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COX-2 Expression Is Upregulated by DNA Hypomethylation after Hematopoietic Stem Cell Transplantation

Racquel Domingo-Gonzalez,* Steven K. Huang,† Yasmina Laouar,‡ Carol A. Wilke,† and Bethany B. Moore†,‡

Hematopoietic stem cell transplantation (HSCT) is commonly used to treat malignant and nonmalignant hematologic disorders (1, 2). Traditionally, a conditioning regimen is implemented before i.v. infusion of hematopoietic stem cells (HSCs) that may consist of chemotherapy with or without total body irradiation (TBI) (2). TBI is itself myeloablative and immununosuppressive, and can affect regions within the body that are not easily accessible by chemotherapeutic agents delivered via the circulation (1–3). Although HSCT has proved to be an effective therapeutic option for malignancy, it is also associated with significant morbidity and mortality (1–5). After 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 development of life-threatening infectious and noninfectious complications (2, 3, 6–9).

The lung is a common target organ posttransplant 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 d after transplant), early postengraftment (30–100 d after transplant), and late postengraftment (>100 d after transplant) (10). Despite full reconstitution or engraftment of donor-derived leukocytes, patients exhibit sustained and enhanced susceptibility to infections posttransplant (6–10). Alveolar macrophage (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 AMs within 4 mo after HSCT, with some deficiency persisting up to 12 mo (15). Thus, impaired innate immune function may explain the prolonged susceptibility to infection observed in posttransplant individuals.

To study the effects of HSCT, our laboratory previously developed a syngeneic bone marrow transplantation (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 immununosuppressive drugs. We have shown that even after full immune reconstitution after syngeneic BMT, donor-derived AMs from BMT mice are defective in phagocytosis and killing compared with mice that did not undergo BMT (11, 16). We discovered this defect is related to decreased cysteinyl leukotrienes and TNF-α production, and increased PGE2 production (11, 17, 18).

Eicosanoids are lipid mediators derived from arachidonic acid, and cells of the myeloid lineage are major producers of both cysteinyl leukotrienes and PGE2 (18, 19). Synthesis of PGs 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, 13, 14, 16). However, in our model of Pseudomonas aeruginosa infection post-BMT, we demonstrated that it was the defect in nonopsonized phagocytosis by AMs post-BMT, rather than PMN function, that 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 four discrete EP receptors that couple to G proteins involved in mediating the intracellular signaling in response to PGE\(_2\) (20, 21). We have shown that binding of the EP2 receptor by PGE\(_2\) results in increased IL-1R-associated kinase expression in AMs, resulting in the inhibition of nonopsonized phagocytosis (14). PGE\(_2\) also induces elevation of the enzyme phosphatase and tensin homolog deleted on chromosome 10, which results in the inhibition of serum-opsonized phagocytosis by AMs (13). 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 AM cell line (MHS) and primary AMs with 5-aza-2′-deoxycytidine (a methyltransferase 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 (22). 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-βRII under the CD11c promoter (CD11c\(^{DN}\)) were provided by Dr. Loaur (University of Michigan) (23, 24). 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.

BMT

Recipient mice received 13.5 Gy 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 (DMEM, 0.1% BSA, 1% penicillin-streptomycin, 1% t-glutamine, and 0.1% amphotericin B). Bone marrow cells (5 × 10\(^4\)) 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). In brief, these cells were collected in lavage fluid consisting of complete medium (DMEM, 1% penicillin-streptomycin, 1% t-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 Kraemer and colleagues (25) and a 989-bp COX-2 promoter (deleted) was amplified using primers to create cloning sites. Supplemental Fig. 1 shows the sequence of the COX-2 promoter and the location of the created cloning sites (KpnI, MluI, and BglII) in gray boxes. Table I 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), whereas the deleted COX-2 promoter was digested with MluI and BglII (New England BioLabs). 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 h. 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 vectors, inserted into the luciferase reporter plasmid pGL3-basic (Promega) were in vitro methylated with CpG methyltransferase M.SssI (New England BioLabs) as specified by the manufacturer for 4 h. Methylation was confirmed by digestion with methylation-sensitive restriction enzyme Smal.

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. In brief, 6.2 × 10\(^4\) MHS cells were cultured in complete media containing RPMI, 10% FCS, 1% penicillin-streptomycin, 1% t-glutamine, and 0.5 mM 2-ME overnight. After 24 h, 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 DNA using lipofectamine LTX and PLUS Reagent (Invitrogen). Transfections were performed with unmethylated or methylated, full or deleted COX-2 luciferase reporter plasmids. Where indicated, cells were treated with 10 μg/ml 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 are listed in Table 1. Each AM sample was pooled from two to three mice and run in duplicate. Average cycle threshold was determined for each sample and normalized to β-actin. Relative gene expression was calculated as described previously (26).

DNA methyltransferase or histone deacetylase 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 histone deacetylase (HDAC) inhibitor trichostatin A (Sigma) for 72 h. Primed AMs were initially stimulated with (1 ng/ml) recombinant murine GM-CSF (R&D Systems, Minneapolis, MN) for 24 h to promote proliferation before receiving the appropriate treatments for the following 72 h.

ELISA/Enzyme immunoassay

MHS cells were cultured at 5 × 10\(^4\) cells/ml in a 24-well plate for 72 h. MHS cells were grown in complete media for 48 h, then switched to serum-free media for 24 h. Supernatants were collected for enzyme immunoassay. Production of PGE\(_2\) and thromboxane B\(_2\) (TXB\(_2\); a metabolite of thromboxane A\(_2\) [TXA\(_2\)]) was measured by enzyme immunoassay (Cayman Chemical, Ann Arbor, MI), according to the manufacturer’s instructions.

Bisulfite conversion and pyrosequencing

DNA was isolated from 5 × 10\(^4\) to 1 × 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 genomic DNA. Bisulfite modification
was performed according to manufacturer’s instructions. In brief, 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 30 s; 50˚C 60 min) × 16 cycles; 4˚C hold. After bisulfite conversion, the PTGS2 (COX-2) promoter was PCR amplified and sequenced on a Pyroseq quencer (Qiagen). The analyzed CpG sites are shown in Supplemental Fig. 1 and color coded by blue, yellow, or pink boxes to denote the regions amplified by different primer pairs for analysis. The analyzed CpG sites are also numbered for clarity. Amplification and sequencing primers for murine PTGS2 were obtained from EpigenDx (assay ADS2001 [sites in yellow] and ADS2002 [sites in pink]; Worcester, MA). Sites in blue were amplified and sequenced using primers designed in Table I. The PCR conditions for determining methylation profile of the DNA using ADS2001 are as follows: 95˚C 15 min; 45 × (95˚C 30 s; 54˚C 30 s; 72˚C 30 s); 72˚C 5 min; 4˚C hold. PCR conditions using ADS2002: 95˚C 15 min; 45 × (95˚C 30 s; 60˚C 30 s; 72˚C 30 s); 72˚C 5 min; 4˚C hold.

P. aeruginosa PA01 preparation and FITC labeling

P. aeruginosa PA01 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 twice in PBS by resuspending cell pellet in 1 ml sterile PBS and transferring into a sterile tube. P. aeruginosa was heat killed by autoclaving for 20 min and resuspended at 10^6 to 10^7 CFU/ml in 0.1 M NaHCO3 (pH 9.2). A total of 0.2 mg/ml FITC (Sigma) in DMSO was added to heat-killed P. aeruginosa and allowed to incubate in dark for 1 h on rocker at room temperature. After FITC labeling, heat-killed P. aeruginosa 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 bronchoalveolar lavage (BAL) were plated at 2 × 10^5 cells/well in 100 μl complete media and cultured overnight on a 96-well, flat-bottom, half-area tissue culture plate (Costar, Corning, NY). The following day, FITC-labeled, heat-killed P. aeruginosa (prepared as described earlier) were added at 500:1 multiplicity of infection. Two hours after incubation at 37˚C in dark, 50 μl 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 nonphagocytosed FITC-labeled bacteria. AM phagocytosis of FITC-labeled P. aeruginosa was measured by real-time RT-PCR (Table I). These results demonstrate that COX-2 is upregulated in BMT AMs when compared with control AMs (Fig. 1A). These results are consistent with our previous observation that COX-2 protein is also increased in AMs post-BMT (14) and confirm that the BMT-induced alterations that enhance COX-2 expression persist in AMs cultured ex vivo.

COX-2 can catalyze the synthesis of numerous PGs. We previously published that prostacyclin (PGI2) is also increased in AMs post-BMT (11). To determine whether TXA2 was also increased, we measured the more stable metabolite TXB2 in control and BMT AMs (Fig. 1B). Taken together, these results suggest that BMT causes a global upregulation of prostanooids.

Results

COX-2 mRNA levels are increased in BMT AMs

PGE2 levels were previously reported to be increased in the plasma of patients undergoing autologous HSCT (27). The increased PGE2 detected in these individuals posttransplantation was independent of conditioning regimen (chemotherapy or radiotherapy). Similar results were previously observed in our established syngeneic BMT mouse model, whereby 13 Gy TBI or a dual-chemotherapy regimen induced a defective pulmonary immune response associated with increased levels of PGE2 produced by lung innate immune cells (11, 16). PGE2 production is dependent on the activity of COX enzymes. To determine whether the increase in PGE2 levels are a result of increased COX-2 expression, we measured COX-2 expression levels from control (untransplanted) and BMT AMs harvested by BAL. After 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 (Table I). These results demonstrate that COX-2 is upregulated in BMT AMs when compared with control AMs (Fig. 1A). These results are consistent with our previous observation that COX-2 protein is also increased in AMs post-BMT (14) and confirm that the BMT-induced alterations that enhance COX-2 expression persist in AMs cultured ex vivo.

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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 Fig. 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 with control AMs at CpG loci within the 5’-UTR and exon 1 (sites 28–34; Fig. 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 (28). 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 PGE2 observed posttransplant. To determine whether DNA methylation of COX-2 contributes to its diminished expression in control non-BMT cells, we treated MHS cells, a murine AM cell line, with increasing concentrations of 5-aza-2’-deoxycytidine for 72 h 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 (Fig. 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 (29), and COX-2 mRNA levels were determined by real-time RT-PCR. Expression patterns of COX-2 transcripts shown in Fig. 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 PGE2 levels (Fig. 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. Fig. 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.

Because 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 (Fig. 5A) and primary AMs (Fig. 5B) stimulated with trichostatin A for 72 h did not show a significant difference in COX-2 expression.
compared with 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 BglII 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. Because 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 h. 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 (Fig. 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 BglII) and deleted (MluI to BglII) 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 this 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 before transfection. As shown in Fig. 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 that was previously analyzed in Fig. 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 (22), and TGF-β1 can induce expression of COX-2 (Fig. 6). To determine whether TGF-β1 could stimulate the transcription of COX-2 from a methylated promoter, we transfected MHS cells as described earlier with the deleted COX-2 promoter-driven firefly luciferase construct that had either been methylated in vitro by M.SssI or left unmethylated. Cells were cotransfected with the SV40-driven Renilla luciferase vector as a control. Cells transfected with the methylated construct were

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Table I. Primers and probes for semiquantitative real-time RT-PCR and PCR

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<td>β-Actin reverse</td>
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<td>COX-2 forward</td>
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**FIGURE 1.** COX-2 mRNA and TXB₂ are increased in BMT mice. (A) AMs were harvested from control and BMT mice at 5 wk 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 TXB₂ by ELISA. n = 4, representative of two experiments. ***p < 0.01.
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 with 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 after reconstitution with donor HSCs from CD11c<sup>dnR</sup> mice**

TGF-β1 can play an important role in both the activation and the suppression of monocytes and macrophages; however, its role in tissue macrophages is primarily immunosuppressive (30). To determine how TGF-β1 may contribute to the defect in BMT AMs, we developed bone marrow chimeras using HSCs from CD11c<sup>dnR</sup> donor mice. These mice express a dominant-negative TGF-β1RII under the control of the CD11c promoter, which results in the generation of TGF-β1–resistant myeloid cells including dendritic cells, macrophages, and NK cells (23). Five weeks post-BMT, AMs were harvested by BAL, and phagocytosis was measured and compared with WT BMT and control mice. As expected, AMs from WT BMT mice displayed defective phagocytosis of *P. aeruginosa* when compared with cells from control mice. However, AMs collected from CD11c<sup>dnR</sup> BMT mice showed enhanced phagocytosis activity compared with WT BMTs (Fig. 8). To determine whether this enhancement in AM function is mediated by a change in COX-2 gene methylation, DNA from CD11c<sup>dnR</sup> BMT AMs was harvested and analyzed for extent of methylation. Interestingly, the methylation of the COX-2 promoter from CD11c<sup>dnR</sup> 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 (Fig. 9).

**Discussion**

We previously showed that AMs post-BMT are defective in phagocytosis and killing of *P. aeruginosa*, a clinically relevant nosocomial pathogen that can afflict patients after HSCT (11, 13, 14, 16, 26). Defective AM function post-BMT is not exclusive to mice receiving TBI but is also seen in mice receiving chemotherapy conditioning regimens (16), and it persists despite immune reconstitution. We have previously shown that both PGE<sub>2</sub> and TGF-β1 are elevated in lungs of BMT mice after immune reconstitution, and both can have immunosuppressive effects (11, 22). We now show that irradiated mice receiving CD11c<sup>+</sup> donor cells that are unable to respond to TGF-β1 exhibit improved AM function (Fig. 8), indicating that TGF-β1 is contributing to the defect in AMs after transplantation. Because the dominant-negative TGF-β1RII is under the CD11c promoter, it is possible that the incomplete rescue of AM function is due to decreased CD11c expression posttransplant (31). The methylation pattern of COX-2 in CD11c<sup>dnR</sup> BMT AMs supports this possibility because we ob-

![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. Representative of two experiments showing similar patterns at the same sites. Numbered CpG sites refer to locations specified in Supplemental Fig. 1. *p < 0.05, **p < 0.01, ***p < 0.001.](http://www.jimmunol.org/)
PGE2 may promote cancer progression and metastasis, as well as cancer is most commonly reported (33). In these circumstances, been associated with a wide range of disease processes, of which

methylation analysis by bisulfite conversion and pyrosequencing. Numbers refer to the same CpG sites noted in Fig. 2.

It is possible, however, that upregulation of TXA2 may have deleted lung innate immune system (11). In this article, we show hematopoietic reconstitution and is intimately linked with a effective lung innate immune system (11). In this article, we show transcription in AMs is elevated in mice post-BMT relative to control AMs. In addition, previously published data showed upregulation of PGE2-specific synthase enzymes post-BMT as well (14). These results correlate with the increased PGE2 levels reported previously in both mice and patients who have undergone HSCT (11, 13, 14, 16, 27). Because other prostanoids (e.g., PG2 and TXA2 [Fig. 1B]) are also increased after transplant, these data indicate that increased COX-2 has a broad effect on downstream effectors. PG2 signaling through its IP receptor on macrophages and neutrophils may play a similar role as PGE2 in inhibiting proper cell function because IP is a G coupled receptor that signals through cAMP (21, 32). Although TXA2 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 Ref. 32).

Uregulation of COX-2 expression and PGE2 production has been associated with a wide range of disease processes, of which cancer is most commonly reported (33). In these circumstances, PGE2 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 (34). The consequence of miR29b upregulation was the destabilization of DNA methyltransferases and the upregulation of COX-2 gene expression secondary to DNA demethylation (34). 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 with 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 (35), and methylation around the transcription start site and in gene exons has been previously described (e.g., in Refs. 34, 36–38). 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 (34, 38). 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 [39], and silencing of the COX-2 gene in human gastric cancers involves hypermethylation of promoter regions upstream of exon 1 [40]). 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 (Fig. 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; Fig. 3B). Furthermore, when methylation patterns were analyzed after 5-aza-2-deoxycytidine treatment, there was a significant decrease in methylation on CpG sites found to be hypomethylated in BMT AMs (Fig. 4). In contrast, HDAC acetylation did not appear to regulate COX-2 expression (Fig. 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 increased 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 (Fig. 6). These data suggest that the reporter vector is regulated similarly to the native gene in MHS cells.
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 that is noted in AMs collected from untransplanted mice likely plays a role in the limited expression of COX-2 and the limited production of PGE2 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 (Fig. 7B). These results suggest that increased levels of TGF-β1, which have been previously reported in BMT mice (22), 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 that is able to stimulate transcription off of unmethylated COX-2 promoters (Fig. 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<sup>-dnR</sup> BMT mice that are unresponsive to 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 that is noted in AMs collected from untransplanted mice likely plays a role in the limited expression of COX-2 and the limited production of PGE2 in these cells under homeostatic conditions.

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TGF-β1 signaling in AMs is highly supportive, and our in vivo methylation patterns of CD11c dnR BMT mice are more similar to control AMs than to WT BMT AMs (Fig. 9). Our results suggest that TGF-β1 may be one mechanism to regulate the increased expression of COX-2 and thus PGE2, 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 CD11c dnR 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 after ionizing radiation and has been implicated in compromised lung immunity (41). Reactive nitrogen species, particularly NO, have been suggested to induce changes in COX within airway epithelium during inflammation (42). In these studies, increasing concentrations of NO were able to induce the production of PGE2 in alveolar epithelial cells. NO and PGE2 expression can be induced by proinflammatory cytokines (42). 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 caused by 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 laboratory have indicated that alveolar epithelial cells may influence AM function through the secretion of soluble factors like GM-CSF (26). Although we have ruled out the contribution of GM-CSF to the induction of COX-2 in this model (26), 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.

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Disclosures

The authors have no financial conflicts of interest.

References


