of the human *NOS2* gene were sequenced. **D.** Individual bisulfite sequencing of CpG sites proximal to the *NOS2* promoter was determined

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in mouse macrophages (MHS cell line, C57Bl6 alveolar macrophages, and C5Bl6 bone marrow-derived macrophages). DNA was isolated, bisulfite conversion performed and the regions sequenced. All CpGs from −755 (relative to TSS) to +636 of the mouse *NOS2* gene were sequenced.

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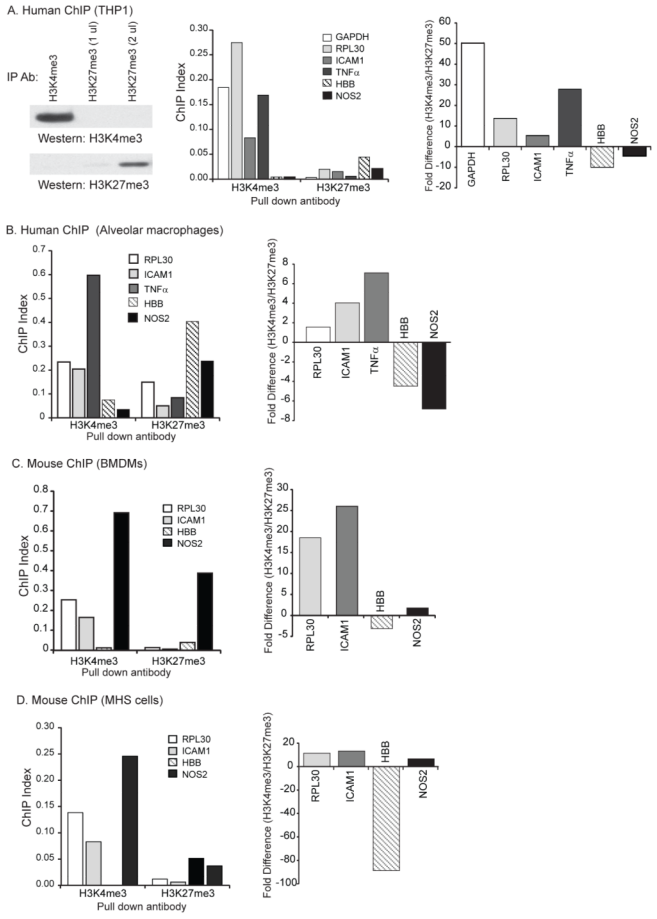


**Figure 4. Human NOS2 gene is associated with silencing H3K27me3 marks; mouse NOS2 segregates with the activating histone mark H3K4me3**

**A.** THP-1 cells were processed for ChIP-Seq. Chromatin-associated DNA was pulled down with antibodies to either the activating histone modification, H3K4me3 or the silencing histone modification, H3K27me3. Shown is a Western analysis of proteins from immunoprecipitations with the two antibodies, demonstrating specific pull down of either H3K4me3 or H3K27me3. DNA was isolated and qRT-PCR performed using primers specific for the region proximal to the TSS of the GAPDH, RPL30, ICAM1, TNFa, HBB and *NOS2*. The graph on the left shows data from a representative experiment (of three) presented as a ChIP Index ($2\% \times 2^{(C(T)\ 2\%\ input\ sample\ -\ C(T)\ IP\ sample)}$). The graph on the right shows a comparison of the H3K4me3 pull down values and the H3K27me3 pull down values (H3K4me3 ChIP Index/H3K27me3 ChIP Index). If the ratio was less than 1, data was converted to a fold change ((1/value)( −1)). **B, C, D.** Identical experiments to those shown in Figure 5A were performed using human alveolar macrophages (B), Bone Marrow-Derived Macrophages from C57Bl6 mice (C) and the murine MHS macrophage cell line (D). The data is presented in the same format as A.

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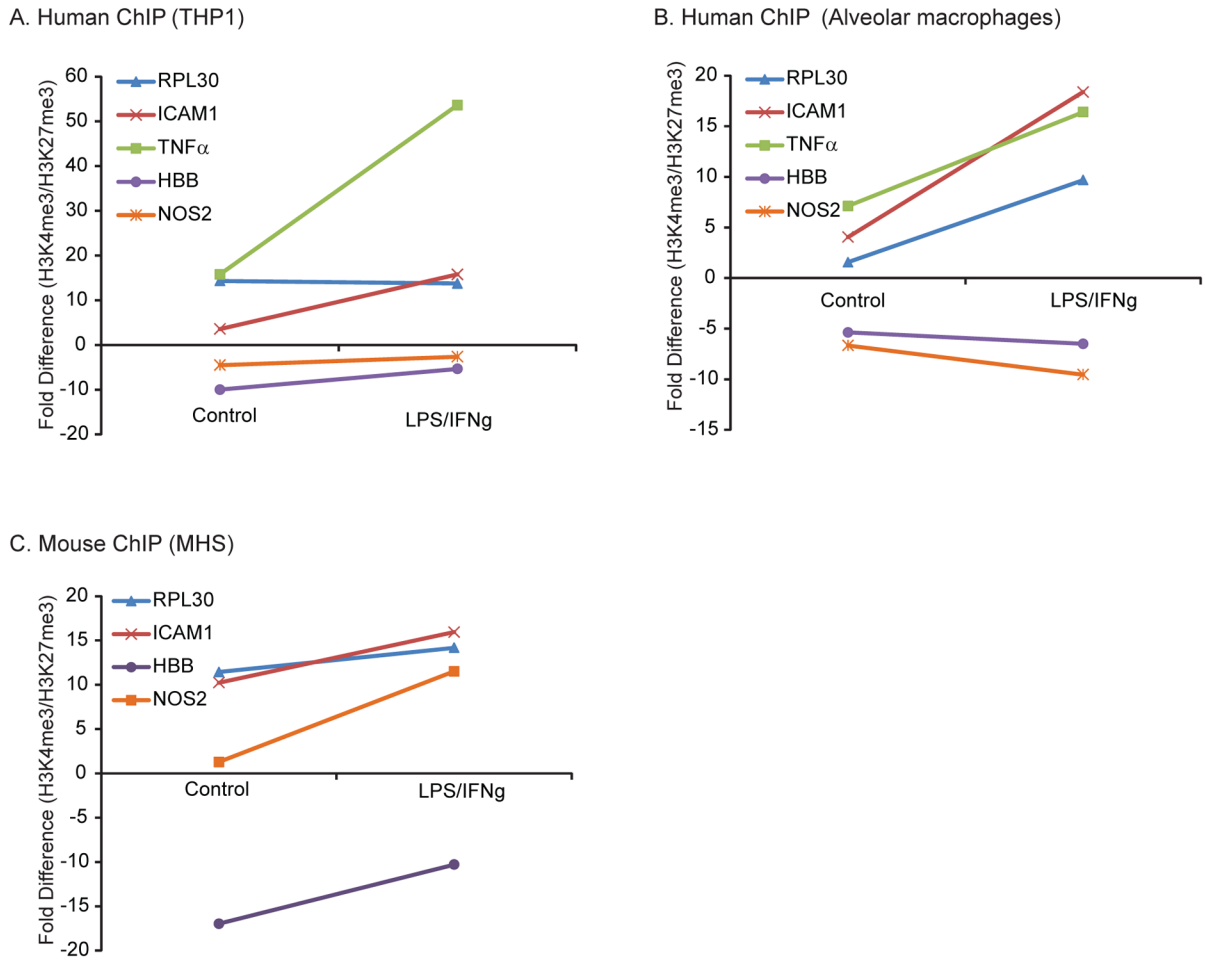


**Figure 5. In human macrophages, LPS/IFNγ exposure does not increase NOS2 gene association with the activating histone mark H3K4me3**

In mouse macrophages, LPS/IFNγ exposure increased H3K4me3 association with the *NOS2* locus. **A.** THP-1 cells were cultured with LPS (100 ng/ml) and IFNγ (100 ng/ml) for 18 hours. DNA and proteins were cross-linked and immunoprecipitation performed with antibodies to H3K4me3 and H3K27me3. DNA was isolated from the immunoprecipitated antibody/protein/DNA complex and qRT-PCR performed for the TSS proximal region of the RPL30, ICAM1, TNFα, HBB and *NOS2* genes. The graph shows fold changes in the ChIP Index ($2\% \times 2^{(C(T)\ 2\%\ input\ sample\ -\ C(T)\ IP\ sample)}$). The data is representative of three separate experiments. **B, C.** Identical studies were performed in human alveolar macrophages and the murine macrophage cell line, MHS. The graph shows fold differences between the amount of gene specific DNA pulled down by the antibody to H3K4me3 and the antibody to H3K27me3. A positive fold difference implies an increase in association of gene promoter with activating histone marks. A negative fold difference implies an increase in association of gene promoter with silencing histone marks.

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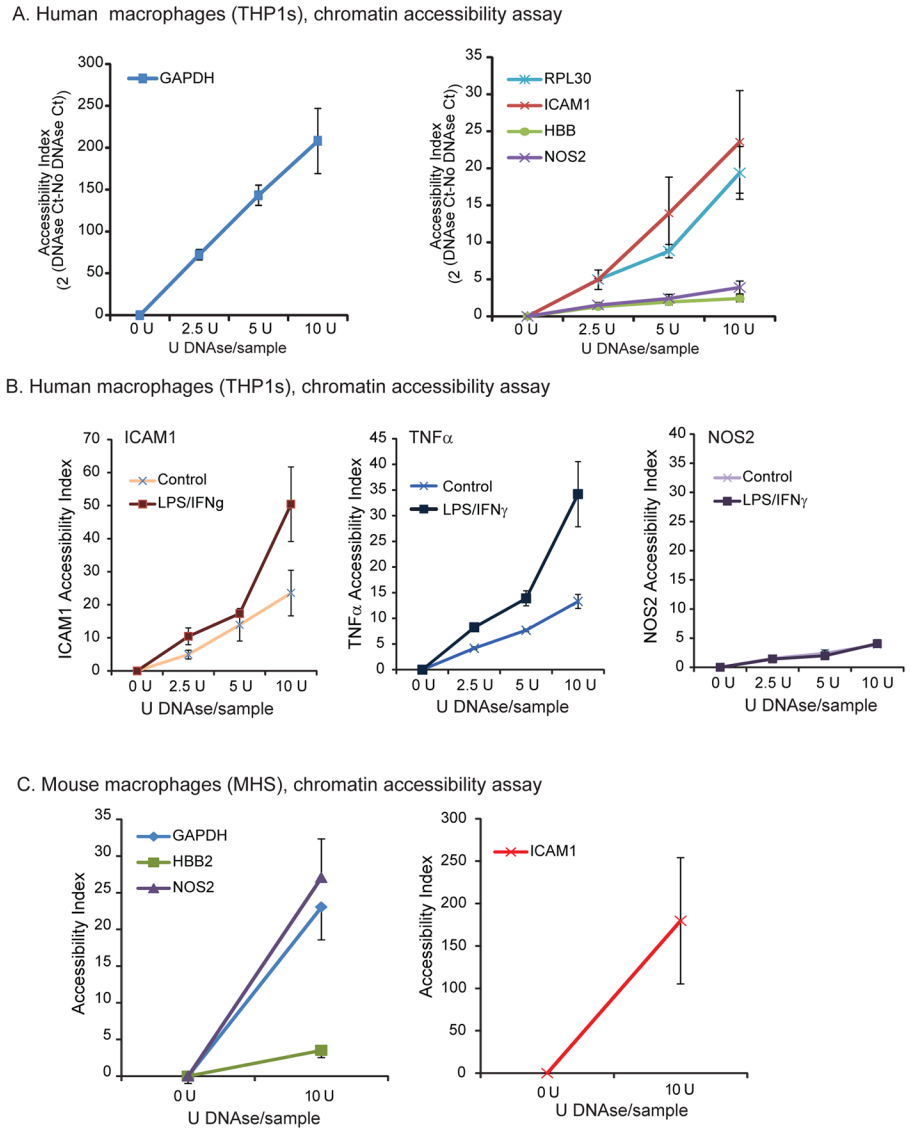


**Figure 6. In human macrophages, NOS2 is a closed gene that remains inaccessible even after inflammatory stimulation**

**A.** Human THP-1 cells were treated with varying amounts of DNAse. DNA was isolated and qRT-PCR performed for GAPDH, RPL30, ICAM1 as representative actively transcribed "open" genes, HBB as a prototypical "closed" gene, and *NOS2* to determine DNAse sensitivity at the loci. **B.** The effect of LPS/IFNγ exposure on chromatin accessibility was determined in THP-1 cells exposed to LPS (100 ng/ml) and IFNγ (100 ng/ml) for 18 hours. Pelleted cells were exposed to increasing levels of DNAse and qRT-PCR performed for ICAM1, TNFa and *NOS2*. Data is presented as Accessibility Index (2 (DNAse Ct-No DNAse Ct)). The data is mean +/− SE of three separate experiments. Significance was evaluated using Student's t-test. **C.** Mouse MHS cells were treated with varying amounts of DNAse. DNA was isolated and qRT-PCR performed for GAPDH, ICAM1, HBB and *NOS2* associated DNA to determine DNAse sensitivity at the loci. In human macrophages, NOS2 has a chromatin accessibility pattern similar to the closed gene HBB and does not become more accessible with stimulation.

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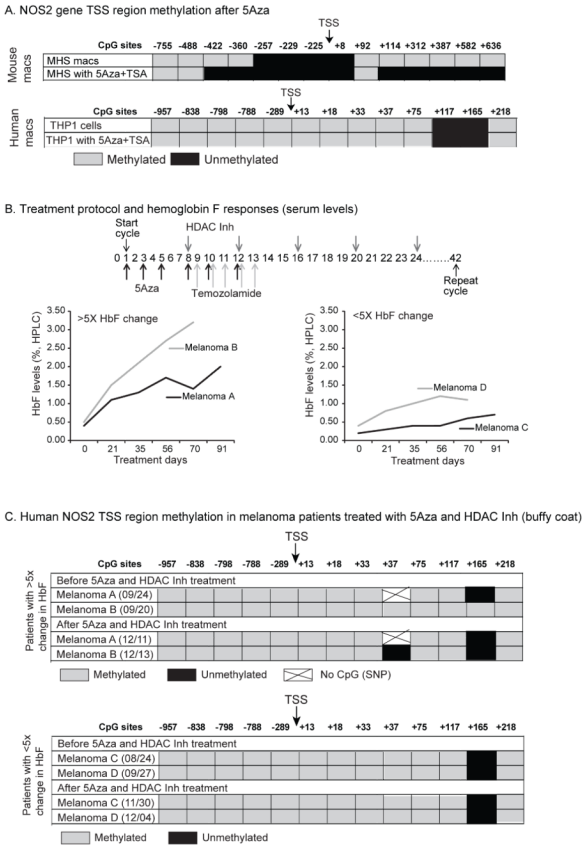


**Figure 7.**

Exposure to a demethylation agent and HDAC inhibitor increases the number of unmethylated CpGs in mouse macrophages, but has no effect on methylation of the *NOS2* TSS region in human macrophages or circulating leukocytes from melanoma patients treated with 5Aza and an HDAC inhibitor. **A.** Mouse (MHS) and human (THP-1) macrophages were treated with 5-aza-dC (1 uM) on days 0 and 2. Trichostatin A was added on day 4 (5ng/ul). Cells were stimulated with IFNγ (100ng/ul), (LPS 100ng/ul) on day 5 and harvested on day 6. Control cells were stimulated on day 5 and harvested on day 6. DNA was isolated with bisulfite conversion and sequencing performed. Sites that sequenced as CG were considered methylated, while those that sequenced as TG were considered unmethylated. All CpGs from −755 (relative to TSS) to +636 of the mouse *NOS2* gene were sequenced. **B.** Melanoma patients with resistant disease were treated at University of Iowa Hospitals and Clinics with 5-aza-dC, temozolamide and a histone deacetylase inhibitor throughout a 42-day cycle. Hemoglobin F (HbF) electrophoresis was performed from blood collected during the treatment. A 5-fold rise in HbF was used as a cut off for establishing expected drug effect. **C.** Whole blood was collected from melanoma patients undergoing experimental therapy outlined above before and after the cycle. DNA was isolated bisulfite conversion and sequencing performed. All CpGs from −957 (relative to TSS) to +218 of the human *NOS2* gene were sequenced.

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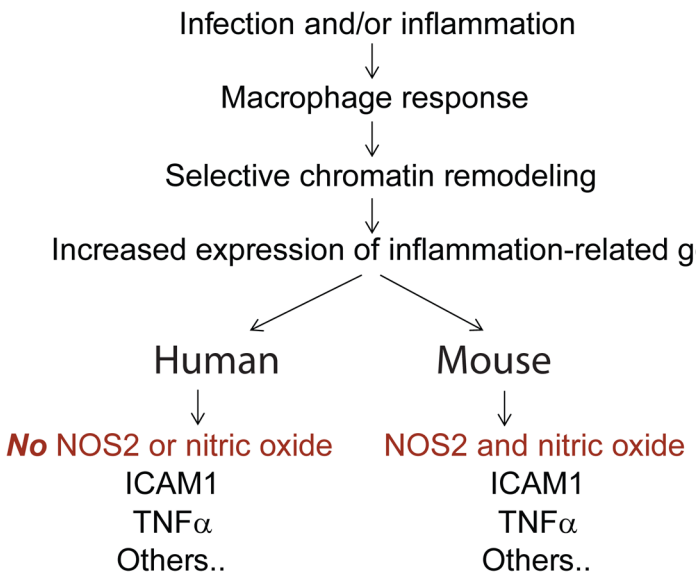


**Figure 8.**
Diagram of study demonstrating effect of *NOS2* silencing on inflammatory response in human macrophages compared to mouse macrophages.