# *COX-2* Expression is Upregulated by DNA Hypomethylation post-Hematopoietic Stem Cell Transplantation[1]

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## Abstract

Hematopoietic stem cell transplant (HSCT) therapy is limited by pulmonary infections. Mice with fully reconstituted hematopoietic compartments, including alveolar macrophages (AMs), post-bone marrow transplantation (BMT) have impaired host defense against Gram negative *Pseudomonas aeruginosa*. Impaired innate immunity is related to increased production of prostaglandin $E_2$ ($PGE_2$) by AMs. Cyclooxygenase (COX)-2 is the rate-limiting enzyme for synthesis of $PGE_2$ from arachidonic acid, and COX-2 expression is elevated in AMs post-BMT. We hypothesized epigenetic mechanisms may be responsible for upregulation of COX-2 in AMs. Using bisulfite sequencing, we observed the 5'-untranslated region and exon 1 of the *COX-2* gene is hypomethylated in the AMs of BMT mice compared to control. COX-2 expression was increased in primary AMs and in the AM cell line (MHS) following treatment with 5-aza-2-deoxycytidine (a methyltransferase inhibitor). Methylation by SssI methyltransferase of a 702 bp region of the *COX-2* promoter including the beginning of exon 1 driving a luciferase reporter silenced luciferase expression. Because transforming growth factor beta (TGF-β1) is elevated in lungs post-BMT, we tested whether TGF-β1 could promote expression of COX-2 in a hypermethylated *COX-2* vector, and observed TGF-β1 induced modest expression of COX-2, suggesting an ability to demethylate the promoter. Finally, BMTs performed with marrow from mice expressing a dominant negative form of the TGF-β receptor on CD11c-expressing cells (which includes AMs) demonstrated improved host defense and AM function. Our findings suggest impaired innate immunity and $PGE_2$ elevation post-BMT are due to hypomethylation of the COX-2 gene which is at least partly regulated by TGF-β1.

## Introduction

Hematopoietic stem cell transplantation (HSCT) is commonly used to treat malignant and nonmalignant hematologic disorders (1, 2). Traditionally, a conditioning regimen is implemented prior to intravenous infusion of hematopoietic stem cells (HSC) which may consist of chemotherapy with or without total body irradiation (TBI) (2). TBI is itself myeloablative and immunosuppressive and can affect regions within the body that are not

easily accessible by chemotherapeutic agents delivered via the circulation (1–3). Although HSCT has proven to be an effective therapeutic option for malignancy, it is also associated with significant morbidity and mortality (1–5). Following either autologous (i.e. recipient HSCs also serve as donor cells) or allogeneic (i.e. related or unrelated donor provides HSCs) HSCT, transplant recipients are susceptible to developing life-threatening infectious and noninfectious complications (2, 3, 6–9).

The lung is a common target organ post-transplant where pulmonary complications account for significant mortality and morbidity in HSCT recipients (2, 3, 6–9). Such complications develop throughout the timeline of pre-engraftment (0–30 days after transplant), early post-engraftment (30–100 days after transplant) and late post-engraftment (>100 days)(10). Despite full reconstitution or engraftment of donor-derived leukocytes, patients exhibit sustained and enhanced susceptibility to infections post-transplant (6–10). AMs are the resident macrophages in the lung and, together with recruited polymorphonuclear leukocytes (PMNs), play an important role in regulating an immune response in the lung (11–14). Previous studies have reported defective phagocytic and bacterial killing function of human alveolar macrophages within 4 months following HSCT, with some deficiency persisting up to 12 months (15). Thus, impaired innate immune function may explain the prolonged susceptibility to infection observed in post-transplant individuals.

To study the effects of HSCT, our laboratory previously developed a syngeneic bone marrow transplant (BMT) murine model that simulates autologous HSCT in humans and allows for a direct approach to study immune reconstitution and function without the confounding effects of graft-versus-host-disease or immunosuppressive drugs. We have shown that even after full immune reconstitution following syngeneic BMT, donor-derived AMs from BMT mice are defective in phagocytosis and killing compared to mice that did not undergo BMT (11, 16). We discovered this defect is related to decreased cysteinyl leukotrienes (cys LTs) and tumor necrosis factor $\alpha$ (TNF$\alpha$) production, and increased prostaglandin $E_2$ (PGE$_2$) production (11, 17, 18).

Eicosanoids are lipid mediators derived from arachidonic acid and cells of the myeloid lineage are major producers of both cys LTs and PGE$_2$ (18, 19). Synthesis of prostaglandins is mediated by the cyclooxygenase (COX) enzymes of which there are two isoforms. COX-1 is a constitutive isoform of COX responsible for basal COX expression required for homeostasis, whereas COX-2 is induced primarily by inflammation (19). PGE$_2$ production post-BMT is attributed to the increased activity of COX-2 and PGE$_2$ negatively regulates the innate immune response (11, 20). In our model, PGE$_2$ and COX-2 expression were found to be elevated post-BMT within AMs and PMNs and this caused functional impairments in the innate immune function of both of these cell types (11, 16, 21, 22). However, in our model of *P. aeruginosa* infection post-BMT, we demonstrated that it was the defect in non-opsonized phagocytosis by AMs post-BMT, rather than PMN function which was responsible for the acute clearance of *P. aeruginosa* (13); thus, we have focused our current studies on regulation of COX-2 expression in AMs.

PGE$_2$ binds to seven transmembrane spanning E prostanoid (EP) receptors, of which there are 4 discrete EP receptors that couple to G proteins involved in mediating the intracellular signaling in response to PGE$_2$ (20, 23). We have shown that binding of the EP2 receptor by PGE$_2$ results in increased IL-1 receptor associated kinase (IRAK-M) expression in AMs resulting in the inhibition of non-opsonized phagocytosis (14). PGE$_2$ also induces elevation of the enzyme phosphatase and tensin homolog deleted on chromosome 10 (PTEN) which results in the inhibition of serum-opsonized phagocytosis by AMs (21). Thus PGE$_2$ is able to act through distinct signaling pathways to compromise host innate immune defense.

Because COX-2 mRNA and protein expression are upregulated in the AMs of mice post-BMT and remain elevated in cells cultured ex vivo (11, 14), we hypothesized that epigenetic mechanisms may be responsible for the upregulation of COX-2. To test this, we sought to determine the methylation status of the *COX-2* gene promoter. Using bisulfite conversion and pyrosequencing, we discovered that the *COX-2* gene was significantly hypomethylated in the 5'-untranslated region (UTR) and exon 1 of AMs from mice post-BMT. Using in vitro assays, we determined that COX-2 mRNA expression is regulated by methylation, as treatment of both a murine alveolar macrophage cell line (MHS) and primary AMs with 5-aza-2'-deoxycytidine (a methyl transferase inhibitor) increased COX-2 mRNA levels by RT-PCR and caused demethylation of the COX-2 gene. Similarly, transfections in MHS cells with methylated COX-2 promoter constructs showed reduced luciferase activity. However, COX-2 promoter activity could be enhanced by treatment with TGF-β1, a cytokine known to be elevated in BMT lungs (24). Thus, our data indicate that epigenetic regulation of *COX-2* is one mechanism driving the observed elevation of both COX-2 and $PGE_2$ in BMT mice and this alteration is regulated in part by TGF-β1.

## Materials and Methods

### Animals

C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice expressing dominant-negative TGF-β receptor II (TGF-βRII) under the CD11c promoter (CD11c[dnR] ) were provided by Dr. Laouar (University of Michigan) (25, 26). Mice were bred and housed under specific pathogen-free conditions and monitored daily by veterinary staff. All mice were euthanized by $CO_2$ asphyxiation. The University of Michigan Committee on the Use and Care of Animals approved these experiments.

### Bone marrow transplantation

Recipient mice received 13.5 Gy of total body irradiation (TBI; orthovoltage x-ray source) split in two fractions, 3 h apart. Bone marrow cells were harvested from donor mice and resuspended in serum-free medium (SFM; DMEM, 0.1% BSA, 1% penicillin-streptomycin, 1% l-glutamine, and 0.1% amphotericin B). Bone marrow cells ($5 \times 10^6$) were administered by tail vein injection into TBI recipient mice. All experiments with BMT mice were performed 5–6 wk post-BMT when mice were fully donor-cell reconstituted. Spleen cells were >94% donor-derived, and AMs were >80% donor-derived at this time point (16).

### Harvesting AMs

Resident AMs from mice were obtained via ex vivo lung lavage, using a previously described protocol (11). Briefly, these cells were collected in lavage fluid consisting of complete medium (DMEM, 1% penicillin-streptomycin, 1% L-glutamine, 10% FCS, 0.1% Fungizone) and 5 mM EDTA. The cells were enumerated by counting on a hemocytometer before plating.

### Molecular cloning of *COX-2* promoter into luciferase expression vector

The full-length (full) murine *COX-2* promoter (1203 bp) DNA as defined in (27) and a 498 bp COX-2 promoter (deleted) was amplified using primers to create cloning sites. Supplemental figure 1 shows the sequence of the COX-2 promoter and the location of the created cloning sites (Kpn I, MluI and Bgl II) in grey boxes. Table 1 shows the primers used to amplify the promoter regions by standard PCR from genomic DNA from AMs. The amplified full *COX-2* promoter DNA was digested with restriction endonucleases *KpnI* and *BglII* (New England BioLabs, Inc.) while the deleted *COX-2* promoter was digested with *Mlu I* and *BglII* (New England BioLabs, Inc.). pGL3-basic luciferase reporter vector

(Promega) was digested with either *KpnI* and *BglII* or *MluI* and *BglII*. The digested DNA and vectors were ligated with T4 DNA ligase for 24 hours. *E. coli* strain MC1061 was made competent by $CaCl_2$ and was transformed with ligated DNA. Positive colonies were selected with Ampicillin (100 mg/mL) and correct insert was verified restriction digestion and PCR.

### In vitro methylation

The COX-2 promoters, both the full and deleted *COX-2* versions, inserted into the luciferase reporter plasmid pGL3-basic (Promega) were in vitro methylated with CpG methyltransferase *M.SssI* (New England BioLabs, Inc.) as specified by the manufacturer for 4 hours. Methylation was confirmed by digestion with methylation-sensitive restriction enzyme *SmaI*.

### In vitro transfections and Dual Luciferase Assay

MHS cells were transfected using Lipofectamine LTX and PLUS Reagent (Invitrogen™) following manufacturer's optimized RAW 264.7 protocol. Briefly, $6.2 \times 10^4$ MHS cells were cultured in complete media containing RPMI, 10% fetal calf serum, 1% penicillin-streptomycin, 1% L-glutamine and 0.5 mM 2-mercaptoethanol overnight. Following 24 hours, MHS cells were transfected with *COX-2* promoter-driven luciferase reporter plasmid and pRL-SV40 in a 50:1 ratio for a total of 0.3 µg of DNA using lipofectamine LTX and PLUS Reagent (Invitrogen™). Transfections were performed with unmethylated or methylated, long or short *COX-2* luciferase reporter plasmids. Where indicated, cells were treated with 10 µg/mL lipopolysaccharide (LPS) or 1 ng/mL porcine TGF-β1.

### Real-time RT-PCR

Real-time RT-PCR was performed on an ABI Prism 7000 thermocycler (Applied Biosystems, Foster City, CA). Gene-specific primers and probes were designed using Primer Express software (PE Biosystems, Foster City, CA) as published previously (11, 12). Sequences for all primers and probes used can be found in Table 1. Each AM sample was pooled from two to three mice and was run in duplicate. Average cycle threshold ($C_T$) was determined for each sample and was normalized to β-actin. Relative gene expression was calculated as described previously (28).

### DNA methyltransferase or histone deacetylase (HDAC) inhibition

AMs or MHS cells were treated with either varying concentrations of the methyltransferase inhibitor 5-aza-2'-deoxycytidine (Sigma, St.Louis, MO) or the HDAC inhibitor Trichostatin A (Sigma, St.Louis, MO) for 72 hours. Primary AMs were initially stimulated with (1ng/mL) of recombinant murine GM-CSF (R&D, Minneapolis, MN) for 24 hours to promote proliferation prior to receiving the appropriate treatments for the following 72 hours.

### ELISA/enzyme immunoassay

MHS cells were cultured at $5 \times 10^5$ cells/ml in a 24-well plate for 72 hours. MHS cells were grown in complete media for 48 hours then switched to serum-free media for 24 hours. Supernatants were collected for enzyme immunoassay (EIA). Production of $PGE_2$ and thromboxane $B_2$ (a metabolite of thromboxane $A_2$) was measured by EIA (Cayman Chemical, Ann Arbor, MI), according to the manufacturer's instructions.

### Bisulfite Conversion and Pyrosequencing

DNA was isolated from $5 \times 10^5$ to $1 \times 10^6$ cells using the Dneasy kit (Qiagen, Orange, CA). The Zymo Research EZ DNA Methylation kit (Irvine, CA) was used to bisulfite modify 500 ng of genomic DNA. Bisulfite modification was performed according to manufacturer's instructions. Briefly, genomic DNA was denatured with Dilution Buffer and further treated

with CT Conversion Reagent. Samples were processed following the alternative incubation conditions recommended by manufacturer whereby samples were incubated in a thermocycler under the following conditions: (95°C 30sec; 50°C 60 min) × 16 cycles; 4°C hold. Following bisulfite conversion, the *PTGS2* (*COX-2*) promoter was PCR amplified and sequenced on a Pyrosequencer (Qiagen). The CpG sites analyzed are shown in Supplementary Figure 1 color coded by blue, yellow or pink boxes to denote the regions amplified by different primer pairs for analysis. The CpG sites analyzed are also numbered for clarity. Amplification and sequencing primers for murine *PTGS2* were obtained from EpigenDx, Inc, Worcester, MA (assay ADS2001 (sites in yellow) and ADS2002 (sites in pink). Sites in blue were amplified and sequenced using primers we designed in Table 1. The PCR conditions for determining the methylation profile of the DNA using ADS2001 are as follows: 95°C 15 min; 45 × (95°C 30 s; 54°C 30s; 72°C 30s); 72°C 5 min; 4°C hold. PCR conditions using ADS2002: 95°C 15 min; 45 × (95°C 30 s; 60°C 30s; 72°C 30s); 72°C 5 min; 4°C hold.

### *P. aeruginosa* PAO1 preparation and FITC-labeling

*P. aeruginosa* PAO1 stock was grown in tryptic soy broth (Difco; BD, Sparks, MD), and the culture concentration was determined via absorbance measurements. For FITC-labeling, a *P. aeruginosa* culture was centrifuged and washed two times by resuspending cell pellet in 1 mL sterile PBS and transferring into a sterile tube. *P. aeruginosa* was heat-killed by autoclaving for 20 minutes and resuspended at $10^9 - 10^{10}$ CFU/mL in 0.1 M NaHCO$_3$ (pH 9.2). 0.2 mg/mL FITC (Sigma, St. Louis, MO) in DMSO was added to heat-killed *P. aeruginosa* and allowed to incubate in dark for 1 hour on rocker at room temperature. Following FITC-labeling, heat-killed *P. aeurginosa* were washed three times and resuspended in 1 mL sterile PBS. Aliquots were prepared and stored at −80°C until use.

### In vitro phagocytosis assay

AMs isolated by BAL were plated at $2 \times 10^5$ cells per well in 100 μl and cultured overnight in complete media on a 96-well, flat-bottomed, half-area tissue culture plate (Costar, Corning, NY). The following day, FITC-labeled heat-killed *P. aeruginosa* (prepared as described above) was added at 300:1 MOI. 2 hours following incubation at 37°C in dark, 50 μL of trypan blue (250 μg/mL in 0.09 M citrate buffer solution; Sigma) was added to each well for 1 min to quench fluorescence of non-phagocytosed FITC-labeled bacteria. AM phagocytosis of FITC-labeled bacteria was measured using a microplate fluorimeter and expressed in arbitrary fluorescence intensity units. For possible differences in AM adherence to tissue culture plate, data were normalized for cell number using a LDH Cytotoxicity Detection Kit (Roche Diagnostics, Indianapolis, IN) as previously described (11).

### Statistical analysis

Statistical significance was analyzed using the GraphPad Prism 5.0 statistical program (La Jolla, CA). Comparisons between two experimental groups were performed using the Student *t* test. Comparisons between 3 or more values used an ANOVA analysis with a post-hoc Bonferroni comparison. A value of $p < 0.05$ was considered statistically significant.

## Results

### *COX-2* mRNA levels are elevated in BMT AMs

PGE$_2$ levels were previously reported to be elevated in the plasma of patients undergoing autologous HSCT (29). The increased PGE$_2$ detected in these individuals post-transplantation was independent of conditioning regimen (chemotherapy or radiotherapy). Similar results were previously observed in our established syngeneic BMT mouse model,

whereby 13 Gy total body irradiation (TBI) or a dual-chemotherapy regimen induced a defective pulmonary immune response associated with elevated levels of $PGE_2$ produced by lung innate immune cells (11, 16). $PGE_2$ production is dependent on the activity of COX enzymes. To determine whether the increase in $PGE_2$ levels are a result of increased COX-2 expression, we measured *COX-2* expression levels from control (untransplanted) and BMT AMs harvested by bronchoalveolar lavage (BAL). Following a 24 h culture with serum-free media, RNA was harvested from AMs and *COX-2* mRNA expression levels were measured by real-time RT-PCR. These results demonstrate that *COX-2* is upregulated in BMT AMs when compared to control AMs (Figure 1A). These results are consistent with our previous observation that COX-2 protein is also elevated in AMs post-BMT(14) and confirm that the BMT-induced alterations which enhance COX-2 expression persist in AMs cultured ex vivo.

COX-2 can catalyze the synthesis of numerous prostaglandins. We previously published that prostacyclin ($PGI_2$) is also elevated in AMs post-BMT (11). To determine if thromboxane A2 was also elevated, we measured the more stable metabolite thromboxane $B_2$ ($TXB_2$), in control and BMT AMs (Fig. 1B). Taken together, these results suggest that BMT causes a global upregulation of prostanoid products.

## BMT AMs are hypomethylated in the 5'-UTR and exon 1 of the *COX-2* gene

To determine whether epigenetic regulation by DNA methylation was involved in the increased expression of *COX-2* mRNA levels observed in Figure 1A, we collected genomic DNA from the AMs of control and BMT mice and performed bisulfite sequencing of the *COX-2* promoter. We analyzed methylation at all 34 CpG sites noted in Supplemental Fig. 1. There was no difference in the methylation status of CpG sites 1–27 in control and BMT AMs (data not shown). However, BMT AMs exhibited significant hypomethylation compared to control AMs at CpG loci within the 5'-UTR and exon 1 (sites 28–34; Figure 2).

## 5-aza-2'-deoxycytidine increases *COX-2* in MHS cells and primary AMs

A chemical analog for cytosine, 5-aza-2'-deoxycytidine, inhibits methylation by blocking DNA methyltransferase activity (30). The methylation patterns of *COX-2* post-BMT indicate that a decrease in methylation of the *COX-2* promoter region may be responsible for the elevation of COX-2 and $PGE_2$ observed post-transplant. To determine whether DNA methylation of *COX-2* contributes to its diminished expression in control non-BMT cells, we treated MHS cells, a murine alveolar macrophage cell line, with increasing concentrations of 5-aza-2'-deoxycytidine for 72 hours or DMSO as a vehicle control. RNA was extracted from these cells and *COX-2* mRNA levels were measured by real-time RT-PCR. *COX-2* expression from MHS cells increased dose-dependently in the presence of the methyltransferase inhibitor (Figure 3A).

We next sought to verify these results in primary AMs from control mice. We collected AMs by BAL and induced proliferation by initially treating the primary AMs with GM-CSF for 24 h. AMs were then treated with 5-aza-2'deoxycytidine for 72 additional hours in the presence of GM-CSF to maintain proliferation (31) and *COX-2* mRNA levels were determined by real-time RT-PCR. Expression patterns of *COX-2* transcripts shown in Figure 3B correlate well with those observed in 5-aza-2'-deoxycytidine-treated MHS cells. Furthermore, treatment of MHS cells with 2.5 μM 5-aza-2'deoxycytidine resulted in increased $PGE_2$ levels (Figure 3C). To determine whether the increase in *COX-2* mRNA levels in MHS cells treated with the methyltransferase inhibitor were in fact due to demethylation of the promoter in the same region noted post-BMT, DNA was harvested from 5-aza-2'deoxycytidine-treated MHS cells and subjected to methylation analysis. Figure 4 shows an overall decrease in methylation on CpG sites 28–34; the same sites found to be

significantly hypomethylated in BMT AMs, supporting the conclusion that the observed effects on *COX-2* expression are due to a change in methylation patterns.

As histone acetylation can affect gene expression, it is possible that *COX-2* can also be influenced by other forms of epigenetic regulation. Thus, both MHS and primary AMs were treated with trichostatin A, an HDAC inhibitor. However, MHS (Figure 5A) and primary AMs (Figure 5B) stimulated with trichostatin A for 72 hours did not show a significant difference in *COX-2* expression compared to control treatment with DMSO. Taken together, these data indicate that the regulation of COX-2 is primarily influenced at the transcriptional level by DNA methylation.

## Cloned *COX-2* luciferase vector shows normal transcriptional regulation

To further study the regulation of COX-2, the full *COX-2* promoter region (from the KpnI to the Bgl II site) was cloned into a pGL3-basic firefly luciferase expression plasmid. MHS cells were then transfected with the cloned *COX-2* promoter-driven firefly luciferase vector and a control SV-40 promoter-driven renilla luciferase vector via lipofectamine. As COX-2 expression is induced by inflammatory stimuli, MHS cells were cultured in the presence or absence of proinflammatory mediators LPS or TGF-β1 for 24 hours. Treatment with both LPS and TGF-β1 showed a significant increase in firefly luciferase over untreated MHS cells, validating the luciferase assay as an assay of regulatable *COX-2* promoter activity (Figure 6). Transfections of the *COX-2* promoter-driven expression vector and control expression were attempted in primary AMs of wild-type mice but despite using high concentrations of plasmid DNA and trying both lipofectamine and electroporation protocols, we were unable to achieve significant luciferase expression (even of the control vector) in primary AMs.

## *In vitro* methylation suppresses *COX-2* promoter activity in MHS cells

Both full (KpnI to Bgl II) and deleted (MluI to Bgl II) *COX-2* promoter constructs were cloned into pGL3-basic firefly luciferase expression plasmids. The deleted *COX-2* promoter contains all CpG sites analyzed in primary AMs by bisulfite conversion and pyrosequencing whereas the full *COX-2* construct contains additional upstream CpG sites outside the range of our current bisulfite analysis. To determine whether methylation of the *COX-2* promoter-driven expression vectors decreases *COX-2* expression, the full-length and deleted *COX-2* promoter expression plasmids were methylated in vitro with a CpG methyltransferase, *M.SssI*. Methylation of the construct was verified by treating the *COX-2*-driven luciferase plasmids with a methylation-sensitive restriction endonuclease, *SmaI* (data not shown). MHS cells were then transfected with methylated or unmethylated *COX-2* firefly luciferase reporter plasmids. The control vector (pRL-SV40) was not treated with the methyltransferase prior to transfection. As shown in Figure 7A, when unmethylated, the deleted *COX-2* promoter was able to stimulate firefly luciferase at levels similar to the full-length construct. This suggests that the deleted *COX-2* promoter construct contains all relevant sequences for ensuring transcription in MHS cells. Furthermore, when methylated, expression driven by either promoter construct was significantly impaired. Taken together, these results demonstrate that methylation of the deleted promoter region is sufficient to impair transcriptional activity and thus indicate that the relevant CpG sites for analysis lie within the deleted promoter region which was previously analyzed in Figure 2.

## TGF-β1 can induce expression of COX-2 from methylated constructs

We have previously shown that BMT mice exhibit increased levels of TGF-β1 in the lung (24), and TGFβ1 can induce expression of COX-2 (Figure 6). To determine whether TGF-β1 could stimulate the transcription of *COX-2* from a methylated promoter, we transfected MHS cells as above with the deleted *COX-2* promoter-driven firefly luciferase construct that

had either been methylated in vitro by *M. Sss1*, or left unmethylated. Cells were co-transfected with the SV40-driven renilla luciferase vector as a control. Cells transfected with the methylated construct were also treated with 1 ng/ml TGF-β1 or vehicle control. As expected, luciferase expression from the methylated *COX-2* construct was impaired when compared to the expression from unmethylated constructs (Fig 7B). However, cells transfected with the methylated constructs exhibited increased luciferase expression when treated with TGF-β1. These data suggest that TGF-β1 may be able to promote the demethylation of the *COX-2* promoter, or may promote the activity of the transcriptional machinery on methylated regions of DNA. Interestingly, LPS was not able to increase the transcriptional activity of the methylated COX-2 promoter (data not shown), suggesting that this effect may be specific to TGF-β1 signaling.

### Improved phagocytosis in AMs from BMT mice following reconstitution with donor HSCs from CD11c^dnR mice

TGF-β1 can play an important role in both the activation and suppression of monocytes and macrophages, however, its role in tissue macrophages is primarily immunosuppressive (32). To determine how TGF-β1 may contribute to the defect in BMT AMs, we developed bone marrow chimeras using HSCs from CD11c$^{dnR}$ donor mice. These mice express a dominant-negative TGF-β1 receptor II under the control of the CD11c promoter which results in the generation of TGF-β1-resistant myeloid cells including dendritic cells, macrophages and natural killer cells (25). Five weeks post-BMT, AMs were harvested by BAL and phagocytosis was measured and compared to WT BMT and control mice. As expected, AMs from WT BMT mice displayed defective phagocytosis of *P. aeruginosa* when compared to cells from control mice. However, AMs collected from CD11c$^{dnR}$ BMT mice showed enhanced phagocytosis activity compared to WT BMTs (Figure 8). To determine whether this enhancement in AM function is mediated by a change in *COX-2* gene methylation, DNA from CD11c$^{dnR}$ BMT AMs was harvested and analyzed for extent of methylation. Interestingly, the methylation of the *COX-2* promoter from CD11c$^{dnR}$ BMT AMs reverted to a pattern more similar to control AMs than WT BMT AMs at some, but not all sites previously found to be demethylated in WT BMT AMs (Figure 9).

## Discussion

We previously showed that AMs post-BMT are defective in phagocytosis and killing of *P. aeuroginosa*, a clinically relevant nosocomial pathogen which can afflict patients following hematopoietic stem cell transplantation (11, 13, 14, 16, 28). Defective AM function post-BMT is not exclusive to mice receiving TBI, but is also seen in mice receiving chemotherapy conditioning regimens (16), and persists despite immune reconstitution. We have previously shown that both PGE$_2$ and TGF-β1 are elevated in lungs of BMT mice following immune reconstitution and both can have immunosuppressive effects (11, 24). We now show that irradiated mice receiving CD11c+ donor cells that are unable to respond to TGF-β1 exhibit improved AM function (Figure 8), indicating that TGF-β1 is contributing to the defect in AMs following transplantation. As the dominant negative TGFβ1 receptor II is under the CD11c promoter, it is possible that the incomplete rescue of AM function is due to decreased CD11c expression post-transplant (33). The methylation pattern of *COX-2* in CD11c$^{dnR}$ BMT AMs supports this possibility as we observed an incomplete restoration of methylation to control patterns (Figure 9).

Overproduction of PGE$_2$ in the lung has been reported following hematopoietic reconstitution and is intimately linked with a defective lung innate immune system (11). Here, we show that *COX-2* transcription in AMs is elevated in mice post-BMT relative to control AMs. Additionally, previously published data showed upregulation of PGE$_2$ specific synthase enzymes post-BMT as well (14). These results correlate with the elevated PGE$_2$

levels reported previously in both mice and patients that have undergone HSCT (11, 13, 14, 16, 29). As other prostanoids [e.g. $PGI_2$ and $TXA_2$ (Figure 1b)] are also elevated after transplant these data indicate that increased COX-2 has a broad effect on downstream effectors. $PGI_2$ signaling through its IP receptor on macrophages and neutrophils may play a similar role as $PGE_2$ in inhibiting proper cell function as IP is a $G_s$–coupled receptor that signals through cAMP (23, 34). While $TXA_2$ is also increased post-BMT, how it affects AM function is unclear. It is possible however that upregulation of TXA2 may have deleterious effects on platelet activation or myocardial ischemia [reviewed in (34)].

Upregulation of *COX-2* expression and $PGE_2$ production has been associated with a wide range of disease processes, of which cancer is most commonly reported (35). In these circumstances, $PGE_2$ may promote cancer progression and metastasis as well as immune suppression. Furthermore, it was recently reported that chronic influenza infection was associated with upregulation of miR29b (36). The consequence of miR29b upregulation was the destabilization of DNA methyltransferases and the upregulation of *COX-2* gene expression secondary to DNA demethylation (36). It is interesting that we have also observed upregulation of miR29b in BMT AMs (data not shown). Thus, it is possible that the process of BMT regulates *COX-2* demethylation via miR29b expression.

To further understand the regulation of *COX-2* expression in our syngeneic murine model for studying innate immune cells post-BMT, we examined methylation patterns of the *COX-2* promoter of transplanted and untransplanted mice. Interestingly, we found that the *COX-2* promoter region analyzed from AMs of transplanted mice was significantly hypomethylated, particularly around the transcription start site and into the first exon, compared to control untransplanted mice. The fact that the deleted *COX-2* promoter drove expression in MHS cells and the fact that no CpG sites located upstream of the transcription start site showed methylation differences, suggests that the region of interest is located near the exon 1 border. Methylation changes of this magnitude have previously been shown to alter EP2 expression (37) and methylation around the transcription start site and in gene exons has been previously described [e.g. in (36, 38–40)]. It is interesting that in human studies related to *Helicobacter pylori*-induced COX-2 expression and influenza-induced COX-2 expression, demethylation occurs at similar sites as those identified in our murine study (36, 40). However, there are examples of regulation at different sites as well (e.g. hepatitis B demethylates *COX-2* in the NFAT sites upstream of the transcription start site (41) and silencing of the *COX-2* gene in human gastric cancers involves hypermethylation of promoter regions upstream of exon 1(42)). When we further explored this epigenetic mechanism of regulation and treated a murine AM cell line with a methyltransferase inhibitor, 5-aza-2'-deoxycytidine, an increase in *COX-2* mRNA levels was detected and this induced expression was dose-dependent with increasing concentrations of 5-aza-2'-deoxycytidine (Figure 3A). A similar response was observed in primary AMs of control untransplanted mice that were treated with either 5-aza-2'-deoxycytidine or vehicle (DMSO, Figure 3B). Furthermore, when methylation patterns were analyzed following 5-aza-2'-deoxycytidine treatment, there was a significant decrease in methylation on CpG sites found to be hypomethylated in BMT AMs (Figure. 4). In contrast, HDAC acetylation did not appear to regulate COX-2 expression (Figure 5). Overall, these results suggest methylation of the promoter and/or beginning of exon 1 is an important mechanism for regulating *COX-2* mRNA levels.

To establish a correlation between elevated levels of mRNA and COX-2 activity in response to methylating or demethylating conditions, the *COX-2* promoter region was cloned into a luciferase vector whereby firefly luciferase activity could be driven by the *COX-2* promoter and serve as a measure of COX-2 expression. MHS cells were successfully transfected, measured by pRL-SV40-driven activity, and luciferase activity of the *COX-2* plasmid was

increased in the presence of proinflammatory LPS and TGF-β1 (Figure 6). These data suggest that the reporter vector is regulated similarly to the native gene in MHS cells. Despite numerous attempts, primary AMs were unable to be transfected by either lipofectamine or electroporation methods. We next determined that a deleted *COX-2* promoter construct (which contained the CpG sites we had analyzed for methylation patterns) was sufficient to induce COX-2 expression in MHS cells. We also studied the effects of methylation on the activity of this construct. *In vitro* methylation of the *COX-2*-driven luciferase vector resulted in decreased *COX-2* promoter activity when transfected into the MHS cells. These results verify that methylation inhibits expression of COX-2, and suggest that the methylation of the *COX-2* gene which is noted in AMs collected from untransplanted mice likely plays a role in the limited expression of COX-2 and the limited production of PGE$_2$ in these cells under homeostatic conditions.

In contrast, treatment of MHS cells transfected with methylated *COX-2* reporter plasmids with TGF-β1 was able to induce modest expression of *COX-2* (Figure 7B). These results suggested that the increased levels of TGF-β1 which have been previously reported in BMT mice (24), may serve to induce *COX-2* demethylation or increase transcription from the methylated promoter in the AMs of BMT mice. Interestingly, this activity was not noted with LPS, a molecule which is able to stimulate transcription off of unmethylated *COX-2* promoters (Figure 6). Thus, these results suggest that the ability to stimulate transcription off of a methylated *COX-2* promoter may be a unique action of TGF-β1 signaling. One caveat to these studies is that we do not know for certain whether the level of methylation achieved by in vitro reactions is similar to the levels noted in vivo in BMT mice. However, our finding of improved host defense in the CD11c$^{dnR}$ BMT mice which are unresponsive to TGF-β1 signaling in AMs is highly supportive, and our in vivo methylation patterns of CD11cdnR BMT mice are more similar to control AMs than to WT BMT AMs (Figure 9).

Our results suggest that TGF-β1 may be one mechanism to regulate the increased expression of *COX-2* and thus PGE$_2$ which occurs in AMs post-BMT. However, it is likely that other signals also occur secondary to conditioning regimen-induced alterations. This is supported by our observation that the phenotype of CD11cdnR BMT mice is only partially restored to control AM levels regarding phagocytosis or methylation patterns. The production of reactive oxygen species has been observed previously following ionizing radiation and has been implicated in compromised lung immunity (43). Reactive nitrogen species, particularly nitric oxide, have been suggested to induce changes in COX within airway epithelium during inflammation (44). In these studies, increasing concentrations of nitric oxide was able to induce the production of PGE$_2$ in alveolar epithelial cells. Nitric oxide and PGE$_2$ expression can be induced by proinflammatory cytokines (44). Therefore, it is possible that the formation of reactive species caused by irradiation or a chemotherapy regimen may also be playing a role in the induction of *COX-2* transcription and expression.

Because irradiation can only directly affect cells that have been irradiated, the fact that unirradiated donor AMs are altered suggests that this impaired AM function may be due to an alteration in the lung microenvironment. Therefore, it is likely that the changes are induced by soluble factors that cause a change in cell behavior or function. Previous data from our lab has indicated that alveolar epithelial cells may influence AM function through the secretion of soluble factors like GM-CSF (28). While we have ruled out the contribution of GM-CSF to the induction of COX-2 in this model (28), it is possible that other soluble factors, in addition to TGF-β1 may trigger the epigenetic changes. This will be an area for future study.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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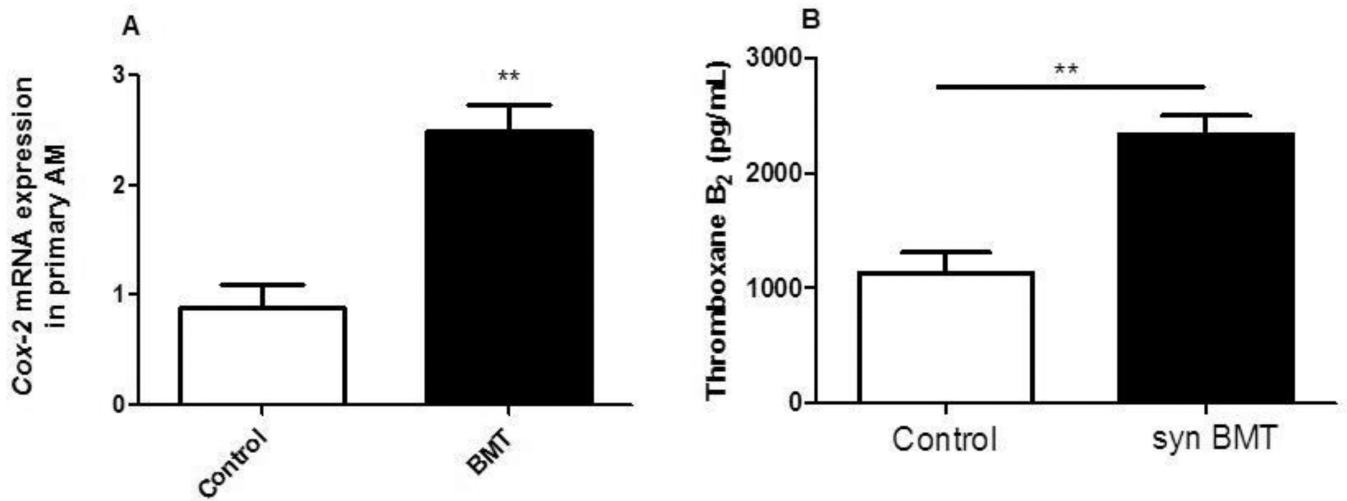
**Figure 1. *COX-2* mRNA and TXB$_2$ are increased in BMT mice**
A: AMs were harvested from control and BMT mice at 5 weeks post-BMT. RNA was isolated and *COX-2* was measured by real-time RT-PCR; n=4, representative of two experiments; B: Supernatants were collected from BMT and control AMs cultured in serum-free media for 24 h and measured for TXB$_2$ by ELISA; n=4, representative of two experiments.
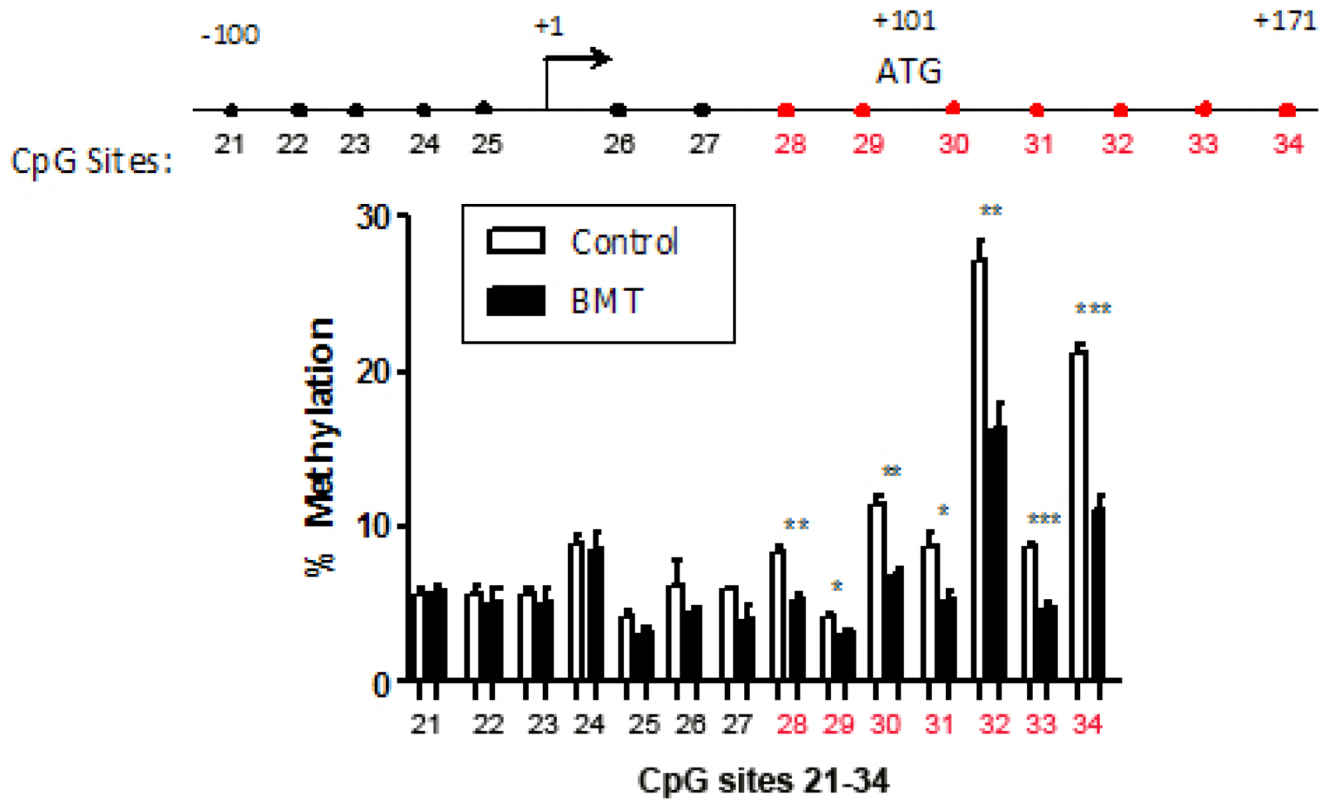
**Figure 2. *COX-2* in BMT AMs is hypomethylated around the first exon start site**
At week 5 post-BMT, AMs from control and BMT mice were harvested, DNA was isolated and bisulfite converted. Cytosine to thymine conversion was detected by pyrosequencing to determine methylation patterns. Control: n=4, BMT: n=3; *p < .05, **p<.01, ***p < .001. Representative of 2 experiments showing similar patterns at the same sites. Numbered CpG sites refer to locations specified in Supplemental Fig.1.
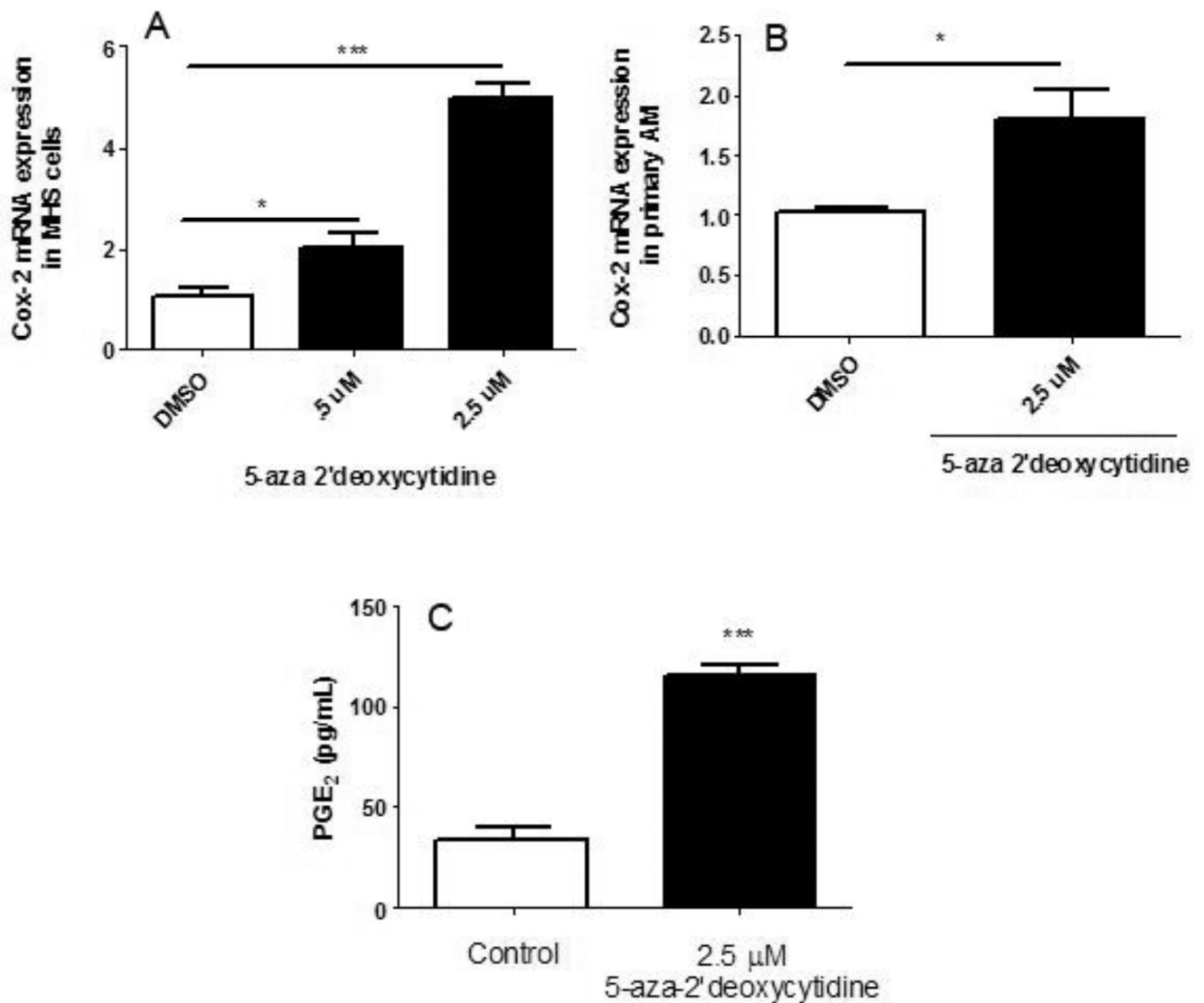
**Figure 3. 5-aza-2'-deoxycytidine increases *COX-2* in MHS cells and primary AMs and PGE$_2$ in MHS cells**

A: $5 \times 10^5$ MHS cells were cultured with 0.5 or 2.5 μM 5-aza 2'-deoxycytidine, or vehicle (DMSO) for 72 hours. RNA was isolated and Cox-2 mRNA levels were analyzed by real-time RT-PCR. Transcripts were normalized to β actin levels (n=4); B: Lungs from untransplanted mice were lavaged and primary AMs were harvested. AMs were initially stimulated with 1 ng/mL GM-CSF for 24 h and further treated with 2.5μM 5-aza-2'-deoxycytidine in the presence of GM-CSF for an additional 72 hours. RNA was isolated and COX-2 mRNA levels calculated by RT-PCR. C: MHS cells ($5 \times 10^5$) were grown in 10% complete media in the presence or absence of 2.5 μM 5-aza-2'-deoxycytidine for 48 hours. Media was aspirated and fresh serum free media with or without 5-aza-2'-deoxycytidine was added for 24 hours. Supernatants were collected and PGE$_2$ ELISA was performed. (n=3); *p<.05, **p<.01, ***p<.001. All data are representative of at least 2 experiments.
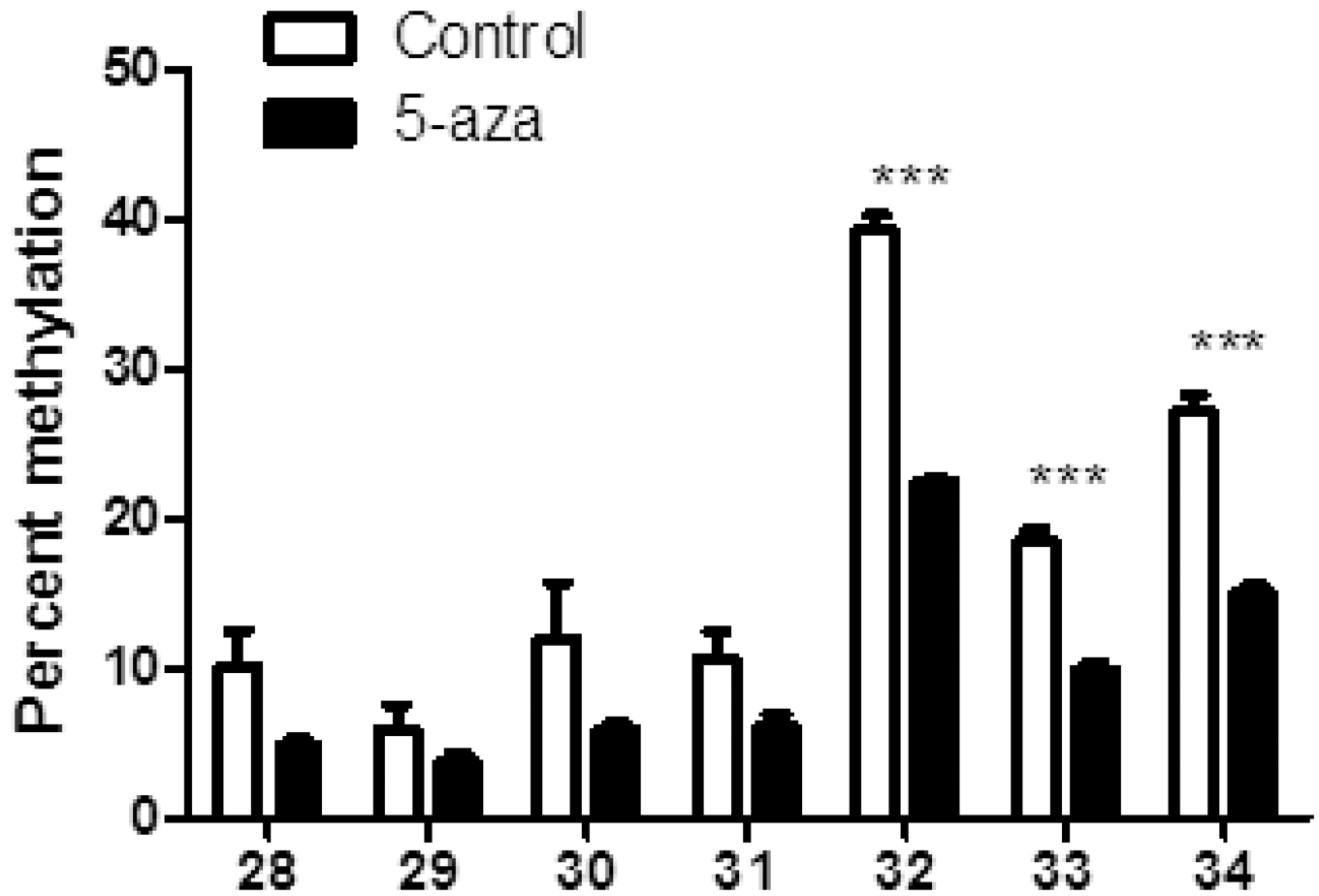
**Figure 4. 5-aza-2'-deoxycytidine treatment alters *COX-2* promoter methylation**
MHS cells ($5 \times 10^5$) were treated with either 2.5 μM 5-aza-2'-deoxycytidine or vehicle (DMSO) for 72 h prior to harvesting DNA for methylation analysis by bisulfite conversion and pyrosequencing; n=3, ***p<.001.
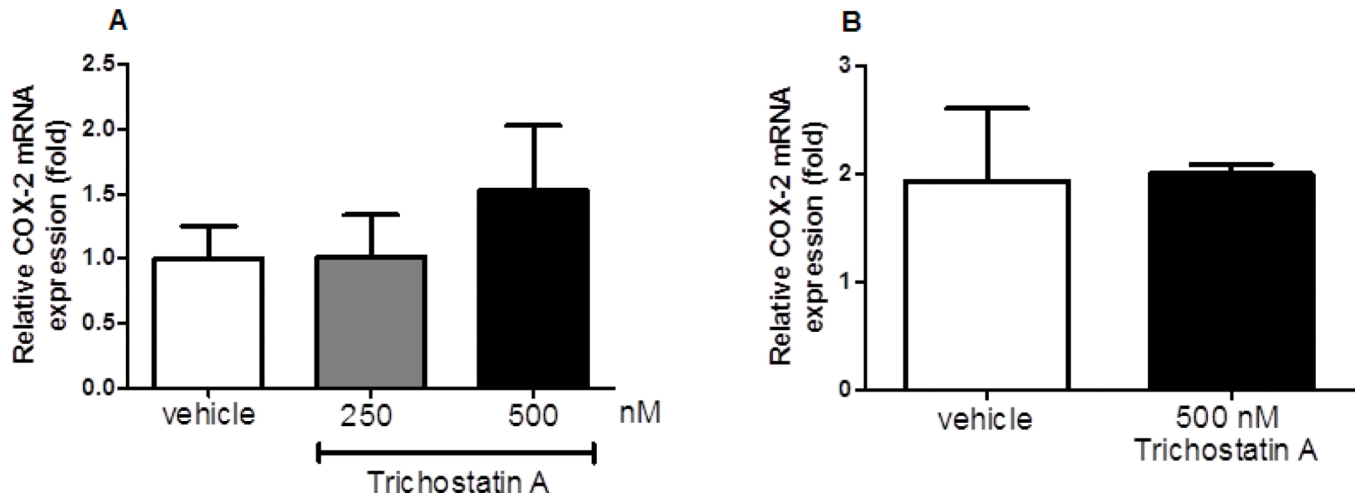
**Figure 5. COX-2 expression is not regulated by histone acetylation**
A: MHS cells ($2 \times 10^5$), B: GM-CSF-treated primary AMs ($2 \times 10^5$) were incubated with either trichostatin A (200 or 500 nM) or vehicle control (DMSO) for 72 h prior to RNA isolation. mRNA expression was measured by real-time RT-PCR; n=3. No changes were significant compared to vehicle.
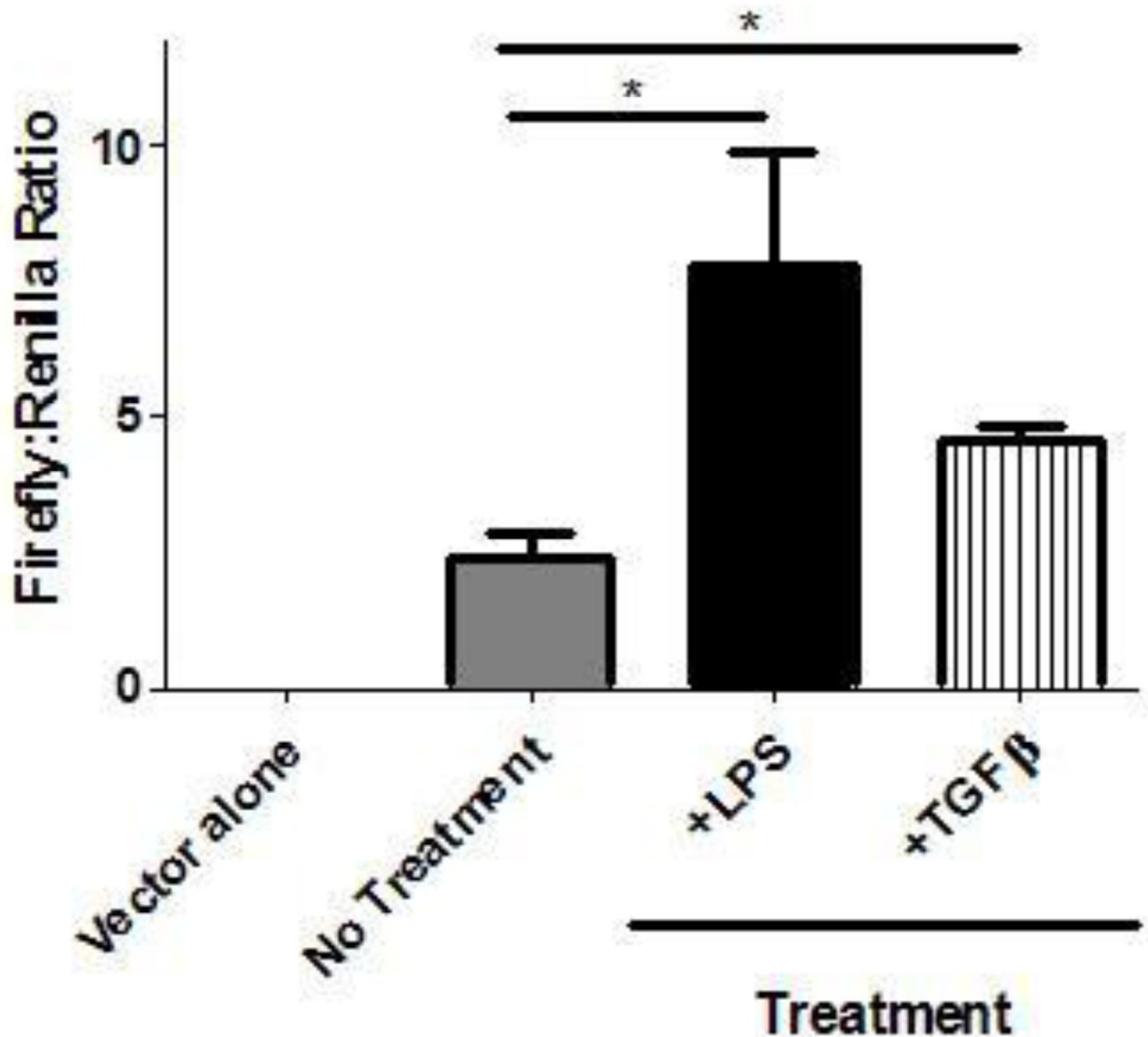
**Figure 6. Transfected *COX-2* promoter-driven luciferase is induced by LPS and TGF-β compared to untreated MHS cells**
MHS cells ($6.2 \times 10^4$) were cultured overnight in the presence or absence of either LPS (10 μg/mL), or porcine TGFβ (1 ng/mL). MHS cells were transfected the next day with either luciferase vector alone without COX-2 promoter insert or the long COX-2 promoter driven-luciferase vector, and pRL-SV40 (control vector) in a 50:1 ratio using lipofectamine LTX and PLUS Reagent. Firefly and renilla luciferase activities were measured and are shown as a ratio. (n=3, representative of 2 experiments; *p<.05).
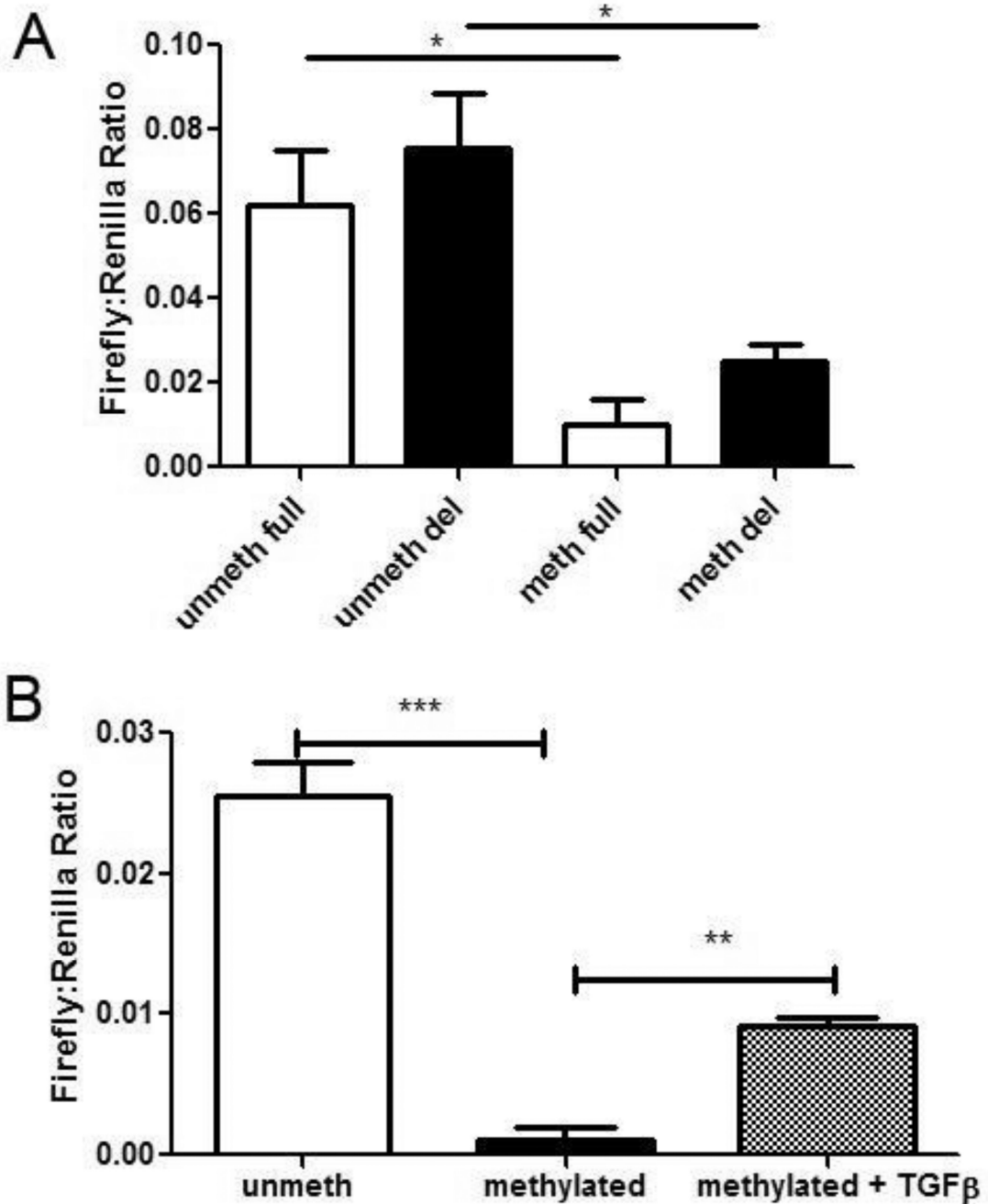
**Figure 7. *COX-2* promoter-driven luciferase expression is decreased following treatment with methyltransferase, but expression is regulated by TGF-β1**
A: Transfections of MHS cells ($6.2 \times 10^4$) were performed with a 50:1 ratio of either methylated or unmethylated, full-length or deleted (del) *COX-2* promoter-driven firefly luciferase plasmid to pRL-SV40 vector using lipofectamine. Firefly:Renilla luciferase activities were quantified by a dual luciferase assay system. (n = 6; *p < .05). B: MHS were cultured overnight in the presence or absence of porcine TGF-β1 (1 ng/mL) and transfected with methylated or unmethylated short COX-2 reporter plasmids and pRL-SV40 and assayed as above (n=3, **p<0.001, ***p<0.001).
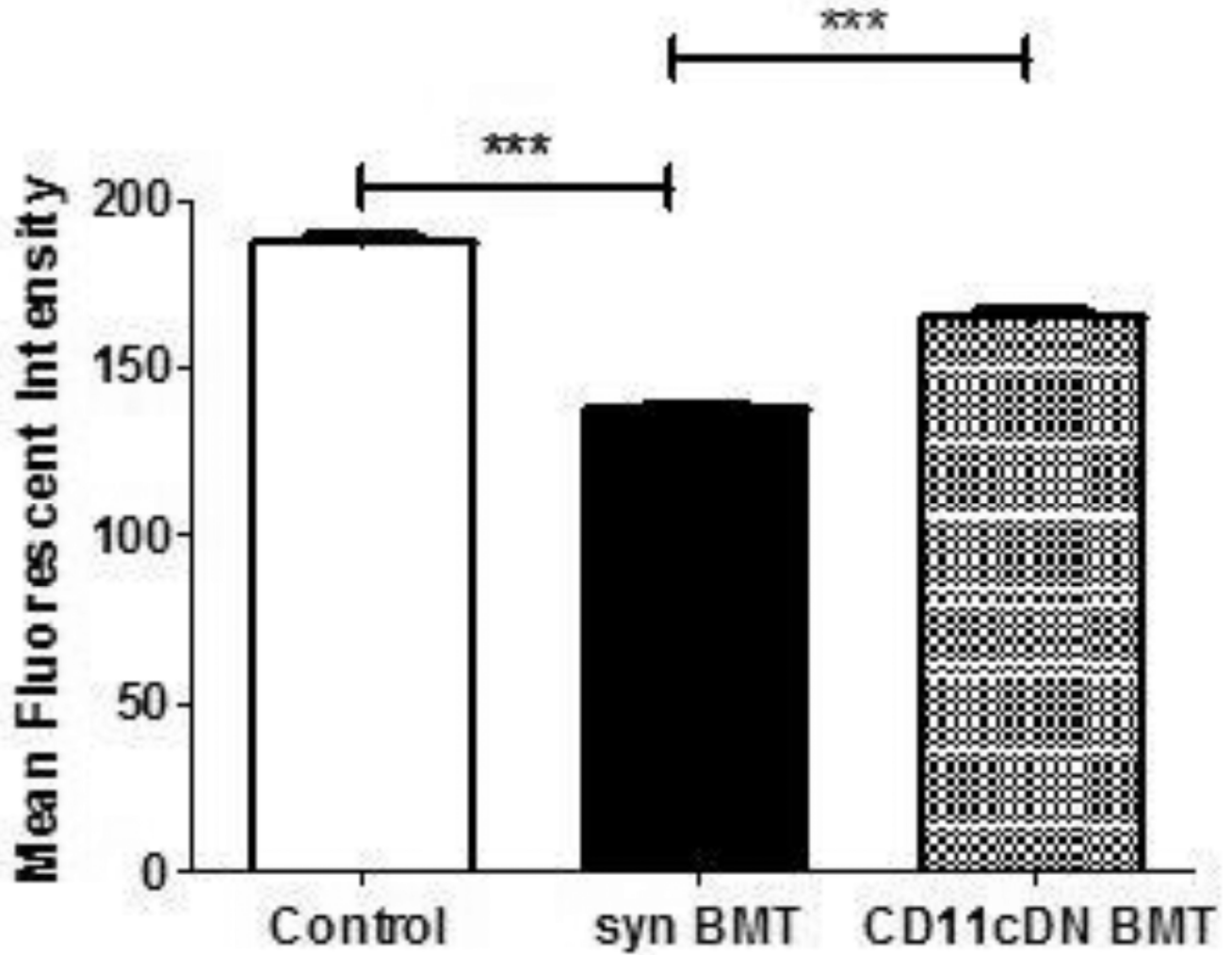
**Figure 8. Improved AM phagocytosis with CD11c$^{dnR}$ bone marrow chimeras**
AMs were harvested by BAL 5 weeks post-transplant from BMT mice reconstituted with either WT or CD11c$^{dnR}$ donor marrow. Phagocytosis of heat-killed FITC-*P. aeuroginosa* was then assessed and compared to AMs from control mice (n=5; ***p<.001).
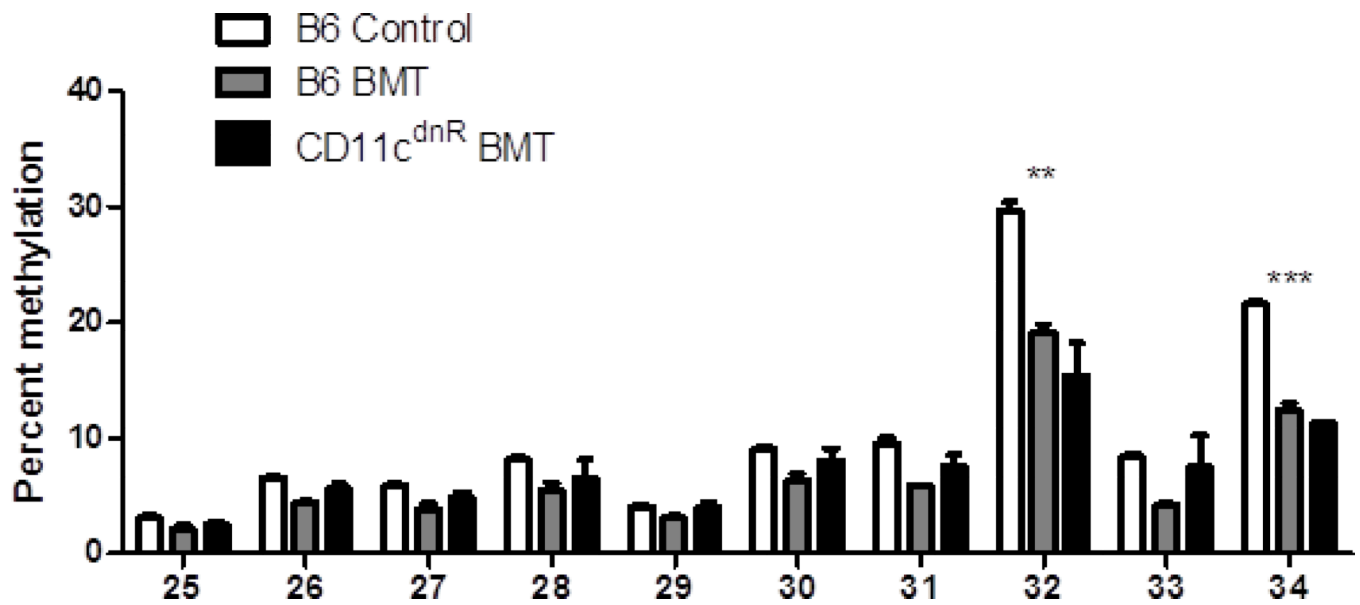
**Figure 9. CD11c^dnR BMT COX-2 promoter methylation is partially rescued**
Following DNA isolation from primary AMs from control, WT BMT and CD11c$^{dnR}$ BMT mice, DNA was subjected to bisulfite conversion and pyrosequencing for methylation analysis; n=5. WT BMT patterns were hypermethylated compared to control AMs at all sites. However, AMs from CD11cdnR BMT mice were not significantly different from control AMs except at CpG sites 32 and 34.

## Table I

Primers and probes for semiquantitative real-time RT-PCR and PCR

| RT-PCR primers and probes | Sequence (5'-3') |
| --- | --- |
| β-Actin forward | CCGTGAAAAGATGACCCAGATC |
| β-Actin reverse | CACAGCCTGGAGGCTACGT |
| β-Actin probe | TTTGAGACCTTCAACACCCCAGCCA |
| COX-2 forward | TGACCCCCAAGGCTCAAAT |
| COX-2 reverse | GAACCCAGGTCCTCGCTTATG |
| COX-2 probe | TTTGCCCAGCACTTCACCCACAGT |
| PCR primers for cloning | Sequence (5'-3') |
| COX-2 forward long | CACAGTAGGAAGGTACCCAACACTATC |
| COX-2 reverse long | CTGCTAGAAAGGAGATCTGAGCCTGAG |
| COX-2 forward short | CCGGTAGCTGTACGCGTGCTCTGAGC |
| COX-2 reverse short | AGATCGCAGATCTGAGCCTGAGG |
| Primers for analysis of CpG methylation on sites 1–6 | Sequence (5'-3') |
| Forward primer: | AGATGTGGATTTTGATAGAGGATATT |
| Reverse primer: | CTACCCTTAACTACCCCAAATAATAC |
| Sequencing primer: | ATTTTATTAAAAATAGAAGAAA |