Epigenetics and cyclooxygenase-2 mediate dysfunction in alveolar macrophages and polymorphonuclear neutrophils post-bone marrow transplantation

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Immunology)
in the University of Michigan
2014

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To my family and friends

Acknowledgments

I am deeply grateful and indebted to you Beth, for your constant guidance, honest feedback and advice, and genuine belief in me. Our conversations and my time in your lab have truly made a difference in my scientific career. I would also like to thank everyone in the Moore lab, especially Carol, who taught me many of the techniques essential to my project and with whom I spent countless hours lavaging mice, Xiofeng for your genuine feedback on my project and practice presentations, and Giovanny Martinez-Colon for your support and collaboration, both on the bench and as a great friend. Thank you to my thesis committee for your guidance, support and contributions to my work. To my collaborators and everyone who supported me scientifically throughout my time here, thank you. I am very lucky to have gotten the chance to do science alongside great scientists. I would like to thank all the friends I have made at the University of Michigan as my experience at U of M would not have been the same without each and every one of you.

Lastly, I would like to thank my family: Mom, Dad, Ben, Eduardo, Daphne, Abuelita, Tia Yenny, Grace, Olga, Melissa, Alex, Tia Sonia, and Richard. Each of you have supported and inspired me in more ways than one and I would not be the person I am without you.

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Abstract

Infectious pulmonary complications limit the success of hematopoietic stem cell transplant (HSCT) therapy in both autologous and allogeneic patients. Susceptibility to pathogens, like *Pseudomonas aeruginosa* and *Staphylococcus aureus*, persists despite successful immune reconstitution. Despite high incidence of infectious pulmonary complications following HSCT, relatively little is known about the mechanisms promoting enhanced susceptibility. Alveolar macrophages (AMs) are the sentinel phagocytes in the lung and following infection polymorphonuclear neutrophils (PMNs) assist in bacterial clearance. Previous human studies implicate AM and PMN impairment post-HSCT. Using a murine syngeneic or allogeneic bone marrow transplant (BMT) model, our studies explore the mechanisms involved in promoting HSCT AM and PMN defects. We show that syngeneic BMT mice display increased susceptibility to both *P. aeruginosa* and *S. aureus,* which correlated with impaired AM function. Altered class A scavenger receptors impaired AM uptake of *P. aeruginosa* but not *S. aureus*, while defective bacterial killing conferred overall susceptibility to these pathogens. Syngeneic BMT AM susceptibility is promoted by upregulation of prostaglandin E_2 (PGE_2) and its rate-limiting enzyme, cyclooxygenase-2 (COX)-2. Studies exploring the etiology of enhanced COX-2 expression revealed a loss in DNA methylation of the COX-2 promoter mediated by transforming growth factor (TGF)-ß-induced miRNA-29b, resulting in elevated COX-2. Previous data show COX-2/PGE_2 impaired PMN bacterial killing but had no effect on phagocytosis post-syngeneic BMT. Here we show that upregulation of COX-2/ PGE_2 inhibits PMN extracellular trap (NET) formation post-syngeneic and allogeneic BMT, a novel finding

that identifies PGE_2 as a physiologically relevant inhibitor of NETosis. Together, these findings highlight the importance of epigenetic changes (DNA methylation and miRNA) in BMT AMs, as they directly and indirectly result in increased COX-2 expression and PGE_2 production. Upregulation of this pathway establishes an immunosuppressive environment in the lung through the inhibition of AM and PMN functions. Moreover, these findings identify potential therapeutic avenues to further explore in HSCT patients.

Chapter 1:

Introduction

1.1 Immune Cells

The mammalian immune system is composed of white blood cells that can originate in the hematopoietic compartment of the bone marrow. Here, self-renewing hematopoietic stem cells (HSCs) give rise to progenitor cells that further differentiate into myeloid and lymphoid cells that make up our immune system, e.g. T cells, B cells, monocytes/macrophages, PMNs, eosinophils, etc. (1). These cells localize to different parts of the body and may remain resident at those sites or may circulate. Macrophages, for example, can strategically reside at different locations in the body and offer rapid protection to the host in the event of an infection (2). PMNs, on the other hand, remain in the peripheral blood, circulating until they receive cues for extravasation from the blood vessels and into the site of infection/injury where they can assist in the proper clearance of invading pathogens (1, 3, 4).

The main functions of the immune system are to differentiate between foreign and host substances (self versus nonself), and to effectively remove the foreign substances. Our immune cells are dynamic and are constantly interacting with their environment and each other. Innate cells like macrophages and dendritic cells are constantly uptaking particles and/or clearing dying cells (5). However, upon recognition of foreign material, like bacteria or viruses, the cells become activated and in an effort to resolve the infection, they produce cytokines and chemokines to effectively recruit and activate other immune cells (6). Recognition of pathogens occurs through the identification of pathogen-associated molecular patterns or PAMPs via

pattern recognition receptors, like toll-like receptors and scavenger receptors (7, 8). Dendritic cells in particular, can process and present antigens acquired from these pathogens on major histocompatibility complex I or II molecules and these dendritic cells then travel to the nearest lymphoid organ, where upon interaction with T and B cells, they assist in their activation and further differentiation into specific T cell subsets or antibody-secreting B cells (1, 3, 4, 7). With both the innate and adaptive arms of the immune system activated, the body is able to resolve the infection and develop memory responses.

1.2 Hematopoietic Stem Cell Transplantation (HSCT) Overview

Early observations made in the 1950s paved the way for the development of HSCT. In 1951, Lorenz et al. showed that animals exposed to radiation could be protected by infusion of bone marrow cells (9). These studies, together with the observation that the deleterious effects of ionizing radiation could be prevented in mice by shielding organs with lead, pushed transplantation forward as a possible therapy for patients suffering from hematologic malignancies (10). HSCT is now used to treat a variety of disorders, including malignant, congenital, and autoimmune disorders (11, 12). Depending on the underlying disease, a patient will receive either an autologous [self donation of HSCs] or allogeneic [human leukocyte antigen (HLA)-matched related or unrelated hematopoietic stem cell donor] transplant (12).

Prior to receiving a graft, HSCT recipients undergo conditioning regimens that include total body irradiation (TBI), chemotherapy, or a combination of TBI and chemotherapy (13, 14). These regimens subject the recipients' organs, including the lungs, to their cytotoxic effects. The hope is to eradicate the malignant or autoimmune cells and replenish the patient with healthy HSCs. If the intensity of the conditioning regimen is myeloablative, repopulation of

hematopoietic cells will predominantly come from the donor HSCs (14, 15). Non-myeloablative conditioning, also known as reduced intensity conditioning (RIC) will allow repopulation from both donor and host HSCs. A recent study analyzing the global use of transplantation reported that autologous HSCT continues to be more highly implemented than allogeneic transplant (16). Although autologous transplant recipients are unlikely to suffer from graft versus host disease (GvHD), which arises when the donor leukocytes mount an immune response against different HLA antigens expressed by the host, autologous transplants performed for hematologic malignancies lack the beneficial effects of graft-versus-leukemia (GvL). GvL reactions can be observed in allogeneic transplantation when the allogeneic donor HSCs recognize and remove leukemic cells within the host by virtue of HLA mismatches (17).

Traditionally, HSCs were aspirated from the bone marrow of donor patients and later infused into recipients. This procedure was often painful for the donor. However, technical advances now allow for mobilization of HSCs into the peripheral blood with the use of the growth factor, granulocyte-colony stimulating factor (G-CSF) (11, 12). Treatment of donors with G-CSF allows HSCs to be collected with a simple blood draw. Additionally, HSCs can also be harvested from umbilical cord blood (18, 19).

1.3 Pulmonary Complications in HSCT Human Patients

Although HSCT has become standard care for malignant and nonmalignant disorders, its success is significantly limited by the different pulmonary complications that arise following either allogeneic or autologous transplantation (12, 20-23). The engraftment process can be characterized by three distinct phases: the pre-engraftment (0-30 days), early post-engraftment (30-100 days), and late post-engraftment phases (beyond 100 days) (23-25). HSCT recipients

are more susceptible to infections prior to engraftment and early after engraftment. During this early time period, the increased susceptibility is mostly due to the incomplete recovery of the immune cell compartment, and is often associated with decreased PMN numbers or function (26). Although engraftment indicates the transplant was successful in reconstituting hematopoietic cell populations, it is evident that this does not correlate with recovery of a fully normal immune system, as patients remain susceptible to diverse complications (including infections) long after engraftment occurs (23, 24, 27). Pulmonary complications, both infectious and noninfectious, have been reported to affect up to 60% of transplant recipients (12, 27). Similarly, autopsy studies performed on both allogeneic and autologous transplant recipients revealed pulmonary complication rates of 80-89% (21). Although allogeneic transplants have increased incidence of infectious pulmonary complications, this may be attributed, in part, to the use of immunosuppressive therapy required to prevent GvHD. Additionally, GvHD can cause mucosal barrier disruptions that also increase susceptibility to pathogens. Interestingly, however, autologous HSCT recipients also continue to suffer from increased susceptibility to pulmonary infections even though this form of transplant does not require immunosuppressive therapies (28).

RIC has evolved as an alternative to the more traditional myeloablative conditioning regimens for individuals that would have otherwise been unable to undergo transplantation as a therapy due to age, co-morbidities, or disorders associated with high-risk non-relapse mortality (29). Interestingly, despite a reduction in intensity regimens, recipients of RIC HSCT display similar incidence of infectious complications and infection-related mortality (30). A retrospective analysis conducted on a cohort of patients over the age of 50 who received either a myeloablative or nonmyeloablative conditioning regimen prior to an allogeneic HSCT found that

patients receiving nonmyeloablative therapy were protected from non-infectious pulmonary complications whereas 40% of patients receiving myeloablative therapy developed these lung problems (31). While Alyea and colleagues saw a reduction in non-infectious complications with nonmyeloablative conditioning, they still noted that infection remained a major cause for nonrelapse mortality in both the myeloablative and nonmyeloablative groups. Furthermore, disease relapse was significantly higher in patients receiving nonmyeloablative conditioning regimens than those receiving myeloablative regimens. Thus, although reduced conditioning therapy does offer some lung protection and therapeutic advantage, myeloablation remains more effective in preventing disease relapse over time.

Taken together, these observations highlight the inherent dysfunction of the immune response following transplant and suggest that some degree of impaired function is independent of the immunosuppressive therapies, GvHD, and possibly even myeloablation. Thus, the susceptibility may be due to phenotypic changes that occur in the alveolar environment. The alveolus is composed primarily of type I and type II epithelial cells (structural cells), as well as alveolar macrophages (AMs) (32). Alveolar epithelial cells (AECs) are vital for proper lung function (i.e. gas exchange, secretion of surfactant) (32) while AMs are the sentinel phagocytes in the alveolus, highlighting their importance in initiating an immune response against an invading pathogen in the lower airways (33). As AMs are integral in the early response to invading pathogens or inhaled materials, it is possible that AMs might be defective post-transplant. The idea that AMs are inherently less functional post-HSCT is supported by previous observations that AMs obtained from pulmonary lavage of allogeneic HSCT patients late in the post-engraftment phase were functionally defective even in the absence of overt pulmonary infection (34). Defects noted in AMs from these patients included defects in chemotaxis,

phagocytosis and killing of *Candida pseudotropicalis* as well as phagocytosis and killing of bacteria including *Staphylococcus aureus* and *Listeria monocytogenes*. This same study suggested that the defects may be primarily confined to the lung as the function of peripheral blood monocytes in these same assays was similar to control patients (34).

Noninfectious complications that arise following transplant include peri-engraftment respiratory distress syndrome, diffuse alveolar hemorrhage, idiopathic pneumonia syndrome, and bronchiolitis obliterans syndrome (21, 23, 27, 28, 35, 36). These occur throughout the different engraftment phases but bronchiolitis obliterans syndrome in particular, is more commonly seen late post-engraftment (35). Malignant relapse or secondary malignancy also complicate successful transplants (12, 37). Often, noninfectious complications can manifest in conjunction with infectious complications (21, 38). Additionally, infections are risk factors for development of some forms of non-infectious lung injury which may occur later, such as idiopathic pneumonia syndrome (39). Importantly, infections with bacterial, viral and fungal pathogens can afflict both allogeneic and autologous HSCT recipients at multiple times post-transplant (23, 40).

Viral infections are common in HSCT patients. Cytomegalovirus (CMV) infections predominate in the late phase of engraftment and are associated with increased mortality (41-43). The advent of prophylactic and antiviral therapy usage has proven to be beneficial in limiting infection in HSCT patients; however, CMV can be particularly difficult to detect and treat (43). One autopsy study revealed that 65% of CMV infections were not diagnosed ante mortem, thus highlighting the frequency of missed detection (44). Thus, HSCT patients are highly susceptible to CMV as well as other herpesviruses (27, 45, 46). Fungal pathogens are one of the major causes of significant morbidity and mortality in HSCT recipients (47). Prior to the use of azole-based anti-fungal prophylaxis, *Candida spp*. were the leading fungal pathogen following HSCT

(48). However, following implementation of this anti-fungal prophylactic treatment, *Aspergillus spp*. have become the predominant fungal pathogens causing invasive fungal infections in the lungs of both autologous and allogeneic HSCT populations (48, 49). When considering bacterial infections, a shorter time to engraftment and the use of broad-spectrum antibiotics have reduced the incidence of bacterial infections, specifically Gram-negative bacteria (50, 51). However, bacterial pneumonias remain a significant cause of mortality in this immunocompromised population (46, 50). *Pseudomonas aeruginosa* (a Gram-negative bacterium) is the most common pathogen isolated within 100 days post-engraftment and is associated with high recurrence rates due to increasing antibiotic resistance (52). *Streptococcus pneumoniae* is the leading Gram-positive bacterium, and together with another Gram-positive agent, *Staphylococcus aureus*, afflicts patients in the early and late post-engraftment phase and causes significant mortality (46, 53) (Figure 1.1).

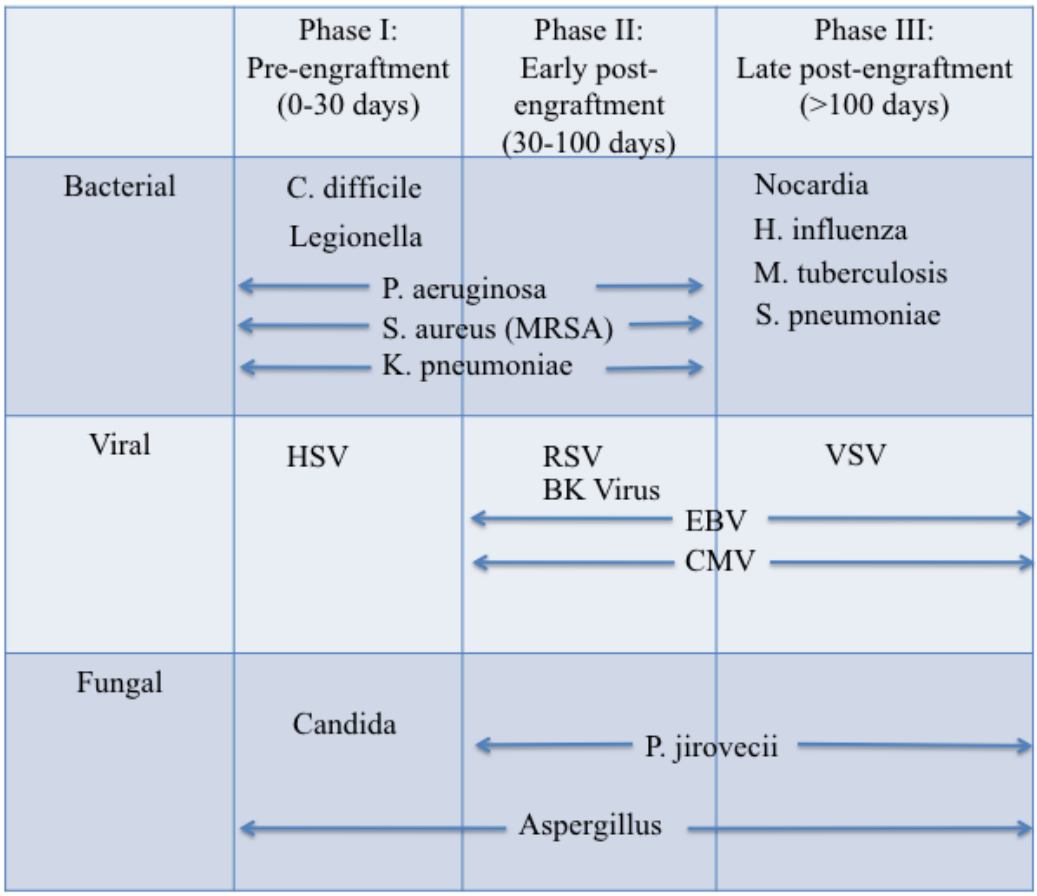

Figure 1.1. Timeline of main infectious pulmonary complications post-HSCT (adapted from Coomes et al. 2011).

As AMs mediate an early immune response to pathogens invading the lower airways and their function has been shown to be impaired post-HSCT, studies have been focused on understanding the mechanisms driving the defects in this cell population. These studies have largely been conducted using animal models of HSCT.

1.4 HSCT Animal Modeling

Murine BMT models for both syngeneic (genetically identical donor), to model human autologous HSCT, and allogeneic (non-identical) transplantation offer the opportunity to study

changes in the pulmonary immune system and microenvironment following transplantation. In these systems, donor cells are harvested from the femur and tibia of genetically inbred mice and introduced into lethally irradiated syngeneic or allogeneic mice to reconstitute ablated hematopoietic compartments. In order to force reconstitution of myeloid cells from hematopoietic donors, myeloablative conditioning is employed. This can be done via TBI or via chemotherapy regimens, but TBI is most common. Turnover of murine lung AMs require radiation levels of at least 9 Gy (54). One note of caution however, is that different strains of mice have differing susceptibilities to TBI dosage. For instance, in our laboratory we routinely use 13 Gy split dose of TBI to myeloablate C57Bl/6 mice and only 10 Gy for the same purpose in Balb/c mice. Addition of splenic T cells along with the donor bone marrow inoculum can hasten reconstitution in syngeneic transplants (55) and can be a source of alloreactive T cells in models of allogeneic BMT hoping to study GvHD or GvL responses (56, 57).

Studies comparing conditioning regimens for their ability to reconstitute AMs in recipient syngeneic BMT mice showed that both dual chemotherapy regimens (4 days of 25 mg/kg busulfan followed by 2 days of 100 mg/kg cyclophosphamide) and 13 Gy TBI regimens induced greater than 50% reconstitution of AMs and spleen cells from donor stem cells by week 5 post-BMT (54). Thirteen Gy TBI, when compared to dual chemotherapy, was the more effective conditioning regimen. With 13 Gy TBI conditioning, donor reconstitution, measured by the percentage of CD45.1 donor cells in CD45.2 recipient mice, was observed at 82% ± 2% in AMs and 95% ± 1% in spleen cells (54). The percentages of donor-derived AMs and spleen cells with dual-chemotherapy were 56% ± 6.2% and 72.3% ± 2.1% (54).

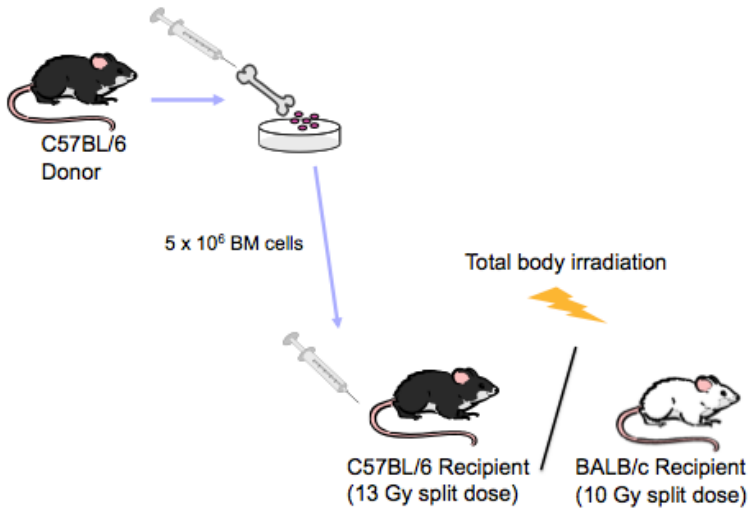

Figure 1.2. Syngeneic and allogeneic bone marrow transplantation animal model.

1.5 Defective pulmonary innate immunity to bacteria

Despite the use of antibiotic prophylaxis, infectious pulmonary complications continue to afflict both autologous and allogeneic HSCT recipients throughout the process of engraftment and well into the late post-engraftment phase (>100days) (46, 53). Although reported to predominantly manifest in the pre-engraftment and early post-engraftment phases, *P. aeruginosa*-driven pneumonia is also seen in some cases more than a year post-initial transplant (53). To understand how alterations following transplantation enhance susceptibility of HSCT patients to bacterial infections, the Moore lab previously exposed syngeneic BMT mice to a sublethal dose of *P. aeruginosa* (PAO1 strain; $5x10^5$ CFU) (24, 55, 58, 59) via intratracheal (i.t.) injection and measured bacterial burden in the lungs and blood following twenty-four hours. Using this model, the lab found that syngeneic BMT mice were more susceptible to bacterial infection as they are unable to effectively clear *P. aeruginosa* (24, 55, 58-60) from the lung and contain higher levels of bacterial dissemination compared to untransplanted, infected control mice.

1.6 Cytokine and eicosanoid dysregulation post-BMT

In humans, bacterial infection is commonly seen in the neutropenic phase due to a slow or incomplete engraftment, or the use of immunosuppressive therapy (23, 24). However, in our syngeneic BMT model, there was prolonged susceptibility to bacterial infection even after full hematopoietic engraftment had occurred. These observations suggested that the environment of the lung post-BMT may be suppressing the function of the innate immune cells which had repopulated the alveolar space. These studies revealed that syngeneic BMT mice are unable to successfully induce tumor necrosis factor (TNF) α or interferon (IFN) γ in the lung following infection with *P. aeruginosa* (55). TNFα in particular, has been shown to be especially important in controlling a *P. aeruginosa* infection (61, 62). Balb/c mice are naturally resistant to *P. aeruginosa* and have been shown to produce higher levels of TNFα in the lung following infection while the susceptible C57BL/6 strain produces significantly lower levels of TNFα post-infection (63, 64). Interestingly, *in vivo* treatment of Balb/c mice with an anti-murine TNFα monoclonal antibody reversed resistance to infection and the anti-TNFα-treated mice exhibited higher bacterial loads in the lung (64). Similarly, a separate study showed that TNFα-knockout mice suffered from significantly higher bacterial burden in the lungs following challenge with *P. aeruginosa* compared to their TNFα-sufficient wild-type controls (65). These data highlight the importance of TNFα in mediating clearance of *P. aeruginosa* and suggest that loss of the mediator post-BMT likely contributes to impaired clearance of this pathogen.

In addition to TNFα, IFNγ is also important for the activation of macrophages. IFNγ functions to prime macrophages to undergo classical activation but does not alone activate macrophages (66, 67). It requires a second signal, provided by either exogenous TNFα or Toll-like receptor (TLR)-induced TNFα (66). However, as both IFNγ and TNFα were decreased in

the alveolar space and TNFα production was decreased by AMs following BMT in response to i.t. challenge with *P. aeruginosa*, it is possible that the lack of activation signals for macrophages contribute to the inability to control and clear bacterial insult post-BMT (55).

Eicosanoids derive from arachidonic acid and are lipid mediators produced by different cell types, including AECs, AMs, and PMNs (58, 68, 69). Prostaglandins and leukotrienes are end-products of arachidonic acid metabolism via the COX and 5-lipoxygenase enzymatic pathways, respectively (69) (Figure 1.2). These eicosanoids have diverse effects on cells. Leukotrienes (LTB_4 and cysteinyl leukotrienes or cysLTs) seem to have an overall pro-inflammatory effect on innate immune cells as they have been shown to promote bacterial phagocytosis and killing in both AMs and PMNs (70-73). Similarly, cysLTs have been shown to be involved in the induction of TNFα production by both AMs and recruited PMNs (74, 75).

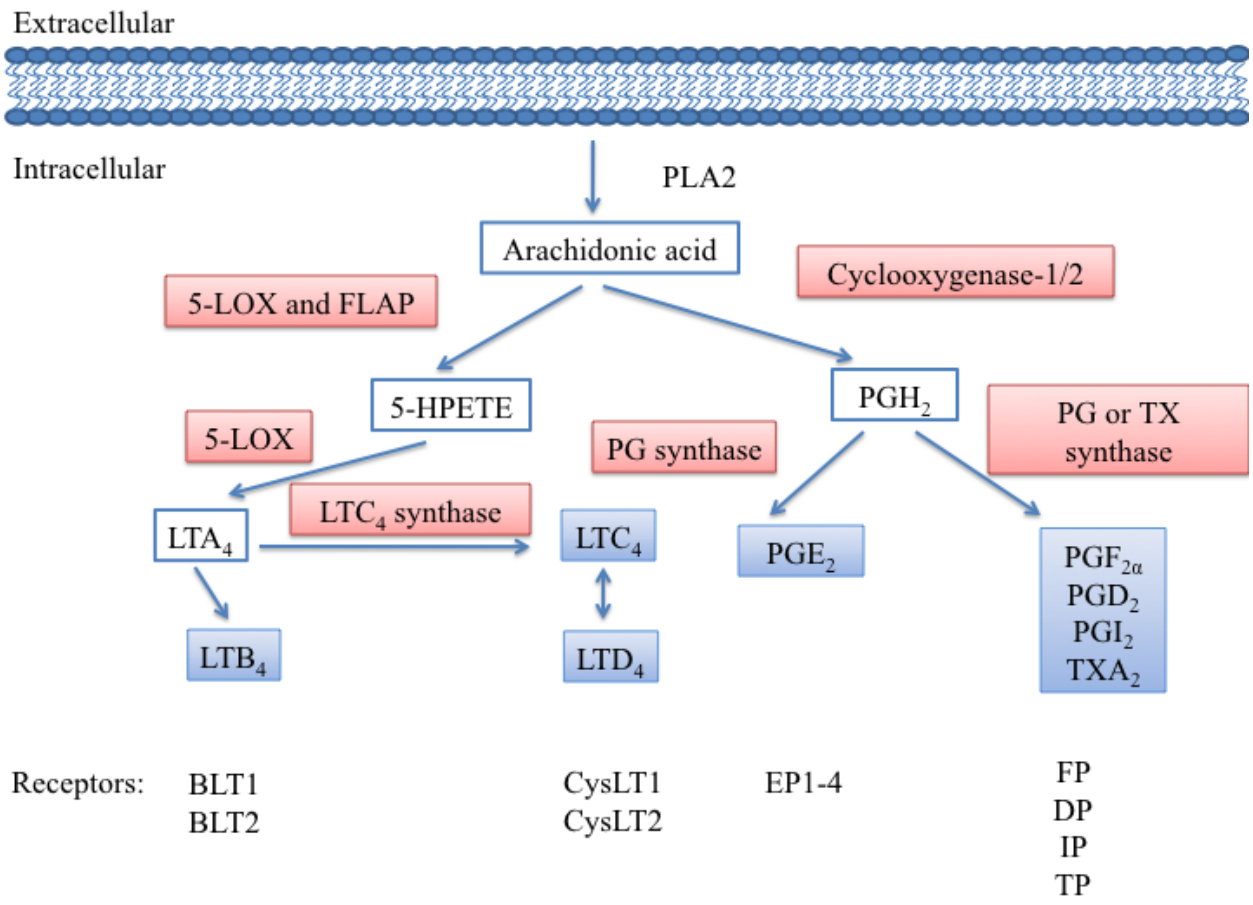

Figure 1.3. Arachidonic acid metabolism pathway.

Studies on the effects of prostaglandins, particularly PGE_2, however, suggest they may negatively regulate innate immune responses (76). PGE_2 signals through four distinct G protein-coupled seven transmembrane spanning E prostanoid (EP) receptors (77, 78). The inhibitory effects of PGE_2 have been attributed to binding of EP2 and EP4. Stimulation of the EP2 or EP4 receptors results in the induction of intracellular cyclic adenosine monophosphate (cAMP) and activation of the downstream cAMP targets: protein kinase A (PKA) and the exchange protein activated by cAMP (Epac-1) (76, 79). Activation of PKA and/or Epac-1 has been shown to impair both AM and PMN function as well as inhibit the production of pro-inflammatory

mediators, leukotrienes and reactive oxygen species (79, 80). Interestingly, PGE_2 has also been shown to induce IL-10, an immunosuppressive cytokine (81, 82).

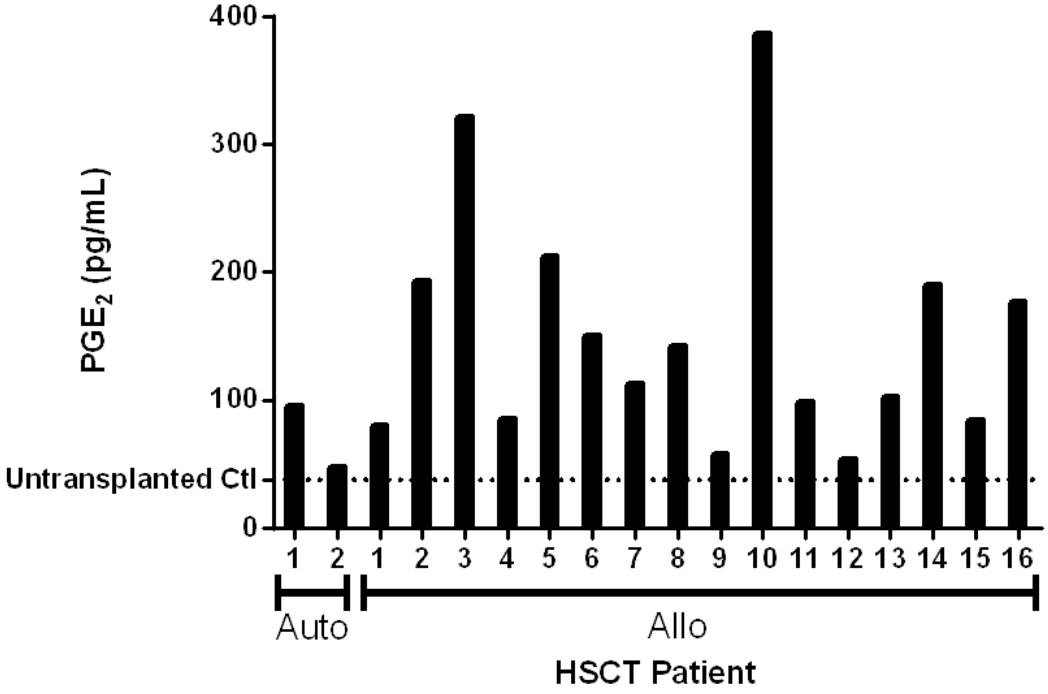

Figure 1.4 PGE_2 is elevated in bronchoalveolar lavage fluid of HSCT patients.

Table 1.1 Healthy and HSCT characteristics for PGE_2 studies shown in Figure 1.4

Patient	Age	Gender	Days post-transplant	Conditioning	Underlying disease
Untransplanted Ctl	49	Male	N/A	N/A	No disease
Auto 1	71	Male	14	BEAM/Rituximab	Mantle cell lymphoma
Auto 2	77	Male	98	BEAM/Rituximab	Diffuse large B cell lymphoma
Allo 1	62	Female	363	FluMel	Precursor B-cell Acute lymphoblastic leukemia

Allo 2	72	Male	699	FluBu/TBI	Acute Erythroid Leukemia
Allo 3	50	Male	615	FluBu/Rituximab	Mantle Cell Lymphoma
Allo 4	71	Male	348	FluBu/TBI	AML/NOS
Allo 5	59	Female	819	FluBu	Acute Myelomonocytic Leukemia
Allo 6	51	Male	344	FluBu	Acute Myeloid Leukemia, NOS
Allo 7	63	Male	2221	FluBu	Chronic Idiopathic Myelofibrosis
Allo 8	57	Male	906	FluBu/TBI	Mantle Cell Lymphoma
Allo 9	68	Female	39	FluBu	Atypical chronic myeloid leukemia
Allo 10	66	Male	118	FluBu	Acute Myeloid Leukemia,NOS
Allo 11	66	Male	631	FluBu	Chronic Idiopathic myelofibrosis
Allo 12	10	Male	51	FluBu/ATG/Thymoglobulin	RAEB-1
Allo 13	58	Male	303	FluBu	Follicular lymphoma, Grade II
Allo 14	38	Female	211	FluMel	Mycosis fungoides
Allo 15	23	Male	800	FluBu/TLI	Acute myeloid leukemia without maturation
Allo 16	65	Male	357	FluBu	Acute Myelomonocytic leukemia

Auto, autologous; Allo, allogeneic; Refractory anemia with excess blasts (RAEB-1); MEL, melphalan; BEAM, BCNU-Etoposide-AraC-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.

PGE_2 has been reported to be elevated in the serum of autologous HSCT recipients (83). This increase in PGE_2 was not specific to a particular conditioning regimen indicating that all HSCT patients are susceptible to overproduction of PGE_2 (83). We have previously reported that PGE_2 is elevated in the lungs of mice following syngeneic BMT (58) and have confirmed this observation in HSCT patients (Figure 1.4, Table 1.1). PGE_2 overproduction in syngeneic BMT mice was localized to AMs, recruited PMNs, and structural cells, whereas cysLTs are decreased in BMT AMs (58). The increased production of PGE_2 is likely to be systemic as BMT peritoneal lavage fluid also displayed higher PGE_2 levels (58). Although it is tempting to suspect PGE_2-induced IL-10 expression may be responsible for the impaired immune environment post-BMT, IL-10 was decreased in the BMT lung following infectious stimuli indicating that the inhibitory effects of PGE_2 were likely independent of the immunosuppressive effects of IL-10 (55). Interestingly, pharmacological inhibition of the COX pathway post-BMT limited PGE_2 production and restored cysLT levels in AMs indicating that PGE_2 negatively regulates cysLTs in the setting of BMT (58). The importance of cysLTs in supporting the clearance and resolution of pulmonary infections post-BMT is observed in mice that received HSCs from granulocyte macrophage colony stimulating factor (GM-CSF)-/- mice (59). GM-CSF-/- BMT mice are defective in cysLT production post-infection and are more susceptible to *P. aeruginosa* infection.

GM-CSF is an important cytokine for regulating innate immune cells, particularly macrophages. Previous studies have shown that GM-CSF is required for effective clearance of *P. aeruginosa* even in untransplanted mice (84). Untransplanted GM-CSF-/- mice had increased bacterial burden compared to wild-type controls. However, the source and amount of GM-CSF is critical to determining host defense function post-BMT. AMs from wild-type syngeneic BMT mice show an overproduction of GM-CSF post-BMT. Interestingly, in human HSCT literature,

elevated production of GM-CSF by AMs during the pancytopenia period was also noted (85). While we initially thought excess AM-derived GM-CSF would be beneficial, this was not the case because the excess GM-CSF in AMs post-BMT drove upregulation of the EP2 receptor on AMs (59). The inhibitory effects of PGE_2 signaling via upregulated EP2 in the wild-type BMT mice impaired bacterial phagocytosis and killing as well as cysLT and TNFα production. When GM-CSF-/- HSCs were used to repopulate WT mice, host defense improved (59). Without AM-derived GM-CSF post-BMT, EP2 receptors were not elevated and mice showed improved host defense despite the fact that PGE_2 levels were still elevated post-BMT. In the absence of EP2 elevations, the GM-CSF-/- BMT mice retained production of cysLTs and TNFα. It is important to stress that improved host defense was only seen in the situation where GM-CSF production was blocked in hematopoietic cells post-transplant. When WT HSCs were transplanted into GM-CSF-/- mice, host defense was once again impaired (59). These data highlight the importance of cross-talk between AECs and AMs post-BMT. AEC-derived GM-CSF is beneficial post-BMT whereas AM-derived GM-CSF is detrimental. In the human HSCT literature mentioned above, elevations of AM-derived GM-CSF in human allogeneic HSCT recipients predicted development of later lung disease (85). This complex homeostatic regulation of GM-CSF means blocking this cytokine needs to be targeted specifically to the AMs to be beneficial. Newer approaches to create microparticle delivery vesicles that are readily uptaken by phagocytes, but not structural cells, may offer a way to deliver antibody or siRNA-based anti-GM-CSF therapies in the future (86).

1.7 Cellular alterations

1.7.1 Alveolar epithelial cells (AECs)

AECs are the structural cells lining the alveolus. Type II AECs are important secretors of pulmonary surfactant, are progenitors to type I AECs that line the majority of the lung, and act as immunomodulators via interactions with immune cells. Exposure to ionizing radiation induces damage to the alveolar epithelial barrier causing changes in gene expression, particularly cytokine expression (87). We have previously shown that upon transplantation, AECs from BMT mice are negatively affected by the transplant process. Specifically, AECs overproduce PGE_2 as well as TGF-β which as discussed above inhibits AM function (58, 88, 89). Furthermore, AECs are defective in the synthesis of GM-CSF (59, 84) which as discussed above is important for modulating AM function. Another important defect that has been described post-BMT is the loss of surfactant protein A (SPA) which is predominantly synthesized by AECs (90, 91). As SPA can serve as a collectin (91) which can bind pathogens to help facilitate phagocytosis via the class A scavenger receptor A (SR-A) (92), loss of SPA post-BMT may be another reason why host defense is impaired in the setting of HSCT. The fact that SPA can bind to SR-AI/II (the receptor that is retained on BMT AMs, see my results in Chapter 3) may suggest that exogenous SPA therapy could prove beneficial post-HSCT.

1.7.2 Alveolar macrophages (AMs)

AMs compose 95% of all leukocytes in the airspaces and thus, are the sentinel phagocytic cells in the lungs (93, 94). They have been implicated as critical innate immune cells for clearance of bacteria as depletion of AMs through clodronate liposomes resulted in impaired host defense against *P. aeruginosa* (95). As AMs are important first responders to infection in the

lung, their function is essential for preventing bacterial dissemination as well as promoting bacterial clearance by engulfment and removal of infectious pathogens, and also by releasing chemokines to stimulate the recruitment and activation of immune cells (94). However, data suggest that BMT AMs demonstrate altered cytokine and eicosanoid production, which impair their innate immune function and predispose the host to infections post-BMT.

AMs have been reported to exhibit decreased ability to phagocytize and kill bacteria following allogeneic HSCT (34). Murine BMT AMs display an impaired ability to phagocytize and kill *P. aeruginosa* (55, 58, 96, 97). This defect has been shown to be dependent on PGE_2 signaling as pharmacological inhibition of the COX pathway through the use of indomethacin can restore productive host defense against bacteria both *in vitro* and *in vivo* post-BMT. Furthermore, blocking the EP2 receptor (using AH 6809 a strong EP2 antagonist with weaker activity at the EP1 and DP1 receptors) in BMT AMs inhibits PGE_2 signaling and restores phagocytosis of *P. aeruginosa* (58). PGE_2 is significantly overproduced by BMT AMs (58). The cause for elevated basal levels of PGE_2 in the AMs post-BMT may be multifactorial. It is possible that differences in differentiation of AMs from donor-derived HSCs versus a resident AM stem cell may influence the amount of PGE_2 observed after transplant (98). However, studies indicate that this cannot fully explain the defect since mice receiving a lower dose of irradiation (8 Gy) contain a large percentage of host-derived AMs (~36%) that also overproduce PGE_2 post-transplant, albeit at approximately a 3-fold lower level than seen in AMs derived from donor HSCs (54). Thus, although the etiology of increased PGE_2 was unclear at this time (and is a question addressed in this dissertation), its importance in mediating impaired AM function was highlighted in our model and experiments exploring mechanisms through which PGE_2 mediates inhibition of AM phagocytosis and bacterial killing post-BMT are discussed below.

Endocytosis of pathogens can occur in a number of ways with two major ways being through opsonized and non-opsonized phagocytosis. As bacterial uptake is impaired post-BMT, we sought to better understand the reasons for this. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) functions to dephosphorylate both protein and lipid targets, and has been traditionally known for its effects on PI3K/AKT signaling through dephosphorylation of phosphatidylinositol (3-5)-triphosphate (PIP_3) (99). Previous studies have shown that PGE_2-induced PTEN activity can inhibit FcγR-mediated phagocytosis and PI3K/AKT activation (100). In the syngeneic BMT model, overproduction of PGE_2 stimulates increased PTEN activity, resulting in decreased serum-opsonized phagocytosis (97). Using myeloid-specific PTEN conditional knockout (PTEN CKO) mice as BMT donors, recipient mice had improved, but not completely restored, host defense to *in vivo* bacterial challenge. This rescued response correlated with recovered serum-opsonized phagocytosis by AMs and improved killing of *P. aeruginosa* by both AMs and PMNs. Interestingly, non-opsonized phagocytosis in AMs was only partially recovered (97). This partial restoration may be due to persistent elevation of the IL-1 receptor-associated kinase-M (IRAK-M) post-BMT.

A member of the IRAK family of serine/threonine kinases, IRAK-M functions as a negative regulator of MyD88-dependent TLR signaling (101). Our BMT model revealed that PGE_2 can upregulate IRAK-M in AMs (96). Furthermore, BMT mice receiving genetically ablated IRAK-M-/- donor HSCs were able to control and clear the bacterial infection similarly to untransplanted controls. This recovery also correlated with rescued TNFα, cysLTs, and AM function (including non-opsonized phagocytosis and killing) (96). Interestingly, these mice retained overproduction of PGE_2 and overexpression of the inhibitory receptor, EP2. Thus, these data suggest that the inhibitory effects of PGE_2 are dependent on IRAK-M and that IRAK-M is a

critical downstream mediator of inhibitory PGE_2 signaling (96). Taken together, these studies suggest that PGE_2 signals through IRAK-M independently of PTEN and increased activity of IRAK-M and PTEN promote differential effects on serum-opsonized and non-opsonized phagocytosis. Furthermore, the PTEN macrophage conditional knock out studies highlight the importance of non-opsonized phagocytic responses in the lung following transplant, as complete recovery of serum-opsonized phagocytosis and bacterial killing did not completely abrogate *in vivo* susceptibility measured 24 h post-infection (97).

Non-opsonized phagocytosis is primarily mediated by scavenger receptors (SRs), specifically Class A SRs (102). Within this family of SRs are SR-AI and II (SR-AI/II), macrophage receptor with a collagenous structure (MARCO), SR with C-type lectin (SRCL), and SR receptor A5 (SCARA5) (103). SR-AI and II are splice variants, and together with MARCO, have been associated with mediating non-opsonized phagocytosis in macrophages (103). Their importance in bacterial clearance is highlighted by the susceptibility of individual MARCO-/- and SR-AI/II-/- mice to clear *S. pneumoniae* (104, 105). Defective phagocytosis of *S. pneumoniae* in SR-AI/II-/- mice has also been reported (104). BMT effects on AM SR profiles and SR contribution to AM function post-BMT were not known previous to this work and are explored in Chapter 3.

1.7.3 Polymorphonuclear neutrophils (PMNs)

A major role for PMNs lies in their ability to be recruited to the lung upon inflammation or infection and assist in the killing and ultimate clearance of pathogens (106, 107). Several studies have looked at the function of human PMNs post-autologous HSCT [reviewed in (108)]. These human studies noted decreased respiratory burst, decreased phagocytosis of *Candida*

albicans, and decreased killing of *S. aureus* (109). Interestingly the role of GM-CSF in restoring PMN function is mixed (109-111). In the context of murine syngeneic BMT, PMNs were shown to have intact phagocytic ability but were defective in their ability to kill the engulfed bacteria (58). Genetic ablation of GM-CSF in donor marrow improved, but did not fully restore, PMN killing of *P. aeruginosa* (59).

PTEN-deficient PMNs have been reported to exhibit enhanced host immune responses (112, 113). Previous studies had shown that alleviating the repressive effect of PTEN on PMNs, augmented PMN chemotaxis (113). Similar to these studies, our PTEN CKO BMT mice had elevated basal levels of PMNs in the alveolar space compared to untransplanted wildtype controls and recovered their ability to kill intracellular bacteria (97). However, despite a rescued PMN phenotype, PTEN CKO BMT mice were still susceptible to acute *P. aeruginosa* infection, highlighting the importance AM function, specifically the need for non-opsonized phagocytosis, post-BMT.

Together with AMs, the impaired function of PMNs can also be attributed to elevated PGE_2, as pharmacological inhibition of PGE_2 synthesis completely resolved their defect (58). Unlike BMT AMs, PMNs do not exhibit a phagocytic defect and IRAK-M is not elevated in recruited PMNs. Thus, it is likely that PGE_2 is mediating impaired killing through PTEN, as we have previously shown that PGE_2 can induce PTEN activity (97). A summary of the changes characterized using the syngeneic BMT model is described in Table 1.2.

Table 1.2. Summary of *observed* cellular alterations following syn BMT

	AECs	AMs	PMNs
Cytokines (baseline and post-infection)	↑ TGF-ß, PGE_2 ↓ GM-CSF	↑ GM-CSF, PGE_2 ↓ cysLTs, TNFα	↑ PGE_2
Function		↓ Phagocytosis Bacterial killing	↓ Bacterial killing
Intracellular signaling		↑ Cyclic AMP, activation of protein kinase A, PTEN, IRAK-M, EP2	Loss of PTEN improves function, IRAK-M levels do not change

1.8 Unanswered questions addressed by my studies

As noted above, at the initiation of my studies, we understood that overproduction of PGE_2 mediated by COX-2 in AMs impaired function, but we did not know why COX-2 was persistently elevated in AMs post-BMT. In addition, we knew that AM phagocytosis of the Gram-negative *P. aeruginosa* was impaired, but it was not known whether this was true for a Gram-positive pathogen like *S. aureus*. It was known that SRs regulated non-opsonized bacterial uptake, but the regulation of SR expression post-BMT was unknown. Finally, while PMNs were known to have bacterial killing defects, it was not known whether this related only to intracellular killing or if other mechanisms of bactericidal activity by PMNs (e.g. induction of NETs) were similarly impaired, and if so, if PGE_2 was also responsible. In the work that will be discussed in this dissertation, we have identified a number of epigenetic modifications that help answer these questions, and as such, the following introductory background is provided.

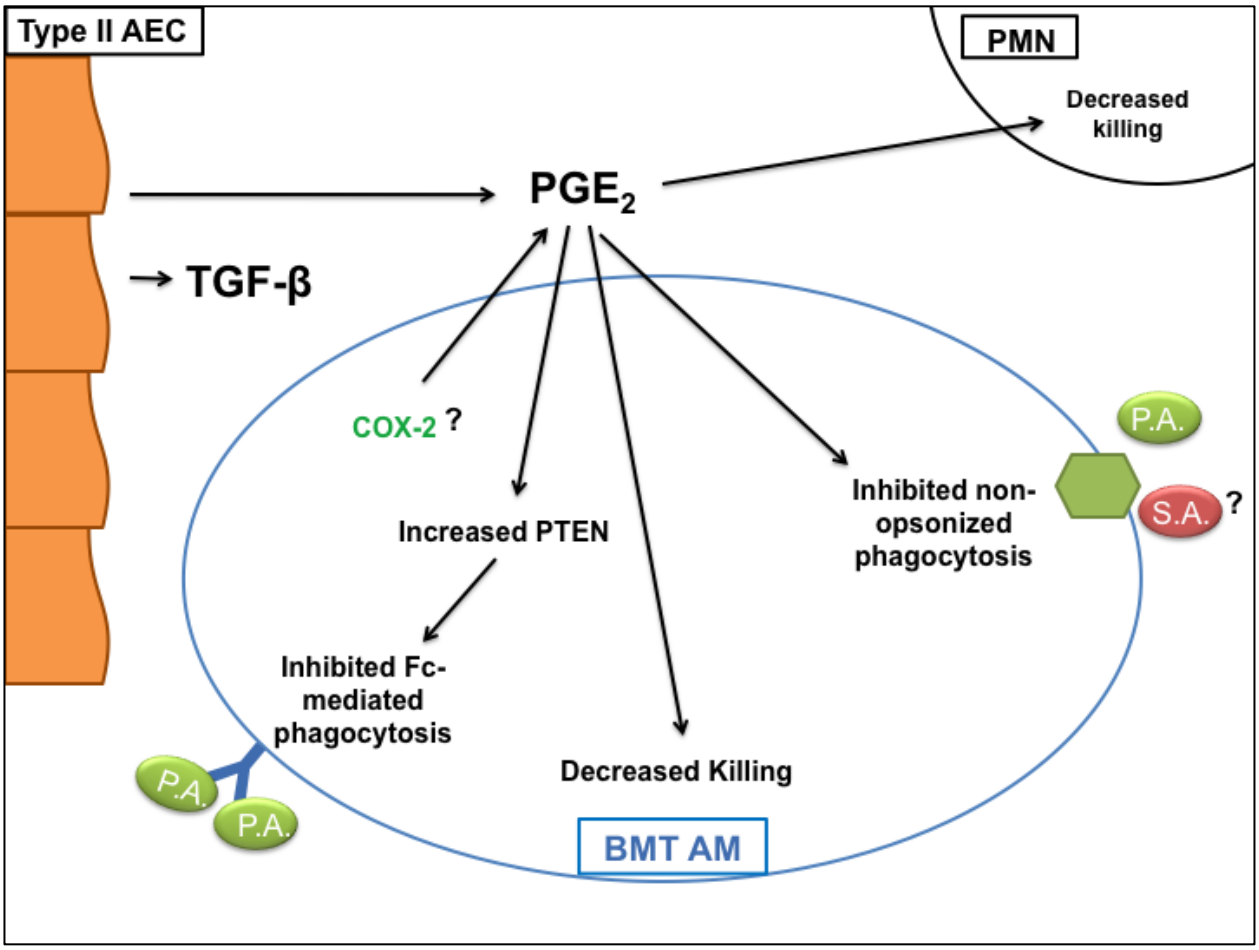

Figure 1.5. Summary of known alveolar macrophage and polymorphonuclear neutrophil dysfunction post-BMT.

1.9 Epigenetics

Epigenetic regulation of gene expression is important for normal biological processes like immune cell development, immune responses, and differentiation from HSCs. Furthermore, it is well understood that epigenetic mechanisms can include DNA methylation, histone modification, and more recently, microRNAs (miRNA). Interestingly, aberrant epigenetic modification can also promote pathology in many diseases like cancer.

Epigenetics can be defined as potentially heritable changes to gene expression that are independent of changes to the DNA sequence (114). Epigenetic regulation can involve DNA

methylation and histone modification. More, recently, miRNA have been included to this category of regulation as some miRNA can regulate various epigenetic-modifying enzymes. In this section, discussion of epigenetic changes in HSCT will be restricted to miRNA effects on DNA methylation.

1.10 DNA Methylation

Epigenetic regulation of gene expression via DNA methylation has been studied extensively due to its importance in regulating diverse biological processes. Though dispensable and nonexistent in some organisms like *Caenorhabditis elegans* (*C. elegans*), DNA methylation is widely conserved across prokaryotes and most eukaryotes (115). Studies in bacteria suggest DNA methylation as an important mechanism for distinguishing self/host DNA from invading phage DNA (116). In mammals, DNA methylation has been extensively studied in natural processes (e.g. embryogenesis) as well as disease processes (e.g. cancer). We now know that in mammals, methylation occurs on DNA whereby a cytosine base is converted to a 5-methylcytosine via *de novo* (DNMT3a/3b) or maintenance (DNMT1) DNA methyltransferases (DNMTs) (117). Although DNA methylation in mammals is primarily regulated by DNMT1, DNMT3a, and DNMT3b, two separate DNMTs have also been identified known as DNMT3L and DNMT2. DNMT3L is implicated in the regulation of DNMT 3a and 3b while DNMT2 has been renamed tRNA aspartic acid methyltransferase for its role in methylated tRNA rather than DNA (117, 118).

Maintenance DNMTs are vital for conserving inherited DNA methylation patterns following cellular replication, while *de novo* DNMTs function to create DNA methylation at new or previously unmethylated CpG sites. DNMT1 has traditionally been highlighted as a

maintenance DNMT. This was primarily due to studies indicating that *de novo* methylation remained intact despite DNMT1 knockout in embryonic stem (ES) cells (119). A separate study had similarly observed that deletion of DNMT1 in these same cells, resulted in a significant reduction in methylated cytosines (120). DNMT3a and DNMT3b, were coined *de novo* DNMTs due to intact inherited DNA methylation patterns in ES cells (121) and overexpression of DNMT3a resulted in increased *de novo* DNA methylation (122). However, other data suggest that these *de novo* DNMTs may exhibit properties of maintenance DNMTs under particular circumstances as in a DNMT1 knockout adenocarcinoma cell line exhibiting intact DNA methylation patterns (123). It is also possible, that other maintenance DNMTs have not yet been identified.

Though DNA is scattered with cytosine bases, the importance of the methylation of cytosines on gene expression localizes to regions of the DNA that are more highly concentrated with cytosine-phosphate-guanine (CpG) dinucleotides, also known as CpG islands (Figure 1.5). The mechanism by which DNMTs target certain CpG islands within genes is not entirely understood.

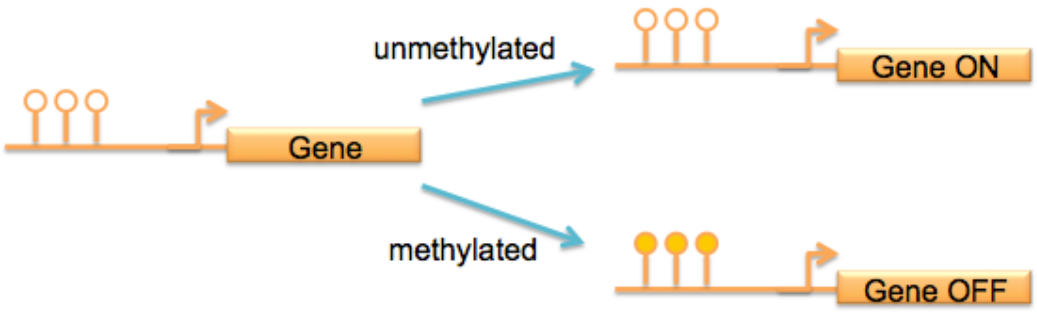

Figure 1.6. Methylation effects on gene expression. CpG sites, or regions where cytosine nucleotides are next to guanine nucleotides, are present in the promoter as well as transcribed regions and can be subjected to regulation by DNA methylation. DNA methyltransferases can

methylate CpG sites resulting in the downregulation of gene expression, whereas decreased methylation (unmethylated) supports genetic expression.

DNA methylation-regulated gene expression has also been studied in the context of disease. ICF (immunodeficiency, centromeric region instability, and facial anomalies) syndrome is a recessive autosomal disease characterized by mutated DNMT3b, resulting in targeted chromosome breakage and decreased serum immunoglobulin levels despite normal B cell counts (124). In idiopathic pulmonary fibrosis, some fibroblasts lose responsiveness to inhibitory effects of PGE_2 signaling via increased DNA methylation of the EP2 receptor (125). In addition, various studies have pointed to dysregulated DNA methylation patterns as cofactors for development of cancer (126). Global DNA hypomethylation and hypermethylation of specific genes, like tumor suppressor genes, was previously observed in tumor cells (127, 128). Hypomethylation of CpG islands in the promoter of COX-2 has been previously associated with increased expression of COX-2 mRNA and contributes to progression of many human cancers (129-131). In gastric cancer, increased expression of COX-2 directly correlated with increased risk of death from gastric cancer compared to patients that did not exhibit induced expression of COX-2 (132). That aberrant COX-2 expression is associated with altered methylation status of CpG regions in the promoter and this suggests an important role in epigenetic regulation of this gene. Taken together, the above data prompted us to examine the DNA methylation patterns of COX-2 post-BMT as part of the studies presented in this dissertation.

1.11 MicroRNAs (miRNAs)

MiRNAs are small, noncoding RNAs that can modulate gene expression post-transcriptionally, affecting mRNA stability as well as inhibiting protein translation. Originally

discovered in *C. elegans* (133), the presence of miRNA has been described in both animal and plant cells. Since the initial description of miRNA, many studies have tried to understand how miRNAs are processed and the mechanism behind RNA interference (RNAi) as a form of regulation of gene expression. In humans, it is estimated that about a quarter of miRNA genes are encoded in intronic regions while others are found clustered throughout the genome (134, 135). We now know that miRNAs arise from noncoding RNA, primarily transcribed by RNA polymerase II. These long noncoding RNA, also known as pri-miRNA, contain stem-loop structures, and go through various processing steps to ultimately yield mature miRNA, roughly about 22 nucleotides in length. Interestingly, multiple miRNA can arise from a single pri-miRNA (136, 137). Cleavage and further processing of these stem-loop structures can occur in one of two identified pathways: the canonical Drosha pathway or a Drosha-independent pathway. The latter pathway has been investigated primarily in *Drosophila* and *C. elegans,* and these studies suggest that some intron-originating miRNA can form pre-miRNA from pri-miRNA via a Drosha-independent splicing mechanism (138, 139). In contrast, the canonical Drosha pathway involves cleavage via a complex containing DiGeorge syndrome critical region gene 8 (DGCR8) and the RNase type III, Drosha. Also known as the microprocessor complex, DGCR8 and Drosha are responsible for mediating the formation of pre-miRNAs from pri-miRNA, the primary miRNA transcript (134, 140). Interaction with exportin-5-Ran-GTP supports transportation of the pre-miRNA to the cytoplasm from the nucleus, where it can be further processed by Dicer, a dsRNase type III, to yield a double stranded miRNA at its mature length. This double stranded miRNA contains a functional strand, (the mature miRNA), which is loaded onto the RNA-induced silencing complex (RISC) together with Argonaute proteins, and a nonfunctional (passenger) strand, which is degraded.

Unlike small interfering RNA (siRNA) which form approximately 21-23 base-paired duplexes that are complementary to any region of the target mRNA and trigger degradation via Argonaute proteins (141), mature miRNA guide the RISC complex via identification of target sequences in 3' untranslated regions (3'UTR) of mRNA to affect mRNA stability and/or inhibit translation (140, 142). It remains the general rule that recognition of a target mRNA by a miRNA requires complete complementarity between the target sequence in the 3'UTR of the mRNA and the 'seed' region, defined as nucleotides 2-8 of the 5' end of the miRNA. However, exceptions to this rule do exist and have been summarized elsewhere (141). Although such exceptions can complicate studies focused on understanding the effects of miRNA on gene expression, it is important to remember that when performing such studies, targeting of the miRNA to the gene sequence under investigation should be verified.

1.12 MiRNA and immune responses

The importance of miRNA on developmental, cellular, and physiological processes was previously underappreciated. However, multiple studies have now shown that miRNA are important for basic biological processes like hematopoiesis as well as regulating immune cell development and immunological responses (143). Initiation of innate immune responses are important not just for the immediate attack on invading pathogens, but also for the further activation of the adaptive immune response, which can confer protective and long-lasting immunity. One way that innate immune cells sense pathogens is through membrane-bound TLRs (7). Binding to these receptors triggers the activation of intracellular signaling cascades that result in a variety of responses, e.g. activation of pro-inflammatory genes. These pathways have been studied extensively. However, what has been more recently observed is that miRNA

can exert regulatory functions that can either support or antagonize these innate immune responses. MiR-146a/b, for example, has been shown to be induced by TLRs expressed on the cell surface and not TLRs bound to intracellular membranes (7, 144). Interestingly, miR-146 targets adaptor proteins important for TLR signaling suggesting that miR-146 functions as a negative regulator of TLR-initiated immune responses (144). MiRNA-mediated gene regulation is extremely complex because of the broad and various mRNA targets. Studies focused on miR-155 regulation of immune responses highlight the complexity of miRNA-mediated regulation.

1.12.1 MiR-155

Both bacterial and viral products, through recognition primarily mediated by TLRs, as well as various proinflammatory cytokines (e.g. IFN-β, TNFα) can induce miR-155 expression in macrophages (144, 145). Furthermore, miR-155 can stabilize TNFα, and thus promote translation of TNFα and support proinflammatory responses, particularly in macrophages (146). Studies on macrophage polarization have shown a role for miRNA regulation (125). In one study, overexpression of miR-155 on tumor-associated macrophages, which typically resemble alternatively activated macrophages and exhibit anti-inflammatory functions, resulted in a switch in polarization to a classically activated macrophage (147). However, similar to miR-146a/b, miR-155 can also serve as a negative regulator of inflammatory responses, as genes involved in LPS-induced signaling have been identified as targets of miR-155 (148). Thus, miRNA can have multifunctional roles and harbor both positive and negative effects on immune responses.

1.12.2 MiR-29b

The miR-29 family is composed of miR-29a, -29b, and 29-c, and all contain identical seed regions, important for mediating the specificity for their targets. Because they harbor the same seed sequence, it is thought that the range of targets is largely overlapping. Interestingly though, members in this family have been shown to differentially regulate target genes, suggesting the possibility that the specific cellular environment or cell type is important in regulating the outcome of miR-29b expression (149, 150). The miR-29 family contains multiple transcription factor binding sites that may regulate expression, however, the exact mechanism(s) driving differential expression of miR-29a/b/c remains elusive. It is thought that perhaps in addition to regulation by transcription factors, miR-29a/b/c expression may also be influenced by rate of decay, as well as alternative splicing of primary RNA (151, 152). Functionally, miR-29 members are particularly interesting. Similar to other miRNAs, they can modulate gene expression directly, through the direct targeting of 3'UTRs with their complimentary miRNA seed regions and often result in decreased target gene expression; however, they are also able to regulate expression indirectly by initially targeting de novo DNA methyltransferases and blocking the DNA methylation of various genes (153, 154). The latter supports the demethylation of genes, resulting in their aberrantly induced expression.

MiR-29 has been associated with mediating multiple cellular effects, including regulation of the extracellular matrix via directly targeting the 3'UTR of extracellular matrix proteins, e.g. collagen, laminin g1, elastin, and integrin β1, and the development of fibrosis in multiple organs (heart, kidney, lung) (149). Further highlighting their importance in disease models, miR-29s have been implicated to play important tumor suppressor roles in cancer as they regulate apoptosis, cell proliferation and differentiation. Expression levels of miR-29s were reported to

correlate with cancer models and progression, as decreased expression of miR-29s were associated with higher incidence of cancer relapse (155, 156). This is not entirely surprising as many miR-29 targets are known oncogenes. Because miRNA expression can be modulated and increased miR-29 expression may improve prognosis, it is an attractive therapeutic avenue for cancer therapy and studies implementing exogenous miR-29 members in acute myeloid leukemia models show promising results (157).

Recently, a role for miR-29 members in regulating immune responses has been suggested. It is well known that macrophage activation as well as T helper 1 (Th1) cell development is supported by interferon-gamma production to enhance immune responses against intracellular pathogens. In a study performed by Ma et al., IFNγ production indirectly correlated with miR-29b expression. Transgenic mice were developed that express a 'sponge' target that effectively competes with miR-29 target genes and thus sequestered miR-29b preventing function *in vivo*. These mice exhibited lower *L. monocytogenes* burden, enhanced Th1 responses, and higher serum IFNγ (158).

1.13 PMN Extracellular Traps (NETs)

Elie Metchnikoff initially observed the existence of PMNs, which he previously called microphagocytes. Since then, PMN immune function was primarily attributed to the engulfment and killing of pathogens through the unloading of the antimicrobicidal and antimicrobistatic granular proteins into the pathogen-containing vacuole (159, 160). However, we now know that PMNs harbor an additional immune mechanism. Originally discovered in 2004 by Brinkmann et al., NETs are a fibrous network composed of chromatin and nucleic and cytoplasmic proteins with antimicrobial properties (160). NETosis is a novel form of cell death, distinct from

apoptosis and necrosis (161), whereby NETs are released and can trap and kill pathogens extracellularly, like *S. aureus* (160). Similarly, NETs were productive in preventing dissemination of *S. pneumoniae* from the lung, but production of endogenous DNases and catalases by a variety of Gram-positive bacteria confer protection against extensive entrapment by facilitating the degradation of NETs (161-164).

The following chapters will detail the methods (Chapter 2) utilized and the results obtained in addressing several pressing questions regarding the regulation of innate immune function post-BMT.

1) Chapter 3: Why are pathogens differentially phagocytized by AMs post-BMT and is this effect mediated by elevated PGE_2 post-BMT? Results of this chapter have been published in the *J.Immunol*.
2) Chapter 4: Is the upregulation of PGE_2 synthesis post-BMT regulated by hypomethylation of the COX-2 gene? Results of this chapter have been published in the *J. Immunol*.
3) Chapter 5: How does TGF-β production post-BMT regulate the expression of COX-2? Results of this chapter have been submitted for publication.
4) Chapter 6: Finally, as PGE_2 is known to impact PMN killing, but not PMN phagocytosis, is this effect mediated by alteration of NETosis? Results of this chapter have been prepared for submission.

Chapter 2:

Materials and Methods

2.1 Animals

Wild type C57BL/6 (B6), SRAI/II-/- mice (C57BL/6 background), and wild type BALB/c were obtained from The Jackson Laboratory (Bar Harbor, Maine). Mice expressing dominant-negative TGF-β receptor II (TGF-βRII) under the CD11c promoter ($CD11c^{dnR}$) in the C57BL/6 background were provided by Dr. Laouar (University of Michigan) (165, 166). 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.

2.2 Human Subjects

Bronchoalveolar lavage cell samples from 14 HSCT patients (both autologous and allogeneic) and six healthy volunteers were used in the microRNA study. Bronchoalveolar lavage cells samples from 21 HSCT patients (both autologous and allogeneic) and one healthy volunteer were used in the PGE_2 study shown in Figure 1.4 and Table 1.1. Studies and consent procedures were performed in accordance with the Declaration of Helsinki at the University of Michigan (HSCT patients) and the VA Ann Arbor Healthcare System (volunteers for miR-29b study) 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 5.1. PMNs from four healthy volunteers (one male and three female) were harvested from peripheral blood and were used in the PMN extracellular traps study. The study outlined here was approved by the University of Michigan IRB and all human subjects gave a written informed consent.

2.3 Bone Marrow Transplantation (BMT)

Recipient mice received 13 Gy of TBI (orthovoltage x-ray source) for syngeneic (B6 donors and recipients) or 10 GY of TBI for allogeneic (B6 donors, BALB/c recipients) BMTs. TBI doses were split in two fractions, 3 hours (h) apart. Whole bone marrow was harvested from the femur and tibia of C57Bl/6 WT or $CD11c^{dnR}$ 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 (5×10^6) were administered by tail vein injection into lethally irradiated C57Bl/6 recipient mice. All experiments with BMT mice were performed 5–6 wk post-BMT. The percentage of donor-derived cells was approximately 95% ±1% in the spleen and 82% ±2% in the lung at this time point, as assessed by transplanting $CD45.1^+$ bone marrow into C57BL/6 $CD45.2^+$ mice (54).

2.4 Harvesting AMs and PMNs via bronchoalveolar lavage (BAL)

Resident AMs from mice were obtained via ex vivo lung lavage, using a previously described protocol (58). 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. PMNs were recruited into the lungs by an i.t. injection of 25 μg/mL of *Pseudomonas*-derived lipopolysaccharide (LPS) (Sigma) in 50 μL doses, diluted in saline. Following 18 h, PMNs were collected by ex vivo lung lavage, as described for resident AMs, or

by bone marrow harvest. However, cells were collected using GIBCO RPMI media with L-glutamine, without phenol red. Red blood cells (RBCs) were lysed with 3 mL of RBC lysis buffer for 2 min on ice, diluted in 10 mL of DPBS, and centrifuged. Pelleted cells were reconstituted and enumerated by counting on a hemocytometer before plating.

2.5 Isolation of human PMN from peripheral blood

PMNs were isolated using a protocol provided by Carolyne Smith (Mariana Kaplan Lab). PMNs were harvested from the peripheral blood of four healthy human volunteers. Peripheral blood was layered over ficoll and spun at 1440 rpm with no acceleration and no break at room temperature for 20 min. The red blood cell layer (bottom layer) containing PMNs were collected and mixed with 20% dextran (1:2 ratio; dextran:red blood cell layer). Mixture was allowed to sit for 15 min at room temperature prior to the addition of PBS. Following 30 min incubation at room temperature, the top layer containing the PMNs were collected, PBS was added, and cells were spun at 1600 rpm with acceleration and brake. RBCs in the cell pellet were removed via addition of 0.2% and 1.8% NaCl in sterile water and incubation at room temperature for 3 min. Cells were spin down at 1600 rpm, washed with PBS. Following a last spin at 1600 rpm, cells were dissolved in RPMI without phenol red (+L-glut).

2.6 *P. aeruginosa* PAO1 and *S. aureus* (MRSA; USA300/NRS384) preparation and FITC labeling

P. aeruginosa PAO1 and *S. aureus* USA300 stock were grown in tryptic soy broth and nutrient broth (Difco; BD), respectively. The culture concentration was determined via absorbance measurements as previously described (55). For fluorescein isothiocyanate (FITC)-labeling, a culture was centrifuged and washed two times by resuspending the pellet in 1 mL sterile PBS and transferring into a sterile tube. The bacteria were 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) in DMSO was added to heat-killed *P. aeruginosa* or *S. aureus* and allowed to incubate in the dark for 1 h on a rocker at room temperature. Following FITC-labeling, heat-killed *P. aeruginosa* or *S. aureus* were washed three times and resuspended in sterile PBS at 6 x 10^9 CFU/ml.

2.7 Intratracheal (i.t.) injection with *P. aeruginosa* or *S. aureus*

A culture of *P. aeruginosa* or *S. aureus* was grown as described above, and an inoculum was prepared. Mice were anesthetized and i.t. injected with 50 μL of inoculum to provide either a sublethal dose of 5 x 10^5 PAO1 CFU as previously described (55, 58) or a sublethal dose of $7x10^7$ USA300 CFU.

2.8 Quantification of bacterial burden in the lung

Mice were euthanized 24 h following i.t. infection with *P. aeruginosa* or *S. aureus*. As previously described (58), lungs were collected from each mouse, homogenized and the bacterial burden of each specimen was assessed by performing a CFU assay. Data are expressed as total CFU per lung.

2.9 Molecular cloning of COX-2 promoter into luciferase expression vector

The full-length (long) murine COX-2 promoter (1179 bp) DNA as defined in (167) and a shorter COX-2 promoter (702 bp) was amplified using primers to create cloning sites shown in Table 2.12 by standard PCR from genomic DNA from AMs. The 1179 bp amplified COX-2 promoter DNA was digested with restriction endonucleases *KpnI* and *BglII* (New England BioLabs, Inc.) while the 702 bp 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 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.

2.10 *In vitro* DNA methylation

The COX-2 promoters , both the longer (1179 bp) and the partially deleted *COX-2* promoter (702 bp), 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 h. DNA Methylation was confirmed by digestion with methylation-sensitive restriction enzyme *SmaI*.

2.11 *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 $x10^4$ 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 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 of DNA using lipofectamine LTX and PLUS Reagent (Invitrogen). Transfections were performed with unmethylated or methylated, entire or partially deleted COX-2 luciferase reporter plasmid for 48 h. Where indicated, cells were treated with 10 μg/mL LPS or 1 ng/mL porcine TGF-β1 for 48 h (together with the transfections).

2.12 Real-time RT-PCR

Gene-specific primers and probes were designed using Primer Express software (PE Biosystems, Foster City, CA) as published previously (58, 168). Sequences for primers and probes used can be found in Table 2.2. SnoR142, snoR202, miR-155, and miR-29b expression were measured using TaqMan® MicroRNA Assays (Applied Biosystems/Life Technologies). Each AM sample was pooled from two to three mice and was run in duplicate. MicroRNA expression was determined by first converting TRIzol-isolated RNA into cDNA using TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems/Life Technologies, Foster City, CA). Real-time RT-PCR was performed on an ABI Prism 7000 thermocycler (Applied Biosystems). Average cycle threshold (C_T) was determined for each sample and was normalized to β-actin or snoR142 or snoR202. Relative gene expression was calculated as described previously (59).

Table 2.12. 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
MARCO forward	CCTGGACGAGTCGGTCAGAA
MARCO reverse	CTTCAGCTCGGCCTCTGTT

MARCO probe	CCAACGCGTCCGGATCATGGGT
SR-AI/II forward	TGAAGGACTGGGAACACTCACA
SR-AI/II reverse	CAGTAAGCCCTCTGTCTCCCTTT
SR-AI/II probe	TTCATTCAAGGGCCTCCTGGACCC
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

2.13 DNA methyltransferase or histone deacetylase inhibition

AMs or MHS cells (an alveolar macrophage cell line) were treated with either varying concentrations of the methyltransferase inhibitor 5-aza-2′-deoxycytidine (Sigma) or the histone deacetylase (HDAC) inhibitor trichostatin A (Sigma) for 72 h. Primary AMs were initially stimulated with (1 ng/ml) recombinant murine GM-CSF (R&D Systems) for 24 h to promote proliferation before receiving the appropriate treatments for the following 72 h.

2.14 ELISA/enzyme immunoassay

MHS cells were cultured at 5×10^5 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), according to the manufacturer's instructions.

2.15 Bisulfite conversion and pyrosequencing

Using the DNeasy Kit (Qiagen), DNA was isolated from ~1 × 10^6 cells or from 2 x10^5 primary AMs previously transfected with control miRNA or miR-29b (30 nM) for 24 h. For DNA methylation studies from miR-transfected AMs, three wells were pooled per sample. The Zymo Research EZ DNA Methylation kit 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) x 16 cycles; 4°C hold. Following bisulfite conversion, the *PTGS2* (*COX-2)* promoter (-449 to +70 from ATG) was PCR amplified and sequenced on a Pyrosequencer (Qiagen). Amplification and sequencing primers for murine *PTGS2* were obtained from EpigenDx, Inc, Worcester, MA (assay ADS2001 and ADS2002). The PCR conditions for determining the methylation profile of the DNA using ADS2001 are as follows: 95°C 15 min; 45 x (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 x (95°C 30 s; 60°C 30s; 72°C 30s); 72°C 5 min; 4°C hold. The primers used for amplification and sequencing of CpG sites 1-6 of the COX-2 promoter were: Forward primer: AGATGTGGATTTTGATAGAGGATATT; Reverse primer: CTACCCTTAACTACCCCAAATAATAC; Sequencing primer: ATTTTATTAAAAATAGAAGAAA.

2.16 *In vitro* phagocytosis assay

AMs isolated by BAL were plated at 2 x 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). The following day, FITC-labeled heat-killed *P. aeruginosa* (prepared as described above) was added at 300:1 MOI. Two h following incubation at 37ºC 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-phagocytized 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) as previously described (58).

2.17 Tetrazolium dye reduction assay of bacterial killing

The ability of AMs from control and BMT mice to kill *P. aeruginosa* and *S. aureus* was quantified using a tetrazolium dye reduction assay, as described elsewhere (72, 169). Briefly, AMs from WT and BMT mice were aliquoted into duplicate 96-well plates: one experimental (37^{o}C) plate and one control (4^{o}C). Cells from both plates were infected with IgG-opsonized *P. aeruginosa* or *S. aureus* (2 x 10^8 CFU/ml; multiplicity of infection (MOI), 50:1) for 30 min at 37^{o}C. The cells on the experimental plate were washed then incubated at 37^{o}C for 2 h while the cells on the control plate were washed then lysed with TSB and 0.5% saponin (Sigma) and placed at 4^{o}C. After 2 h, the cells from the experimental plate were lysed with 0.5% saponin in tryptic soy broth. Both plates were then incubated at 37^{o}C for 3 h (*P. aeruginosa*) or 4 h (*S. aureus*). Five mg/ml of MTT (Sigma) was added to each plate and incubated for 30 min or until purple precipitate was visible. Solubilization solution was added to dissolve formazan salts and

the absorbance was read at 595 nm. Results were expressed as percentage of survival of ingested bacteria normalized to the percent of control, where the A_{595} experiment values were divided by the average of the A_{595} control values. Survival of ingested bacteria = (A_{595} experimental plate/A_{595} control plate) x 100%. The results were expressed as "percent of control" to indicate the percentage of survival of ingested bacteria normalized to the percentage of control.

2.18 Flow Cytometry

5×10^5 primary AMs from untransplanted control and BMT mice were stained for flow cytometry using a primary antibody (2 μg/mL) against the cell surface receptor MARCO (Hycult Biotech) after incubation with anti-CD16/CD32 (FcBlock; BD Biosciences). Fluorochrome-conjugated secondary antibody, donkey anti-rat PE, (Jackson Immunoresearch) was used to detect the primary antibody. Cells were resuspended in PBS and fixed with 4% paraformaldehyde. Data were analyzed using flow cytometry analysis software (FlowJo, version 7.5; Tree Star, Inc., Ashland, OR).

2.19 *In vitro* miRNA transfection

Primary AMs were harvested by BAL from untransplanted C57BL/6 mice as described above. $2x10^5$ cells were transfected with the antagomir of miR-155 (Dharmacon/Thermo Fisher Scientific) or anti-miR™ miRNA inhibitor negative control #1 (Ambion®/Life Technologies) for 24 h when measuring mRNA expression and 48 h for phagocytosis assays. Transfections were performed using Lipofectamine® RNAiMAX Reagent (Invitrogen/Life Technologies) as described by the manufacturer. Briefly, antagomir or control miR were diluted in Opti-MEM® I Reduced Serum Media (Life Technologies) and mixed with Lipofectamine® RNAiMAX Reagent prior to transfecting the cells.

2.20 Sytox Green Fluorescence Assays

PMN extracellular traps were quantified using a cell-impermeable nucleic acid dye, Sytox Green (Invitrogen). The protocol was generously provided by Dr. Mariana Kaplan and modified for adequate measurement of NETosis in our model. Briefly, PMNs were recruited into the lungs as described above. Using a Costar black, clear bottom, non-treated, flat bottom 96-well plate, 100,000 cells in 200 μL of RPMI 1640 without Phenol Red were plated for 20 min at 37°C 5% CO_2. Following removal of non-adherent cells, treatments were added with 0.2 μM Sytox to the adhered cells for 5 h (mice) or 3 h (human). One hundred microliters of 4% paraformaldehyde was carefully added to the plate and the plate was stored at 4°C overnight, covered with aluminum foil (to protect from the light). The next day, the plate was brought to room temperature, spun down to remove air bubbles, and fluorescence was measured at an excitation wavelength of 485 nm, and an emission wavelength of 525 nm. Sytox assays do not distinguish NETs from other types of cell death. To date, no publications have described a Sytox assay that is able to do this. Thus, all Sytox assays should be confirmed with immunofluorescence studies.

2.21 Immunofluorescence studies

The immunofluorescence protocol for the visual detection of NETs was provided by Dr. Mariana Kaplan. Briefly, PMNs from mice or humans were collected as described above. Using coverslips previously coated with 0.001% poly-lysine for 20 min at room temperature, 200,000 cells in RPMI 1640 without phenol red were seeded for 20 min at 37°C 5% CO_2. Treatments were added directly for 7 h (mice) or 3 h (human), fixed with 4% paraformaldehyde, washed with PBS, and blocked with 0.2% gelatin in PBS overnight at 4 °C. Primary antibody (myeloperoxidase, MPO, 1:500; PMN elastase, NE, 1:100) were diluted in blocking solution and

incubated for 30 min. Following a washing step, secondary antibody (1:250; diluted in blocking solution) was added for 30 min at 37°C, 5% CO_2. Following two washing steps, Hoescht, diluted in PBS (1:1000), was added for 10 min at room temperature. Upon removal of Hoescht and a washing step, the coverslips were allowed to dry prior to their placement onto a slide containing a drop of Prolong Gold. Slides were allowed to dry overnight, prior to any microscope analysis.

2.22 Western Blot Analysis for NETs

PMNs were recruited into the lung as described above (Section 2.4). PMNs were plated at 200,000 cells in 300 μL (per well) Gibco RPMI with L-glutamine, without phenol red in a 24 well plate. PMNs receiving PGE_2 treatment were pretreated with PGE_2 during adherence. Following 30 min adherence and pretreatment, supernatants were pulled and treatments were added for 5 h. Cells were incubated at 37°C with 5% CO_2. Following 5 h, supernatants were pulled and 30 μg of DNase 1 (from bovine pancreas; Sigma) was added (DNase was made fresh each time and resuspended in 50 mM Tris-HCl, 10 mM Mg Cl, pH=7.5 as recommended by Sigma). Supernatants were incubated with DNase 1 for 1 h at 37°C. Following DNase treatment, acetone precipitation was performed as described by Thermo Scientific. Briefly, cold (-20°C) acetone was added to each sample at four times the volume (1200 μL of acetone was added to each 300 μL sample). Samples were vortexed and incubated overnight at -20°C. The following day, samples were centrifuged for 10 min at 15,000 x g. Supernatants were decanted carefully so as not to dislodge the pellet. The pellet was incubated at room temperature for 5 min, uncapped, to allow for the evaporation of leftover acetone. Pellets were dissolved in Pierce RIPA buffer (Thermo). To determine protein concentrations, Pierce BCA protein assay kit (Thermo) was used. Samples were diluted to a final concentration of 20 μg of total protein per sample with 6x

laemmli SDS loading buffer containing β-mercaptoethanol. Following electrophoresis, a wet transfer was performed using a PVDF membrane. Blocking was performed using 5% milk-TBS-tween for 1 h at room temperature. The primary polyclonal rabbit anti-human myeloperoxidase (Dako, Denmark) antibody was used at 1:500 and incubated with the membrane overnight in 5% milk-TBS-tween at 4°C. Secondary antibody was incubated at 1:250 for 1 h rocking in 5% milk-TBS-tween at room temperature. Chemiluminescence was determined by 3 min incubation with working solution of super signal west pico chemiluminscence substrate (Thermo) in a 1:1 ratio. Detection was recorded using autoradiography films.

2.23 Pharmacological Agents

Indomethacin was used as a non-selective cyclooxygenase inhibitor (Sigma). PF-04418948, a specific EP2 antagonist was generously supplied by Pfizer through their compound transfer program (170, 171). The protein kinase A (PKA)-specific cAMP analog 6-Bnz-cAMP and the guanine nucleotide exchange protein (Epac)-1-specific camp analog 8-pCPT-2'-O-Me-cAMP were purchased from Biolog Life Science Institute, diluted in dimethyl sufloxide (DMSO) and were used at 500 μM doses.

2.24 Statistical analysis

To determine statistical significance, the GraphPad Prism 6.0 statistical program was utilized. Comparisons between two experimental groups were determined using the Student *t* test and error bars denote the standard error of the mean. Comparison between three or more groups utilized one-way ANOVA analyses followed by a Bonferroni post-test and standard error of the mean were graphed. A p value <0.05 was considered statistically significant.

Chapter 3:

Prostaglandin E_2-induced changes in alveolar macrophage scavenger receptor profiles differentially alter phagocytosis of *P. aeruginosa* and *S. aureus* post-bone marrow transplantation

3.1 Background

HSCT is a widely used treatment to address a variety of inherited and genetic disorders in patients. Though effective, HSCT patients show increased susceptibility to numerous complications, many involving the lung, in the months to years following treatment (27, 28, 172). Infectious and non-infectious complications arise in HSCT recipients despite differences in stem cell source (172, 173). Autopsy reports found 80-89% pulmonary complication rates in both allogeneic and autologous HSCT patients (21, 173) and a recent study found that pulmonary complications manifested in more than 25% of autologous HSCT recipients, with the majority of the complications being infectious (28).

Bacterial pulmonary complications in HSCT recipients manifest at different times post-transplant and are varied in pathogen species (Gram-positive vs. Gram-negative). Although allogeneic transplant recipients develop pulmonary infectious complications more frequently (potentially due to barrier disruptions secondary to GvHD), autologous HSCT patients also experience infectious complications and there is no difference in the types of infections that manifest in autologous and allogeneic HSCT recipients in the first 30 days post-transplant (pre-engraftment phase) (23). While it is not surprising that infections are common in the pre-

engraftment phase (174), or in allogeneic HSCT patients on immunosuppressive therapy, it is less understood why infections have also been reported to occur in autologous HSCT recipients in the late post-transplant phase (175). Both Gram-negative and Gram-positive infections can be problematic in HSCT patients. Prophylactic antibiotics have lowered the incidence of *Pseudomonas aeruginosa* infection, but invasive *Pseudomonas* remains a concern in this population (52, 176). Additionally, infections caused by Gram-positive bacteria, particularly the *Streptococcus* and *Staphylococcus* species, have risen as predominant infections in HSCT recipients (23).

In order to better understand the immunological alterations that may characterize innate immune cells in the lung and predispose the host to bacterial infection post-engraftment, our lab developed a syngeneic (syn) BMT animal model. This model allows for an investigation of transplant-related immune alterations that are not caused by immunosuppressive drugs or impaired barrier function secondary to graft vs. host disease. We previously demonstrated that C57BL/6 mice that have received a syn BMT are more susceptible to infection by Gram-negative *P. aeruginosa* (55). Increased susceptibility to *P. aeruginosa* was related to overproduction of PGE_2 post-BMT (58). Elevations of PGE_2 post-BMT impaired AM phagocytosis and killing of ingested bacteria, and also limited PMN-mediated killing (58). Some of the effects of PGE_2 signaling on AMs post-BMT were to upregulate IRAK-M and activation of PTEN (96, 97). These changes limited inflammatory cytokine production and non-opsonized and opsonized phagocytosis of *P. aeruginosa* as well as bacterial killing.

The current studies were undertaken to determine whether syn BMT mice also displayed increased susceptibility to a Gram-positive, *S. aureus* infection and if so, whether the mechanisms of susceptibility were related to PGE_2 production and impaired phagocytosis

mediated by SRs. Similar to our previous studies with *P. aeruginosa*, we find syn BMT mice are more susceptible to *S. aureus* and this is related to overproduction of PGE_2. Surprisingly however, phagocytosis of *S. aureus* is enhanced, not diminished post-BMT. Our mechanistic studies identify PGE_2-induced changes, including alterations in miRNAs which alter the functional profile of scavenger receptors to differentially regulate phagocytosis. Despite the fact that phagocytosis of *S. aureus* is improved post-BMT, bacterial killing is impaired, explaining the overall defect in host defense.

3.2 Results

Syn BMT mice are more susceptible to S. aureus

To determine the susceptibility of BMT mice to *S. aureus*, untransplanted (control) and syn BMT mice were i.t. injected with a methicillin-resistant strain of *S. aureus* or with the PAO1 strain of *P. aeruginosa* for comparison. Lungs were harvested 24 h post-infection for CFU analysis. BMT mice were more susceptible to both *P. aeruginosa* (Figure 3.1A) and *S. aureus* (Figure 3.1B) pulmonary infections.

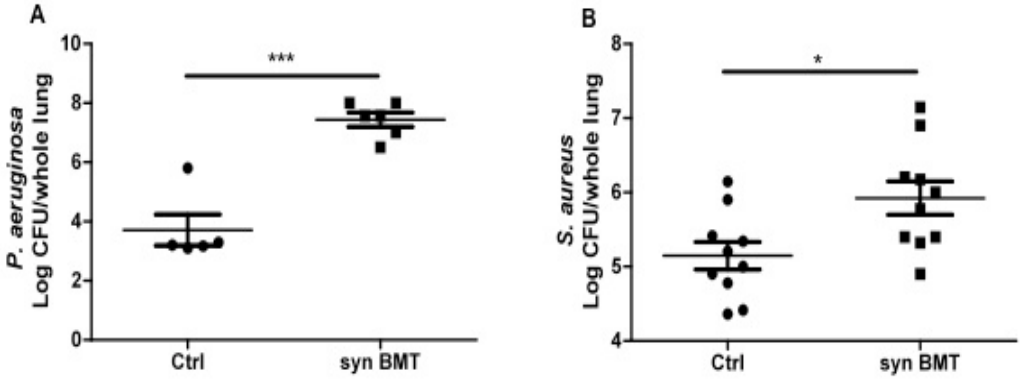

Figure 3.1. Syn BMT are more susceptible to *P. aeruginosa* and *S.* aureus. Untransplanted control (Ctrl) or syn BMT mice received (A) *P. aeruginosa* (n=5-6/group) or (B) *S. aureus* (n=10/group) i.t. and lungs were collected for CFU analysis 24 h later; **p*<0.05, ****p*<0.001.

Syn BMT AMs exhibit a defect in phagocytosis of P. aeruginosa, but enhanced phagocytosis of S. aureus

Because AMs are the predominant immune cell in the lung during an initial infection (177), we hypothesized that AM function was compromised in a BMT setting. Using AMs harvested from BAL of control and syn BMT mice, phagocytosis of *P. aeruginosa* by AMs was

measured. As we have previously published (58), syn BMT AMs exhibited decreased phagocytosis of FITC-*P. aeruginosa* compared to controls (Figure 3.2A). Surprisingly, syn BMT AMs incubated with FITC-*S. aureus* displayed augmented phagocytosis compared to controls (Figure 3.2B).

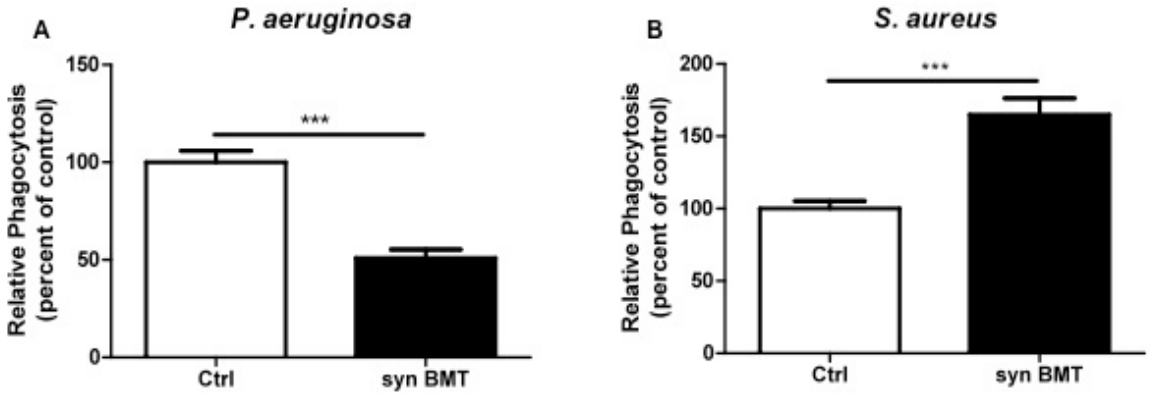

Figure 3.2. Syn BMT AMs exhibit defective phagocytosis of *P. aeruginosa* but not *S. aureus*. Primary AMs were harvested and phagocytosis of (A) FITC-P. aeruginosa or (B) FITC-S. aureus was determined (n=5-10 per group). Data are normalized to LDH levels per well to standardize for cell numbers. Results are presented relative to the average of untransplanted control values set to 100%; ***$p<0.001$.

SR profiles are altered post-BMT

Class A SRs are pattern recognition receptors reported to play important roles in the recognition of non-opsonized Gram-positive and –negative bacteria and endogenous ligands (178). Studies have shown that MARCO, a class A SR, is important for *in vivo* host response against *Streptococcus pneumoniae,* a Gram-positive bacterium (105), and SR-A I/II have been previously reported to recognize *S. aureus* (179). However, it remains unclear which SR is important for recognition of *P. aeruginosa* in the lung. As MARCO and SRAI/II have been implicated in the recognition and initiation of an immune response within macrophages (178),

we wanted to determine whether the difference in phagocytosis between *P. aeruginosa* and *S. aureus* could be explained by an alteration in SR expression on AMs. Using RNA isolated from control and BMT AMs, MARCO mRNA expression decreased post-BMT (Figure 3.3A, left) while SR-AI/II mRNA increased (Figure 3.3B) compared to control. A decrease in MARCO surface expression was confirmed by flow cytometry (Figure 3.3A, right), correlating with MARCO mRNA analysis. Commercially available antibodies against SRAI/II do not recognize the allele in the C57BL/6 mice, precluding our ability to measure protein expression of this receptor by flow cytometry in our model.

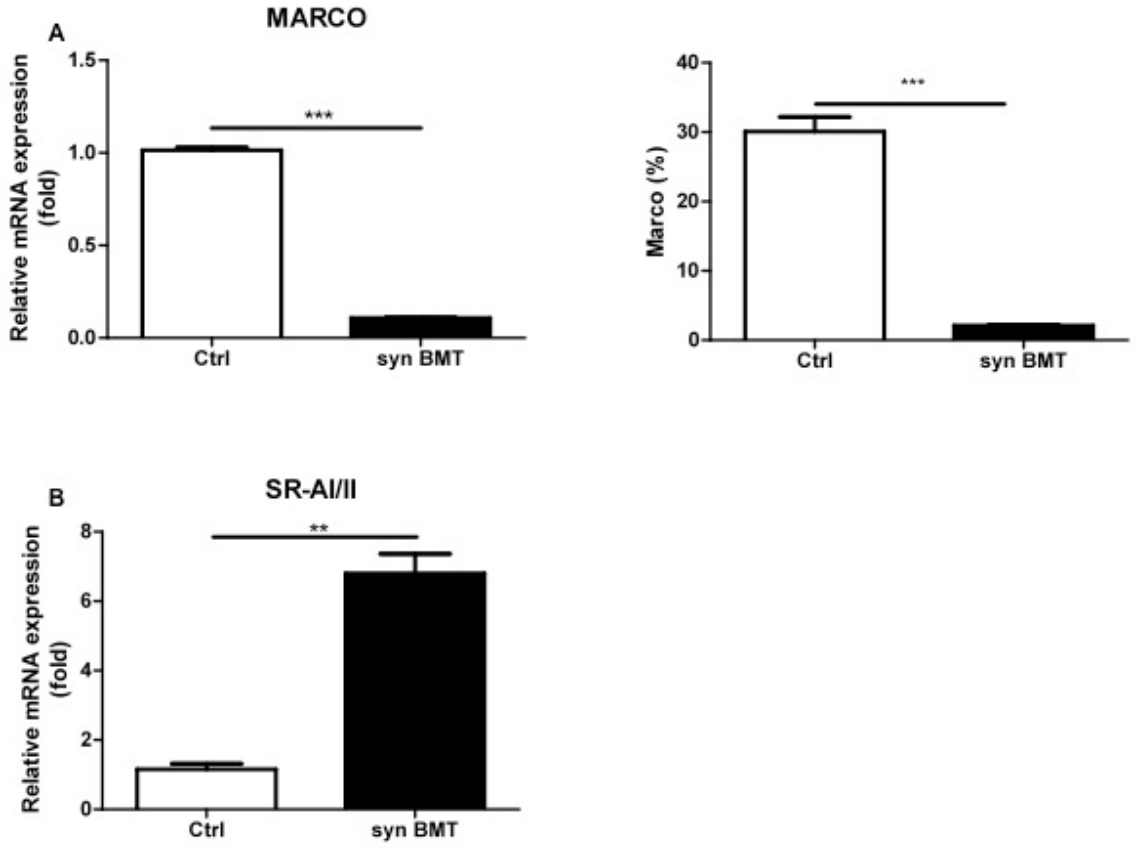

Figure 3.3. MARCO is decreased on syn BMT AMs while SR-AI/II is increased. AMs were harvested from untransplanted control B6 and syn BMT mice and MARCO (A, left) mRNA was detected by real-time RT-PCR. Expression of one control AM sample was set to n=1 for

comparisons, n=3. (A, right) MARCO protein levels were detected by flow cytometry, n=3. (B) SR-AI/II mRNA expression was measured by real-time RT-PCR. Analysis was performed as described with MARCO above, n=3. $**p<.01$, $***p<.001$.

MARCO is negatively regulated by PGE_2 and is important for P. aeruginosa phagocytosis

To further understand how an altered SR profile on AMs post-BMT contributes to AM function, control AMs were treated with class A SR inhibitors and AM phagocytic capacity against non-opsonized *P. aeruginosa* was determined. Interestingly, soluble MARCO (sMARCO) inhibited internalization of *P. aeruginosa* significantly compared to no treatment (no txt) and treatment with control compounds poly C and chondroitin sulfate ($ChSO_4$) (Figure 3.4A). Furthermore, the decrease in AM phagocytosis induced by sMARCO was comparable to the inhibition noted with the pan class A SR inhibitors [fucoidan and polyinosinic acid (poly I)], suggesting that MARCO may be the primary SR for *P. aeruginosa* phagocytosis by AMs. Cytochalasin D (cyto D) is an inhibitor of actin polymerization and was included as a negative control for phagocytosis. Although scavenger receptor with C-type lectin (SRCL) mRNA expression decreased in syn BMT AMs (data not shown), blocking SRCL did not affect uptake of non-opsonized *P. aeruginosa* in control AMs (Figure 3.4A) suggesting that this receptor is not used for *P. aeruginosa* uptake. Soluble mannan did not inhibit *P. aeruginosa* engulfment either, suggesting the mannose receptor is not involved in recognition of *P. aeruginosa* (not shown). Finally, Figure 3.4B shows that control AMs treated with sMARCO exhibited a similar defect in engulfment of *P. aeruginosa* as syn BMT AMs when compared to untreated control AMs. This highlights MARCO as the dominant *P. aeruginosa*-recognizing SR in AMs.

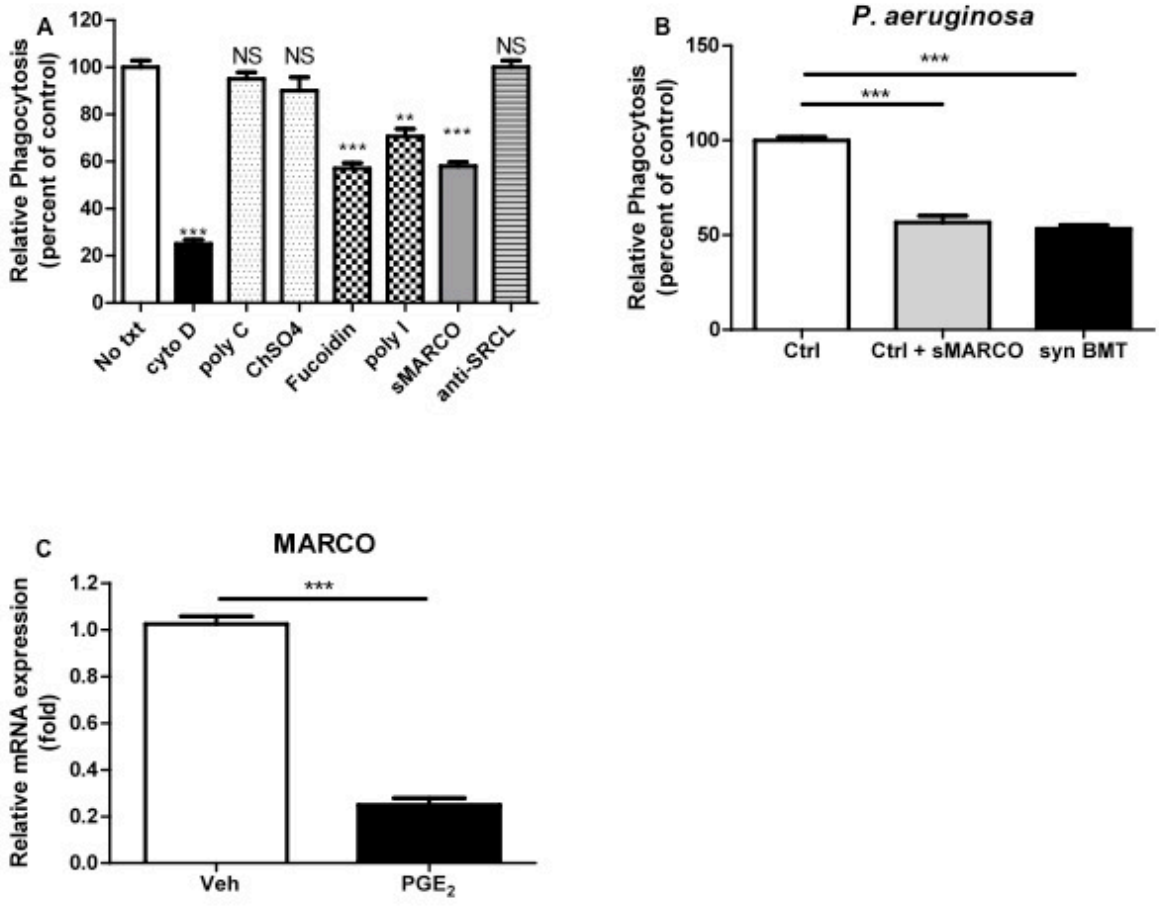

Figure 3.4. MARCO is necessary for *P. aeruginosa* phagocytosis and expression is regulated by PGE_2 post-BMT. (A) AMs were harvested and pretreated with pan class A SR inhibitors (fucoidin, poly I and DxSO4) or the control compounds poly C and ChSO4, as well as with anti-SRCL or soluble MARCO and phagocytosis of *P. aeruginosa* was measured. Cytochalasin D (cyto D) inhibits phagocytosis by limiting actin polymerization. Values are presented relative to the no treatment (no txt) control set to 100% for one sample; n=3 per group. (B) Phagocytic ability of control, soluble MARCO-treated control, or syn BMT AMs was measured. Results are presented relative to the average of untransplanted control values set to 100%, n=3. (C) AMs from control mice were treated for 24 h with 1μM PGE_2 or control DMSO (veh). MARCO mRNA levels were determined by real-time RT-PCR. Expression of one control AM sample was set to n=1 for comparisons; n=3. *p<0.05, ***p<0.001.

Our laboratory has previously shown that the defect in AMs post-BMT is, in part, mediated by upregulated COX-2 expression and overproduction of PGE_2. Treatment with

indomethacin, a COX inhibitor, rescues host defense in BMT mice *in vivo* and restores the ability of BMT AMs to phagocytose *P. aeruginosa* (58). However, whether decreased MARCO post-BMT is driven by PGE_2 remains unknown. Therefore, untransplanted AMs were treated with 1 μM PGE_2 or vehicle DMSO *in vitro.* Following 24 h, RNA was harvested and real-time RT-PCR analysis of MARCO mRNA expression indicated that decreased MARCO expression is PGE_2-driven (Figure 3.4C).

SR-AI/II mediates phagocytosis of S. aureus, but not P. aeruginosa by AMs

Syn BMT AMs exhibited increased *S. aureus* phagocytosis compared to control AMs (Figure 3.2A) and SR-AI/II mRNA expression was increased post-BMT (Figure 3.3B). To determine whether the observed AM phagocytosis of *S. aureus* may be due to increased SR-AI/II on macrophages following transplant, chinese hamster ovary (CHO) cells (which do not express class A SRs) were transfected with SR-AI. Transfected and untransfected CHO cells were measured for internalization of *S. aureus*. SR-AI-transfected CHO cells were more efficient at phagocytizing *S. aureus* than untransfected CHO cells (Figure 3.5A left), while *P. aeruginosa* internalization was unaffected by SR-AI transfection in CHO cells (Figure 3.5A right). These data verify that SR-AI is utilized for the recognition of *S. aureus* and explain why SR-A elevations do not influence *P. aeruginosa* uptake. To confirm this result post-BMT, AMs harvested from BMT mice that received HSCs from SR-AI/II-/- mice (SR-AI/II-/- BMT) were unable to phagocytize *S. aureus* compared to WT BMT AMs (Figure 3.5B).

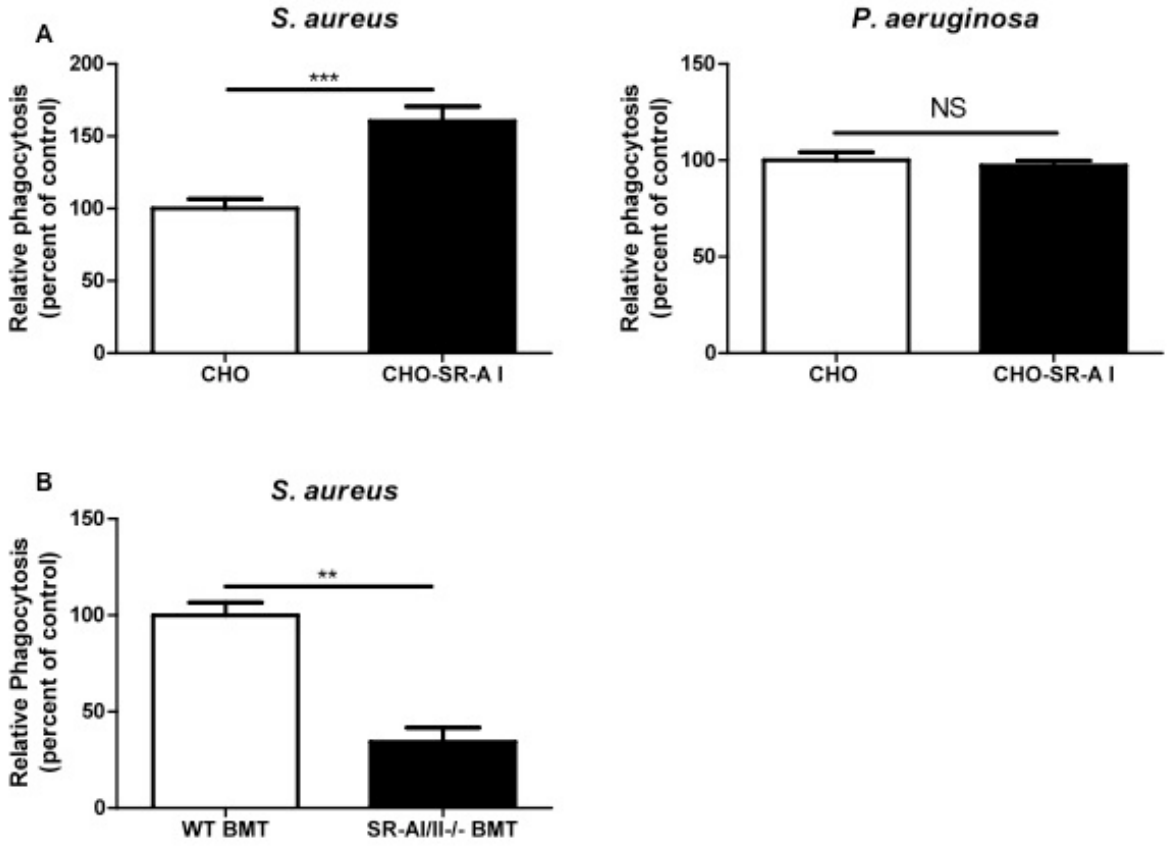

Figure 3.5. SRAI/II is important for AM phagocytosis of *S. aureus*. (A) Phagocytosis of FITC-*S. aureus* (left) or FITC-*P. aeruginosa* (right) by control CHO or SR-A-I-transfected CHO cells was measured. Results are presented relative to the average of control CHO values set to 100%; n=12/group; (B) Phagocytosis of FITC-*S. aureus* by WT BMT (n=4) or SR-A-/- BMT (n=3) AMs was measured. Data are normalized to LDH levels per well. Results are presented relative to the average of WT BMT values set to 100%; (C) SR-AI/II expression measured in AMs ($2x10^5$) treated with 10 μM of PGE_2 or DMSO (Veh) for 24 h; n=3, **p<.01, ***p<.001.

SR-AI/II negatively regulates MARCO expression

The fact that the phagocytic ability of AMs from SR-AI/II-/- BMT mice was not completely abolished (Figure 3.5B) suggested that there may be some compensatory upregulation of MARCO expression in these mice which allowed phagocytosis of *S. aureus*. MARCO has previously been shown to mediate *S. aureus* uptake (180, 181). To determine if this were true, we compared the percent of MARCO-expressing AMs in untransplanted WT and

SR-AI/II-/- mice (Figure 3.6A). Interestingly, loss of SR-AI/II resulted in compensatory upregulation of MARCO, suggesting a possible negative role of SR-AI/II on MARCO expression. To study the effect of this *in vivo*, we created all combinations of chimeric BMT mice. In Figure 3.6B, untransplanted control C57BL/6 mice or SR-AI/II-/- mice were compared to BMT mice created with the following donor→host combinations: WT→WT, SR-AI/II-/- →SR-AI/II-/- or SR-AI/II-/-→WT. When looking at *in vivo* host defense against *S. aureus*, non-transplanted SR-AI/II-/- mice had similar lung CFU as control C57BL/6 mice, presumably reflecting adequate MARCO expression in each strain at baseline. All BMT combinations were more susceptible than untransplanted mice, but there was no difference between the BMT groups. This suggests that uptake of *S. aureus* is mediated by either SR-AI/II or MARCO post-BMT as long as either receptor is available. In contrast, when looking at *in vivo* responses to P. *aeruginosa*, a bacterium that can be recognized by MARCO but not SR-A, the SR-AI/II-/-→WT mice (which likely have a compensatory MARCO upregulation) showed decreased lung and blood CFU burden when compared to the WT→WT BMT mice. There was no difference between WT→WT and WT→SR-AI/II-/- BMT mice.

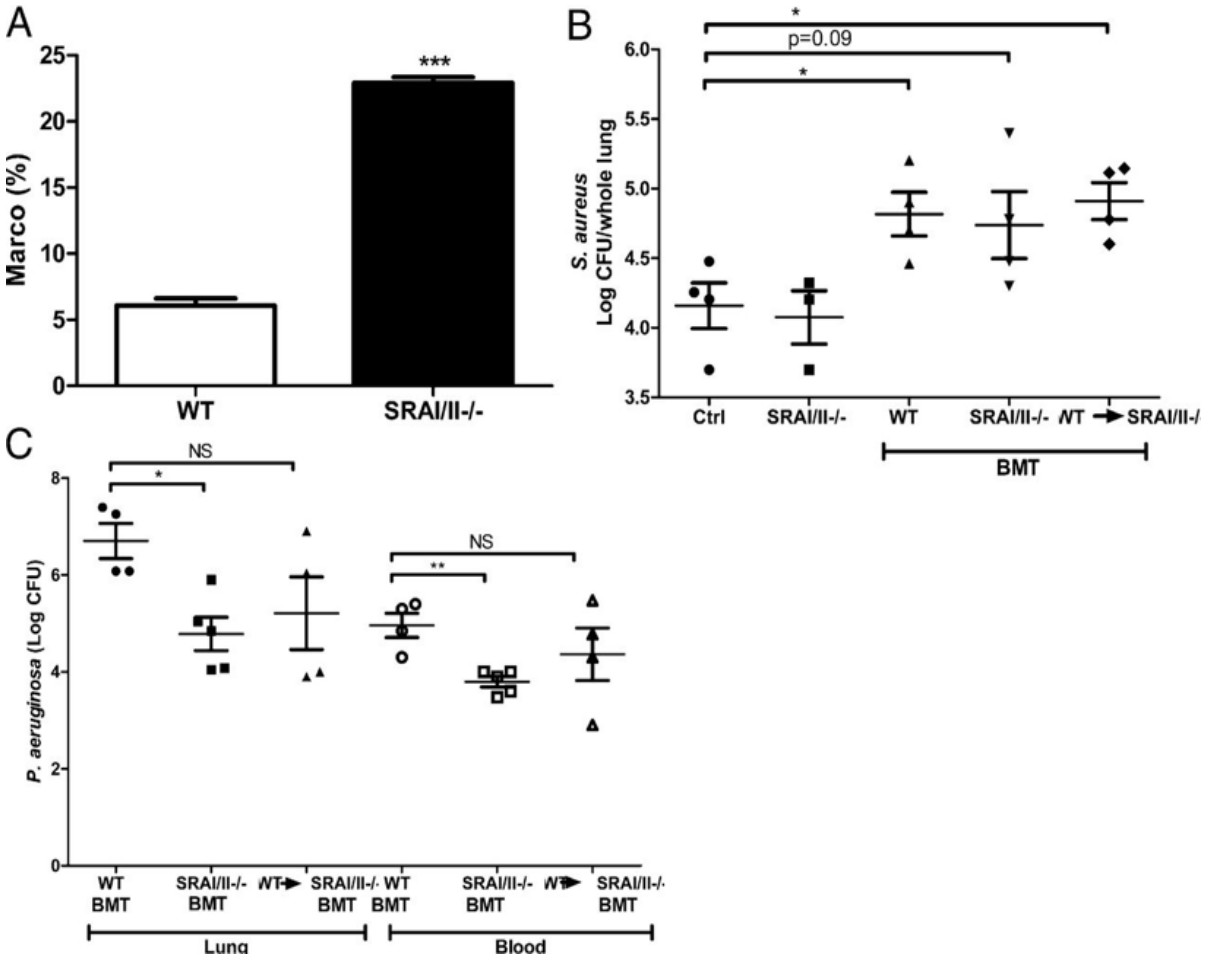

Figure 3.6. SR-AI/II negatively regulates MARCO expression. (**A**) MARCO protein expression was determined on AMs from untransplanted SR-AI/II$^{-/-}$ mice by flow cytometry (***n*** = 3 individual mice). Untransplanted B6 (Ctrl), untransplanted SR-AI/II$^{-/-}$, or chimeric BMT mice were challenged *in vivo* with *S. aureus* (n = 3–4) (**B**) or *P. aeruginosa* (n = 4–5) (**C**) via i.t. injection 24 h prior to lung and blood harvest for CFU analysis. *p < 0.05, **p < 0.01, ***p < 0.001.

SR-AI/II expression is regulated by PGE_2 and miR-155 post-BMT

Figure 3.5 suggests SR-A plays a prominent role in the recognition and phagocytosis of *S. aureus*. However, why SR-AI/II is upregulated post-BMT is unclear. To determine whether PGE_2 signaling could upregulate SR-AI/II, control AMs were treated with PGE_2 *in vitro* and SR-AI/II mRNA expression was analyzed by real-time RT-PCR. Figure 3.7A demonstrates that PGE_2 signaling increases SR-AI/II mRNA expression. Elevations in SR-AI/II mRNA in AMs

post-BMT (Figure 3.3B)(which have a high basal PGE_2 production (58)) and in exogenous PGE_2-treated macrophages (Figure 3.7A), suggest that changes in the transcriptional machinery or actions of key microRNAs indeed play a role in PGE_2-enhanced SR-AI/II expression. To our knowledge, this receptor does not have a cyclic AMP responsive binding element (CREB) (the main transcription factor induced by cAMP) secondary to PGE_2 signaling within the promoter region. Rather, SR-A type I contains a predicted target sequence in its 3' UTR for miR-155 (TargetScan). Thus, we hypothesized that miRNAs are involved in SR regulation post-BMT. Since SR-AI/II mRNA expression increased post-BMT and PGE_2 treatment, the data suggested the loss of a regulatory miRNA.

Analysis of miRNA expression profiles with a focused miRNA array revealed miR-155 as the only miRNA significantly downregulated (Table 3.1). To confirm that miR-155 was in fact decreased after BMT, miR-155 expression was determined by real-time RT-PCR using miR-155-specific primers. Our data show that there was a 6-fold decrease in miR-155 expression in syn BMT (Figure 3.7B) correlating well with the PCR array data. To determine whether the decrease in miR-155 expression was related to overproduction of PGE_2 in the lung after BMT, control AMs were harvested and treated with 10 μM PGE_2 for 24 h. MiR-155 mRNA expression decreased upon treatment with PGE_2 compared to AMs treated with DMSO (vehicle; Veh) control (Figure 3.7C). These data suggest that PGE_2 can negatively regulate miR-155 and it is likely that overexpression of PGE_2 in the lung following BMT is acting to inhibit miR-155. However, studies blocking PGE_2 (EP2 antagonist) in BMT AMs and then measuring miR-155 would be needed to confirm that PGE_2 signaling is promoting downregulation of this miRNA.

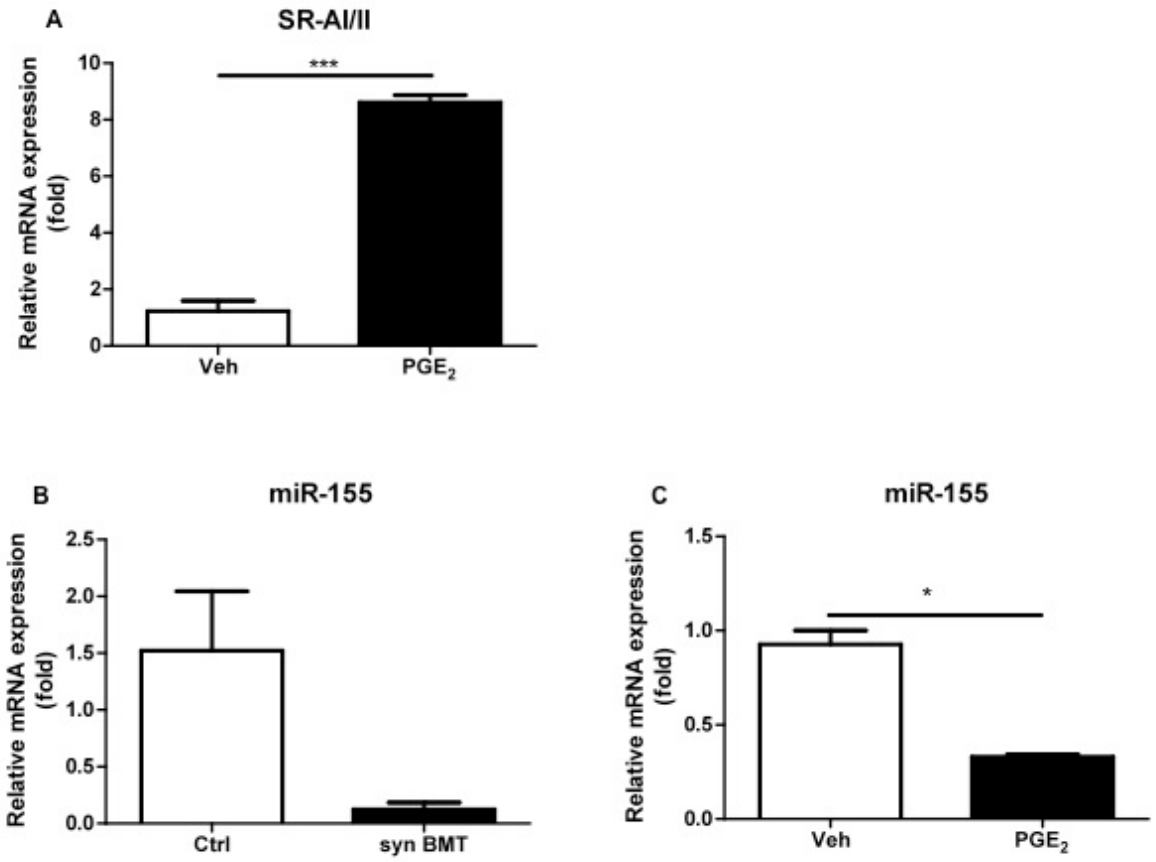

Figure 3.7. miR-155 mRNA expression is decreased in syn BMT AMs. (A) SR-AI/II expression was measured in AMs treated with10 μM PGE_2 or DMSO (Veh) for 24 h (n=3). Expression of one control AM samples was set to n=1 for comparisons. (B) miR-155 expression was measured by real-time RT-PCR in syn BMT AMs (n=2 samples pooled from multiple mice). Expression was normalized to snoRNA142 and one control AM sample was set to n=1 for comparison. (C) Primary AMs were treated with 10 μM PGE_2 or control DMSO (Veh) for 24 h prior to RNA isolation and cDNA conversion. miR-155 expression was compared to snoR142 (n=2 samples pooled from multiple mice); *p<0.05, ***p<0.001.

MiR-155 regulates SR-AI/II expression and S. aureus phagocytosis

Because miR-155 is decreased post-BMT, regulation of SR-AI/II by miR-155 was further explored. An antagomir of miR-155 (anti-miR-155) was transfected into control AMs to inhibit miR-155 endogenous expression. SR-AI/II mRNA expression was increased upon transfection of

anti-miR-155 compared to cells transfected with a control antisense oligomer (anti-miR) (Figure 3.8A). As SR-AI/II was shown in Figure 3.5 to be important for *S. aureus* internalization, we were interested in understanding whether a decrease in miR-155 would have a functional effect on *S. aureus* internalization. AMs transfected with anti-miR-155 at increasing concentrations were measured for their ability to phagocytize *S. aureus*. Figure 3.8B shows *S. aureus* internalization increased dose-dependently with anti-miR-155.

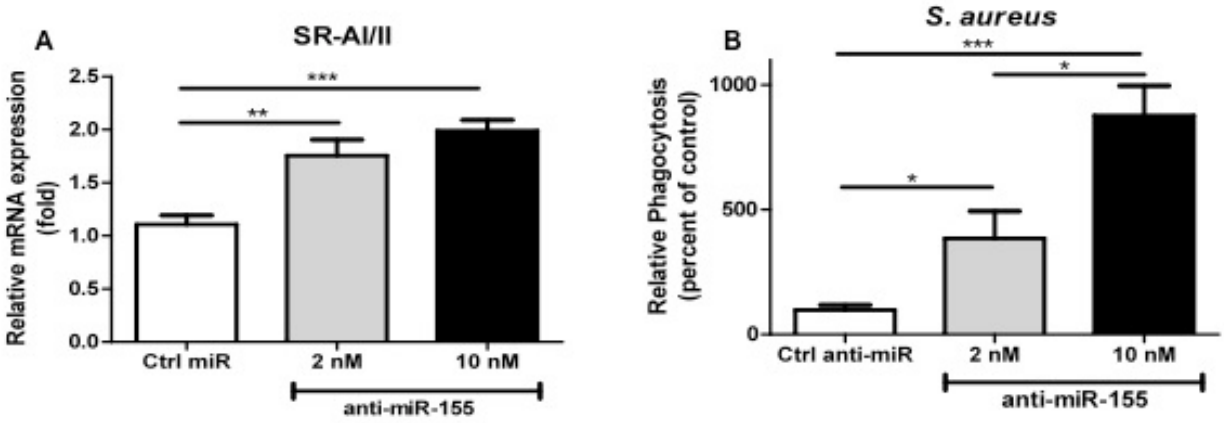

Figure 3.8. Anti-miR-155-transfected AMs exhibit increased expression of SR-AI/II and increased phagocytosis of FITC-SA . (A) Primary AMs were transfected with either control anti-miR (10 nM) or anti-miR-155 (2 or 10 nM) for 24 h prior to harvesting RNA. SR-AI/II mRNA expression was determined by real-time RT-PCR and the expression of one control AM sample was set to n=1 for comparison; n=3-4 from 2 combined experiments. (B) Primary AMs were transfected with either control anti-miR (10 nM) or anti-miR-155 (2 or 10 nM) for 48 h prior to the two h incubation with FITC-SA for phagocytosis measurement. Values are normalized to LDH levels per well. Results are presented relative to the average of control values set to 100%; n=3-4; *p<0.05, **p<0.01.

Syn BMT AMs are compromised in intracellular killing of P. aeruginosa and S. aureus

Bacterial clearance by AMs relies not only on phagocytosis of the pathogen, but also on intracellular killing of the organism. While a defect in phagocytosis, as shown in Figure 3.2A, would increase susceptibility to *P. aeruginosa,* AMs collected from syn BMTs are also unable to

kill *P. aeruginosa* (Figure 3.9A) as we have previously published (58). Because the BMT mice are more susceptible to *S. aureus in vivo* (Figure 3.1B), yet display enhanced phagocytosis of this Gram-positive bacteria (Figure 3.2B), we examined the ability of BMT AMs to kill ingested *S. aureus* and found that killing of this pathogen was impaired in syn BMT AMs when compared to control AMs (Figure 3.9B). Thus, killing defects likely account for the *in vivo* susceptibility to infection despite enhanced phagocytosis. Taken together, it is interesting to speculate that because both phagocytosis and killing are defective for *P. aeruginosa*, this could explain the approximately 4-log increase in bacterial susceptibility to this pathogen in BMT mice *in vivo*, whereas the improved phagocytosis of *S. aureus* moderates the killing defect to account for an approximate 1 log difference in susceptibility to this pathogen in BMT mice *in vivo*.

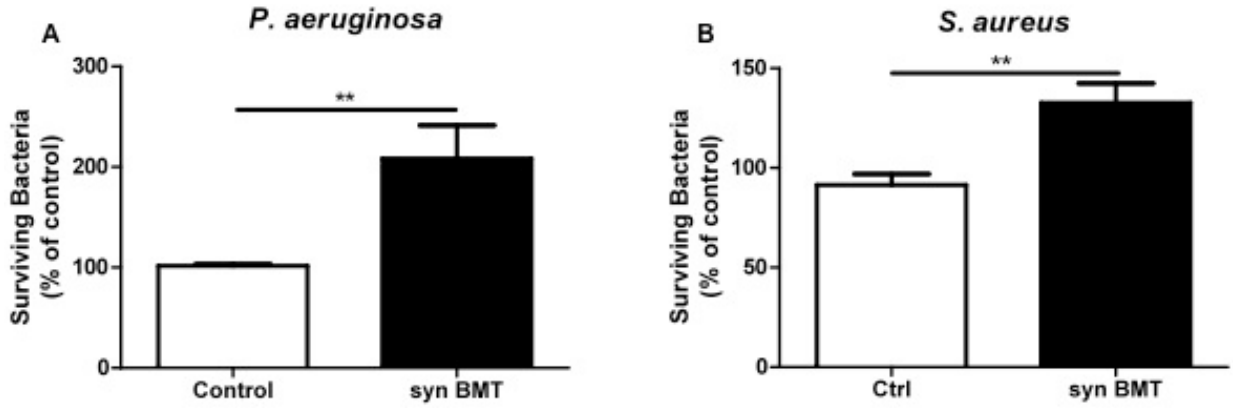

Figure 3.9. Syn BMT mice have defective killing of *P. aeruginosa* and *S. aureus.* Primary AMs from untransplanted control B6 and syn BMT mice were assessed for killing of serum-opsonized (A) *P. aeruginosa* (n=8-10) and (B) *S. aureus* (n=4-6). ***$p<0.001$.

3.3 Discussion

Syn BMT mice show increased *in vivo* susceptibility to both *S. aureus* and *P. aeruginosa*, with the latter observations confirming previously published data (55, 58). While this impaired host response can be explained by an inability of BMT AMs to engulf *P. aeruginosa*, the same is not true with *S. aureus*. In fact, BMT AMs exhibit enhanced phagocytosis of *S. aureus*. This difference suggested that receptors mediating phagocytosis may have been altered following transplantation. Although MARCO and SR-AI/II have both been reported to have the ability of recognizing Gram-positive and –negative bacteria (105, 179, 180, 182) and SR-AI/II has previously been linked to recognition of *S. aureus* (179), it was unclear which receptor was important for *P. aeruginosa* in control cells, and nothing was known about the regulation of these SRs post-BMT. Here, we show that MARCO is significantly downregulated at both the mRNA and protein levels, while SR-AI/II mRNA expression is increased post-BMT. These data indicate that MARCO is important for *P. aeruginosa* internalization as elevated levels of SR-AI/II were unable to rescue phagocytosis of this Gram-negative pathogen in BMT AMs or CHO cells. This observation was further supported by the finding that control AMs pre-treated with soluble MARCO were unable to phagocytize *P. aeruginosa* as efficiently.

A surprising result was the enhanced phagocytosis of *S. aureus* given that BMT mice were more susceptible to infection *in vivo*. As SR-AI/II can regulate *S. aureus* phagocytosis (179), elevations in SR-AI/II may explain this result. Here we show that CHO cells expressing SR-AI only, were able to phagocytize *S. aureus* but not *P. aeruginosa*. Furthermore, recipient mice reconstituted with SR-AI/II-/- donor marrow showed a reduction in *S. aureus* phagocytosis when compared to mice transplanted with wild-type (C57BL/6) marrow. These data support the importance of SR-AI/II for *S. aureus* and MARCO for *P. aeruginosa* phagocytosis.

Interestingly, the fact that phagocytosis of *S. aureus* in the SR-AI/II-/- BMT mice was not completely abolished suggested there may be some compensatory upregulation of MARCO in these mice as MARCO has been shown to recognize *S. aureus* (180, 181). These data highlight an interesting inhibitory role of SR-AI/II on MARCO expression. SR-AI/II-/- untransplanted mice have a higher percentage of MARCO positive cells in the absence of infection. Therefore, it is also possible that the overexpression of SR-AI/II observed post-BMT may contribute to the decreased expression of MARCO in addition to the PGE_2 signaling. We believe the inhibitory effects of SR-AI/II on MARCO may not be immediate as transfection of the anti-miR-155 which upregulated SR-AI/II expression within 48 h did not yet alter MARCO expression, whereas PGE_2 stimulation reduced MARCO in 24 h. Ultimately, SR-AI/II-/- BMT mice may upregulate MARCO to improve their host defense against both pathogens, which is consistent with the observations in. The co-regulation of these receptors is complex, but on the whole our *in vivo* results are consistent with the conclusion that MARCO is uniquely responsible for *P. aeruginosa* uptake while *S. aureus* can be recognized by either MARCO or SRAI/II. Our findings of SR-AI/II inhibition of MARCO expression are also consistent with previously published data that suggest SR-AI/II may have an inhibitory role on inflammatory responses (183, 184).

MicroRNA analysis of BMT AMs indicated that altered miRNA expression may be responsible for the upregulation of SRAI/II post-BMT. Specifically, miR-155 expression was decreased 6-fold and SR-AI contains a putative target sequence for miR-155 in its 3'UTR. As miRNAs generally function to destabilize or inhibit protein translation of the targets they regulate, we hypothesized decreased expression of miR-155 would be responsible for the increase in SR-AI/II observed post-BMT. Anti-mir-155-transfected AMs showed a significant increase in SR-AI/II expression. To determine whether this correlated with the functional

phenotype observed after transplant, anti-mir-155-transfected AMs measured for phagocytosis of *S. aureus* exhibited a dose-dependent increase in *S. aureus* internalization. This provided evidence that after transplant it is likely that the elevation of SR-AI/II was regulated by decreased miR-155 expression. As PGE_2 could inhibit miR-155 and induce SR-AI/II, it is possible that the overproduction of PGE_2 post-BMT promotes overexpression of SR-AI/II by negatively regulating its inhibitor, miR-155. PGE_2 also decreases MARCO, but the mechanism does not appear to involve miR-155. This finding provides new insight into intracellular regulation of scavenger receptors by microRNA. A recent study suggested miR-155 as a novel therapeutic target for improving graft vs. host disease (185). This is likely to also cause an inhibition of SR-AI/II which may impair phagocytosis of some pathogens, and the long term effects of this treatment on MARCO expression would need to be evaluated.

COX-2 and PGE_2, are elevated following transplantation (58, 68) and inhibition of COX-2 by indomethacin treatment rescues host defense (58). PGE_2 production is not influenced by SR expression as PGE_2 levels were similarly elevated in all chimeric BMT mice in this study (not shown), however PGE_2 can dramatically alter SR profiles and also inhibit bacterial killing. Here, we show that *in vitro* treatment with PGE_2 decreases miR-155 and MARCO, while increasing SR-AI/II expression. Although increased SR-AI/II expression enhances *S. aureus* phagocytosis, syn BMT mice remain susceptible to bacteria *in vivo*.

Further investigation showed that effective killing of both *P. aeruginosa* and *S. aureus* was impaired in syn BMT mice compared to their untransplanted controls. Therefore, a better understanding of how killing is impaired remains an area of future investigation. We have previously shown that BMT AMs express an immature phenotype (low CD11a and CD11c, high CD11b) (55) and that the BAL fluid of BMT mice contains aberrant mediator expression

(elevated IL-6, GM-CSF, and PGE_2; decreased TNFα, IFN-γ, H_2O_2 and leukotrienes) (55, 58, 59, 88). Overall, BMT AMs appear impaired in their ability to become activated, which is likely important for intracellular bacterial killing. Phagocytosis and killing function is rescued upon treatment with indomethacin (58) indicating that the impairment is due to overexpression of the COX pathway. Microarray analysis also revealed increased expression of miR-27b (15-fold) and -29b (20-fold) (Table 3.1).

Table 3.1 SABiosciences miRNA microarray from syn BMT AMs.

miRNA	Fold Regulation	miRNA	Fold Regulation
let-7a	1.4679	**miR-295**	**2.0338**
let-7b	**4.8116**	**miR-297b-3p**	**3.2249**
let-7c	**4.1459**	miR-29a	1.0757
let-7d	1.4218	**miR-29b**	**23.5058**
let-7e	1.2061	miR-29c	1.3683
let-7f	1.9867	**miR-301a**	**2.4024**
let-7g	1.7013	**miR-301b**	**2.4154**
let-7i	1.8176	**miR-302b**	**2.4645**
miR-106a	**2.4503**	**miR-302d**	**2.6332**
miR-106b	1.5479	miR-30a	1.5919
miR-1192	**2.4645**	miR-30b	1.9533
miR-126-5p	**3.0021**	miR-30c	1.9452
miR-128	1.4976	miR-30d	1.8535
miR-130a	1.1271	miR-30e	1.196
miR-130b	1.4905	miR-322	-1.125
miR-140	1.913	miR-325	1.874
miR-144	-1.545	**miR-338-5p**	**2.9612**
mir-155	**-3.0057**	**miR-340-5p**	**2.9737**
miR-15a	-1.3451	**miR-369-3p**	**2.0637**
miR-15b	**2.1809**	**miR-374**	**3.4715**
miR-16	**2.1352**	**miR-384-5p**	**2.4645**
miR-17	1.8385	**miR-410**	**3.6968**
miR-181a	-1.0598	miR-429	-1.1171
miR-181b	1.7489	**miR-466b-3-3p**	**7.6226**
miR-181c	-1.1716	**miR-466d-3p**	**7.0595**
miR-181d	**2.5132**	**miR-466d-5p**	**2.4645**
miR-182	**3.6386**	**miR-466f-3p**	**2.7299**
mir-186	1.9054	miR-466k	1.7422
miR-195	1.8842	**miR-495**	**2.4645**
miR-19a	**3.072**	miR-497	1.876
miR-19b	**2.0852**	**miR-590-3p**	**2.4645**

miR-200c	-1.1613	**miR-669f**	**6.0483**
miR-20a	1.8742	miR-669h-3p	1.1649
miR-20b	**2.321**	miR-669k	1.2967
miR-221	**3.5526**	**miR-692**	**2.0717**
miR-222	**3.9053**	**miR-694**	**2.2755**
miR-23a	**2.0364**	miR-712	1.8727
miR-23b	**4.0324**	**miR-721**	**2.4645**
miR-26a	1.7291	miR-743a	1.2518
miR-26b	1.6209	miR-743b-3p	1.3453
miR-27a	1.4935	**miR-876-3p**	**2.4645**
miR-27b	**15.9657**	**miR-9**	**6.1375**
miR-291a-3p	-1.2185	miR-93	1.0083
miR-294	1.173	**miR-98**	**2.2096**

Blue indicates significant upregulation of miRNA expression. Red indicates significant downregulation miRNA expression.

NFkB suppresses miR-29b expression (158) and miR-27b was found to inhibit NF-kB translocation into the nucleus (186) supporting the idea that these miRNA function in an anti-inflammatory and perhaps functionally suppressive manner and that their expression pattern is influenced by BMT. Interestingly, miR-29b has been shown to directly regulate DNA methyltransferases and affect COX-2 expression in lung epithelial cells(187). Thus, upregulation of miR-29b may be promoting COX-2 overexpression in BMT AMs. Here we show that PGE_2 can decrease miR-155 and affect SR-AI/II expression. Furthermore, miR-155 has been shown to regulate TNFα by mRNA stabilization. TNFα is important for macrophage activation and immune function, and it is decreased post-BMT (59). Our results suggest that miR-155 loss post-BMT may also play a role in destabilizing TNFα. These differences related to dysregulated miRNA expression, may, in part, be responsible for the defective killing post-BMT.

Taken together, our results show that AMs undergo significant functional alterations in the setting of syn BMT. Our current studies highlight the fact that elevated PGE_2 leads to alterations in the scavenger receptor profile (decreases MARCO and increases SR-AI/II via downregulation of miR-155). These epigenetic changes differentially affect phagocytosis of *P.*

aeruginosa and *S. aureus*; however, bacterial killing of both pathogens is impaired post-BMT. It is important to note that these changes observed in AMs post-BMT are only seen in the setting of myeloablative conditioning. Tarling et al. suggested that AMs derive from a lung resident stem cell that is naturally radio-resistant (98). At lower doses of irradiation, AMs likely repopulate from these radio-resistant host stem cells and only upon myeloablative conditioning (8-13 Gy, or using high dose chemotherapy combinations) did AM reconstitution derive from the donor bone marrow resulting in the PGE_2-induced impaired function (54). Thus, interventions to limit PGE_2 production post-HSCT or reverse these epigenetic alterations may improve outcomes for patients developing bacterial infections. Furthermore, as PGE_2 is a product of COX-2, future studies will explore the etiology of COX-2 overexpression in BMT AMs.

Chapter 4:

Cyclooxygenase-2 expression is upregulated by DNA hypomethylation after hematopoietic stem cell transplantation

4.1 Background

HSCT is commonly used to treat malignant and nonmalignant hematologic disorders (12, 25). Traditionally, a conditioning regimen is implemented prior to intravenous infusion of HSC which may consist of chemotherapy with or without TBI (25). 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 (12, 25, 188). Although HSCT has proven to be an effective therapeutic option for malignancy, it is also associated with significant morbidity and mortality (12, 25, 188-190). 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 (25, 28, 53, 176, 188, 191)

The lung is a common target organ post-transplant where pulmonary complications account for significant mortality and morbidity in HSCT recipients (25, 28, 53, 176, 188, 191). 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) (23). Despite full reconstitution or engraftment of donor-derived leukocytes, patients exhibit sustained and enhanced susceptibility to infections post-transplant (23, 28, 53, 176, 191). AMs are the resident macrophages in the lung and, together with recruited PMNs, play an important role in

regulating an immune response in the lung (58, 96, 97, 168). 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 (34). 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 syn 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 GvHD or immunosuppressive drugs. We have shown that even after full immune reconstitution following syn BMT, donor-derived AMs from BMT mice are defective in phagocytosis and killing compared to mice that did not undergo BMT (54, 58). We discovered this defect is related to decreased cysLTs and TNFα production, and increased PGE_2 production (24, 58, 68).

Eicosanoids are lipid mediators derived from arachidonic acid and cells of the myeloid lineage are major producers of both cysLTs and PGE_2 (24, 192). Synthesis of prostaglandins is mediated by the 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 (192). PGE_2 production post-BMT is attributed to the increased activity of COX-2 and PGE_2 negatively regulates the innate immune response (58, 76). 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 (54, 58, 96, 97). 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* (24); thus, we have focused our current studies on regulation of COX-2 expression in AMs.

PGE_2 binds to seven transmembrane spanning 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 (76, 193). We have shown that binding of the EP2 receptor by PGE_2 results in increased IRAK-M expression in AMs resulting in the inhibition of non-opsonized phagocytosis (96). PGE_2 also induces elevation of the enzyme PTEN which results in the inhibition of serum-opsonized phagocytosis by AMs (97). Thus PGE_2 is able to act through distinct signaling pathways to compromise host innate immune defense.

Because COX-2 expression is upregulated in the AMs of mice post-BMT and its expression remains elevated in culture (54, 58, 59, 96, 97), we hypothesized that epigenetic mechanisms may be responsible for the upregulation of COX-2. To test this, we sought to determine the DNA 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'-UTR and exon 1 of AMs from mice post-BMT. Using *in vitro* assays, we determined that COX-2 mRNA expression is regulated by DNA 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. 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 (88). Thus, our data indicate that epigenetic regulation of the COX-2 gene 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-β.

4.2 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 (83). 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 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 (54, 58). 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 mRNA levels in control (untransplanted) and BMT mice. AMs were collected by BAL and COX-2 mRNA expression levels were measured by real-time RT-PCR. These results demonstrate that COX-2 mRNA is elevated in BMT AMs when compared to control AMs (Figure 4.1).

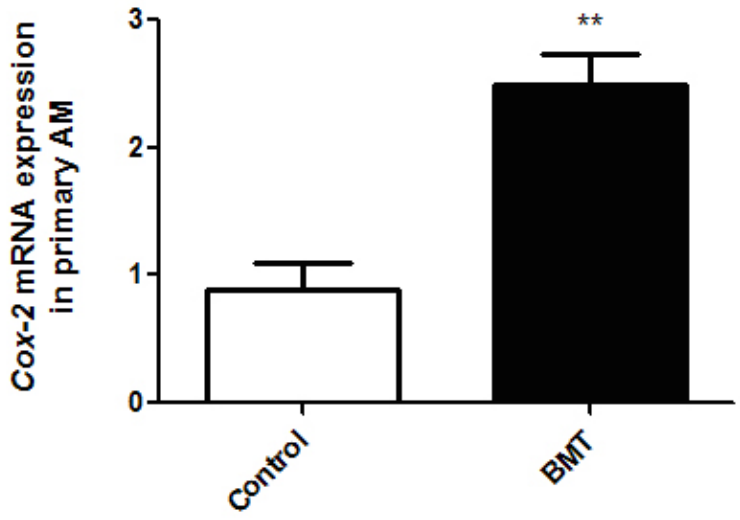

Figure 4.1. COX-2 mRNA is increased in BMT mice. AMs were harvested from control and BMT mice at 5 weeks post-BMT. RNA was isolated and COX-2 mRNA was measured by real-

time RT-PCR. Samples were normalized to β-actin. (n=4, $**p<0.01$); Representative of two experiments.

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 4.1, we collected genomic DNA from the AMs of control and BMT mice and performed bisulfite sequencing of the COX-2 promoter. BMT AMs exhibited significant hypomethylation compared to control AMs at CpG loci within the 5'-UTR and exon 1 (Figure 4.2). CpG sites found between -449 and -100 of the promoter region located upstream of the transcription start site did not show any differences in DNA methylation between the control and BMT AMs (data not shown).

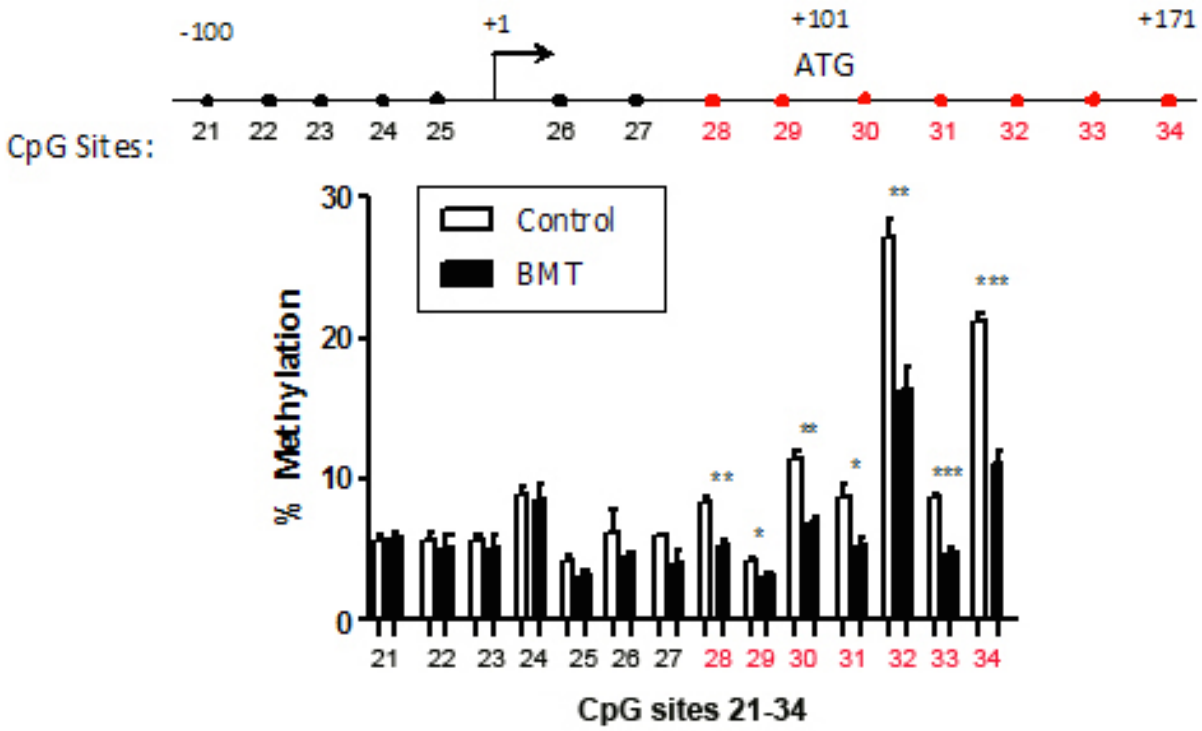

Figure 4.2. COX-2 in BMT AMs is hypomethylated around the first exon start site. At week 5 post-BMT, AMs from control and BMT mice were harvested, DNA was isolated and bisulfite converted. Cytosine to thymine conversion was detected by pyrosequencing to determine DNA

methylation patterns. Control: n=4, BMT: n=3; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Representative of 2 experiments showing similar patterns at the same sites.

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

A chemical analog for cytosine, 5-aza-2'-deoxycytidine inhibits DNA methylation by blocking DNA methyltransferase activity (194). The DNA 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 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 mRNA expression from MHS cells increased dose-dependently in the presence of the methyltransferase inhibitor (Figure 4.3A).

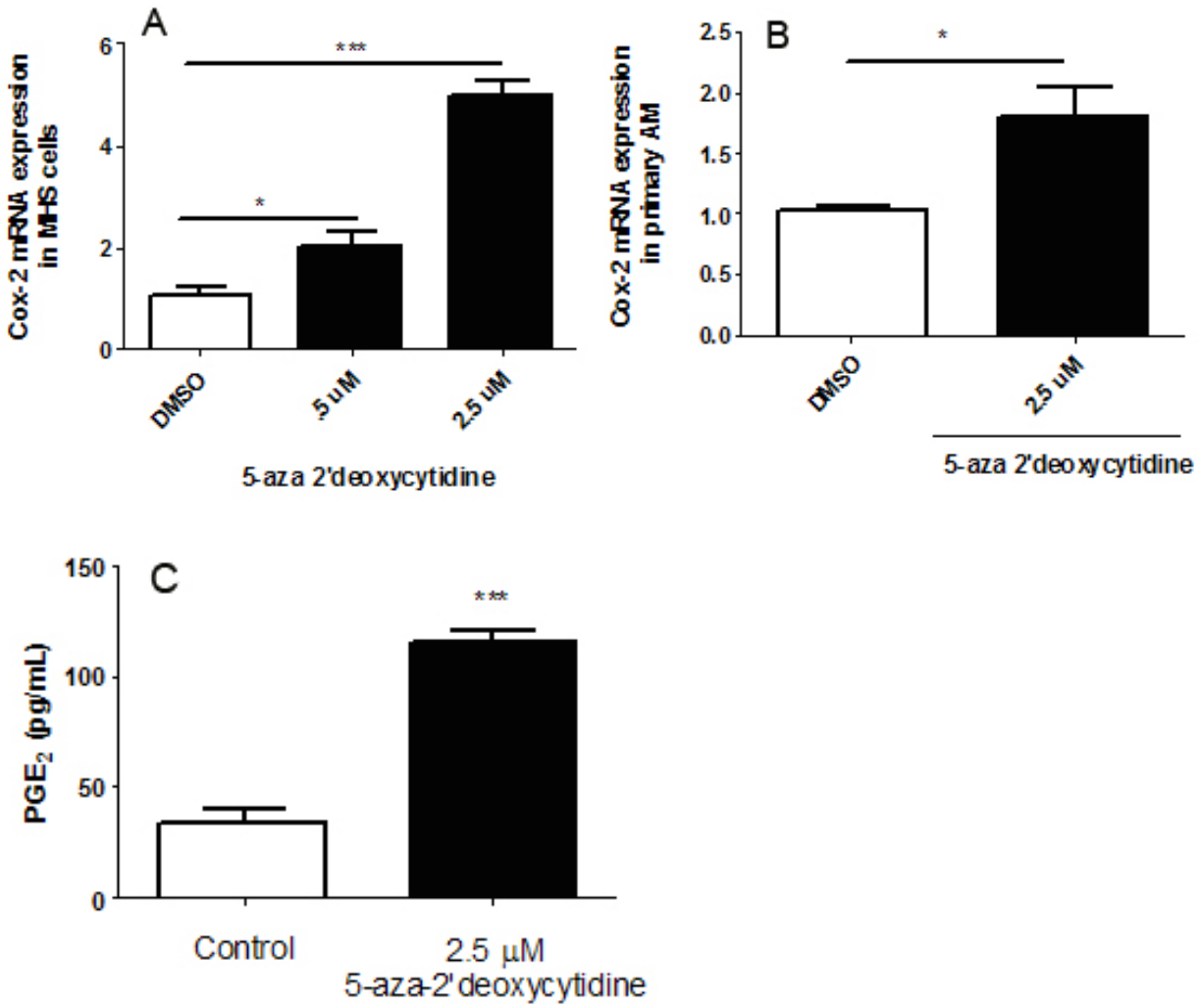

Figure 4.3. 5-aza-2'deoxycytidine increases COX-2 mRNA in MHS cells and primary AMs and PGE_2 in MHS cells. (A) $5x10^5$ MHS cells were cultured with 0.5 or 2.5 µM 5-aza 2' deoxycytidine, or vehicle (DMSO) for 72 h. RNA was isolated and COX-2 mRNA levels were analyzed by real-time RT-PCR. Transcripts 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 2 days and further treated with 2.5µM 5-aza-2'deoxycytidine in the presence of GM-CSF for an additional 72 h. RNA was isolated and COX-2 mRNA levels calculated by RT-PCR. (C) MHS cells (5×10^5) were grown in10% complete media in the presence or absence of 2.5 µM 5-aza-2'deoxycytidine for 48 h. Media was aspirated and fresh serum free media with or without 5-aza-2'deoxycytidine was added for 24 h. Supernatants were collected and PGE_2 ELISA was performed. (n=3); *p<0.05, **p<0.01, ***p<0.001. All data are representative of at least 2 experiments.

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 2 days. AMs were then treated with 5-aza-2'deoxycytidine for 72 additional h in the presence of GM-CSF to maintain proliferation (195) and COX-2 mRNA levels were determined by real-time RT-PCR (Figure 4.3B). Expression patterns of COX-2 transcripts from primary AMs shown in Figure 4.3B correlate well with the results from DNA demethylation studies in MHS cells. Furthermore, treatment of MHS cells with 2.5 μM 5-aza-2'deoxycytidine resulted in increased PGE_2 levels (Figure 4.3C). Taken together, these data indicate that the regulation of COX-2 is heavily 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 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 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 molecules LPS or TGF-β1 for 48 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 COX-2 promoter activity (Figure 4.4). 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.

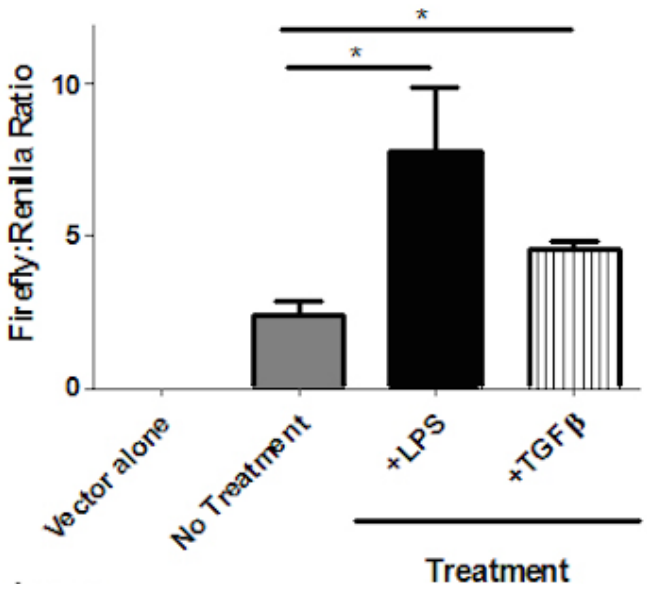

Figure 4.4. Transfected COX-2 promoter-driven luciferase is induced by LPS and TGF-β compared to untreated MHS cells. MHS cells (6.2×10^4) were cultured overnight in the presence or absence of either LPS (10 μg/mL), or porcine TGFβ (1 ng/mL). MHS cells were transfected the next day with either luciferase 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); *$p<0.05$

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

Both long and short COX-2 promoter constructs were cloned into pGL3-basic firefly luciferase expression plasmids. The shorter COX-2 promoter contains only those CpG sites analyzed in primary AMs (beginning at -449 to +70) by bisulfite conversion and pyrosequencing whereas the long COX-2 construct contains additional upstream CpG sites outside the range of our current bisulfite analysis. To determine whether DNA methylation of the COX-2 promoter-driven expression vectors decreases COX-2 expression, the long and short COX-2 promoter expression plasmids were methylated *in vitro* with a CpG methyltransferase, *M.SssI*. 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 4.5A, when unmethylated, the short COX-2 promoter was able to stimulate firefly luciferase at levels similar to the long construct. This suggests that the short 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 DNA methylation of the short promoter region is sufficient to impair transcriptional activity and indicate that the relevant CpG sites for analysis lie within the short promoter region which was previously analyzed in Figure 4.2.

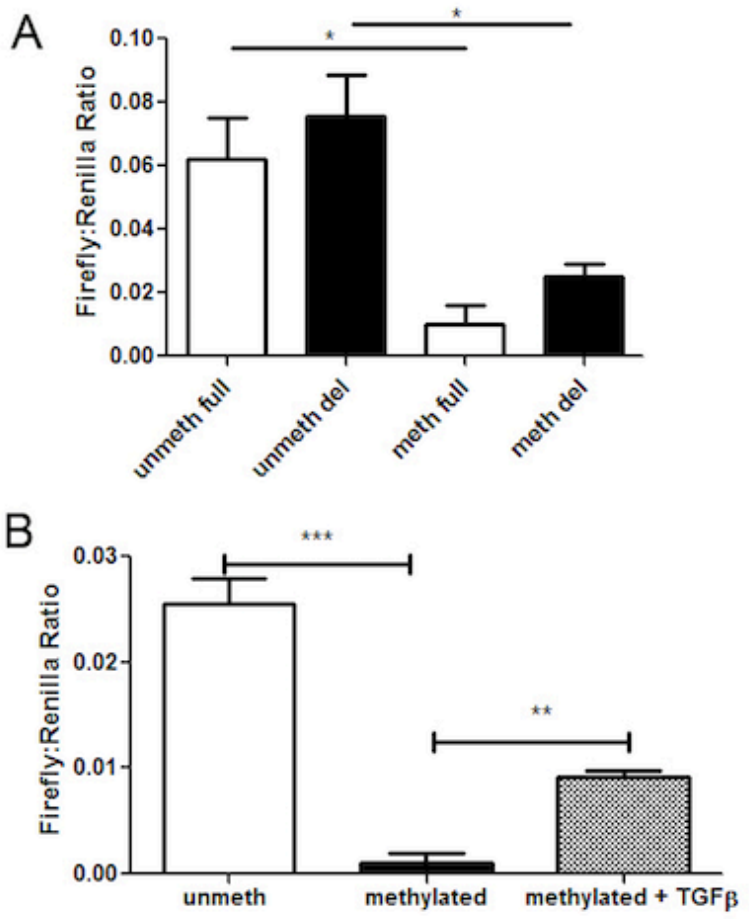

Figure 4.5. COX-2-driven luciferase expression is decreased following treatment with methyltransferase, but expression is regulated by TGF-β1. (A) Transfections of MHS cells (6.2×10^4) were performed with a 50:1 ratio of either methylated or unmethylated, full (1179 bp) or partially deleted (702bp) COX-2 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 umethylated COX-2 reporter plasmids (702 bp) and pRL-SV40 and assayed as above (n=3, $**p<0.001$, $***p<0.001$).

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 (88). To determine whether TGF-β1 could stimulate the transcription of COX-2 from a methylated promoter, we transfected MHS cells as above with the short 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 (Figure 4.5B). 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 DNA 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 (Figure 4.6), suggesting that this effect may be specific to TGF-β1 signaling.

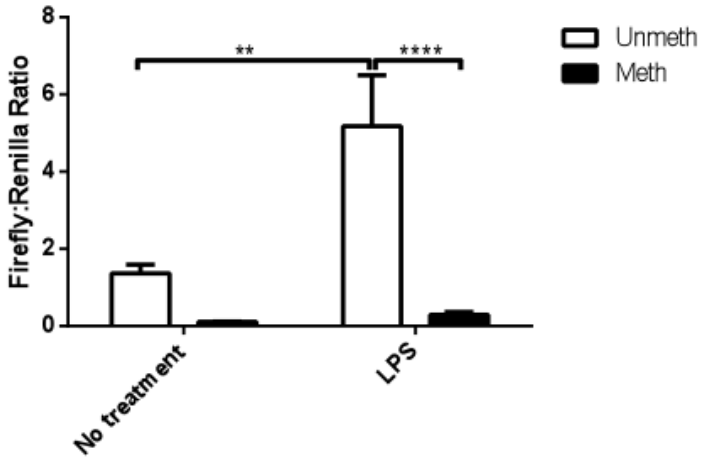

Figure 4.6 LPS does not induce COX-2-mediated luciferase from methylated COX-2 plasmid. MHS cells were transfected with an umethylated or methylated COX-2 firefly luciferase plasmid and a renilla luciferase plasmid (control transfection) in a 50:1 ratio and treated with 10 μg/mL LPS for 48h (n=2 combined experiments); ***p*<0.01, *****p*<0.0001.

Improved phagocytosis and partial restoration of COX-2 DNA methylation 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 (196). 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 (165). 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 4.7).

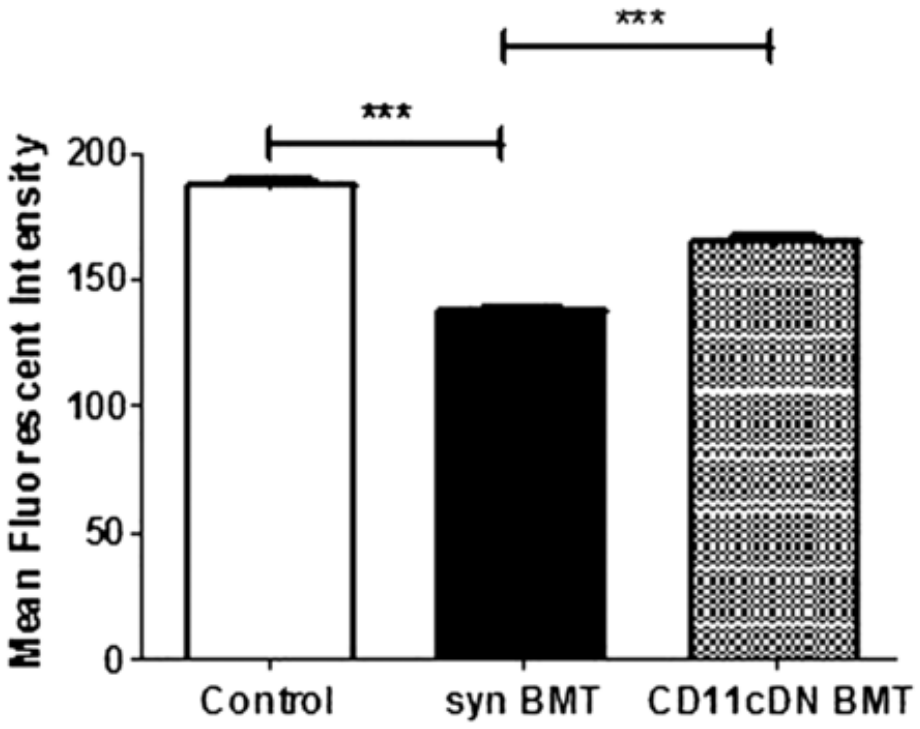

Figure 4.7. Improved AM phagocytosis with CD11c^{dnR} bone marrow chimeras. AMs were harvested by BAL 5 weeks post-transplant from BMT mice reconstituted with either WT or CD11c^{dnR} donor marrow. Phagocytosis of heat-killed *P. aeruginosa* was then assessed and compared to AMs from control mice (n=5; ***p<0.001).

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 DNA 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 4.8).

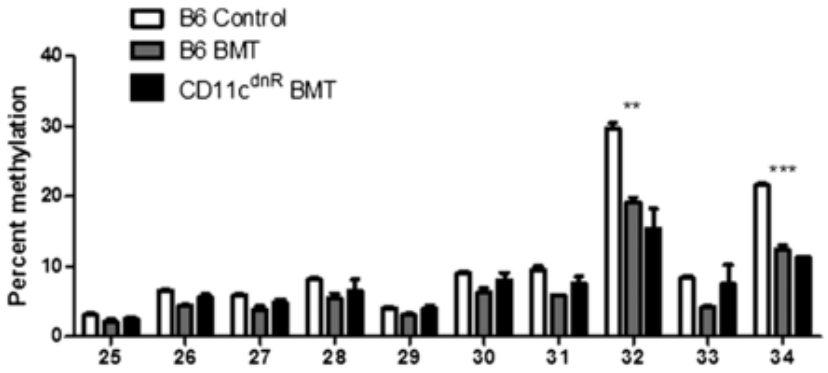

Figure 4.8. CD11c^{dnR} BMT COX-2 promoter methylation is partially rescued. After DNA isolation from primary AMs from control, WT BMT, and CD11c^{dnR} BMT mice, DNA was subjected to bisulfite conversion and pyrosequencing for DNA methylation analysis. $n = 5$. WT BMT patterns were hypermethylated compared with control AMs at all sites. However, AMs from CD11c^{dnR} BMT mice were not significantly different from control AMs except at CpG sites 32 and 34. $^{**}p < 0.01$, $^{***}p < 0.001$.

4.3 Discussion

We previously showed that AMs post-BMT are defective in phagocytosis and killing of *P. aeruginosa*, a clinically relevant nosocomial pathogen which can afflict patients following hematopoietic stem cell transplantation (54, 58, 59, 96, 97). Defective AM function post-BMT is not exclusive to mice receiving TBI, but is also seen in mice receiving chemotherapy conditioning regimens (54), 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 (58, 88). We now show that irradiated mice receiving CD11c+ donor cells that are unable to respond to TGF-β1 exhibit improved AM function (Figure 4.6), indicating that TGF-β1 is contributing to the defect in AMs following transplantation. Overproduction of PGE_2 in the lung has been reported following hematopoietic reconstitution and is intimately linked with a defective lung innate immune system (58). Here, we show that COX-2 transcription in AMs is elevated in mice post-BMT relative to control AMs. These results correlate with the elevated PGE_2 levels reported previously in both mice and patients that have undergone HSCT (54, 58, 83, 96, 97), and give insight to a possible transcriptional dysregulation of COX-2 expression.

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 (197). In these circumstances, PGE_2 may promoter cancer progression and metastasis as well as immune suppression. Furthermore, it was recently reported that chronic influenza infection was associated with upregulation of miR-29b (187). The consequence of miR-29b upregulation was the destabilization of DNA methyltransferases and the upregulation of COX-2 gene expression secondary to DNA demethylation (187). It is interesting that we have also observed upregulation

of miR-29b in BMT AMs (Table 3.1 and Chapter 5). Thus, it is possible that the process of BMT regulates COX-2 DNA demethylation via miR-29b expression.

To further understand the regulation of COX-2 expression in our syngeneic murine model for studying innate immune cells post-BMT, we examined DNA 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 702 bp COX-2 promoter drove expression in MHS cells and the fact that no CpG sites located upstream of the transcription start site showed DNA methylation differences suggests that the region of interest is located near the exon 1 border. DNA methylation changes of this magnitude have previously been shown to alter EP2 expression (154) and methylation around the transcription start site and in gene exons has been previously described [e.g. in (187, 198-200)]. It is interesting that in human studies related to *Helicobacter pylori*-induced COX-2 expression and influenza-induced COX-2 expression, DNA demethylation occurs at similar sites as those identified in our murine study (187, 200). 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 (201) and silencing of the COX-2 gene in human gastric cancers involves DNA hypermethylation of promoter regions upstream of exon 1 (202). 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. A similar response was observed in primary AMs of untransplanted control mice that were treated with either 5-aza-2'-deoxycytidine

or vehicle. Overall, these results suggest DNA 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 expression 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. These data suggest that 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 shortened COX-2 promoter construct (which contained the CpG sites we had analyzed for DNA methylation patterns) was sufficient to induce COX-2 expression in MHS cells. We next studied the effects of DNA methylation on the activity of this construct. *In vitro* DNA 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 DNA 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. These results suggested that the increased levels of TGF-β1which have been previously reported in BMT mice (88), may serve to induce COX-2 DNA demethylation or increase transcription from the methylated promoter in the

AMs in the BMT mice. Interestingly, this activity was not noted with LPS, a molecule which is able to stimulate transcription off of unmethylated COX-2 promoters. 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 DNA 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.

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 occur secondary to conditioning regimen-induced alterations. The production of reactive oxygen species has been observed previously following ionizing radiation and has been implicated in compromised lung immunity (203). Reactive nitrogen species, particularly nitric oxide, have been suggested to induce changes in COX within airway epithelium during inflammation (204). 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 (204). 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 affected 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 (59). While we have ruled out the contribution of GM-CSF to the induction of COX-2 in this model (59), 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.

Chapter 5:

Transforming Growth Factor-β induces microRNA-29b to promote murine alveolar macrophage dysfunction post-bone marrow transplantation

5.1 Background

As an effective therapy for a variety of malignant and inherited disorders, 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, transplant patients receive their own hematopoietic stem cells (autologous HSCT) or receive stem cells from HLA-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 (205). However, despite differences in conditioning regimens or application of immunosuppressive therapy, HSCT patients exhibit increased susceptibility to pulmonary complications both pre- and post-engraftment (23, 28, 206, 207). The associated morbidity and mortality associated with microbial infection is further confounded by antibiotic-resistant bacteria (52).

During the pre-engraftment 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 post-engraftment (30-100 d after transplant) and the late post-engraftment (> 100 d after transplant) phase, where the immune compartment has been reconstituted by donor-derived cells (21, 28). Previous studies, including autopsy reports, have revealed that pulmonary complications are a significant cause of death for transplant patients and a predictor for poor survival (21). Thus, understanding why these pulmonary complications arise is vital to survival and quality of life for HSCT patients.

Existing in the alveolar compartment between air and lung tissue, alveolar macrophages (AMs) compose more than 90% of the cells retrieved by bronchoalveolar lavage in a healthy individual and represent the first line of defense in the lung (177). Following HSCT, however, phagocytosis and bacterial killing by human AMs is significantly compromised (34), suggesting that impaired AM responses support prolonged sensitivity to infection following transplantation. To investigate the causes for impaired innate immune responses, our lab utilized a syngeneic murine model of BMT as a representation of autologous transplantation. This model sheds light on the underlying effects of transplantation on immune cell function independent of immunosuppressive therapy or development of GvHD (54).

Using this model, we previously reported that BMT mice were more susceptible to Gram-negative (*Pseudomonas aeruginosa*) and Gram-positive (*Staphylococcus aureus*) pneumonia (55, 60). This increased susceptibility was at least partially attributed to functionally defective AMs (54, 58-60, 68, 88, 96, 97, 208). As AMs are the sentinel phagocytes in the lung, their ability to recognize an invading pathogen, recruit immune cells to the infected lung, and control/clear the

invading pathogen is important for preventing widespread infection and alveolar damage. However, murine AMs post-BMT exhibit impaired phagocytosis and bacterial killing similarly to human HSCT AMs (34, 88, 208). These defects were attributed to overexpression of COX-2 and subsequent increased production of its downstream effector, PGE_2. Previous observations also noted elevated PGE_2 in the serum of autologous HSCT patients (83).

Eicosanoids are lipid mediators derived from arachidonic acid metabolism. There are two isoforms of the COX enzymes: COX-1 and COX-2 (192). Constitutively expressed, COX-1 is important for mediating homeostatic effects while COX-2 is upregulated by inflammatory signals. Metabolism of arachidonic acid by COX-1/COX-2 is necessary for the generation of PGE_2. PGE_2 can be recognized by EP receptors (EP1-4) (76, 193, 209). In our BMT model, EP2 and EP4 are significantly overexpressed and binding of PGE_2 via these receptors, particularly EP2, results in inhibition of macrophage function (58). In BMT AMs, basal COX-2 expression is significantly higher than in untransplanted control AMs (58, 89) which we previously showed was due to hypomethylation of CpG sites within the COX-2 promoter (89). Hypomethylation and increased COX-2 expression has previously also been shown in cancer (201, 210). In our model, COX-2 hypomethylation was a result of increased TGF-β signaling (89). Disruption of TGF-β signaling resulted in partial restoration of DNA methylation patterns in COX-2 as well as AM function, i.e. phagocytosis (89). However, the mechanism by which TGF-β mediated this effect was unknown.

MiRNA are short, noncoding RNA sequences roughly 22 nucleotides in length and have been previously dubbed as “fine tuners” of immune responses (211, 212). MiRNA are post-transcriptional regulators that can inhibit mRNA stability and/or protein translation by binding to 3’UTRs of their target genes (211, 212). Preliminary studies revealed miR-29b was the most

upregulated miRNA in BMT AMs when cellular RNA was analyzed on the mouse inflammation miRNA RT^2profile miRNA array from SABiosciences (Table 3.1). Interestingly, miR-29b contains predicted target sites within the 3'UTR of the de novo DNMTs, DNMT3a and -3b (TargetScan) (187, 213-215). DNMTs are responsible for the transfer of methyl groups onto CpG residues (216). Here, we determine the role of miR-29b in the ability of TGF-β to induce COX-2 promoter hypomethylation in BMT AMs.

5.2 Results

Increased miR-29b and decreased DNMT expression in AMs post-BMT

We previously showed that increased TGF-β signaling in AMs post-BMT was responsible for hypomethylation of COX-2, and that inhibition of TGF-β signaling in AMs post-BMT resulted in hypermethylation of the COX-2 promoter (89) resulting in decreased expression of COX-2, and restored AM function (89). 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. As DNMTs are responsible for the transfer of methyl groups onto CpG sites, we measured the expression of maintenance (DNMT1) and *de novo* (DNMT3a and -3b) DNA methylation enzymes following BMT. The expression of all three DNMTs was decreased two-fold compared to untransplanted controls (Figure 5.1A). We originally ran a microarray analysis to look for alterations in miRNA that may regulate DNMTs and found miR29b to be elevated (Table 3.1). Thus, we verified using real-time RT-PCR that AMs from BMT mice express higher levels of miR29b than do AMs from control mice (Figure 5.1B).

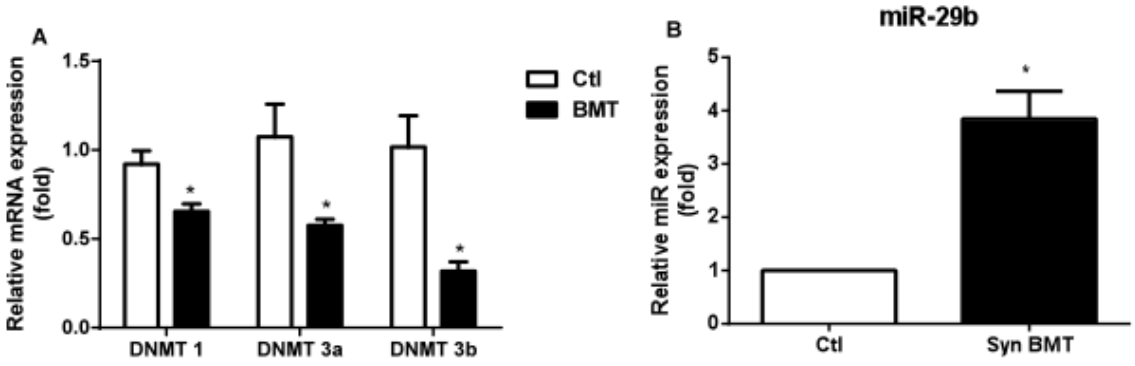

Figure 5.1. MiR-29b and DNA methyltransferase (DNMT) expressions are altered post-BMT. Untransplanted control and BMT AMs were measured for (A) DNMT 1, 3a, and 3b (n=3-4) or (B) miR-29b (n=2-3) by real-time RT-PCR; *$p<0.05$.

Similarly, AMs from both autologous and allogeneic HSCT patients exhibited high expression of miR-29b relative to the U6 control when compared to AMs collected from normal healthy volunteers (Figure 5.2). Interestingly, high expression levels of miR-29b were noted even in patients more than two years post-HSCT when compared to healthy controls. HSCT patient characteristics are outlined in Table 5.1. Thus, these data support our murine model as an appropriate representation of the alterations observed in HSCT patients and suggest sustained elevation of miR-29b.

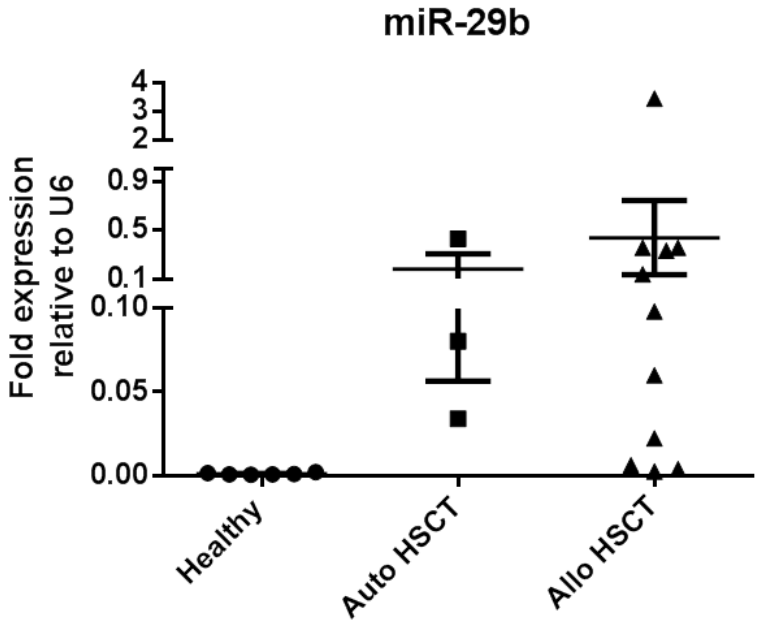

Figure 5.2. MiR-29b is overexpressed in HSCT patients. Expression by human AMs of miR-29b relative to U6 was determined in autologous (n=3 auto) and allogeneic HSCT patients (n=11 allo) and compared to healthy individuals (n=6).

Table 5.1. Healthy and HSCT patient characteristics for miR-29b studies.

Patient	Age	Gender	Days post-transplant	Conditioning	Underlying disease
Control 1	58	Female	N/A	N/A	No disease, never smoker
Control 2	20	Male	N/A	N/A	No disease, never smoker
Control 3	30	Female	N/A	N/A	No disease, never smoker
Control 4	39	Female	N/A	N/A	No disease, never smoker
Control 5	54	Female	N/A	N/A	No disease, never smoker
Control 6	20	Female	N/A	N/A	No disease, never smoker
Allo 1	65	Male	357	FluBu	Acute myelomonocytic leukemia (AML)
Allo 2	60	Male	768	Flu/Bu/TLI	Chronic lymphocytic leukemia, b-cell/Small lymphocytic lymphoma
Allo 3	60	Male	10	FluBu	Multiple Myeloma
Allo 4	57	Female	341	FluBu/TBI	Acute undifferentiated leukemia
Allo 5	63	Female	10	FluBu	Acute lymphoblastic leukemia
Allo 6	34	Female	617	FluBu	AML
Allo 7	52	Female	552	CloBu	AML
Allo 8	21	Male	2179	FluCy/TBI	Precursor B-cell ALL
Allo 9	25	Female	176	FluBu, ATG/thymoglobulin	Myelodysplastic Syndrome (MDS)
Allo 10	60	Female	758	FluBu	AML/mixed lineage leukemia
Allo 11	42	Female	4137	BAC	MDS
Auto 1	59	Male	19	MEL	Multiple Myeloma –IgG

Auto 2	57	Female	174	BEAM	Anaplastic large-cell lymphoma, T/null cell, primary systemic type
Auto 3	35	Male	12	Cy, ATG/thymoglobulin	Systemic sclerosis

MEL, melphalan; BEAM, BCNU-Etoposide-AraC-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.

TGF-β upregulates miR-29b and downregulates DNMT expression

Previous data showed elevated levels of TGF-β in BMT lungs compared to untransplanted controls (88). Furthermore, TGF-β promotes upregulation of COX-2 (204, 217). To determine whether TGF-β-induced COX-2 promoter hypomethylation was due to increased miR-29b and decreased DNMT, primary AMs from wild-type untransplanted mice were treated with TGF-β for 24 h prior to RNA isolation. Real-time RT-PCR analysis revealed that TGF-β-treated AMs exhibited decreased DNMT3a and -3b expression (Figure 5.3A) and increased expression of miR-29b (Figure 5.3B; white bars). To further verify the effects of TGF-β on DNMT expression, $CD11c^{dnR}$ mice expressing a dominant-negative TGF-βRII regulated by CD11c promoter activity were used. Under these conditions, cells expressing CD11c (i.e. dendritic cells, macrophages, natural killer cells) would be unresponsive to TGF-β signaling. AMs from $CD11c^{dnR}$ mice did not show significant changes in miR-29b (Figure 5.3B; black bars) or DNMT (Figure 5.3C) 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.

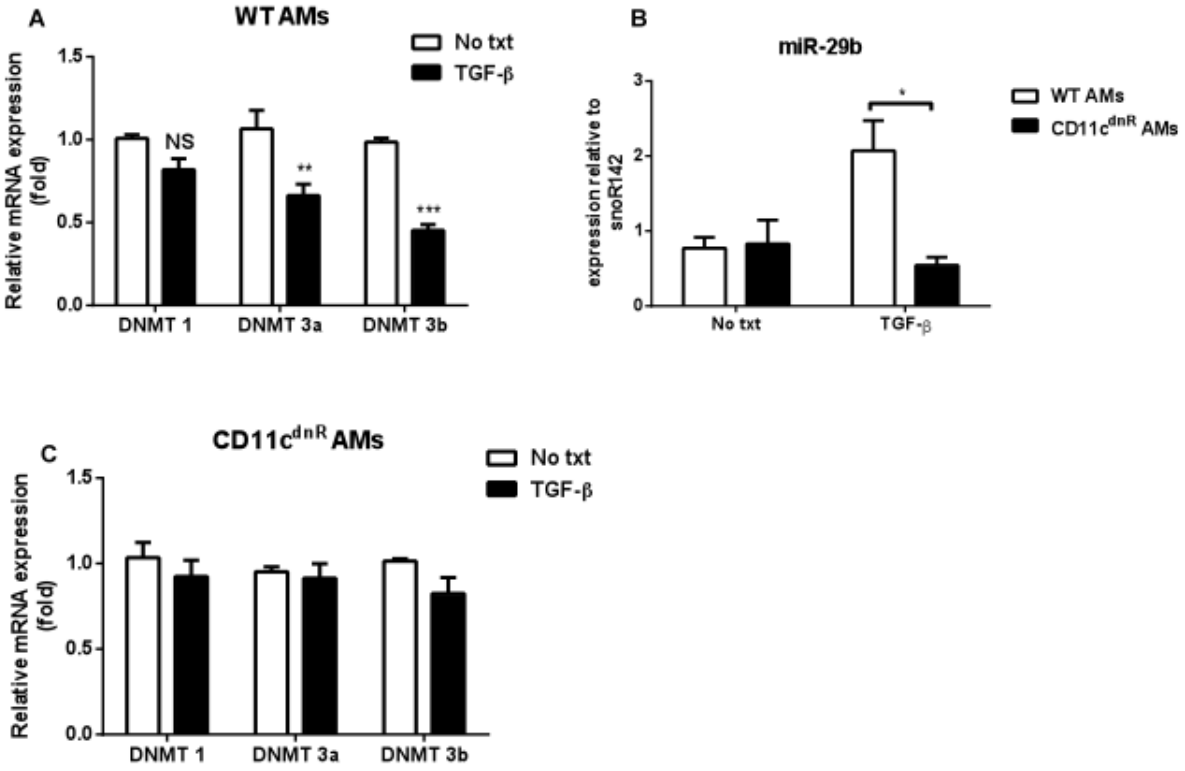

Figure 5.3. TGF-β promotes altered miR-29b and DNMT expression. (A) Primary untransplanted control AMs (WT AMs) (n=4-8) and (B) WT or CD11c^{dnR} AMs (n=2-3) or (C) CD11c^{dnR} AMs (n=3-5) were treated with 2 ng/mL TGF-β for 24 h and DNMT 1, 3a, 3b or miR-29b expression was measured by real-time RT-PCR; *p<0.05, **p<0.01, ***p<0.001.

MiR-29b downregulates DNMT expression and upregulates the COX-2 pathway

DNMT 3a and 3b contain a miR-29b target sequence in their 3'untranslated region (TargetScan) (187). To determine whether the altered expression 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, DNMT 3a and 3b as well as DNMT1 expression decreased significantly (Figure 5.4A). These data indicate that miR-29b negatively regulates DNMTs in AMs.

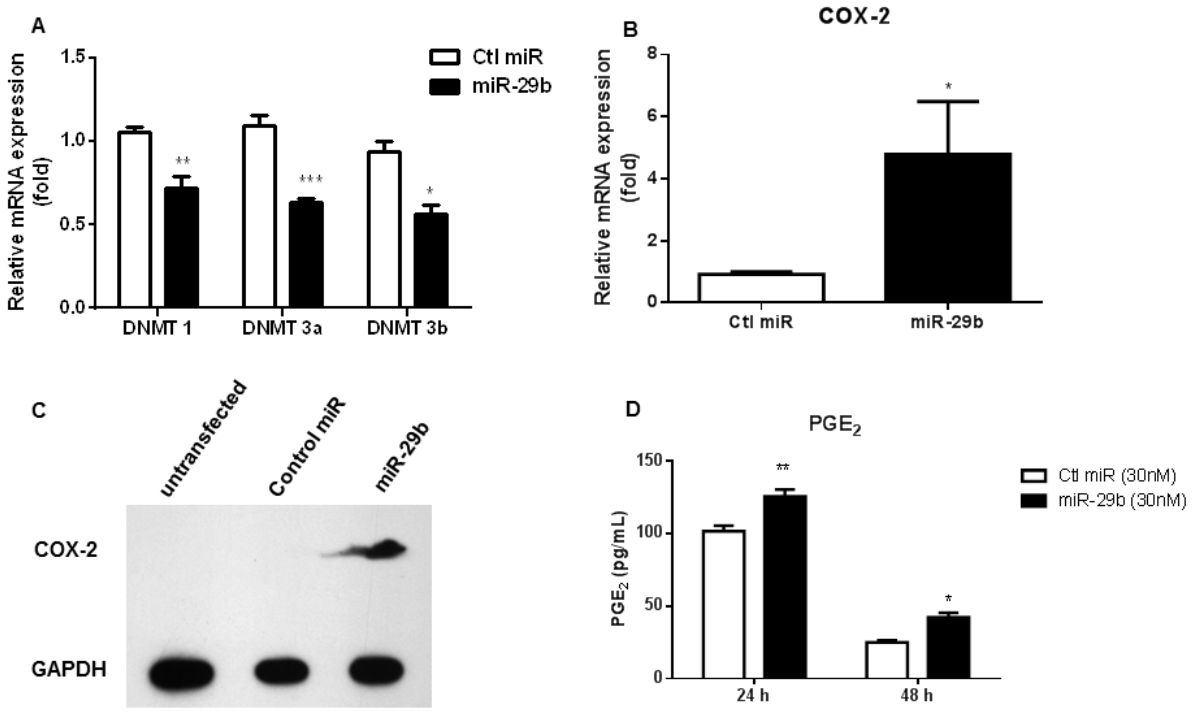

Figure 5.4. MiR-29b overexpression in primary AMs inhibits DNMT expression and upregulates COX-2/PGE_2. (A) Primary AMs were transfected with 30 nM miR-29b or a control scrambled miRNA at the same concentration for 24 h prior to measuring DNMT expression by real-time RT-PCR (n=2-4). MiR-29b or a control scrambled miR (30 nM) were transfected into primary wild-type AMs for 48 h prior to measuring COX-2 expression by (B) real-time RT-PCR (n=2-4) or (C) Western blot (pooled from n=3-4), and (D) PGE_2 was measured following miR29b transfection by ELISA (n=3); *p<0.05, **p<0.01; ***p<0.001.

Increased activity of COX-2 and its downstream effector, PGE_2, were previously shown to promote the defective functional phenotype in BMT AMs (54, 58-60, 68, 88, 89, 97, 208). Furthermore, TGF-β promotes COX-2/PGE_2 (217) (Figure 5.5) and miR-29b (Figure 5.4B) while decreasing DNMT expression (Figure 5.4A). To determine if miR-29b could affect the COX pathway, primary AMs from WT mice were transfected with miR-29b. In response to transient

miR-29b overexpression, COX-2 mRNA (Figure 5.4B) and protein (Figure 5.4C) were upregulated. Increased PGE_2 production in miR-29-transfected AMs following 24 and 48 h transfections indicate that the upregulation of COX-2 resulted in increased COX-2 activity (Figure 5.4D).

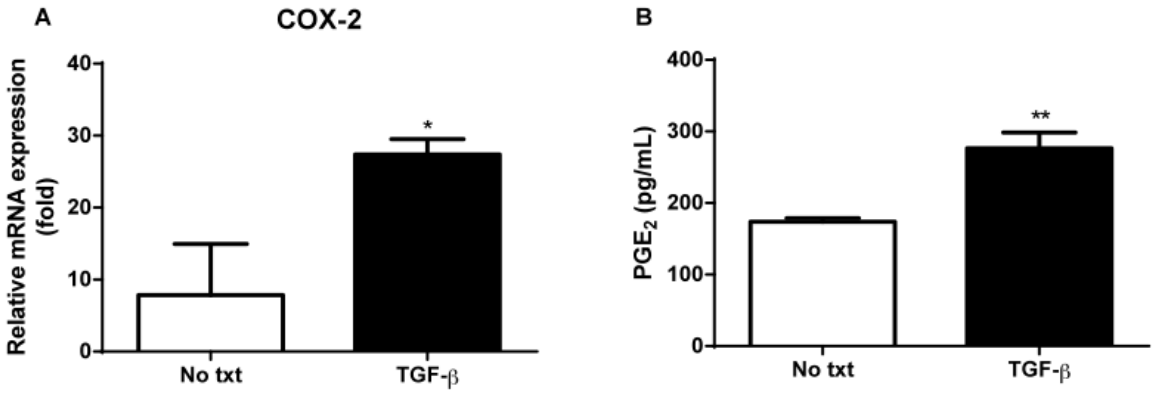

Figure 5.5. TGF-β upregulates COX-2 in primary AMs. Primary AMs were treated with 5 ng/mL TGF-β for 24 h. (A) COX-2 mRNA was measured by real-time RT-PCR following RNA extraction by TRIzol (n=4) and (B) PGE_2 released in the supernatant was measured by ELISA (n=4); *p<0.05, **p<0.01.

Since TGF-β increased expression of miR-29b and decreased expression of DNMT3a and -3b, we sought to determine whether miR-29b is responsible for the hypomethylation of COX-2 in BMT AMs (89). Primary AMs were transfected with miR-29b and COX-2 promoter methylation was determined by bisulfite conversion and pyrosequencing. The location of CpG sites 1-6 is outlined in Figure 5.6A. COX-2 was hypomethylated in CpG sites 1-6 following miR-29b-transfection compared to AMs transfected with a control miR (Figure 5.6B).

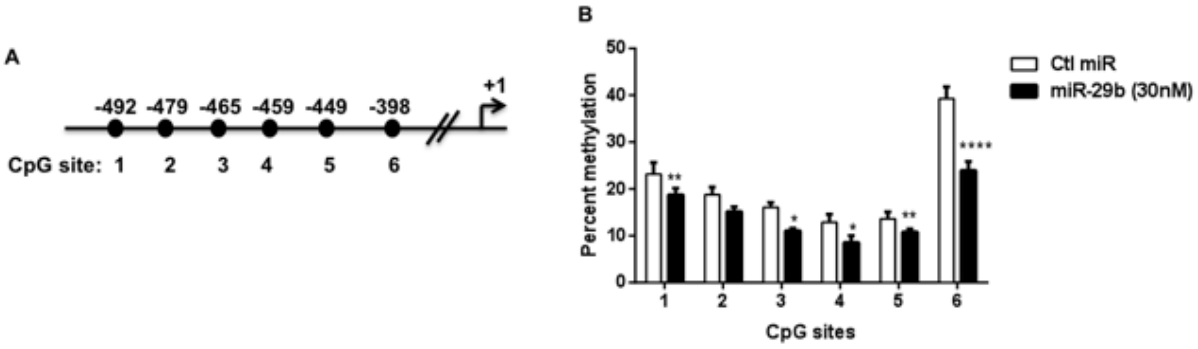

Figure 5.6. MiR-29b overexpression promotes hypomethylation of COX-2. Primary AMs were transfected with miR-29b or control scrambled miR (30 nM) for 48 h prior to bisulfite conversion and pyrosequencing analysis of CpG sites 1-6 (n=4 separate experiments). The locations of the depicted CpG sites in the COX-2 promoter are depicted above the graph. The transcription start site is denoted by the "+1"; **p*<0.05. ***p*<0.01, *****p*<0.0001.

This decreased DNA methylation pattern at CpG sites 1-6 was similarly observed in BMT AMs (Figure 5.7A) and primary AMs treated with a methyltransferase inhibitor, 5-aza-2'-deoxycytidine (Figure 5.7B). While our previous results suggested that CpG sites near exon 1 were hypomethylated in BMT AMs (89), we did not see these sites regulated by miR29b transfection (data not shown).

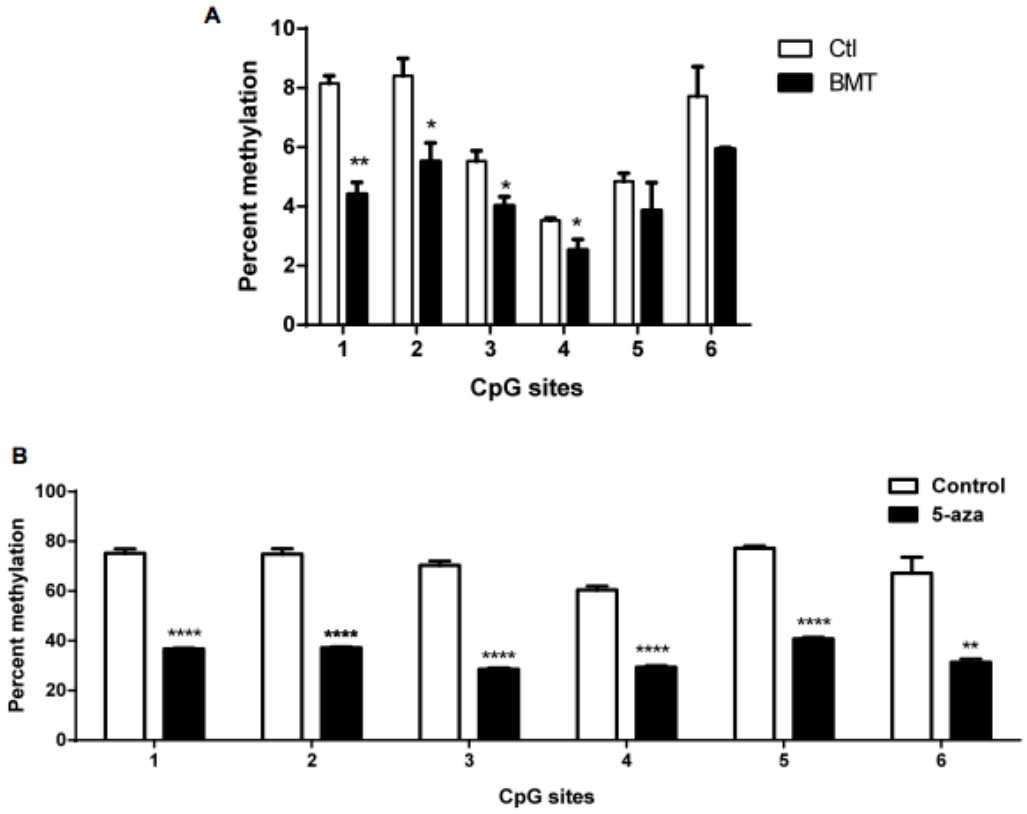

Figure 5.7. BMT AMs exhibit decreased COX-2 methylation at CpG sites 1-6. (A) DNA methylation of upstream CpG sites 1-6 were measured in untransplanted control and BMT AMs (n= 2 separate experiments). (B) Primary wild-type AMs harvested by BAL were initially supplemented with GM-CSF (1ng/mL) for 24 h prior to the addition of 2.5 μM 5-aza-2'deoxycytidine (5-aza) for 72 h. DNA 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.

Inhibition of TGF-β rescues bacterial killing defect in BMT AMs

As the data thus far points to a TGF-β-induced dysregulation of miR-29b and DNMTs in BMT AMs, the effects of TGF-β on AM function post-BMT were determined. Killing of phagocytized *S. aureus* was measured in BMT and untransplanted control AMs. BMT AMs exhibited impaired bacterial killing compared to control AMs, supporting previous publications (58, 96, 208). However, reconstituting the alveolar compartment with donor AMs insensitive to

TGF-β (CD11c^{dnR} BMT) rescued this killing defect (Figure 5.8). These data suggest that TGF-β signaling mediates the functional defect seen in AMs post-BMT.

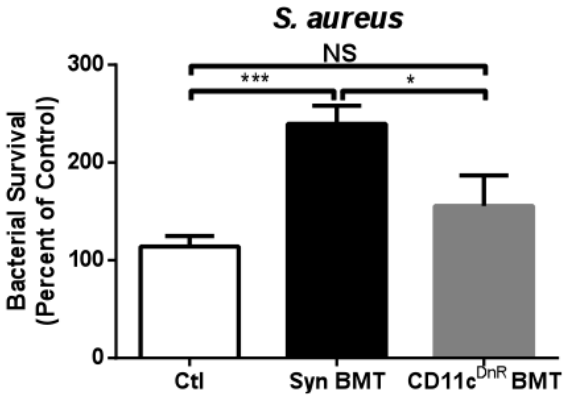

Figure 5.8. CD11c^{dnR} BMT AMs exhibit intact bacterial killing of *S. aureus*. AMs from untransplanted control, B6 BMT (Syn BMT), and CD11c^{dnR} BMT mice were measured for their ability to kill *S. aureus* intracellularly (n=5-8); *p<0.05, ****p<0.0001.

MiR-29b compromises effective bacterial killing in BMT AMs

To determine the functional effects of miR-29b post-BMT, primary BMT AMs were transfected with an antagomir of miR-29b (antimiR-29b) or a control miR, and measured for their ability to clear *S. aureus* intracellularly. Compared to untransplanted control AMs, BMT AMs allowed increased intracellular bacterial survival (Figure 5.9A). However, upon inhibition of miR-29b (antimiR-29b), bacterial killing was restored in BMT AMs to levels similar to control AMs transfected with a control miRNA (Figure 5.9A). Interestingly, transient transfection of miR-29b in control AMs was sufficient to drive a defective functional response comparable to control transfected BMT AMs (Figure 5.9B). Previous data support an integral role for COX-2 in mediating the bacterial killing defect in BMT AMs by upregulating the production of PGE_2. Thus, we investigated whether inhibition of bacterial clearance caused by overexpression of miR-29b was due 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 PGE_2 signaling with a novel EP2 antagonist (EP2a; PF-04418948) (170, 171) similarly restored miR-29b-transfected AM function (Figure 5.9B).

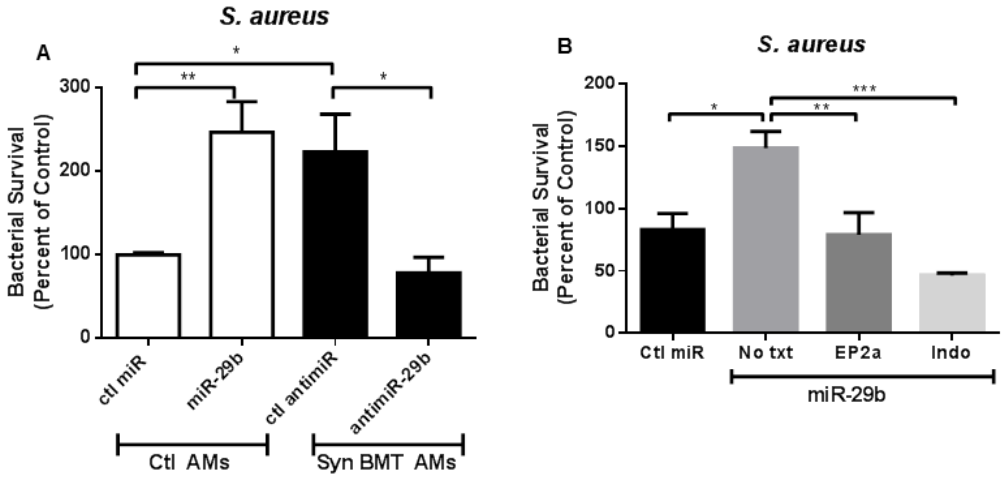

Figure 5.9. MiR-29b compromises bacterial killing in AMs through PGE_2 signaling. (A) AMs from untransplanted control mice were transfected with a control miR (30 nM) or miR-29b (30 nM), and BMT mice were transfected with a control antimiR (10 nM) or the antagomiR (10 nM) for 48 h prior to measuring bacterial killing of *S. aureus* (n=5-8). (B) *S. aureus* killing by miR-29b-transfected wild-type AMs was determined in the presence or absence of EP2 antagonist (PF-04418948; 10nM) or indomethacin (5µM) and compared to AMs transfected with a control miR (n=4-5); *p<0.05, **p<0.01, ***p<0.0001.

5.3 Discussion

The results presented here show that DNMT1, DNMT3a and DNMT3b expression are diminished in AMs post-BMT, and that TGF-β downregulates DNMT expression in control AMs. $CD11c^{dnr}$ AMs are insensitive to TGF-β and thus expression of DNMT isoforms are unaffected by TGF-β. MiR-29b on the other hand was increased in AMs post-BMT, and in control AMs treated with TGF-β. The effects on DNMT expression are miR-29b-dependent as wild-type AMs transfected with miR-29b, but not control miRNA revealed decreased DNMT expression. As mentioned earlier, miR-29b contains putative binding sites in the 3'UTR of DNMT 3a and 3b. A previous study confirmed these binding sites and showed direct binding of miR-29b on the 3'UTR (187). A previous study also showed that miR-29b can indirectly down-regulate DNMT1 by targeting *Sp1* (153). Thus, our results showing that miR29b 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 where TGF-β negatively regulates miR-29b (218-220). These conflicting reports highlight the tissue-specific nature of TGFβ regulation of miR-29b.

HSCT patients suffer from pulmonary complications that commonly manifest as infectious pneumonias (12, 23, 28). Interestingly, observations made in HSCT patients note impaired function in the innate immune compartment (i.e. AMs and PMNs) (26, 34, 83). 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 and kill engulfed bacteria (55, 58, 88, 208). These defects were primarily mediated by the overproduction of PGE_2 as inhibition of PGE_2 or the COX-2 pathway

rescued bacterial phagocytosis and killing by BMT AMs *in vitro* (58). These data highlight the importance of the COX-2 pathway and its effector, PGE_2 in mediating the defects observed in our BMT model. However, the mechanism for enhanced basal expression of COX-2 remained elusive. Recently, we published that the COX-2 promoter was significantly hypomethylated in BMT AMs, allowing for enhanced COX-2 expression (89). Interestingly, BMT AMs insensitive to TGF-β exhibited partially restored DNA methylation and phagocytic function (89). This study sought to shed light on a possible mechanism by which TGF-β could affect immune responses in BMT mice through regulating DNA methylation of COX-2.

The data presented show that overexpression of miR-29b in primary AMs induces COX-2 and PGE_2 by inhibition of DNMTs, resulting in the hypomethylation of COX-2. Previous studies have implicated COX-2 and subsequently, PGE_2, in mediating immunosuppressive effects on macrophages (221-224). We have shown that the inability of BMT AMs to function effectively in response to bacteria is due to the elevated basal PGE_2 levels (58, 60). Consequently, understanding the source for upregulation of PGE_2 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 the 3) hypomethylation of COX-2, and the subsequent 4) COX-2-dependent synthesis of PGE_2. In turn, PGE_2 binding to elevated EP2 receptors on AMs post-BMT impairs phagocytosis and bacterial killing. TGF-β has previously been characterized to have dual effects on COX-2 (217, 225-228). A study in human non-small cell lung cancer A549 cells showed that TGF-β was able to downregulate COX-2 (227). However, the bulk of the literature has shown induction of COX-2 in response to

TGF-β in models using mammary epithelial cells (229), human mesangial cells (217), and macrophages in muscle tissue (228). Activation of ERK1/2, p38 MAPK, and PI3K signaling pathways are suggested to mediate the TGF-β-dependent activation of COX-2 (217). Here we show that overexpression of miR-29b in primary AMs induces COX-2 and PGE_2 by indirectly promoting the hypomethylation of COX-2 through its regulatory effects on DNMTs. At least one source of elevated TGFβ in the lungs post-BMT is from alveolar epithelial cells (88). BMT AMs show hypomethylation on CpG sites 1-4 and near exon 1 of COX-2 (89), however, miR-29b induces hypomethylation on the former CpG sites and not the latter. Previous studies showed that the COX-2 gene contained an NF-kB, a C/EBP, and a cAMP response element-1 (CRE-1) site in the promoter, however, Kang and colleagues identified three additional functional elements (CRE-2, an AP-1 site, and an E-box overlapping CRE-1) (230, 231). Interestingly, our DNA 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 (231). Thus, it is possible that despite changes in DNA methylation at CpG sites near exon 1 of COX-2 (89) following transplantation, hypomethylation of the more upstream CpG sites observed both post-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 *in vitro* and overexpression of miR-29b in primary AMs alone inhibits proper bacterial clearance *in vitro*. These effects are due to the miR-29b-induced PGE_2 as blocking PGE_2 signaling (using an EP2 receptor antagonist) and the COX pathway (using indomethacin) are 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 HSCT patients as both autologous and allogeneic HSCT patients exhibit increased miR-29b expression.

Taken together, these data reveal a novel pathway that regulates the immunosuppressive effects of COX-2 and highlights miR-29b as a potential therapeutic target for improving pulmonary immune responses in HSCT patients. 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 (232). Currently there are no FDA-approved miRNA therapies available, however, commercial development of miRNA therapies are underway and are in pre-clinical stages for the treatment of non-small cell lung cancer, prostate cancer, muscle and cardiovascular disease, and fibrosis (232, 233). Thus, the possibility of delivering miR-29b antagomirs to AMs is a promising strategy for targeted immunoregulatory therapy for HSCT patients who are likely to suffer from infectious pulmonary complications.

Chapter 6:

Prostaglandin E_2 signaling through Epac-1 inhibits PMN extracellular trap formation

6.1 Background

PMNs are derived from myeloid precursors (234, 235). Along with macrophages, they are innate immune cells and are important mediators in acute immune responses. Aside from their segmented nucleus, PMNs contain cytoplasmic granules formed during maturation from the promyelocyte stage and primarily remain in circulation, awaiting activating signals (234, 235). There are three types of granules, each carrying proinflammatory proteins. The "primary" or azurophilic granules are peroxidase-positive and contain myeloperoxidase; the "secondary" or specific granules are peroxidase-negative, and carry proteins like lactoferrin; finally, the "tertiary" or gelatinase granules contain enzymes like matrix metalloproteinase-9 (234-237). The latter, unlike the former two, contain relatively few antimicrobials and instead, seem to function as storage for enzymes like gelatinase. These granules are important for mediating PMN immune responses against pathogens, both intracellular and extracellular.

In response to inflammation, the circulating PMN migrates to the site of infection through adherence to the activated endothelium, supporting extravasation. At the site of infection PMNs can phagocytize the invading pathogen, and granule fusion with the phagosome exposes the phagosomal cargo to azurophilic and specific granule antimicrobial enzymes. Importantly, fusion of specific granules and the phagosome is particularly important for formation of reactive oxygen species as flavocytochrome b558, a component of the NADPH oxidase machinery, is present on specific granular membranes (235, 238). Thus, production of the reactive oxygen

species together with primary and secondary granular proteins is important for creating the antimicrobial milieu for efficient intracellular killing. Similarly, these granules can also bind to the plasma membrane, activate NADPH oxidase machinery, and release their bactericidal components to the extracellular space (234).

More recently, the production of extracellular traps has provided another mechanism by which PMNs can capture and kill pathogens extracellularly. Considered to be an active form of cell death, PMN extracellular traps or NETs are web-like structures composed of decondensed chromatin, histones, and granular proteins (160, 235, 239). It is thought that NETs function to trap pathogens and kill via the localized concentration of antimicrobials that decorate these structures. Although the mechanisms regulating NET formation or NETosis are not entirely clear, NADPH oxidase, myeloperoxidase (MPO) and PMN elastase (NE) activity are required (240-242). Moreover, patients with chronic granulomatous disease (CGD), a congenital disorder affecting the NADPH oxidase system, exhibit impaired NET formation (243). Phorbol myristate acetate (PMA)-induced NETosis studies similarly show that upon inhibition of NADPH oxidase activity with diphenylene iodonium (DPI), NETs production significantly decreases compared to DPI-untreated PMNs, highlighting the importance of respiratory burst in the regulation of NETs (161).

Hematopoietic stem cell transplantation (HSCT) is an effective therapy for the treatment of malignant and congenital disorders. However both autologous (self donor stem cells) and allogeneic (HLA-matched donor stem cells) transplant patients exhibit higher susceptibility to infectious pulmonary complications long after transplant (23, 28, 173). Despite immune reconstitution, persistent susceptibility to infection suggests functional immune defects. Alveolar macrophages constitute more than 90% of the immune cells in the alveoli and are

important for the initiation of host responses against invading pathogens (244-246). One way that AMs are able to clear infection is through the recruitment and activation of circulating PMNs, key innate cells important for combating fungal and bacterial pathogens. HSCT patients are more susceptible to *P. aeruginosa* and *S. aureus*, pathogens also afflicting CGD patients (108). Interestingly, PMNs from bone marrow transplant (BMT) patients exhibit impaired superoxide production, bactericidal function and chemotaxis (26).

Using a murine BMT model, we have previously published that bactericidal function against both *P. aeruginosa* and *S. aureus* is impaired (55, 58, 60). As NETs have been previously shown to effectively kill both *S. aureus* and *P. aeruginosa* (160, 161, 247), it is unclear whether this observed bactericidal defect could be attributed to impaired NETosis following transplantation. We previously published that the defect in PMN function was partly due to overproduction of PGE_2 (58). PGE_2 is an effector of the cyclooxygenase enzyme, of which there are two isoforms (basal COX-1 and inducible COX-2) (197). The rate-limiting enzyme of PGE_2 synthesis, COX-2, is upregulated in murine BMT PMNs (58). Interestingly, inhibition of COX with indomethacin rescued the functional defect and intracellular bacterial killing improved (58). As intracellular bacterial killing relies on pathways shared with NETosis (e.g. NADPH oxidase for superoxide production), it is possible that NET formation is also impaired post-BMT. Aside from the requirement of NADPH oxidase activity and autophagy (248) to promote NETosis, regulation of this process is not fully understood. Additionally, no previous studies have documented any factors that may serve to limit this process physiologically. Here, we propose a novel role for PGE_2 as an inhibitor of effective NET production.

6.2 Results

NETs are impaired post-syn/allo BMT

Previously, our lab published that syn BMT PMNs phagocytized *P. aeruginosa*, similarly to untransplanted control PMNs. However, intracellular bacterial killing was impaired. As NETs are web-like structures made of chromatin, nuclear proteins and cytoplasmic granule proteins that contain antimicrobial functions, it is also possible that NETs are impaired. To determine whether the capacity to NET was deficient or intact, NETosis was measured via sytox green fluorescence detection, as sytox binds to extracellular DNA. Following recruitment into the lung by intratracheal LPS injections, PMNs were treated with PMA and NETosis was compared to untreated PMNs. PMA, a known inducer of NETosis, was able to stimulate NETs in untransplanted control PMNs; however, PMA-treated syn and allo BMT PMNs were unable to NET as effectively as untransplanted controls (Figure 6.1A and 6.1B). These observations were supported by immunofluorescence studies that showed little NETosis across mouse groups when untreated (Figure 6.1C, first row), but upon PMA-induction, extensive and intact NETs were seen in untransplanted controls while in both syn and allo BMTs, NETs were significantly decreased and appeared structurally less intact (Figure 6.1C, second row). BMT PMNs were able to form NETs or NET-like structures that appeared shorter and more fragile than untransplanted controls (Figure 6.1C). Interestingly, the impaired NETosis following syn BMT extended to NETosis in response to physiologically relevant stimuli like *Streptococcus pneumonia* (Figure 6.1A)

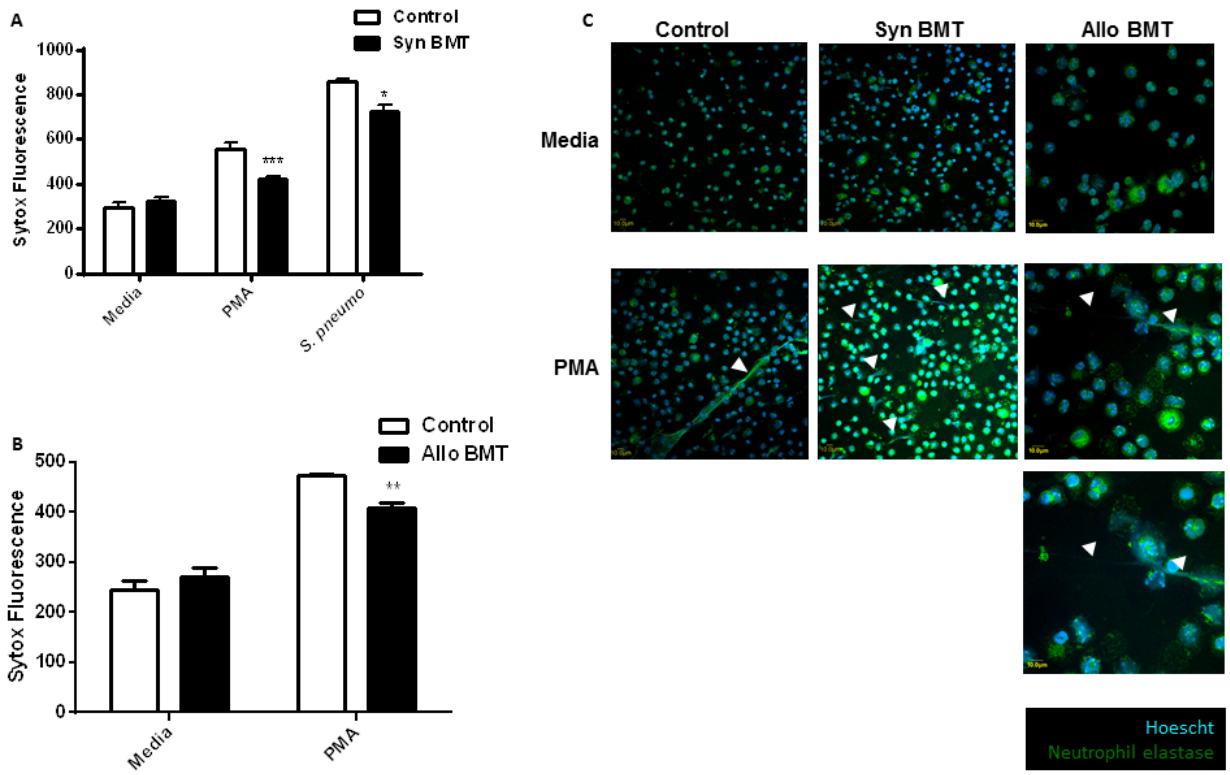

Figure 6.1. NETosis is impaired post-syn and allo BMT. LPS-recruited PMNs from the lung of (A) syn BMT (n=4-6) or (B) allo BMT (n=3) and untransplanted control mice were stimulated for 5 h with PMA (100 nM), *Streptococcus pneumoniae* (*S. pneumo*), or left untreated and NETosis was measured by sytox green fluorescence. (C) NETosis following 7 h PMA treatment in LPS-recruited PMNs from untransplanted control, syn BMT, and allo BMT mice was visualized by immunofluorescence via staining of DNA (Hoechst) and PMN elastase (control and syn BMT 20x; allo BMT 40x magnification); arrowheads show colocalization of neutrophil elastase and DNA in NET structures, $^{**}p<0.01$, $^{***}p<0.001$.

Indomethacin restores NET formation

Impaired AM and PMN function post-BMT were previously shown to be primarily mediated by the overexpression of the COX pathway. Thus, to determine whether NETosis was negatively regulated by increased COX activity, syn BMT mice were intraperitoneally injected with indomethacin, a COX inhibitor. Interestingly, *in vivo* inhibition of COX rescued impaired

NETosis post-syn BMT (Figure 6.2A). Immunofluorescence studies again confirmed the effects of indomethacin on NETosis, as *in vivo* inhibition of COX was able to visibly enhance NET production in syn BMT PMNs compared to untransplanted PMNs (Figure 6.2B). Similarly, NETosis was also increased in allo BMT mice upon COX inhibition with indomethacin (Figure 6.2C).

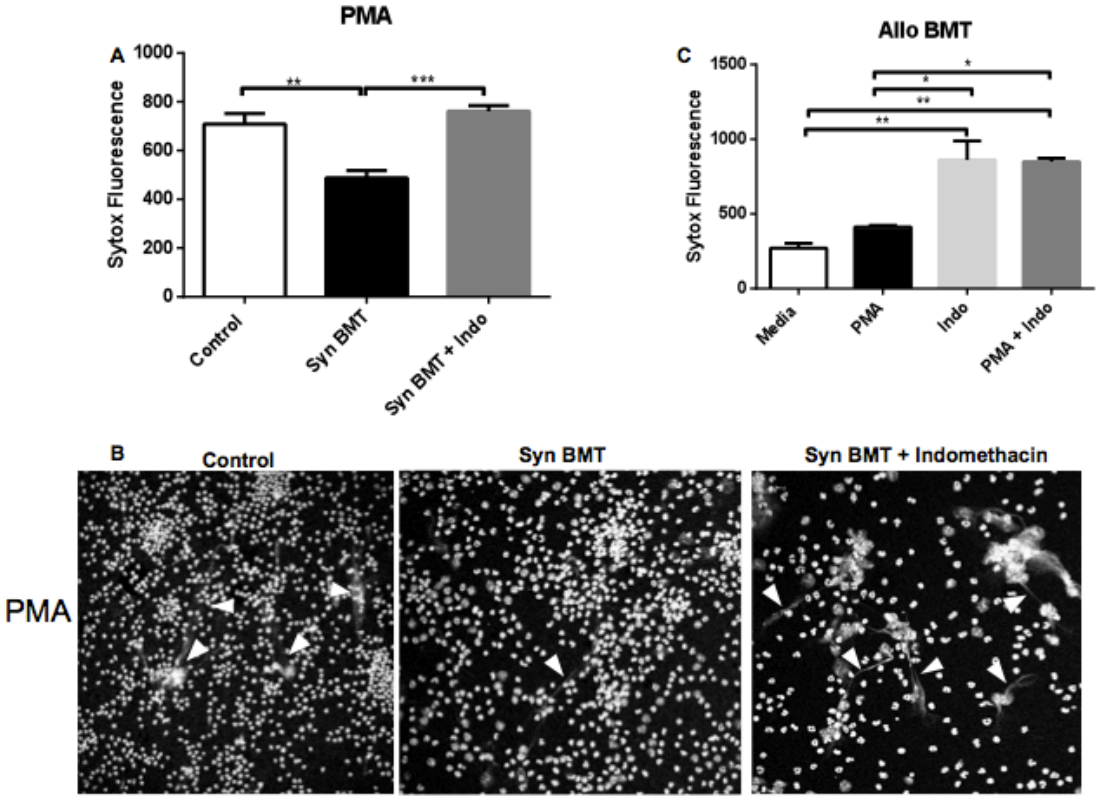

Figure 6.2. Indomethacin rescues impaired NETosis. LPS-recruited pulmonary PMNs from untransplanted control, syn BMT, or syn BMT mice following i.p. injection with indomethacin (1.2 mg/kg) were treated with PMA (100 nM) or left untreated for 5 h and NETosis was measured via (A) sytox fluorescence (n=4-5) or (B) immunofluorescence studies (gray scale of Hoechst DNA staining at 20x magnification). (C) Sytox fluorescence were performed in LPS-recruited allo BMT PMNs to measure NETosis following 5 h *in vitro* treatment with indomethacin (10 μM), PMA (100 nM), PMA and indomethacin, or left untreated (n=4-6); arrowheads denote NET structures, *p<0.05, **p<0.01, ***p<0.001.

PGE_2 inhibits PMA-induced NETs

Activation of the COX pathway results in increased synthesis of PGE_2. Our lab has shown that PGE_2 promotes functional defects in AMs and PMNs. Thus, to further understand the role of the COX pathway in the regulation of NETosis, we focused our studies to effects of PGE_2. Interestingly, *in vitro* treatment with PGE_2 was able to decrease NET release in response to a known inducer of NETs, PMA (Figure 6.3A), suggesting an immunosuppressive role for PGE_2 in the context of NETosis. This was confirmed with immunofluorescence studies that showed a significant absence of clear and intact NET formation upon the simultaneous treatment of PMA with PGE_2 (Figure 6.3B, right). PMA-treated untransplanted control PMNs recruited into the lung displayed extensive NET release (Figure 6.3B, middle).

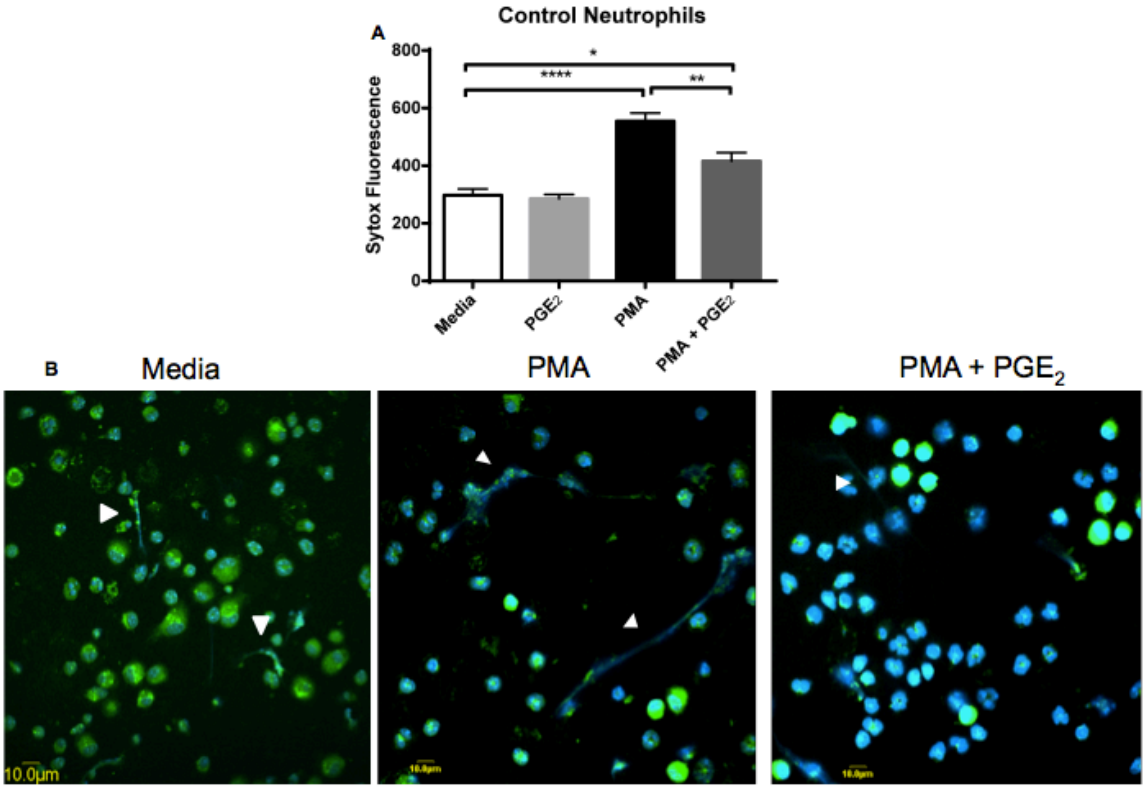

Figure 6.3. PGE_2 inhibits PMA-induced NETs. LPS-recruited PMNs from untransplanted control mice were stimulated with (A) PGE_2 (10 μM), PMA (100 nM), PMA + PGE_2 or left untreated for 5 h and measured for NETosis via sytox fluorescence (n=4-6) or (B) PMA, PMA + PGE_2, or untreated for 7 h and NETs were visualized by immunofluorescence (Hoechst, DNA; FITC, PMN elastase; 60x magnification); arrowheads show colocalization of neutrophil elastase and DNA in NET structures, *p<0.05, **p<0.01, ***p<0.001.

Inhibition of PGE_2 signaling restores NET production

Although Figure 6.7 supports an inhibitory effect for PGE_2 on NET release, the role of PGE_2 on NETs in the context of transplantation remained unclear. PGE_2 signals through EP receptors 1-4 and our lab previously showed that EP2 was enhanced on BMT AMs. Furthermore, inhibition of EP2 rescued multiple defects observed in BMT AMs. Thus, to determine whether the impaired NETs observed in BMT PMNs was primarily due to PGE_2, BMT PMNs were treated with a novel EP2 receptor antagonist (provided by Pfizer). Interestingly, treatment with the EP2 antagonist was able enhance NETosis in both Syn and Allo BMT PMNs (Figure 6.4A and 6.4B). PGE_2 signaling is mediated through PKA or Epac-dependent pathways. Thus, to shed light on the PGE_2 signaling important for mediating the inhibitory effects on NETs, PKA and Epac agonists were used. Activation of the PKA pathway did not inhibit PMA-induced NETs, however, the Epac agonist was able to effectively block NET production. Furthermore, PKA and Epac agonists together, similarly inhibited PMA-induced NETosis similarly as the Epac agonist with PMA alone (Figure 6.4C). These results suggest that in murine PMNs, EP2 activation of Epac is responsible for the inhibition of NETosis.

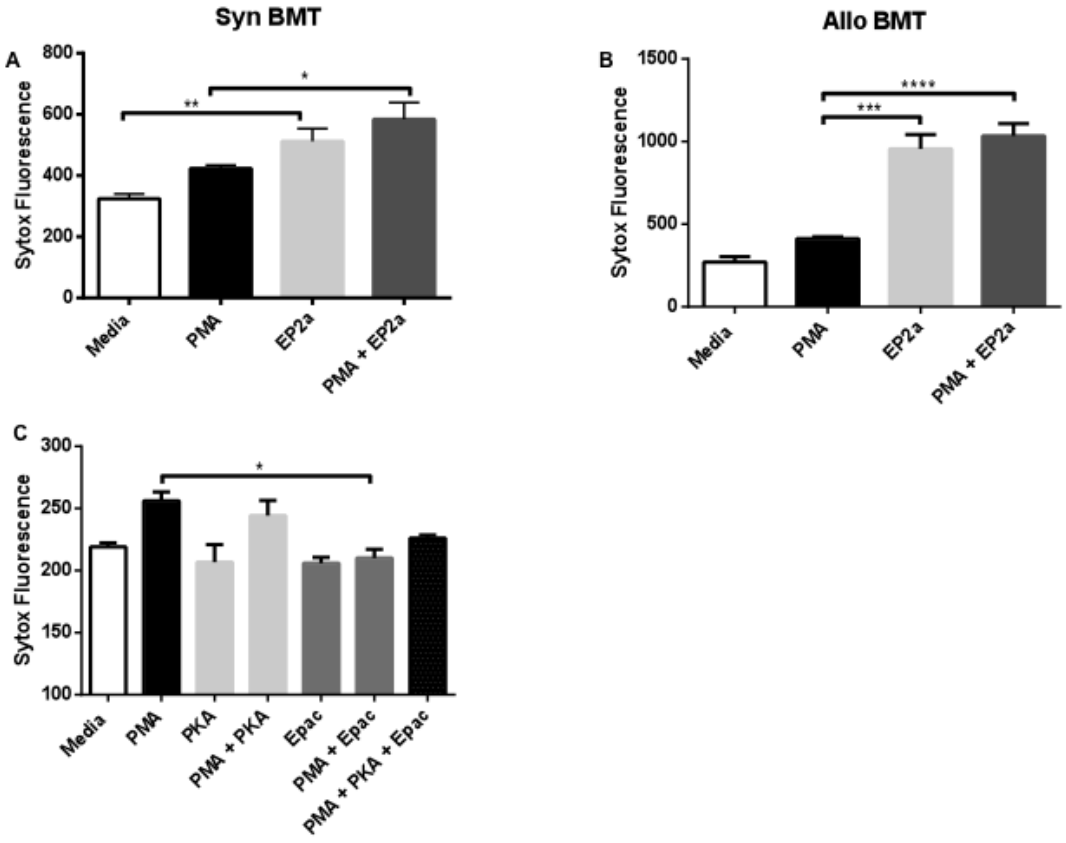

Figure 6.4. Inhibition of PGE_2 signaling rescues NET production and inhibition is mediated by Epac, but not PKA activation in mice. LPS-recruited PMNs from (A) syn (n=5-6) and (B) allo (n=4-5) BMT mice were treated with PMA (100 nM), an EP2 antagonist (EP2a, 1 nM), or PMA and EP2a for 5 h and NETs were measured by sytox fluorescence. (C) Effects of downstream PGE_2 signaling on NETosis were determined with 5 h PKA (500 μM) and Epac (500 μM) agonist treatments on untransplanted control PMNs. NET production was determine by sytox fluorescence (n=4-6); *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Blocking PGE_2 signaling rescues NETs in both autologous and allogeneic HSCT patients

Our murine BMT model suggests an impairment of NETosis in BMT PMNs mediated by PGE_2 signaling primarily via Epac. To understand its relevance in HSCT patients, the effect of PGE_2 on NETosis was measured in PMNs harvested from a bronchoalveolar lavage sample of an autologous HSCT patient (Figure 6.5A) and from peripheral blood from an allogeneic HSCT

patient (Figure 6.5B). Blocking PGE_2 signaling via the EP2 receptor was able to enhance NET production, measured by increased sytox fluorescence. These data were compatible with the data collected from our murine BMT model.

NETosis in human PMN is inhibited by both PKA and Epac activation by EP2 signaling

To further validate our murine findings, human untransplanted healthy PMNs were collected from peripheral blood. Following purification, they were treated with PMA, PGE_2, and/or Epac or PKA agonists. Similar to our murine data, PMA was able to induce NETs. This induction was decreased both by concurrent treatment with PGE_2 an Epac agonist or a PKA agonist (Figure 6.5C). These sytox findings correlated with immunofluorescence studies. NETs were undetected in PGE_2-treated and untreated human PMNs (Figure 6.6; Rows 1 and 2). Upon treatment with PMA, release of web-like DNA structures colocalizing with PMN elastase indicated formation of NETs (Figure 6.6; Row 3). However, NETs were visually absent in response to PGE_2 treatment, despite addition of PMA (Figure 6.6; Row 4). These results highlight that PGE_2 signaling via Epac can limit NETosis in murine PMNs; however, both Epac and PKA signaling may be involved in inhibition of human PMN NETosis.

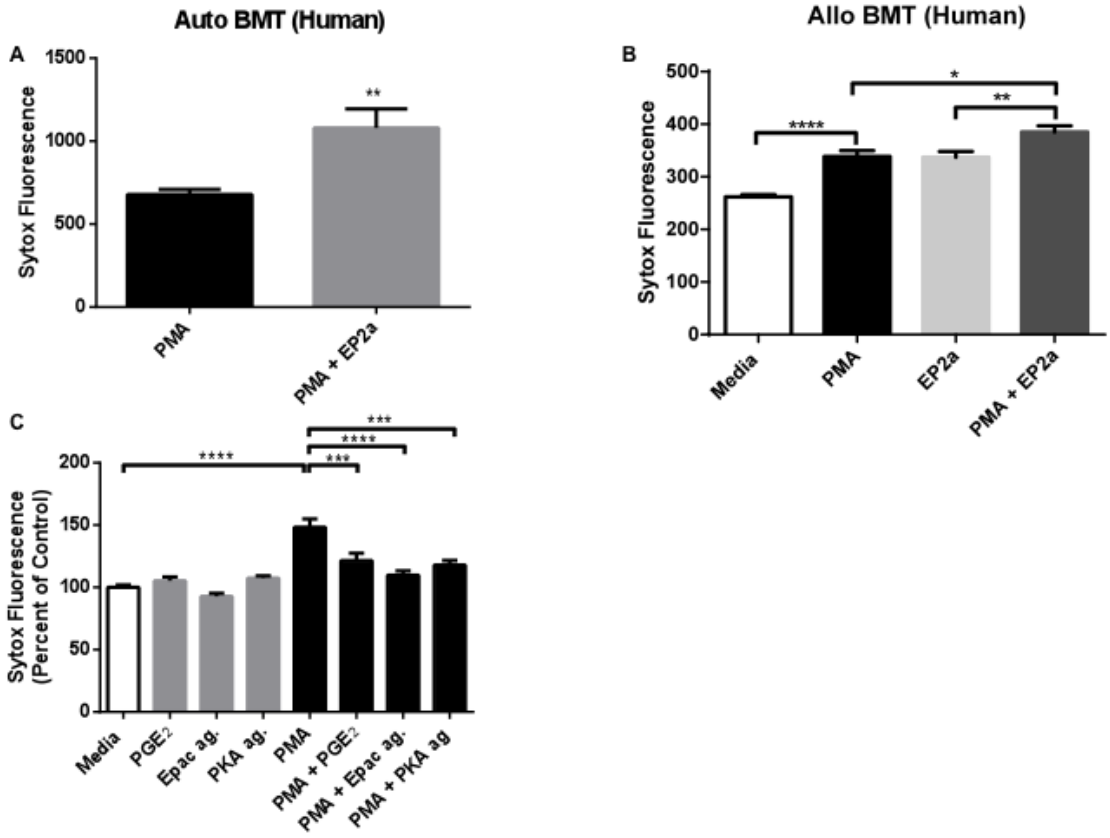

Figure 6.5. Blocking PGE_2 signaling rescues NETs in both autologous and allogeneic HSCT patients. NETosis was measured by sytox fluorescence using PMNs from (A) a bronchoalveolar lavage of an autologous transplant patient (n=5) and (B) peripheral blood from an allogeneic patient (n=10-12) following 2.5-3 h *in vitro* treatment with PMA (100 nM), EP2a (1 nM), or PMA + EP2a. (C) PMNs from n=2 healthy human volunteers were harvested from peripheral blood and treated with PGE_2, Epac agonist (Epac ag., 500 μM), PKA agonist (PKA ag., 500 μM), PMA, PMA + PGE_2, PMA + Epac ag., PMA + PKA ag., or left unstimulated for 3 h and NETosis was measured by sytox fluorescence (n=4 experimental replicates); *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

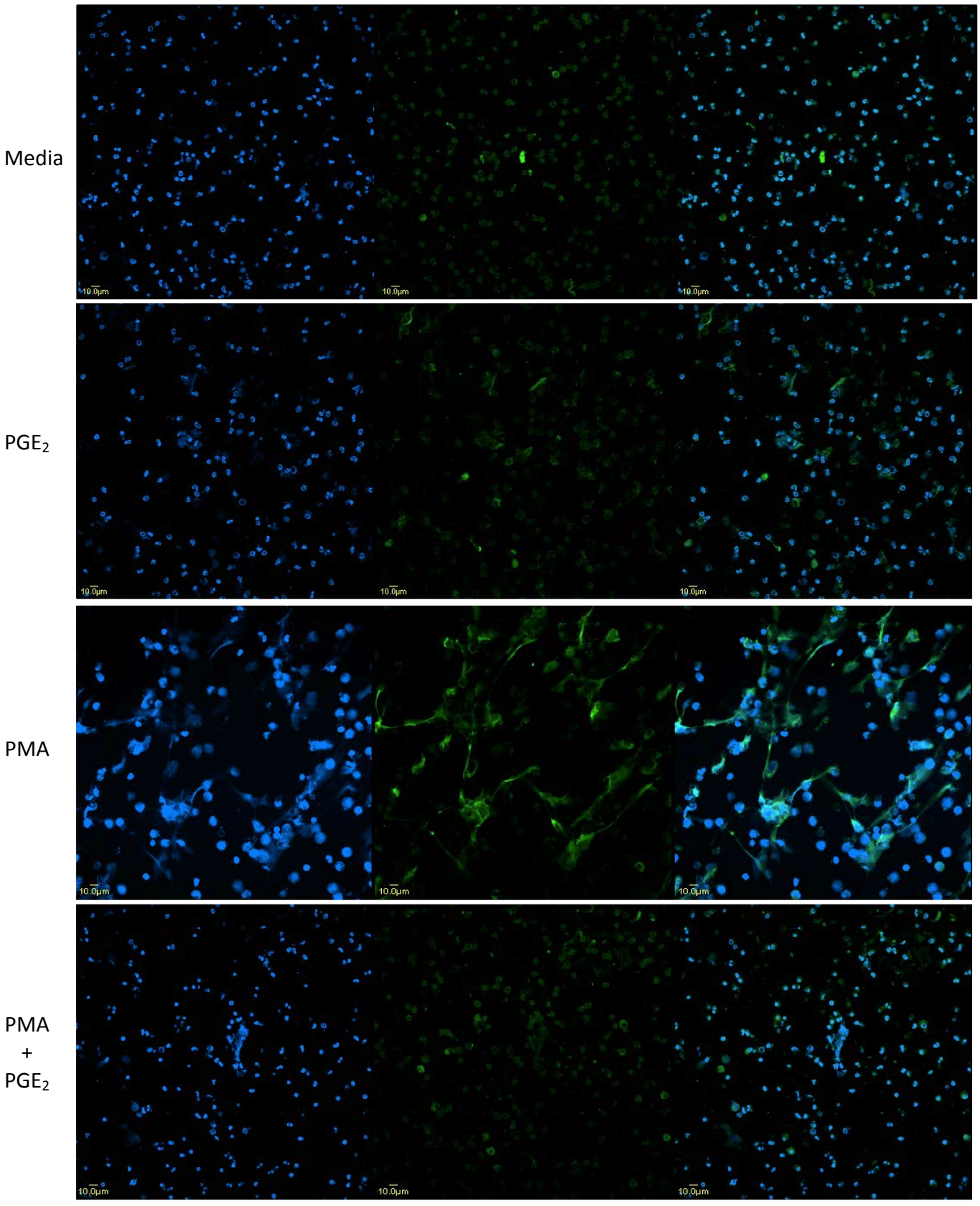
DNA
N. elastase
Colocalization
Media
PGE2
PMA
PMA
+
PGE2
10.0µm

Figure 6.6. PGE_2 inhibits PMA-induced NETosis in human PMNs. Human PMNs were harvested from n=4 healthy volunteers and stimulated with PGE_2 (10 μM), PMA (100 nM), PMA + PGE_2, or left untreated for 3 h. NETosis was measured by staining for DNA (Hoescht) and PMN elastase (N. elastase; FITC) and visualized via immunofluorescence (40x magnification).

6.3 Discussion

HSCT patients suffer from a myriad of infectious pulmonary complications, likely due to impaired alveolar macrophage and PMN function. PMN function has been shown to be particularly important for host response to pulmonary infection. We have previously shown that PMNs are unable to clear engulfed pathogens. Here we show that PMNs recruited to the lung exhibit impaired NETosis in response to PMA following either syngeneic or allogeneic BMT. Consistent with previous findings suggesting defective PMN function post-BMT is dependent on overexpression of COX-2 and PGE_2 signaling. Here we show that NETosis is similarly inhibited post-BMT, as inhibition of the COX pathway alone both *in vivo* and *in vitro* rescued impaired NETosis.

Multiple changes in the lung have been reported (e.g. elevated IL-6, GM-CSF; decreased TNFα, IFNγ, leukotrienes) (55, 59, 88, 208) which may contribute to immunosuppressive effects on PMN function and NETosis. Furthermore, induction of COX-2 may result in the overproduction of multiple downstream effectors (e.g. thromboxane). However, PGE_2 treatment decreased NETosis in control PMNs despite the presence of a known NET inducer, PMA, indicating that negative NETosis regulation via upregulation of COX-2 was mediated by PGE_2 signaling. This was further supported by the specific inhibition of the EP2 receptor using an EP2 antagonist. Both syngeneic and allogeneic PMNs exhibited rescued NETosis following a block in PGE_2-EP2 signaling. This was partly surprising as we previously measured EP4 receptor expression to be 4.7-fold higher in BMT PMNs while EP2 was 2.2-fold higher compared to untransplanted controls (58). However, we see that an EP2-specific antagonist was able to mediate a protective effect. Thus it is possible that inhibition by PGE_2 is mediated mostly through EP2, EP2 and EP4 are performing redundant inhibitory functions, or that blocking

signaling through EP4 along with EP2 may illustrate an additive effect. Still these data reveal PGE_2 as an important negative regulator of NETosis.

As PGE_2 intracellular signaling relies on PKA and Epac-dependent pathways (79), here we show that PGE_2 negatively regulates NETosis in mice through induction of the Epac pathway as an Epac agonist, but not a PKA agonist, inhibited NETosis similarly to PGE_2 treatment. Thus, these data illustrate a novel pathway by which NETs can be negatively regulated. Similarly, PMNs collected from the bronchoalveolar lavage of an autologous HSCT patient exhibit enhanced NETosis upon inhibition of PGE_2-EP2 signaling. Interestingly, PMNs collected from the peripheral blood of an allogeneic HSCT patient exhibited similar results. These data indicate that the negative effects of PGE_2 signaling are independent of the location of the PMN and the type of transplant. What was different between the murine and human studies however was that the inhibitory effects of PGE_2 are both Epac- and PKA-dependent in human PMNs. The reasons for this species divergence is currently unclear. We previously showed that hydrogen peroxide, a product of reactive oxygen species synthesis, was decreased in the murine lung post-BMT. As ROS production is required for NETosis, it is possible that Epac-dependent inhibition of NETosis is mediated through the inhibition of ROS. Recently, a renal ischemia-reperfusion study found that activation of Epac reduced mitochondrial ROS production via interaction with Rap1 in tubular epithelial cells (249). Thus it is possible that Epac signaling upstream of NAPDPH oxidase or mitochondrial ROS production mediates inhibition of NETs. Furthermore, the effect of PGE_2 and Epac on autophagy is unknown. Remijsen et al. 2011 reported the requirement for both ROS and autophagy in promoting induction of NETs. Thus, whether autophagy is defective in BMT PMNs remains to be determined. Additionally, whether there is differential regulation of these downstream pathways by Epac and PKA in humans remains to be determined.

Still, these observations implicate an important regulatory role for PGE_2 in NETosis. Additionally, they offer a potential therapeutic avenue by which NETosis can be inhibited, particularly in diseases whereby uncontrolled induction of NETs promote disease exacerbation, as in the case of systemic lupus erythematous (SLE) (250-252).

Chapter 7:

Conclusion

7.1 Summary of results

7.1.1 Scavenger Receptor Studies

The observations that human HSCT patients harbored defective AM function and that PGE_2 was elevated in circulation of HSCT patients, propelled our studies to characterize and investigate the link between these two observations (34, 83). Using a murine model, our lab previously showed that AM phagocytosis was impaired and that this was mediated by elevated PGE_2 in the lungs (58). These experiments were performed using *P. aeruginosa*, a Gram-negative bacterium. However, HSCT patients are also susceptible to Gram-positive bacteria like *S. pneumoniae* and *S. aureus* (208). Here we show differential phagocytosis of pathogens post-BMT is dependent on the pathogen. AM phagocytosis of *P. aeruginosa* was indeed decreased, as was previously published; however, *S. aureus* phagocytosis by BMT AMs was enhanced (60). These differences were attributed to dysregulation of scavenger receptors, particularly MARCO was reduced and SR-A was upregulated. These studies highlight the importance of MARCO for *P. aeruginosa* recognition and SR-A for *S. aureus* recognition. Importantly, we show that changes in scavenger receptor expression were due to the effects of PGE_2. PGE_2 was shown to decrease MARCO and increase SR-A, correlating with the profile obtained post-BMT (60).

In addition, we show that PGE_2 is able to mediate these effects by inhibiting miR-155, a negative regulator of SR-A (60). Blocking miR-155 through transfection of the antagomiR of

155 in primary AMs, increased SR-A mRNA levels, supporting a role for miR-155 in destabilizing SR-A mRNA. Interestingly, inhibition of miR-155 in primary AMs resulted in increased phagocytosis of *S. aureus.* Thus, PGE_2 signaling inhibits miR-155 and supports overexpression of SR-A. However, the mechanism for how PGE_2 mediates downregulation of MARCO remains unclear. Despite reduced phagocytosis, BMT mice remain more susceptible to *S. aureus*, a result that we found correlated with defective bacterial intracellular killing (60).

7.1.2 COX-2 hypomethylation studies

We have shown that AM and PMN dysfunction is mediated by overproduction of PGE_2 in the BMT lung (58). As PGE_2 overproduction is a result of increased COX-2, our studies focused on understanding the etiology for its upregulation. Using bisulfite conversion and pyrosequencing analysis, we show that COX-2 was significantly hypomethylated in its promoter and into the first exon (89). The effects of COX-2 DNA methylation were further explored by using a COX-2 promoter-driven luciferase plasmid. *In vitro* DNA methylation of the COX-2 promoter-driven luciferase plasmid silenced luciferase activity following transfection in MHS cells. Interestingly, DNA methylation-induced inhibition of luciferase expression was overcome by TGF-β, suggesting that TGF-β was able to demethylate the luciferase plasmid. That TGF-β was able to demethylate COX-2 was supported by bisulfite conversion and pyrosequencing studies using $CD11c^{dnR}$ BMT mice. $CD11c^{dnR}$ BMT AMs exhibited partially rescued DNA methylation of CpG sites that were significantly hypomethylated in WT BMT mice. Interestingly these same $CD11c^{dnR}$ BMT AMs exhibited rescued phagocytosis function, compared to untransplanted control and WT BMT AMs. Thus these studies showed that the overexpression of COX-2 post-BMT was mediated by dysregulation of the DNA methylation of CpG sites in COX-

2. Furthermore, changes in DNA methylation were due to TGF-β, previously shown in our lab to be overexpressed in BMT lungs (88, 253).

7.1.3 TGF-β and miR-29b studies

Our lab has shown that increased COX-2 and PGE_2 promote impaired AM responses (58). We now know TGF-β signaling post-BMT can promote hypomethylation of the COX-2 gene. Here, we provide mechanistic insight into how this occurs and show that TGF-β induces miR-29b while decreasing DNMT 1, DNMT 3a and DNMT 3b in AMs post-BMT. De novo DNMT 3a and 3b were decreased upon transient transfection of miR-29b, resulting in decreased DNA 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 transfection 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 PGE_2 as miR-29b-transfected AMs treated with an EP2 antagonist abrogated the impaired bacterial killing. We also demonstrate that HSCT patients exhibit increased miR-29b expression and also increased PGE_2 expression; thus, these studies highlight miR-29b in driving defective AM responses and identify this miRNA as a potential therapeutic target. Our AM findings, tying the projects together, are shown below, in a working model.

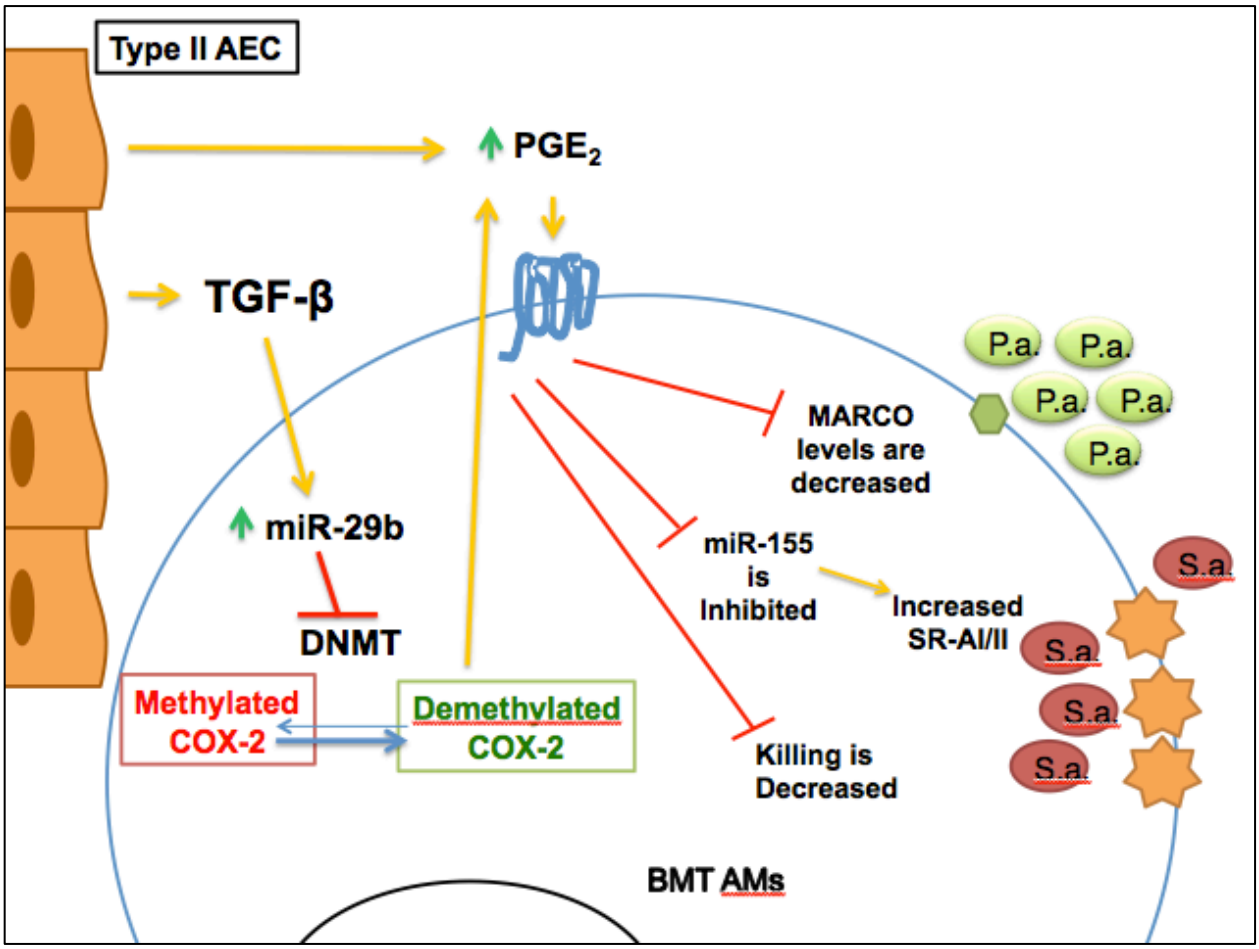

Figure 7.1. Effects of TGF-β-induced expression of COX-2 on BMT AMs: A model. Elevated TGF-β in the lung, likely produced by type II alveolar epithelial cells, signal to BMT AMs resulting in the upregulation of miR-29b. Elevated miR-29b targets DNMTs to allow for hypomethylation of COX-2. Decreased DNA methylation of COX-2 enhances COX-2 expression and induces PGE_2 synthesis. Increased PGE_2 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, decreased miR-155, impaired phagocytosis, and defective bacterial killing).

7.1.4 PGE_2 and NETosis studies

NETs have emerged as important DNA structures, decorated with granular proteins like myeloperoxidase and PMN elastase, with a role in trapping and killing invading pathogens (160). Defects in their production have been implicated to contribute to an impaired innate immune

response against bacterial infections (243). Furthermore, using our BMT model, we previously reported that PMNs over express COX-2 and PGE_2 and exhibit defective bacterial killing following BMT (58). To further characterize the defects in BMT PMNs, we measured their capacity to NET. Here we show that PMNs recruited into the lung following either syn or allo BMT exhibit a reduced ability to produce NETs upon treatment with PMA. This impaired NET formation was rescued following treatment with indomethacin, a pharmacological inhibitor of the COX pathway, both *in vitro* and *in vivo*. The defect is likely secondary to PGE_2 elevations post-BMT and signaling via the EP2 receptor.

Treatment of BMT PMNs with an EP2 receptor antagonist (PF-04418948) restored NET formation whereas exogenous PGE_2 treatment could limit NET formation in control PMNs. PGE_2 signaling involves PKA and Epac-dependent pathways. Interestingly, treatment with an Epac agonist, but not a PKA agonist, mimicked the inhibition on NETosis post-PMA in the same way as exogenous PGE_2 treatment in mice. Human PMNs also showed the ability of PGE_2 to limit NETosis, but in this case, inhibition could be mediated by both Epac and PKA. The reasons for this species-specific dichotomy are currently unclear. These findings suggest that enhanced activity of the COX pathway leading to PGE_2 elevations suppresses NET formation post-HSCT, thus providing significant insight into why HSCT recipients exhibit impaired innate immunity to bacterial pathogens.

7.2 Critical Review and Future directions

7.2.1 Scavenger receptor studies

Scavenger receptor studies demonstrate that MARCO is important for *P. aeruginosa* while SR-A is utilized for *S. aureus* recognition. Our studies have primarily focused on the measurement of MARCO levels at baseline, both mRNA and cell-surface expression, at which

time point expression of both is decreased. However, MARCO expression has previously been characterized as inducible (102, 181). Thus it is possible that upon infection, MARCO expression may increase and we may have missed this observation by only looking at baseline analyses. Thus, future experiments could include the careful analysis of MARCO expression (mRNA and protein) upon stimulation with bacteria or LPS post-BMT.

SR-A expression has been linked to promoting polarization of macrophages to an alternatively activated (M2) phenotype (254, 255). Prototypical M2 macrophages harbor an overall anti-inflammatory phenotype and are important for wound healing (5). Upregulation of SR-A in M2 macrophages is thought to facilitate apoptotic cell clearance (5), however how it might negatively regulate macrophage clearance of intracellular bacteria remains unclear. Furthermore, whether AMs post-BMT harbor a phenotype aligned with classical or alternatively activated macrophages has remained unexplored and may provide interesting insight into the mechanisms driving decreased immune responses in the lung. Finally, SR-A expression was upregulated upon transfection of antimiR-155. Our data suggested that PGE_2-mediated decrease in miR-155 resulted in the overexpression of SR-A. However, further experiments could have fortified this argument by investigating whether overexpression of miR-155 in BMT AMs decreased SR-A expression and *S. aureus* phagocytosis compared to control miR-transfected BMT AMs.

The work presented here highlights the importance of miRNA in macrophage function. We showed that miR-155 expression is decreased while miR-29b is increased. We reported that decreased miR-155 resulted in increased SR-A and increased *S. aureus* phagocytosis by BMT AMs. Recently, it has been shown that miR-155 can induce autophagy by targeting Rheb, a negative regulator of autophagy (256). Autophagy is a tightly controlled self-degradative process

utilized by host cells to maintain cellular homeostasis (257). It has also been shown to play a role in host responses, promoting engulfment and degradation of intracellular bacteria that may otherwise escape detection (i.e. *Mycobacterium tuberculosis*) (256, 258). Furthermore, miR-155 was also shown to promote phagosome maturation (256). Thus, future studies could explore whether autophagy defects that are secondary to miR-155 loss may also help to explain the inhibition of bacterial killing that occurs post-BMT. In fact, there is recent evidence that autophagy is critical for killing of *P. aeruginosa* (259) and thus, how PGE_2 regulates autophagy could be an important area for future study.

Type II alveolar epithelial cells produce and secrete pulmonary surfactant, a mixture of phospholipids, cholesterol, and proteins (260). The proteins associated with pulmonary surfactant are surfactant proteins (SP) A, B, C, and D, which can be subdivided into hydrophilic and hydrophobic classes (260). However, the most abundant surfactant protein is SP-A. SP-B and SP-C have been more closely associated with promoting adsorption of surfactant lipids onto the lung surface (260, 261). Aside from their role in lowering surface tension in the alveolus, SP-A, as well as SP-D, have more recently been shown to play important roles in modulating immune responses (262) via recognition of inhaled pathogens. Interestingly, SP-A knockout mice exhibit increased susceptibility to various bacterial (e.g. *P. aeruginosa*) and viral (e.g. respiratory syncytial virus) pathogens (263-266). It has been shown that SP-A can function in an opsonin-like fashion through the recognition of carbohydrates present on pathogens and interaction with macrophages to promote immune responses (267). In addition, Kuronuma and colleagues showed SP-A but not SP-D enhanced uptake of *S. pneumoniae* in AMs via interaction with SR-A (92). As SR-A is enhanced on BMT AMs, it is possible that opsonizing bacteria that are not normally dependent on SR-A for phagocytosis with SP-A may prove beneficial. Thus,

future studies may explore the possibility of utilizing SP-A as a method for enhancing uptake of *P. aeruginosa* by BMT AMs through SP-A and SR-A interaction.

Previous studies have shown that SP-A binds *P. aeruginosa* flagellin and enhances uptake of the bacteria (268-270). Furthermore, it is well-known that SP-A can induce respiratory burst in rat and human macrophages (91, 271, 272). It is possible that addition of surfactant may not only promote effective phagocytosis of *P. aeruginosa,* but also activation of macrophages, as previous data in our lab showed that BMT AMs have impaired respiratory burst. Currently surfactant preparations are given to pre-term infants to improve lung ventilation, but at this time the preparation that is approved for human use contains only the lipid components and not the protein components. Our work suggests that a surfactant therapy that was composed of both lipid and SPA could improve host defense in these infants. Potentially, HSCT patients might only need treatment with the nebulized SPA as no current evidence suggests that the lipid fraction of surfactant is dysregulated in this more mature population.

7.2.2 Hypomethylation studies

These data explore the importance of DNA methylation as a regulator of COX-2 expression. TGF-β is also identified as a cytokine driving the demethylation of COX-2 as DNA methylation of COX-2 is maintained in $CD11c^{dnR}$ BMT mice. However, BMT mice have multiple dysregulated pathways. Therefore, to clarify the role of TGF-β, studies measuring COX-2 DNA methylation using untransplanted $CD11c^{dnR}$ mice and control mice treated with or without TGF-β would fortify the BMT findings. Furthermore, we had previously described that BMT mice exhibit higher susceptibility to both *P. aeruginosa* and *S. aureus.* If TGF-β does in fact mediate COX-2 expression, then $CD11c^{dnR}$ BMT mice should be protected and exhibit intact immune responses against both pathogens. While we studied TGF-β as a regulator of miR29

expression, we did not determine whether TGF-β also contributed to the downregulation of miR-155. Perhaps the biggest weakness with the hypomethylation data is that the changes noted in percent DNA methylation of each CpG residue were relatively small. It is hard to know what percent of DNA methylation is functionally important. It is also hard to know, from our work, whether all BMT AMs showed a similar degree of DNA methylation or whether we were studying a population with heterogenous DNA methylation patterns (some cells showing hypomethylation, but others not). Future studies exploring the effects of individual or combinatorial mutation of these CpG sites in the context of BMT, TGF-β, and miR-29b transfection would provide insight into which CpG sites are critical sites affected by changes in DNA methylation that regulate COX-2 expression. This could be done by creating site directed mutations in particular CpG sites and then testing expression plasmids carrying mutated promoters for activity following transfection of AMs or MHS cells.

Kang et al. showed that the CRE-2 site within the COX-2 promoter was an important regulatory site for COX-2 expression (231). Interestingly, the CRE-2 site and not the other sites (e.g. AP-1, CRE-1, NF-kB) that have been previously characterized coincided with the CpG sites that were significantly hypomethylated post-BMT and in response to miR-29b transfection and 5-aza-2'deoxycytidine treatment. Future studies could explore whether the hypomethylation of these CpG sites supports COX-2 transcription from this CRE-2 site. This could be achieved by mutating the sequence of the CRE-2 site without disturbing the CpG sites, and measuring COX-2 expression.

7.2.3 MiR-29b studies

The human cohort used in these studies measures miR-29b expression in HSCT patients

relative to non-smoker healthy human controls. However, the smoking status in the HSCT population is not known. A precedent study suggests miR-29b can be altered in response to smoking (273), however this study focused on the effects of smoking on plasma microvesicles and miRNA (including miR-29b), and did not extend their studies to cells within the lung. Thus, future studies could carefully select control samples and HSCT patient samples to exclude any factors that may confound the results while at the same time providing beneficial information on miR-29b post-HSCT.

The murine BMT studies identify a miR-29b-dependent pathway in mediating TGF-β effects on COX-2 DNA methylation. Most of the literature reveals an inhibitory effect of TGF-β on miR-29b; however, these studies were not performed using AMs (218-220). Thus, experiments exploring TGF-β induction of miR-29b could have been characterized in multiple macrophage populations (e.g. bone marrow-derived, peritoneal, etc.) to see if this phenomenon is cell-type or tissue specific or both. TGF-β intracellular signaling via SMADs and their effects on miR-29b may elucidate a more specific pathway by which TGF-β can upregulate miR-29b in some cell types but not others. Perhaps in other cell types inhibitory SMAD7 is induced, whereas this does not occur in AMs. Furthermore, as miR-29b is elevated post-BMT, studies utilizing loss-of-function models of miR-29b may provide further proof that miR-29b is critical for TGF-β-mediated induction of COX-2.

Classical knockout mice of miR-29b (274) could have been utilized as donor HSCs and TGF-β induction of COX-2 could have been further analyzed. If TGF-β were still able to induce COX-2 expression even in a miR-29b-/- mouse, this would suggest that there are other pathways by which TGF-β exerts its effects. Most likely this would be at the level of increasing gene transcription, but potentially TGFβ could also regulate mRNA stability to increase COX-2 levels.

Alternatively, use of GS29 mice (transgenic mouse expressing a 'sponge' target to compete with endogenous miR-29b targets), first described in 2011 by Feng and colleagues (158), could be used as donors for stem cell transplantation and COX-2 expression and promoter methylation could be analyzed.

We also explored the effects of miR-29b on COX-2 DNA methylation. We reported that miR-29b induces small but significant hypomethylation of CpG sites 1-6 but not 28-34. Interestingly, $CD11c^{dnR}$ BMT AMs exhibited hypomethylation of CpG sites 28-34, however, sites 1-6 were not examined, as primers measuring sites 1-6 had not yet been designed. Thus, studies measuring DNA methylation of CpG sites 1-6 in $CD11c^{dnR}$ BMT AMs are warranted to confirm how TGF-β-induced miR-29b ultimately affects COX-2 expression. Furthermore, miR-29b-specific changes in COX-2 post-BMT may be supported by DNA methylation studies following transfection of BMT AMs with antimiR-29b. Still, taken together, the data we have generated do suggest that perhaps CpG sites 1-6 are the more important sites mediating COX-2 induction. It is also curious that our DNA methylation studies identified two independent regions for regulation by TGF-β, one in the upstream promoter and one near exon 1. One site is regulated by miR-29b, but the other is not. Given that miR-29b inhibits DNMTs, it is hard to know why there would be sequence specificity. One possibility is that TGF-β is a much stronger stimulus to inhibit DNMTs than is miR-29b, thus the magnitude of DNMT suppression may be greater in response to the cytokine, thus leading to both sites being targeted. Alternatively, perhaps the induction of gene transcription or translation near the first exon that is triggered by TGF-β stimulation creates a more accessible site for DNMT action near the first exon. Transfection of miR-29b would not necessarily be expected to upregulate the same transcriptional and translational machinery that TGF-β does. It is also possible that the

downstream CpG sites are not as important in mediating COX-2 expression as the upstream CpG sites within the COX-2 promoter. This would be supported by the fact that miR-29b transfection was able to induce COX-2 mRNA and protein by hypomethylating CpG sites 1-6 and not CpG sites 28-34. Future experiments could potentially investigate the importance of these different CpG sites as described in the future directions section for the hypomethylation studies.

Overexpression of miR-29b post-BMT indirectly supports increased COX-2, and ultimately promotes PGE_2 expression and the downstream effects of PGE_2 (e.g. impaired bacterial killing) on BMT AMs. However, miR-29b can have other downstream targets that may also be important for propagating AM function in response to infection. Recently, a study investigating mechanisms driving chemoresistance and relapse of ovarian cancer identified a correlation between miR-29b and autophagy-related protein 9A (ATG9A) (275). Furthermore, a bioinformatic analysis predicting possible targets for miR-29b identified ATG9A (276). However, neither study confirmed ATG9A was in fact a target for miR-29b or studied miR-29b effects on autophagy. ATG9A was recently shown to be required for the formation of the isolation membrane, later developing into the autophagosome (277). Thus, it is possible that overexpression of miR-29b in AMs post-BMT may inhibit ATG9A. In a cell with the inability to initiate the production of an isolation membrane, autophagy is inhibited, and this may be a mechanism that also impairs bacterial killing.

Alternatively, miR-29b can be inhibiting autophagy through a separate pathway. In 2012, Tumaneng et al. described their novel findings on the molecular mechanism associated with Yes-associated protein (YAP) 1, a transcriptional co-activator in the Hippo pathway, and its regulation on organ size (278). They suggested that miR-29b induces mammalian target of rapamycin, or mTOR, activity. mTOR is a known inhibitor of autophagy (279, 280), thus miR-

29b upregulation of mTOR may be another mechanism causing defective autophagy in BMT AMs, and as we speculated above, autophagy may be critical to bacterial killing. Future studies could focus on delineating the mechanism(s) driving PGE_2-mediated inhibition of autophagy. Overcoming this negative regulation may rescue impaired bacterial killing of bacteria like *S. aureus.*

7.2.4 NETosis studies

NETosis studies described here utilize PMNs recruited to the lung following intratracheal injection of LPS. It has been previously shown that LPS can induce NETosis, thus our method of recruiting PMNs to the lung may induce NETosis on its own. This highlights a limitation to this study, as PMNs do not commonly exist in the lung without recruitment. To get around this, one possibility would be to measure NETosis *in vivo* in response to NET-inducing stimulation, i.e. using killed or live bacteria, and exogenous PGE_2, to measure PGE_2 inhibitory effects on NETosis. NETs could then be determined via immunohistochemistry on formalin-fixed lung sections and stained for NET-associated proteins like myeloperoxidase and neutrophil elastase, as well as histones (281). These *in vivo* studies would also provide insight into the importance of NETosis in BMT and untransplanted control mice in providing protection against invading pathogens. Another possibility would be to harvest PMNs from the bone marrow. Although these cells would not be exposed to the lung-specific environment, this would allow for the study of PGE_2 effects on NETosis in the absence of a stimulatory agent, like LPS.

Quantification of NETs in this study has been particularly difficult to assess, as most of the existing methods for measuring NETosis are indirect. Furthermore, distinguishing cell death versus NETosis can be particularly challenging and requires immunofluorescence imaging for the identification of NET formation. One way to differentiate between NETosis and other modes

of cell death could be through the measurement of MPO, NE, or histones (NETosis), and caspase activation, Bid cleavage, cytochrome c release, or and HMGB1 (apoptosis and necrosis) (282). Recently, Brinkmann and colleagues described a semi-automatic protocol for NET quantification (239). This method involves the use of anti-chromatin antibodies and relies on the observations that these antibodies bind decondensed chromatin more efficiently than condensed chromatin. Relating detection of this subnucleosomal complex with the anti-chromatin antibodies to total cell number through Hoescht 33342 staining allowed the quantification of the percentage of netting PMNs. Thus, this may be another way in which NETs can be directly quantified through immunofluorescence studies.

Here we show how PGE_2 negatively regulates NETosis in BMT PMNs and untransplanted control PMNs. Our studies also reveal that this inhibition is Epac-dependent in mice. However, how PGE_2 is able to promote NETosis through Epac is not entirely clear. As ROS synthesis is decreased in BMT PMNs, it is possible that PGE_2 is able to inhibit ROS. ROS is required for proper NETosis, thus this may be a mechanism by which PGE_2 inhibits NETs. We would also need to determine whether regulation of ROS is differentially affected by Epac and PKA in humans, as both of these mediators inhibited NETosis in human PMNs. Alternatively, a role for autophagy in mediating NET production has recently been proposed. Inhibition of autophagy, despite intact NADPH oxidase activity, impaired NET induction (248). Thus, it is possible PGE_2 may be downregulating autophagy in PMNs post-BMT. Preliminary studies investigating autophagy-induced NETosis in our BMT model revealed that syn BMT PMNs were unable to NET as efficiently as untransplanted control cells upon rapamycin treatment. Rapamycin targets mTOR and relieves inhibition of autophagy promoted by mTOR (Figure 7.2). Thus, these preliminary data support a possible role for PGE_2 in blocking autophagy.

Furthermore, studies exploring Epac-dependent effects on ROS and autophagy would be beneficial in understanding the defect in BMT PMNs. Focus in this area may identify possible pathways that can be targeted in the future. It should be noted however that PMNs present a particularly tough cell type for studies of autophagy as the inducer of NETosis due to their short half-life in culture.

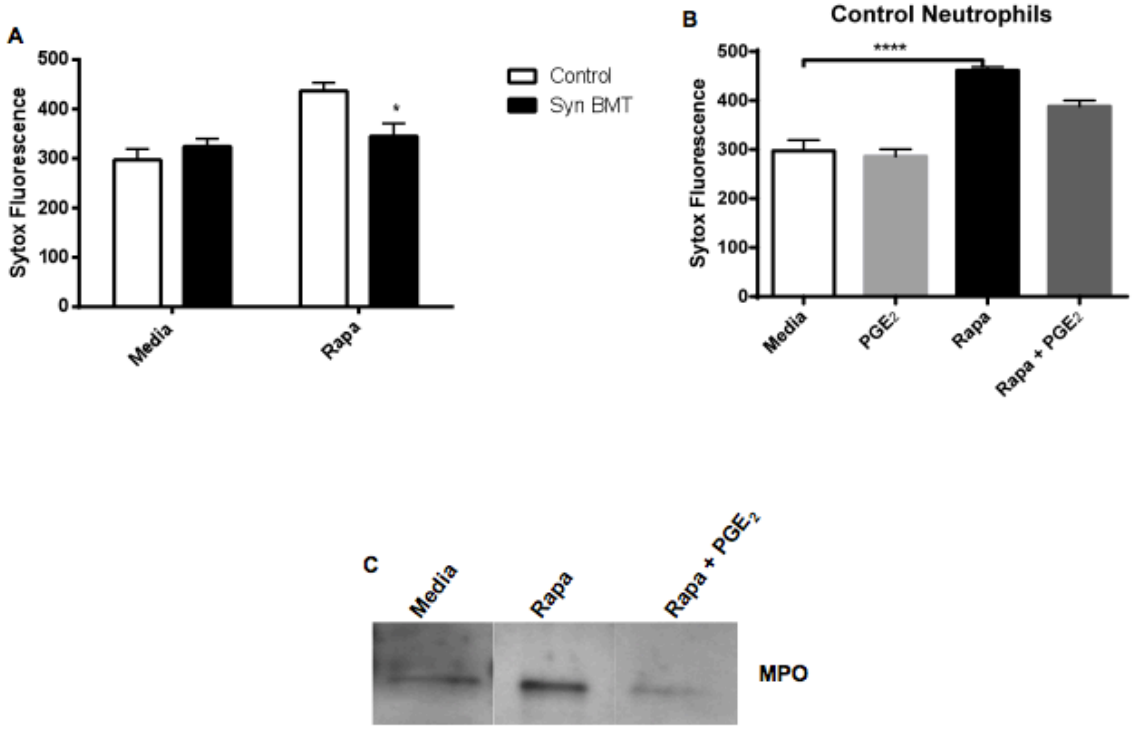

Figure 7.2. PGE_2 inhibits autophagy-induced NETosis. (A) LPS-recruited PMNs from the lungs of Syn BMT and untransplanted control mice were treated with rapamycin (200 nM) or untreated (media) for 5 h and NETosis was measured via Sytox fluorescence (n=6-12 wells). (B) Untransplanted control PMNs were collected from BAL of LPS-challenged mice and treated with PGE_2 (10 μM), rapamycin (200 nM), or both. NETosis was measured via Sytox fluorescence (n=4-6 wells). (C) LPS-recruited PMNs from untransplanted control mice were treated with rapamycin (200 nM) or rapamycin and PGE_2 (10 μM) for 5 h prior to treatment of

the supernatants with DNase I, acetone precipitation, and western blot analysis; *$p<0.05$, ****$p<0.0001$.

7.3 Contributions to the field/Final remarks

Taken together, our studies use a murine BMT model to explore mechanisms in BMT AMs and PMNs that contribute to host susceptibility to infections. We show the importance of SRs in mediating phagocytosis of *P. aeruginosa* and *S. aureus*, pathogens that afflict HSCT patients. Furthermore, we show that miR-155 is a negative regulator of SR-A and that in the context of transplantation, overexpression of PGE_2 inhibits miR-155, allowing elevated SR-A. This novel finding has implications in other disease models as miR-155 is a multifunctional miRNA and has been shown to be important for development of immune cells, initiation of immune responses, as well as macrophage polarization (125, 147). We also explore BMT PMN NETosis and identify a novel role for PGE_2 in negatively regulating NET induction. As the defects observed in BMT AMs and PMNs are mediated through the upregulation of COX-2 and PGE_2, the etiology of COX-2 overexpression was also investigated. Our findings highlight the importance of TGF-β and miRNA in epigenetic regulation of gene expression and promoting dysfunction of BMT AMs.

TGF-β is a pleiotropic cytokine and targeting TGF-β may have adverse effects on patients as TGF-β action is context-specific and cell-specific (283). Reductions in TGF-β may induce autoimmunity. Here we show the importance of TGF-β1 in modulating BMT AM immune responses. Thus, while our results suggest that anti-TGF-β therapies may be beneficial, they must be entered into with caution. Therapies targeting different components of the TGF-β signaling pathway as well as TGF-β isoforms are under development or in clinical trials (283). Along with infectious complications, pulmonary pneumonitis and fibrosis also occurs in HSCT

patients (41). Aberrant TGF-β1 production by alveolar epithelial cells promotes fibrotic responses and severely compromises lung function (88, 284). Thus, potential targeting of TGF-β in HSCT patients could have two-fold benefit (improved immunity and reduced fibrosis) but as mentioned before, would require careful targeting. Differential targeting of TGF-β isoforms would likely decrease the magnitude of adverse effects and inhibition of TGF-β1 expression or utilization of drugs like LY2383770 which is a TGF-β1 ligand-selective blocking antibody from Ely Lilly rather than blocking TGF-β signaling as a whole may provide more specific protection.

As TGF-β targeting strategies remain to be optimized, here we identify multiple other potential targets for therapeutic development for the enhancement of immune responses that may prove beneficial to HSCT patients. Use of indomethacin or COX-2 inhibitors may reverse the immunosuppressed state of AMs in HSCT patients and provide protection against the development of bacterial pneumonias. Alternatively, use of the EP2 antagonist examined in these studies would similarly augment HSCT AM immune responses. Furthermore, we previously discussed the importance of SP-A in opsonizing bacteria and enhancing bacterial uptake by macrophages. Thus, utilizing SP-A to promote phagocytosis of *P. aeruginosa* by BMT AMs through SP-A and SR-A interaction may be a useful method to overcome decreased uptake of pathogens that do not depend on SR-A for engulfment. MiRNAs comprise a new class of RNAi that contain attractive properties as targets for therapeutic development. Although therapies targeting miR-29b specifically remain in their infancy, there is promise in this area highlighted by the development of Miravirsen (SPC3649), an antimiR drug candidate that inhibits miR-122. Miravirsen is currently in clinical trials for the treatment of hepatitis C virus (285). Thus, the work highlighted here provides multiple avenues by which HSCT AM immune defects may be overcome and productive immune responses promoted.

Finally, perhaps the most important thing that can be done next is to advance all of our murine studies into AMs and PMNs isolated from HSCT patients. While we provide data that miR29 is regulated in HSCT patients, we have not yet verified that other changes, e.g. MARCO expression are as expected in human HSCT patients. If we determine that human AMs and PMNs mirror the defects noted in our BMT studies (as we anticipate they will) we will be well poised to move forward with experiments to determine drug efficacy.

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