Transforming growth factor-β induces microRNA-29b to promote murine alveolar macrophage dysfunction after bone marrow transplantation

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1Graduate Program in Immunology, University of Michigan Medical School, Ann Arbor, Michigan; 2Pulmonary and Critical Care Medicine Division, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan; 3Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan; 4Research Service, VA Ann Arbor Healthcare System, Ann Arbor, Michigan; 5Medical Service, VA Ann Arbor Healthcare System, Ann Arbor, Michigan; 6Department of Pediatrics, Division of Hematology-Oncology, University of Michigan Medical School, Ann Arbor, Michigan

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Domingo-Gonzalez R, Wilke CA, Huang SK, Laouar Y, Brown JP, Freeman CM, Curtis JL, Yanik GA, Moore BB. Transforming growth factor-β induces microRNA-29b to promote murine alveolar macrophage dysfunction after bone marrow transplantation. Am J Physiol Lung Cell Mol Physiol 308: L86–L95, 2015. First published October 31, 2014; doi:10.1152/ajplung.00283.2014.—Hematopoietic stem cell transplantation (HSCT) is complicated by pulmonary infections that manifest posttransplantation. Despite engraftment, susceptibility to infections persists long after reconstitution. Previous work using a murine bone marrow transplant (BMT) model implicated increased cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2) in promoting impaired alveolar macrophage (AM) responses. However, mechanisms driving COX-2 overexpression remained elusive. Previously, transforming growth factor-β (TGF-β) signaling after BMT was shown to promote hypomethylation of the COX-2 gene. Here, we provide mechanistic insight into how this occurs and show that TGF-β induces microRNA-29b (miR-29b) while decreasing DNA methyltransferases (DNMT)1, DNMT3a, and DNMT3b in AMs after BMT. De novo DNMT3a and DNMT3b were decreased upon transient transcription of miR-29b, resulting in decreased methylation of the COX-2 promoter and induction of COX-2. As a consequence, miR-29b-driven upregulation of COX-2 promoted AM dysfunction, and transcription of BMT AMs with a miR-29b inhibitor rescued the bacterial-killing defect. MiR-29b-mediated defects in BMT AMs were dependent on increased levels of PGE2, as miR-29b-transfected AMs treated with a novel E prostaglandin receptor 2 antagonist abrogated the impaired bacterial killing. We also demonstrate that patients that have undergone HSCT exhibit increased miR-29b; thus these studies highlight miR-29b in driving defective AM responses and identify this miRNA as a potential therapeutic target.

AS AN EFFECTIVE THERAPY for a variety of malignant and inherited disorders, hematopoietic stem cell transplantation (HSCT) offers a curative option for many patients. Advances in conditioning regimens and stem cell acquisition have widened the accessibility and implementation of HSCT. Traditionally, patients that get a transplant either receive their own hematopoietic stem cells (autologous HSCT) or receive stem cells from human leukocyte antigen-matched, related, or unrelated donors (allogeneic HSCT) following myeloablative conditioning regimens. More recently, reduced-intensity conditioning regimens have enabled older patients or patients unable to undergo conventional conditioning regimens the opportunity to receive stem cell transplantation for their conditions as well (16). However, despite differences in conditioning regimens or application of immunosuppressive therapy, patients undergoing HSCT exhibit increased susceptibility to pulmonary complications both before and after engraftment (2, 3, 48, 57). The associated morbidity and mortality associated with microbial infection is further confounded by antibiotic-resistant bacteria (23). During the preengraftment phase, susceptibility to infection is likely due to the neutropenia commonly associated with this stage. However, less clear is why patients continue to exhibit sustained susceptibility to bacterial infections throughout the early postengraftment phase (30–100 days after transplant) and the late postengraftment phase (>100 days after transplant), when the immune compartment has been reconstituted by donor-derived cells (2, 47). Previous studies, including autopsy reports, have revealed that pulmonary complications are a significant cause of death for patients with transplants and a predictor for poor survival (47). Thus understanding why these pulmonary complications arise is vital to survival and quality of life for patients undergoing HSCT.

Existing in the alveolar compartment between air and lung tissue, alveolar macrophages (AMs) compose >90% of the cells retrieved by bronchoalveolar lavage (BAL) in a healthy individual and constitute the first line of defense in the lung (35). Following HSCT, however, phagocytosis and bacterial killing by human AMs are significantly compromised (59), suggesting that impaired AM responses support prolonged sensitivity to infection following transplantation. To investigate the causes for impaired innate immune responses, our laboratory utilized a syngeneic murine model of bone marrow transplantation (BMT) as a surrogate of autologous transplantation. This model sheds light on the underlying effects of transplantation on immune cell function independent of immunosuppressive therapy or development of graft-vs.-host disease (28). Using this model, we previously reported that BMT mice were more susceptible to Gram-negative (Pseudomonas aeruginosa, P. aeruginosa) and Gram-positive (Staphylococcus aureus, S. aureus) pneumonia (18, 42). This increased susceptibility was at least partially attributed to functionally defective AMs (5–7, 14, 18, 19, 26–28). As AMs are the sentinel phagocytes in the lung, their ability to recognize an
invading pathogen, to recruit immune cells to the infected lung, and to control or clear the invading pathogen is important for preventing widespread infection and alveolar damage. Similarly to human HSCT AMs, murine AMs after BMT exhibit impaired phagocytosis and bacterial killing (14, 19, 59). Specifically, phagocytosis of *P. aeruginosa* is diminished because of reduced levels of the macrophage receptor with collagenous structure (MARCO) on AMs after BMT, whereas phagocytosis of *S. aureus* is actually increased because of elevations of scavenger receptor A (18). Despite these differences in phagocytosis, however, BMT AMs display defects in intracellular killing of both of these pathogens (18). These defects were attributed to overexpression of cyclooxygenase (COX)-2 and increased production of its downstream effector, prostaglandin E2 (PGE2). Previous observations also noted elevated PGE2 in the serum of patients undergoing autologous HSCT (11).

Eicosanoids are lipid mediators derived from arachidonic acid metabolism. There are two isoforms of the COX enzymes: COX-1 and COX-2 (10). Constitutively expressed, COX-1 is important for mediating homeostatic effects, whereas COX-2 is upregulated by inflammatory signals. Metabolism of arachidonic acid by COX-1/COX-2 is necessary for the generation of PGE2. PGE2 can be recognized by G protein-coupled E prostanoid (EP) receptors (EP1–4) (4, 40, 54). In our BMT model, EP2 and EP4 are significantly overexpressed, and binding of PGE2 via these receptors, particularly EP2, results in inhibition of macrophage function (5). In BMT AMs, basal COX-2 expression is significantly higher than in untransplanted control AMs (5, 17), which we previously showed was attributable to hypomethylation of CpG sites within the COX-2 promoter (17). Hypomethylation and increased COX-2 expression have previously also been shown in cancer (39, 60). In our model, COX-2 hypomethylation was a result of increased transforming growth factor-β (TGF-β) signaling (17). Disruption of TGF-β signaling resulted in partial restoration of methylation patterns in COX-2 as well as AM function, i.e., phagocytosis (17). However, the mechanism by which TGF-β mediated this effect was unknown.

Mature microRNA (miRNA) are short, noncoding RNA sequences about 22 nucleotides in length, previously dubbed as “fine tuners” of immune responses (25, 50). MiRNA are posttranscriptional regulators that can inhibit mRNA stability and/or protein translation by binding to 3’ untranslated regions (UTR) of their target genes (25, 50). Our study presented here identifies the microRNA miR-29b as significantly upregulated in BMT AMs. Interestingly, miR-29b contains predicted target sites within the 3’UTR of the de novo DNA methyltransferases (DNMT), DNMT3a and DNMT3b (TargetScan, Cambridge, MA) (12, 20, 22, 55), and has been shown to directly target DNMT3a and DNMT3b (20) while indirectly targeting DNMT1 by targeting a transactivator of DNMT1, Sp1 (21). DNMTs are responsible for the transfer of methyl groups onto CpG residues and for maintaining methylation patterns (29). Previous studies in nonmyeloid cells have shown that TGF-β inhibits miR-29b in a SMAD3-dependent manner (62), and, conversely, upregulation of miR-29b was shown to inhibit TGF-β signaling (61). However, as TGF-β is a pleiotropic cytokine, it is not surprising that TGF-β may mediate differential cell-specific effects on miR-29b. Here, we demonstrate that TGF-β-induced miR-29b leads to COX-2 promoter hypomethylation in BMT AMs.

**Materials and Methods**

**Human subjects.** BAL cell samples from 14 patients undergoing HSCT (both autologous and allogeneic) and 6 healthy volunteers were used in this study. Studies and consent procedures were performed in accordance with the Declaration of Helsinki at the University of Michigan (patients undergoing HSCT) and the VA Ann Arbor Healthcare System (volunteers) and were approved by their respective Institutional Review Boards. All human subjects gave their written informed consent before any research procedures. Patient and healthy donor characteristics are outlined in Table 1.

**Animals.** CD11c<sup>dnr</sup> mice that express a dominant-negative TGF-βRII driven by CD11c promoter were previously described (33, 34). Wild-type (WT) C57Bl/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were bred and housed under veterinary-monitored pathogen-free conditions. Mice were euthanized by CO2 asphyxiation. The experiments in which the mice were utilized were approved by the University of Michigan Committee on the Use and Care of Animals.

**BMT.** BMT was performed as described previously (28). Briefly, whole bone marrow was harvested from the femur and tibia of C57Bl/6 WT or CD11c<sup>dnr</sup> donor mice, and 5 × 10<sup>6</sup> marrow cells were introduced via tail vein injection into lethally irradiated C57Bl/6 recipients. A fractionated 13-Gy dose of total body irradiation from an X-ray orthovoltage source was administered to recipient C57Bl/6 mice for ablation of the recipient hematopoietic compartment. Experiments were performed 5 wk posttransplant, a time point when reconstitution with donor-derived cells is 95 ± 1% in the spleen and 82 ± 2% in the lung using CD45.1 and CD45.2 congenic mice (28).

**Harvesting AMs via BAL.** Primary or resident AMs were obtained from C57Bl/6 mice via ex vivo BAL as previously described (5). Briefly, complete media (DMEM, 1% penicillin-streptomycin, 1% l-glutamine, 10% FCS, 0.1% fungizone) and 5 mM EDTA were used to lavage the lung. Cells collected in the lavage fluid were counted on a hemocytometer before being plated. Previous studies showed >95% purity in AM cultures as determined by modified Wright-Giemsa staining following 1-h adherence (5).

**Tetrazolium dye reduction assay of bacterial killing.** The tetrazolium dye reduction assay (43, 49) was used to determine the ability of miRNA-transfected AMs to kill *S. aureus* (USA300/NRS384). This assay was chosen because it normalizes for differences in phagocytosis, such as is seen after BMT. AMs from untransplanted control or BMT mice were harvested by BAL and aliquoted into duplicate 96-well plates (1 experimental and 1 control plate). Cells from both plates were infected with serum-opsonized (Abcam, Cambridge MA) *S. aureus* at a multiplicity of infection of 50 for 30 min at 37°C. Both plates were washed, and the experimental plate was further incubated for 2 h at 37°C while the control plate was lysed with 0.5% saponin in tryptic soy broth and placed at 4°C. Following 2 h, the AMs on the experimental plate were lysed with 0.5% saponin in tryptic soy broth and incubated on ice for 5 min. Both plates were then incubated for 4 h at 37°C, with shaking at 200 revolution/min. Both plates then received 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma, St. Louis, MO) and were incubated for 30 min. Following the formation of a purple precipitate, solubilization solution was added to dissolve the formazan salts. Absorbance was read at 595 nm, and bacterial survival was calculated as follows: survival of ingested bacteria = (A<sub>595</sub> experimental plate/A<sub>595</sub> control plate) × 100%. The results were expressed as a percentage of control to indicate the percentage of survival of ingested bacteria normalized to the percentage of control.

**Real-time RT-PCR.** Primer Express software (PE Biosystems, Foster City, CA) was used to generate gene-specific primers and probes as previously described (17). The designed primer and probe sequences can be found in Table 2. Primers/probes used for miRNA expression analysis were purchased from Applied Biosystems/Life Technologies (Foster City, CA) and measured using
Table 1. Characteristics of patients undergoing HSCT

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Days Posttransplant</th>
<th>Conditioning</th>
<th>Underlying Disease</th>
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<tr>
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<tr>
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<td>N/A</td>
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<tr>
<td>Control 6</td>
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<tr>
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<td>AML</td>
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<td>Allo 2</td>
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<td>AML</td>
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<td>BEAM</td>
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Allo, allogeneic hematopoietic stem cell transplantation (HSCT); Auto, autologous HSCT; MEL, melphalan; BEAM, BCNU-Etoposide-Ara-C-Melphalan; Flu, fludarabine; Bu, busulfan; TLI, total lymphocyte infusion; TBI, total body irradiation; Clo, clofarabine; Cy, cytoxan; ATG, anti-thymocyte globulin; BAC, bendamustine and cytarabine; AML, acute myelomonocytic leukemia; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndrome.

Pharmacological agents. Indomethacin was used as a nonselective COX inhibitor (Sigma). PF-04418948, a specific EP2 antagonist, was generously supplied by Pfizer (New York, NY) through their compound transfer program (1, 8). The methyltransferase inhibitor, 5-aza-2’-deoxycytidine, was used on primary WT AMs harvested by BAL to determine the effects of methylation on gene expression. Cells were initially supplemented with granulocyte-macrophage colony-stimulating factor (1 ng/ml) for 24 h before the addition of 2.5 μM 5-aza-2’-deoxycytidine for 72 h.

Statistical analysis. To determine statistical significance, the GraphPad Prism 6.0 statistical program (La Jolla, CA) was utilized. Comparisons between two experimental groups were determined using the Student’s t-test. Comparison between three or more groups utilized ANOVA analyses followed by a Bonferroni posttest. A P value <0.05 was considered statistically significant.

RESULTS

Increased miR-29b and decreased DNMT expression in AMs after BMT. We previously showed that increased TGF-β signaling in AMs after BMT was responsible for hypomethylation
of COX-2 and that inhibition of TGF-β signaling in AMs after BMT induced hypermethylation of the COX-2 promoter (17), resulting in decreased expression and restored AM function (17). To determine how TGF-β might result in COX-2 hypomethylation, we investigated the expression of various DNMT isoforms in AMs from control (untransplanted) and syngeneic BMT mice. Because DNMTs are responsible for the transfer of methyl groups onto CpG sites, we measured the expression of maintenance (DNMT1) and de novo (DNMT3a and DNMT3b) methylation enzymes following BMT. The expression of all three DNMTs was significantly decreased compared with untransplanted controls (14). We previously showed elevated levels of TGF-β expression.

Primary AMs from WT untransplanted mice were treated with LPS. Thus we verified using real-time RT-PCR that AMs from BMT mice express higher levels of miR-29b than do AMs from control mice (Fig. 1A). Similarly, AMs from patients undergoing autologous and allogeneic HSCT exhibited high expression of miR-29b relative to the U6 control compared with AMs collected from normal healthy volunteers (Fig. 1C). Interestingly, high expression levels of miR-29b were noted even in patients more than 2 yr after HSCT compared with healthy controls. Thus these data support our murine model as an appropriate representation of the alterations observed in patients undergoing HSCT and suggest sustained elevation of miR-29b.

TGF-β upregulates miR-29b and downregulates DNMT expression. Previous data showed elevated levels of TGF-β in BMT lungs compared with untransplanted controls (14). Furthermore, TGF-β promotes upregulation of COX-2 (41, 45). To determine whether TGF-β-induced Cox-2 hypomethylation was attributable to increased miR-29b and decreased DNMT, primary AMs from WT untransplanted mice were treated with TGF-β for 24 h before RNA isolation. Real-time RT-PCR analysis revealed that TGF-β-treated AMs exhibited decreased DNMT3a and DNMT3b expression (Fig. 2A) and increased expression of miR-29b (Fig. 2B; open bars). To further verify the effects of TGF-β on DNMT expression, we also used CD11c<sup>dnR</sup> mice expressing a dominant-negative TGF-βRII regulated by CD11c promoter activity. In these mice, cells expressing CD11c (i.e., dendritic cells, macrophages, natural killer cells) would be unresponsive to TGF-β signaling. AMs from CD11c<sup>dnR</sup> mice did not show significant changes in miR-29b (Fig. 2B; solid bars) or DNMT (Fig. 2C) expression in response to TGF-β stimulation. Loss of altered DNMT and miR-29b expression in AMs insensitive to TGF-β supports TGF-β as the mediator of these transcriptional changes.

**miR-29b downregulates DNMT expression and upregulates the COX-2 pathway.** DNMT3a and DNMT3b contain a miR-29b target sequence in their 3′UTR (TargetScan) (20). To determine whether the altered expressions of DNMTs were a result of direct regulation by miR-29b, primary AMs from WT mice were transiently transfected with miR-29b for 24 h. Following overexpression of miR-29b, DNMT3a and DNMT3b as well as DNMT1 expression decreased significantly (Fig. 3A). These data indicate that miR-29b negatively regulates DNMTs in AMs. To determine whether miR-29b could affect the COX pathway, we transfected miR-29b into primary AMs from WT mice. In response to transient miR-29b overexpression, COX-2 mRNA (Fig. 3B) and protein (Fig. 3C) were upregulated. Increased PGE<sub>2</sub> production in miR-29b-transfected AMs following 24- and 48-h transfections indicate that the upregulation of COX-2 resulted in increased COX-2 activity (Fig. 3D). Increased activity of COX-2 and hence greater expression of its downstream effector, PGE<sub>2</sub>, were previously shown to promote the defective functional pheno-

![Fig. 1. DNA methyltransferase (DNMT) expression decreases in murine bone marrow transplant (BMT) alveolar macrophages (AMs), and microRNA (miR)-29b increases in murine BMT and human patients undergoing autologous and allogeneic hematopoietic stem cell transplantation (HSCT). Murine untransplanted control AMs and BMT AMs were assayed for DNMT1, DNMT3a, and DNMT3b (n = 3–4) (A) or miR-29b (n = 2–3) (B) by real-time RT-PCR. Syn, syngeneic. C: expression by human AMs of miR-29b relative to U6 was determined in patients with autologous (n = 3, auto) and allogeneic HSCT (n = 11, allo) and compared with healthy individuals (n = 6); *P < 0.05.](http://ajplung.physiology.org/)
type in BMT AMs (5–7, 13, 17–19, 26, 28). Furthermore, TGF-β/H9252 promotes these increases in COX-2, PGE2 (45) (Fig. 4), and miR-29b (Fig. 2B) while decreasing DNMT expression (Fig. 2A).

Next, because TGF-β increased expression of miR-29b and decreased expression of DNMT3a and DNMT3b, we sought to determine whether miR-29b was responsible for the hypomethylation of COX-2 CpG sites 1–6 (diagrammed in Fig. 5A).
in BMT AMs (17). We transfected primary murine AMs with miR-29b and determined COX-2 promoter methylation using bisulfite conversion and pyrosequencing. COX-2 was hypomethylated in CpG sites 1–6 following miR-29b transfection compared with AMs transfected with a control miR (Fig. 5B). This decreased methylation pattern at CpG sites 1–6 was similarly observed in BMT AMs (Fig. 5C) and in primary murine AMs treated with a methyltransferase inhibitor, 5-aza-2’-deoxycytidine (Fig. 5D). Whereas our previous results suggested that CpG sites downstream near exon 1 were also hypomethylated in BMT AMs (17), we did not see these sites regulated by miR-29b transfection (data not shown).

Inhibition of TGF-β rescues the bacterial killing defect in BMT AMs. Next, we investigated the effects of TGF-β on AM bactericidal function after BMT. Killing of phagocytized S. aureus was measured in AMs from BMT mice and untransplanted control mice. BMT AMs exhibited impaired S. aureus killing compared with control AMs, supporting a previous study whereby BMT mice exhibited higher susceptibility to S. aureus in vivo, which correlated to impaired killing by BMT AMs (18). However, reconstituting the alveolar compartment with donor AMs insensitive to TGF-β (CD11cddB BMT) rescued this killing defect (Fig. 6). These data suggest that TGF-β signaling mediates the functional defect seen in AMs after BMT.

**Fig. 4.** TGF-β upregulates COX-2 in primary AMs. Primary murine AMs were treated with 5 ng/ml TGF-β for 24 h. A: COX-2 mRNA was measured by real-time RT-PCR following RNA extraction by TRIzol (n = 4). B: PGE₂ released in the supernatant was measured by ELISA (n = 4); *P < 0.05, **P < 0.01, unpaired Student’s t-test.

**Fig. 5.** miR-29b overexpression promotes hypomethylation of COX-2. A: the locations of the CpG sites analyzed in the COX-2 promoter are shown in the schematic, with the transcription start site denoted by the +1. B: primary AMs from untransplanted mice were transfected with miR-29b or control scrambled miR (each at 30 nM) for 48 h before bisulfite conversion and pyrosequencing analysis of CpG sites 1–6 (n = 4 separate experiments). C: methylation of upstream CpG sites 1–6 were measured in untransplanted control and BMT AMs (n = 2 separate experiments). D: primary WT AMs harvested by bronchoalveolar lavage were initially supplemented with granulocyte-macrophage colony-stimulating factor (1 ng/ml) for 24 h before the addition of 2.5 μM 5-aza-2’-deoxycytidine (5-aza) for 72 h. Methylation of CpG sites 1–6 were determined by bisulfite conversion and pyrosequencing (n = 3–4); *P < 0.05, **P < 0.01, ****P < 0.0001 vs. control miR transfection at same CpG site (B), untransplanted control mice (C), or vehicle treatment (D), unpaired Student’s t-test.
miR-29b compromises effective bacterial killing in BMT AMs. To determine the functional effects of miR-29b after BMT, primary BMT AMs were transfected with an antagomir of miR-29b (anti-miR-29b) or a control miR and measured for their ability to kill *S. aureus* intracellularly. Compared with control AMs, BMT AMs transfected with a control miR exhibited increased intracellular bacterial survival (Fig. 7A). However, upon inhibition of miR-29b (anti-miR-29b), bacterial killing was restored in BMT AMs to levels similar to control AMs transfected with a control miRNA (Fig. 7A). Interestingly, transient transfection of miR-29b in control AMs was sufficient to drive a defective functional response comparable to control transfected BMT AMs (Fig. 7A). Previous data support an integral role for COX-2 in mediating the bacterial killing defect in BMT AMs by upregulating the production of PGE2. Thus we investigated whether inhibition of bacterial clearance caused by overexpression of miR-29b was attributable to miR-29b-dependent upregulation of the COX-2 pathway. Treatment of miR-29b-transfected AMs with indomethacin, a COX inhibitor, rescued the defective phenotype. Interestingly, blocking PGE2 signaling with a novel EP2 antagonist (EP2a; PF-04418948) (1, 8) similarly restored miR-29b-transfected AM function (Fig. 7B).

**DISCUSSION**

The results presented here collectively support the hypothesis that TGF-β/H9252 impairs dysfunction of AM after BMT via miR-29b-induced hypomethylation of the COX-2 promoter and consequent increased intrapulmonary production of PGE2. Why TGF-β/H9252 is overproduced by alveolar epithelial cells following BMT is unclear; however, it is possible that radiation-induced epithelial cell injury may promote elevated TGF-β/H9252 levels. Here, we showed that expression of DNMT1, DNMT3a, and DNMT3b is diminished in murine AMs after BMT and that TGF-β/H9252 downregulates DNMT expression in control AMs but not in AM of CD11cdnR, which are insensitive to TGF-β/H9252. miR-29b, on the other hand, was increased in AMs after BMT and in control AMs treated with TGF-β/H9252. The effects on DNMT expression are miR-29b dependent as WT AMs transfected with miR-29b, but not control miRNA, revealed decreased DNMT expression. As mentioned earlier, miR-29b has putative binding sites in the 3'-UTR of DNMT3a and DNMT3b. A previous study confirmed these binding sites and showed direct binding of miR-29b on the 3'-UTR (20). A previous study also showed that miR-29b can indirectly downregulate DNMT1 by targeting Sp1 (21). Thus our results showing that miR-29b transfection can also inhibit DNMT1 correlate with this earlier observation. However, the effects of TGF-β on miR-29b in our system are different than previous studies in other cell types.

**Fig. 6.** CD11cdnR BMT AMs exhibit intact bacterial killing of *Staphylococcus aureus* (*S. aureus*). AMs from untransplanted control mice, B6 BMT mice (Syn BMT), and CD11cdnR BMT mice were measured for their ability to kill *S. aureus* intracellularly (n = 5–8); *P < 0.05, ***P < 0.001, 1-way ANOVA.

**Fig. 7.** miR-29b compromises bacterial killing in AMs through PGE2 signaling. A: AMs from untransplanted control mice were transfected with a control miR (30 nM) or miR-29b (30 nM), and BMT mice were transfected with a control anti-miR (10 nM) or the antagonor (10 nM) for 48 h before the measurement of bacterial killing of *S. aureus* (n = 5–8). B: *S. aureus* killing by miR-29b-transfected WT AMs was determined in the presence or absence of E prostanoid receptor 2 (EP2) antagonist (PF-04418948; 10 nM) or indomethacin (5 μM) and compared with AMs transfected with a control miR (n = 4–5); *P < 0.05, **P < 0.01, ***P < 0.0001, 1-way ANOVA.
where TGF-β negatively regulates miR-29b (44, 58, 62). These conflicting reports highlight the tissue-specific nature of TGF-β regulation of miR-29b.

Patients undergoing HSCT suffer from pulmonary complications that commonly manifest as bacterial pneumonias (2, 3, 15). Interestingly, observations made in patients undergoing HSCT note impaired function in the innate immune compartment (i.e., AMs and neutrophils) (11, 59, 63). Using a murine model of BMT to understand the correlation between these defective innate immune cells and the increased susceptibility of these patients, we previously published that BMT AMs are unable to phagocytize *P. aeruginosa* and kill engulfed *P. aeruginosa* or *S. aureus* well (5, 13, 18, 42). These defects were primarily mediated by the overproduction of PGE2, as demonstrated by rescue of *P. aeruginosa* phagocytosis and killing of both *P. aeruginosa* and *S. aureus* in BMT AMs by inhibition of PGE2 or the COX-2 pathway in vitro (5). These data highlight the importance of the COX-2 pathway and its effector, PGE2, in mediating the defects observed in our BMT model. However, the mechanism for enhanced basal expression of COX-2 pathway and its inhibitor, PGE2, in mediating the defects observed in our BMT model is still unelusive. Recently, we published that the COX-2 promoter was significantly hypomethylated in BMT AMs, allowing for enhanced COX-2 expression (17). Interestingly, BMT AMs insensitive to TGF-β exhibited partially restored methylation and phagocytic function (17). This study sought to shed light on a possible mechanism by which TGF-β could affect immune responses in BMT mice through regulating methylation of COX-2.

The data presented show that overexpression of miR-29b in primary AMs induces COX-2 and PGE2 by inhibition of DNMTs, resulting in the hypomethylation of COX-2. Although the effects on methylation following transfection of miR-29b are rapid, these observation are supported by previous publications highlighting changes in PGE2 production following 24-h transfection of A549 cells with siRNA against DNMT1, DNMT3a, or DNMT3b (20) and DNMT activity following 48-h transfection of a lung fibroblast cell line with siRNA against DNMTs (17). Furthermore, a study investigating gene methylation in breast cancer cells following transfection with miR-29b showed significant changes in methylation following 72-h transfection of pre-miR-29b (52). Whereas pre-miRs require further processing by RNA polymerase III within the cell, the transfection of mature miRs do not and can directly interact with the RNA-induced silencing complex without further processing. Previous studies have implicated COX-2 and subsequently PGE2 in mediating immunosuppressive effects on macrophages (31, 32, 37, 53). We have shown that the inability of BMT AMs to function effectively in response to bacteria is attributable to the elevated basal PGE2 levels (5, 18). Consequently, understanding the source for upregulation of PGE2 would identify pathways that could potentially be targeted for therapy.

Here we propose that elevated TGF-β in the alveolar space of BMT mice 1) signals the upregulation of miR-29b, which then 2) targets DNMTs, resulting in 3) hypomethylation of COX-2 and 4) the subsequent COX-2-dependent synthesis of PGE2. In turn, PGE2 binds to elevated EP2 receptors on AMs after BMT and impairs phagocytosis of MARCO-dependent pathogens and bacterial killing mediated by MARCO or scavenger receptor A (Fig. 8). TGF-β has previously been characterized to have dual effects on COX-2 (24, 36, 45, 51, 56). A study in human nonsmall cell lung cancer A549 cells showed that TGF-β was able to downregulate COX-2 (56). However, the bulk of the literature has shown induction of COX-2 in response to TGF-β in models using mammary epithelial cells (41), human mesangial cells (45), and macrophages in muscle tissue (51). Activation of ERK1/2, p38 MAPK, and phosphatidylinositol 3-kinase signaling pathways are suggested to mediate the TGF-β-dependent activation of COX-2 (45). BMT AMs show hypomethylation on CpG sites 1–4 and near exon

![Fig. 8. Model of the effects of TGF-β-induced miR-29b on BMT AMs. Elevated TGF-β in the lung, likely produced by type II alveolar epithelial cells, signals to BMT AMs, resulting in the upregulation of miR-29b. Elevated miR-29b targets DNMTs to allow for hypomethylation of COX-2. Decreased methylation of COX-2 expression and induces PGE2 synthesis. Increased PGE2 by BMT AMs can signal in an autocrine and paracrine fashion via EP2 receptors to mediate its inhibitory effect on AM function [altered scavenger receptor (SR) profiles, impaired phagocytosis, and defective bacterial killing].](http://ajplung.physiology.org/ by 10.220.33.5 on November 6, 2017)
miR-29b in primary AMs alone inhibits proper bacterial killing, and overexpression of miR-29b in primary AMs alone inhibits proper bacterial killing. Despite changes in methylation at CpG sites near CRE-1 following transplantation, hypomethylation of the COX-2 enhancer binding protein, and a cAMP response element-1 (CRE-1) site in the promoter; however, Kang and colleagues identified three additional functional elements (CRE-2, an activator protein 1 site, and an E-box overlapping CRE-1) (30, 38). Interestingly, our methylation data following miR-29b transfection show significant hypomethylation in CpG sites that overlap with CRE-2 but not the other sites. Moreover, deletion of the CRE-2 site resulted in 50% reduction of LPS-induced COX-2, highlighting the importance of this regulatory site in COX-2 expression (30). Thus it is possible that, despite changes in methylation at CpG sites near exon 1 of COX-2 (17) following transplantation, hypomethylation of the more upstream CpG sites observed both after BMT and in response to miR-29b are the critical CpG sites that result in enhanced COX-2.

We also show that blocking miR-29b in BMT AMs is sufficient to rescue the bacterial killing defect, and overexpression of miR-29b in primary AMs alone inhibits proper bacterial clearance. These effects are due to the miR-29b-induced PGE2, as blocking PGE2 signaling (using an EP2 receptor antagonist) and the COX pathway (using indomethacin) is able to rescue the killing defect despite overexpression of miR-29b. These data highlight an immunoregulatory role for miR-29b on AM function. More importantly, they can be translated to patients undergoing HSCT, as patients with both autologous and allogeneic HSCT exhibit increased miR-29b expression.

Taken together, these data highlight miR-29b as a potential therapeutic target for improving pulmonary immune responses in patients undergoing HSCT. Although miRNA biology is not fully understood, the knowledge gathered thus far and the progress made in the development of strategies to block or enhance miRNA activity have propelled the therapeutic promise of these small RNAs (9). Currently there are no FDA-approved miRNA therapies available; however, commercial development of miRNA therapies are underway and are in preclinical stages for the treatment of nonsmall cell lung cancer, prostate cancer, muscle, cardiovascular disease, and fibrosis (9, 46). Thus the possibility of delivering miR-29b antagonirs to AMs is a promising strategy for targeted immunoregulatory therapy for patients undergoing HSCT, who are likely to suffer from infectious pulmonary complications.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.


