Impaired Pulmonary Host Defense Against *Pseudomonas aeruginosa* Following Bone Marrow Transplantation

by

Leah L. N. Hubbard

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Doctoral Committee:

Associate Professor Bethany B. Moore, Chair
Professor Nicholas W. Lukacs
Professor Wesley Dunnick
Associate Professor Eric S. White
Assistant Professor David Aronoff

To my family and Sophia

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AC	adenylyl cylcase
AM	alveolar macrophage
AP-1	activator protein 1
APC	antigen presenting cell
ATP	adenosine triphosphate
BAL	bronchoalveolar lavage
BMT	bone marrow transplantation
C	complement
cAMP	cyclic adenosine monophosphate
CKO	conditional knockout
CM	complete media
COX	cyclooxygenase
CR	complement receptor
CRAMP	cathelicidin-related antimicrobial peptide
Cys-LT	cysteinyl leukotriene
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EP	E prostanoid
EPAC	exchange protein activated by cAMP
FcγR	Fc gamma receptor
GPCR	G protein coupled receptor

GVHD	graft-versus-host disease
HSCT	hematopoietic stem cell transplant
i.t.	intratracheal
Ig	immunoglobulin
IKK	IκB kinase
IL	interleukin
IRAK	interleukin-1 receptor associated kinase
KO	knockout
LT	leukotriene
MAP	mitogen activated protein
MARCO	macrophage receptor with collagenous structure
MBL	mannose-binding lectin
MIP	macrophage inflammatory protein
MyD88	myeloid differentiation primary response gene 88
NFκ-B	nuclear factor κ B
NK	natural killer
NLR	NOD-like receptor
NO	nitric oxide
PAMP	pathogen-associated molecular pattern
PG	prostaglandin
PI3K	phosphatidylinositol 3-kinase
PIP_2	phosphatidylinositol-4,5-bisphosphate
PIP_3	phosphatidylinositol-3,4,5-triphosphate
PRR	pattern recognition receptor
PTEN	phosphastase and tensin homolog on chromosome 10

ROS	reactive oxygen species
SFM	serum-free media
SHIP	src homology 2-containing inositol phosphatase
SP	surfactant protein
SRBC	sheep red blood cell
TBI	total body irradiation
TLR	toll-like receptor
TNF	tumor necrosis factor
TRAF6	tumor necrosis factor receptor associated factor 6
TREM	triggering receptor expressed on myeloid cells
TRIF	TIR-domain-containing adaptor inducing interferon-β
WT	wild type

ABSTRACT

Hematopoietic stem cell transplant (HSCT) patients are especially susceptible to lung infections, despite immune reconstitution. In a mouse model of syngeneic bone marrow transplantation (BMT), we previously reported that prostaglandin E_2 (PGE_2) is overproduced in lungs of BMT mice, significantly impairing pulmonary host defense against *Pseudomonas aeruginosa*. This impairment in pulmonary host defense post-BMT is also marked by diminished alveolar macrophage (AM) function. These data were generated using total body irradiation (TBI) as a means to ablate host bone marrow. Therefore, we wanted to determine whether the use of clinically relevant chemotherapy regimens to ablate host bone marrow would result in similar findings. We compared donor-cell reconstitution, pulmonary host defense, and PGE_2 production among control and BMT mice conditioned with either TBI or chemotherapy. Dual chemotherapy conditioned mice had a lower frequency of donor-derived cells compared to TBI; however, both groups displayed similar impairment in AM host defense and bacterial clearance following *P. aeruginosa* pneumonia. Furthermore, impairment in AM host defense was directly related to overproduction of PGE_2 in both groups. These data suggest that compared to TBI, use of chemotherapy conditioning results in similar PGE_2-mediated suppression of pulmonary host defense in BMT mice, despite reduced efficiency of donor-cell reconstitution. To determine a mechanism for PGE_2-mediated suppression of pulmonary host defense post-BMT, roles for inhibitory molecules interleukin-1 receptor associated kinase (IRAK)-M and phosphatase and tensin homolog

on chromosome 10 (PTEN) were examined. We found that IRAK-M expression and PTEN phosphatase activity were elevated in BMT AMs relative to control AMs, and this was related to AM overproduction of PGE_2. Furthermore, genetic ablation of IRAK-M in the bone marrow of BMT mice restored host defense against *P. aeruginosa*. Similarly, WT mice transplanted with myeloid-specific PTEN knockout (KO) bone marrow also displayed improved host defense against *P. aeruginosa*. Despite overproduction of PGE_2, AM phagocytosis, killing, and production of proinflammatory mediators were restored in the absence of either IRAK-M or PTEN post-BMT. Overall, these data demonstrate that the absence of either IRAK-M or PTEN in the hematopoietic compartment post-BMT enhances pulmonary host defense and mitigates AM sensitivity to the inhibitory effects of PGE_2.

CHAPTER 1: Introduction

The Mammalian Immune System

The mammalian immune system is a complex network of innate and adaptive immune cells which arise from hematopoietic stem cell progenitors in the bone marrow (1, 2). Innate immune cells are derived from myeloid and lymphoid progenitors and consist of macrophages, monocytes, dendritic cells, natural killer (NK) cells, and granulocytes such as neutrophils, eosinophils, and basophils. T and B lymphocytes make up the adaptive arm of the immune system and are derived from lymphoid progenitors (1, 2). The purpose of the immune system is to discriminate between self and non-self antigens and to eliminate anything recognized as non-self via cell-mediated and humoral responses. In the event that a particular antigen is recognized as non-self, such as an invading pathogen or tumor antigen, the host elicits an inflammatory response mediated initially by antigen presenting cells (APCs) of the innate immune system (such as dendritic cells and tissue macrophages) (3, 4). Pathogen-associated molecular patterns (PAMPs) on microbes, as well as certain endogenous proteins released from tumors and damaged tissues, engage pattern recognition receptors (PRRs) expressed on the surface of APCs. Binding of these "danger signals" to PRRs activates a number of signaling pathways to induce transcription and subsequent release of proinflammatory cytokines and chemokines (3, 5). Consequently, this proinflammatory milieu recruits more innate

immune cells to the site of infection (such as neutrophils or eosinophils) and facilitates activation of the adaptive immune system for antigen-specific clearance of pathogen and the resolution of tissue damage (6).

Hematopoietic Stem Cell Transplantation

Malignancy or defective function of bone marrow and bone marrow-derived cells can severely disrupt multiple aspects of immune homeostasis, responsiveness, and resolution. Examples of such disorders include: severe combined immunodeficiency, leukemia, sickle cell anemia, glycogen storage disease, and multiple myeloma (7). Treating such malignant and non-malignant disorders of the immune system often requires hematopoietic stem cell transplantation (HSCT).

The process of HSCT involves ablation of host bone marrow and reinfusion of the host with his/her own bone marrow (autologous HSCT), genetically identical bone marrow (syngeneic HSCT), or bone marrow from a histocompatible, HLA matched donor (allogeneic HSCT) (8). Preparative regimens used to ablate host bone marrow typically involve a fractionated dose of radiation (known as total body irradiation; TBI), treatment with high dose chemotherapy drugs (such as cyclophosphamide and/or busulfan), or a combination of both TBI and chemotherapy (7). In most cases, ablating the host bone marrow via these methods eradicates the underlying disease or malignancy of the immune system and allows for engraftment of transplanted donor stem cells. HSC grafts are composed of immature and mature hematopoietic cells and can be obtained directly from the bone marrow, umbilical cord blood, or peripheral blood stem cells mobilized from the bone marrow (7, 8).

Effectiveness of engraftment (or potency) is dependent on the amount of cells present in the graft that express the CD34 antigen— a well characterized cell surface marker for cells of hematopoietic origin. Moreover, immune potency is determined based on the number of certain immune cell types present in the graft including: CD3 cells, natural killer cells, and dendritic cells (9). The greater the number of these immune cell populations within the graft, the faster the kinetics of immune reconstitution. In allogeneic HSCT, T cell depletion is often performed to reduce the risk of graft-versus-host disease (GVHD), a condition where donor T cells recognize certain host minor and major MHC antigens as foreign and mount an immune response against host cells expressing these antigens. However, depletion of donor T cells can lead to other complications, such as increased risk of graft rejection and delayed reconstitution of T and B cells (9).

Immune Reconstitution and Functional Recovery

Following HSCT, donor-derived stem cells undergo hematopoiesis to reestablish the entire immune system. The innate immune compartment is the first to emerge following HSCT (8, 10, 11). Neutrophils are the first leukocytes to recover between weeks two and four post-HSCT; however, restored host defense function in neutrophils is not achieved until as early as two months post- HSCT (8). Full reconstitution of monocytes and tissue macrophages ranges between several weeks to months post-HSCT. Monocyte numbers are restored around day 41 post-HSCT, and tissue macrophages (such as in the lung) are restored nearly three months post-HSCT (12). The kinetics of DC reconstitution varies greatly depending on the HSC donor source and the presence of

GVHD (13-15). DC subsets can be found as early as two weeks post-HSCT; however, the functional recovery of donor-derived DCs is not well characterized. NK cells recover in cellularity between one to two months post-HSCT, and functional recovery is reported to coincide with complete reconstitution of this particular immune cell type (8, 10, 12).

The kinetics of reconstitution and the functional recovery of B cells and T cells are fairly well described. B cell numbers are reestablished three months post-HSCT; however, immunoglobulin (Ig) production to new and previously encountered antigens is not fully restored for as long as two years post-HSCT (10, 16). Mature CD3 T cells begin to emerge approximately 15 days post-HSCT, and like B cells, reach normal counts by three months post-HSCT. However, in terms of T cell subsets, CD4 T cell numbers are decreased relative to non-transplant patients and do not reach counts that can provide effective immunity for as long as six to twelve months post-HSCT (11). CD8 T cell reconstitution occurs more rapidly than CD4 T cell reconstitution, with total counts recovering within a few months following HSCT (8, 10). As with certain innate immune cell types, functional recovery of B and T cell subsets can vary depending on the HSC donor source, T cell depletion of graft, and the presence GVHD (8).

Outcomes of HSCT

Although there is significant therapeutic benefit from use of HSCT to treat malignant and genetic abnormalities of the immune system, the success of HSCT is often limited due to a number of pre- and post-transplant related complications (17). In particular, patients face a number of infectious complications following HSCT, due to

delays in immune reconstitution, defective immune function, onset of GVHD, and use of immunosuppressive drug therapy (7, 8). Furthermore, use of TBI and chemotherapy preparative regimens typically induces many of the non-infectious complications observed in HSCT patients, such as severe organ toxicity (7). The 100 day mortality rate has been reported to range from 10 to 40% in allogeneic transplant patients. Mortality in these patients results mainly from GVHD, multiorgan failure, and interstitial pneumonitis (18). Autologous transplant recipients have a 100 day mortality range of 5 to 20% with the major causes of death being relapse of underlying disease and second cancer (18). Infections are reported in about half of all patients within the first two years after HSCT, and mortality rates can be as high as 50% depending on the type of infection, HSC source, and the presence of GVHD (19).

Non-Infectious Complications of HSCT

Non-infectious complications following HSCT can be either acute or chronic and often result from GVHD or toxicity caused by the type of conditioning regimen used (7). One major non-infectious complication is mucositis. Mucositis is painful ulceration of the mucous membranes of the intestinal tract and oral cavity and affects the majority of HSCT patients receiving TBI, high-dose chemotherapy, and certain anti-GVHD drug therapies (20, 21). Sinusoidal obstructive syndrome of the liver is another major non-infectious complication (22), where conditioning-induced damage of venules and sinusoids leads to obstruction of hepatic circulation, hepatocyte injury, and multiorgan failure (22). Non-infectious pulmonary complications are also a significant problem following transplant and can include: idiopathic pneumonia syndrome, bronchiolitis

obliterans, and pulmonary edema (18). GVHD is another serious complication of allogeneic HSCT and may occur in the gastrointestinal tract, skin, or lungs (7). Major consequences of acute GVHD include: gastrointestinal problems, jaundice, rash, and delayed immune reconstitution and responsiveness to infection. On the other hand, chronic GVHD is characterized by malabsorption, loss of self-tolerance, and generalized immunosuppression (7, 23). Moreover, steroids and other drugs used to suppress T cell responses during GVHD cause severe immunodeficiency and further increase the risk of infection (24-26).

Infectious Complications of HSCT

Infectious risk and type of infection following HSCT are generally dictated by the HSC source, conditioning regimens, the presence of GVHD, and post-transplant therapy (9). Specific infectious risks are divided among three phases following HSCT: pre-engraftment (day 0 to 30), early post-engraftment (day 30 to 100), and late post-engraftment (day 100 and beyond) (9). Pre-engraftment is characterized by neutropenia (a severe deficit in the number of functional neutrophils) (9, 17) and mucosal injury (7). These conditions increase the risk for both Gram-negative and Gram-positive bacterial infections of the lung, skin, urogenital and intestinal tract, and oral cavity (9). In particular, the mortality rate for bacterial infections of the lung is especially high, ranging from 22 to 29% for BMT patients (27). Nearly half of all bacterial infections post-HSCT are Gram-negative infections, and are predominately caused by *Pseudomonas aeruginosa* and *Klebsiella pneumonia* (9). Of the Gram-positive bacterial infections in HSCT patients, nearly half are caused by *Staphylococcus epidermidis*, one third are caused by

viridians streptococci, and the remaining are caused by *Staphylococcus aureus*, *Legionella,* and various others (9, 27). Invasive fungal infections, such as Candida, are also present during this phase, with the lung being a primary site of infection (28, 29). Viruses also pose a major risk for infection (9, 30). During this phase, human herpesviruses -1, -2, and -6 commonly reactivate within 1 to 2 weeks post-HSCT in seropositive individuals (9, 27). Lung-associated viruses such as influenza, respiratory syncytial virus, and adenovirus also pose infectious risks during pre-engraftment (27).

Infectious risk reduces dramatically during the early post-engraftment phase and even more so during the late engraftment phase, due to resolved neutropenia and reconstitution of many immune cell types (9, 30). However, deficiencies in immune function as well as the presence of GVHD during the post-engraftment phases render HSCT recipients susceptible to infection. Bacterial and fungal infections remain common at catheter sites as well as in the lung (9, 18). Pulmonary infections caused by cytomegalovirus and Epstein-Barr virus are also reported during these stages (9, 18, 30).

P. aeruginosa **Pneumonia**

Pulmonary complications occur in up to 60% of HSCT patients, and infectious pneumonia remains one of the leading causes of mortality (19). In particular, *P. aeruginosa* acute lung infection is reported to have a mortality rate of as high as 40% in some studies (31). This high incidence is likely due to the ubiquitous and opportunistic nature of *P. aeruginosa*. *P. aeruginosa* is present throughout the environment including: water systems, air, soil, and vegetation (32). *P. aeruginosa* has a poor ability to adhere to and colonize normal, healthy epithelia; however, if epithelial surfaces become damaged

by burns, chemicals, infection, nutrient deprivation, or other insults, *P. aeruginosa* can readily establish infection (33). As a result, *P. aeruginosa* is common cause of infection for immunocompromised individuals, such as HSCT patients. Moreover, infectious risk in these individuals is exacerbated given the intrinsic resistance of *P. aeruginosa* to a variety of antibiotics and disinfectants (32, 33). Therefore, it is important to understand normal host defense against this pathogen, particularly in the lung, and how it may be altered in the setting of HSCT.

Pulmonary Immunity and Host Defense Against *P. aeruginosa*

During respiration, the lung is exposed to various airborne pathogens and particles. This includes bacteria, mycobacteria, fungi, viruses, and particulate matter (5). Consequently, the host has evolved various mechanical and immune defenses to trap and clear away pathogens and debris that enter into airways (4). Mechanical host defenses are the physical barriers encountered by particles and pathogens as they enter the airways. These barriers include upper airway structures, cilia, and mucus which trap inhaled agents (4). However, particles less than 5 μm in diameter can easily pass through these mechanical structures of the upper airways and enter the terminal airways (4). To prevent colonization of pathogens which bypass these mechanical structures, immune defenses play a critical role. These defenses include various antimicrobial factors, humoral opsonins, proinflammatory mediators, resident and recruited lung phagocytes, structural cells, and antigen-specific B- and T-cell responses.

Pulmonary host defense against *P. aeruginosa* is initially mediated by airway epithelial cells and resident alveolar macrophages (AMs) (32). During infection, *P.*

aeruginosa trapped in the mucosal lining of the upper and lower respiratory tract is recognized by opsonins and PRRs on AM and epithelial cell surfaces. These events trigger AM phagocytosis/killing and production of antimicrobial factors, cytokines, and chemokines from AMs and structural cells (3, 32). Neutrophils are quickly recruited to the site of infection and are essential for complete phagocytic clearance and bacterial killing of *P. aeruginosa* (6). Additionally, monocytes are also recruited from the circulation and given that the phagocytic ability of this cell type is rather limited, their primary role appears to be the production of proinflammatory mediators and antimicrobial factors (3, 34). Collectively, these innate immune defenses work together to clear *P. aeruginosa* lung infection.

Antimicrobial Factors

Defensins

Defensins are a family of single-chain, cationic peptides ranging in molecular weight from 2 to 6 kDa. Defensins are broken up into three groups (α, β, and θ) based on the sequence relationship between the three intramolecular disulfide bonds formed by six cysteine residues within the peptide (35). α-defensins have been identified in both humans and rodents and are produced by neutrophils, certain macrophages, and Paneth cells in the small intestine (36-38). β-defensins have been identified in virtually all mammals studied and are produced mainly by epithelial cells of the lung, skin, intestine, and urogenital tract. θ-defensins are not well described and have only been identified in phagocytes from primates (35, 39).

Defensins are produced under basal conditions and in response to infection or proinflammatory cytokine stimulus. In mice, β-defensin is upregulated by lung epithelial cells following bacterial infection or treatment with tumor necrosis factor (TNF)-α, IL-1β, or LPS (35). Defensins can inactivate and kill both Gram-negative and Gram-positive bacteria, fungi, and enveloped viruses (4). The mechanism of direct defensin microbial killing/inactivation is unknown; however, it is thought that defensins permeabilize microbial membranes by inserting into phospholipid membranes and forming pores that result in microbial lysis (35). In particular, human β-defensin 2 is critical for bacterial killing of *P. aeruginosa* and is rapidly induced in response to infection (40). Other important functions of defensins include: enhancing macrophage phagocytosis, enhancing bronchoepithelial cell IL-8 production for neutrophil recruitment, increasing monocyte TNF-α and IL-1 production, and decreasing monocyte IL-10 production (4, 35).

Lactoferrin

Lactoferrin is a 80 kDa glycoprotein that has been detected in both humans and mice (41). Lactoferrin is stored and released from secondary granules of neutrophils and inhibits microbial growth by limiting the amount of free iron available for microorganism metabolism and growth (42). Lactoferrin is known to inhibit the growth of Gram-negative bacteria and fungi and to prevent replication of viruses, such as CMV (42). Lactoferrin can also directly kill and inhibit biofilm formation of bacteria, such as *P. aeruginosa* (43). Although the mechanism of direct microbial killing is not known, it is

thought that lactoferrin binds to the surface of microbes and induces microbial lysis by permeablizing the outer membrane (42).

Cathelicidins

Cathelicidins are bipartite molecules with a conserved N-terminal domain and a highly variable antimicrobial C-terminal domain (44). The single cathelicidin identified in humans is hCAP-18, and proteolytic cleaveage of this peptide produces the antimicrobial product, LL-37. The murine cathelicidin, cathelicidin-related antimicrobial peptide (CRAMP), has strong homology to LL-37 (44). LL-37 and CRAMP are both found in secondary granules of neutrophils and are also produced by epithelial cells (such as in the lung) during injury and infection (41). LL-37 production has also been reported in T cells, NK cells, B cells, γδ-T cells, and monocytes. The antimicrobial functions of cathelicidins include: binding to LPS to reduce pathogen toxicity and direct killing of bacteria, such as *P. aeruginosa*, via undescribed mechanisms. Moreover, LL-37 acts as a chemotactic factor for monocytes and neutrophils via binding to the G protein coupled receptor (GPCR), peptide-like receptor-1, which is expressed on these immune cells (44).

Lysozyme

Lysozyme is a 14 kDa protein that permeablizes bacterial cell walls via enzymatic and non-enzymatic mechanisms. Enzymatic antimicrobial properties of lysozyme involve the breakdown of the bacterial cell wall component, peptidoglycan (41, 42). The mechanism for non-enzymatic bacterial cell wall lysis is not known. Lysozyme is produced by epithelial cells, neutrophils, and macrophages/monocytes and is present

within phagocytic compartments (42). While humans have only one lysozyme gene, mice have two lysozyme genes (lysozyme-M and -P), which are differentially expressed. Lysozyme M is expressed in phagocytes and epithelial cells, and lysozyme P is expressed in Paneth cells in the small intestine (41). Lysozyme is highly effective against Gram-positive species; however, lysozyme alone is ineffective in microbial killing of Gram-negative species and requires the activity of other antimicrobial factors, such as lactoferrin and antibody-complement, to degrade the outer layer of Gram-negative cell walls (41). Nevertheless, studies have demonstrated the importance of lysozyme in pulmonary host defense against *P. aeruginosa*. For example, over-expression of lysozyme in the lung enhances bacterial clearance, reduces bacterial dissemination to the blood, and improves survival rates following *P. aeruginosa* infection in mice (45).

Opsonins

Collectins

Collagenous C-type lectins, or collectins, are a large group of multimeric carbohydrate-binding proteins. Collectins typically aggregate into trimers to form a lectin domain that recognizes highly conserved sugar patterns on viral, bacterial, yeast, and fungal pathogens (4). Collectin binding to microbial targets results in opsonization and clustering of pathogens. This ultimately neutralizes the pathogen and enhances phagocytic uptake (46). There are three major types of collectins including: surfactant proteins (SPs), lung collectin, and mannose-binding lectin (MBL). The major group of collectins in the lung, however, are the SPs (47).

There are two known SPs in human and mouse that function in lung immune responses: SP-A and SP-D. Both SP-A and SP-D are produced by type II alveolar epithelial cells (AECs) and Clara cells. SP-D is also produced in other tissues, such as the intestine (46). SP-A and SP-D have multiple immunomodulatory functions. One major function of SPs in the lung is to dampen immune responsiveness to ubiquitous pathogens (48, 49). Furthermore, SP-A and -D can bind to toll-like receptors (TLR)-4 and -2 and inhibit TLR-mediated proinflammatory cytokine production (50-52). However, some studies have demonstrated that surfactants are capable of inducing inflammatory responses. SP-A has been reported to stimulate both scavenger and mannose receptor expression in AMs, to enhance macrophage and neutrophil chemotaxis, and to stimulate cytokine and reactive oxygen species (ROS) production in phagocytes (46). Furthermore, SP-A and -D bind to AMs with high affinity and enhance phagocytic uptake of *P. aeruginosa* (53). It has also been reported that deficiency of SPs in mice severely impairs bacterial clearance following *P. aeruginosa* lung infection (54, 55).

Complement

The complement system is composed of more than 20 plasma and cell membrane proteins that have important functions in both innate and adaptive immunity (56). Complement proteins circulate as zymogens and become activated in cascade fashion in response to inflammatory stimuli. Three complement activation pathways have been described: the classical pathway, the MBL or lectin pathway, and the alternative pathway (56). In the classical pathway, the complement protein C1q binds to antibodies bound to the surface of pathogens. This interaction activates a serine protease cascade that results

in proteolytic cleavage of the complement protein C3 into C3a and C3b (57). Following C3 cleavage into C3a and C3b, C3b undergoes a conformational change that allows for binding and opsonization of pathogens. On the pathogen surface, C3b is further cleaved into smaller fragments that are recognized by specific complement receptors (CR) expressed on phagocyte cell surfaces (57). These receptors include: CR1 through CR4 and CRIg. The lectin pathway is activated following binding of MBL or ficolins to mannose and other sugars on pathogen surfaces. This interaction initiates cleavage of C4 and C2 complement proteins and formation of C4b and C3b for pathogen opsonization (57). The alternative pathway involves spontaneous cleavage of C3 to form C3a and C3b (56). Another consequence of activation of all three pathways is the formation of C5a protein, which can function in concert with C3a as a chemokine to recruit phagocytes to sites of infection (58). Complement proteins C5 through C9 are also known to form a cascade that leads to assembly of a membrane-attack complex. This complex mediates microbial lysis by generating pores in the pathogen cell wall (58). Recognition and phagocytic clearance of *P. aeruginosa* via the complement pathway are described in subsequent sections.

Immunoglobulin

Igs, also called antibodies, are proteins composed of four polypeptide chains (two light and two heavy) connected by two disulfide bonds (59). Igs are the central factor of adaptive humoral immunity, and following antigen stimulation, Igs are generated and optimized for functional activity by mature B cells and plasma cells. There are five different isotype classes of human Igs: IgG, IgA, IgM, IgE, and IgD. IgG and IgA are

further broken down into several subclasses (59). The main functions of Igs in immunity are the mediation of classical complement activation (IgG1/2/3 and IgM), alternative complement activation (IgA, IgD, and IgG1/2/3), Fc receptor-mediated phagocytosis (IgG1/3, and IgA), inflammatory mediator release from granulocytes (IgE), cytotoxicity (IgG1/3), and receptor-mediated transport across mucosa (IgA and IgM) (59). During *P. aeruginosa* infection in mice, increases in antigen specific IgG1 and IgG2b are detected (60). This may be critical for chronic or repeated *P. aeruginosa* infections, where IgG can opsonize *P. aeruginosa* and facilitate Fc gamma receptor (FcγR)-mediated phagocytosis.

TLRs

TLRs are a well described group of PRRs that belong to the TLR/Interleukin-1 receptor (IL-1R) or TIR superfamily. More than a dozen TLRs have been identified in humans and mice to date (61). Both TLRs and IL-1Rs have a common intracellular TIR domain; however, TLRs have a leucine-rich repeat motif in the extracellular domain, while IL-1Rs have an immunoglobulin containing extracellular domain (61, 62). Activation of TLRs and IL-1Rs recruits the adaptor molecule myeloid differentiation primary response gene 88 (MyD88) to their TIR domains. Members of the interleukin-1 receptor associated kinase (IRAK) family and various other proteins bind to MyD88 and form an adaptor complex that activates signaling pathways for induction of proinflammatory gene transcription (61, 63). TLR3 signaling, however, is not mediated through MyD88 but the adaptor protein, TIR-domain-containing adaptor inducing interferon-β (TRIF). TLR4 signaling can also occur via MyD88 or TRIF (61).

Cytoplasmic PRRs have also been identified and include NOD-like receptors (NLRs) and retinoic-acid-inducible gene I-like RNA helicases.

During *P. aeruginosa* lung infection, TLRs and NLRs expressed by lung epithelial cells and phagocytes are engaged and induce the production of proinflammatory mediators and antimicrobial factors (3, 5). Specific roles for MyD88, TLR2, and TLR4 have been identified in pulmonary host defense against *P. aeruginosa.* Mice deficient in expression of MyD88, TLR2, or TLR4 display increased bacterial burden and decreased neutrophil influx during *P. aeruginosa* pneumonia (64-66). Additionally, the cytoplasmic PRR, NOD1, has been shown to bind to *P. aeruginosa* peptidoglycan, and deficient expression of NOD1 reduces proinflammatory cytokine production in cells inoculated with *P. aeruginosa* (67).

Proinflammatory Mediators

Key inflammatory mediators produced by AMs and AECs in response to infectious stimulus include: TNF-α, interleukin (IL)-1, IL-8 or macrophage inflammatory protein (MIP)-2, IL-10, prostaglandin E_2 (PGE_2), and leukotrienes (LT) such as LTB_4 (4, 68). Production of TNF-α and IL-1 is critical for induction of chemokines, such as IL-8, which recruit neutrophils to the site of infection (69). LTB_4 is also produced in response to infectious stimulus for neutrophil recruitment (70). IFN-γ is a critical activator of AMs, serving to enhance microbial killing and further TNF-α release (71). PGE_2 and IL-10 are anti-inflammatory mediators also produced during infection that inhibit

proinflammatory cytokine production, phagocytosis, and microbial killing in phagocytes (72, 73).

Multiple proinflammatory mediators are produced during *P. aeruginosa* lung infection. Proinflammatory cytokines detected in bronchoalveolar lavage (BAL) fluid following acute *P. aeruginosa* lung infection in mice include: TNF-α, IL-1β, IFN-γ and IL-6 (74). The eicosanoids, PGE_2 and cysteinyl-LTs (cys-LTs), are also detectable at high levels in lung (68). Chemokines IL-8/MIP-2, KC, and JE and their receptor CXCR2 are also elevated following infection (74). Of these mediators, TNF-α appears to be the most critical during *P. aeruginosa* pneumonia, as inhibition of TNF-α impairs neutrophil recruitment to the lung and bacterial clearance (75-77). It has also been reported that IFN-γ and granulocyte-colony stimulating factor (G-CSF) may enhance TNF-α production in the lung to increase phagocytosis and clearance of *P. aeruginosa* (78).

Cellular Mediators

AMs and Monocytes

Macrophages are mononuclear, phagocytic cells and mature from monocytes that migrate from the blood stream into the tissues (79). Macrophages are the largest phagocyte population in the alveolar space and interstitium of uninfected lungs (3, 80). Alveolar macrophages make up to 95% of immune cells present in the submucosal layer of the alveolar space. Consequently, AMs are the first cellular line of defense against inhaled pathogens and are critical in the recognition and clearance of both opsonized and non-opsonized pathogens (4, 5). In addition, AMs produce a number of inflammatory

mediators to recruit immune effectors (such as neutrophils and monocytes) and to coordinate immune responses (3, 6).

AM and monocyte recognition and clearance of bacterial pathogens invading lung is a complex process involving several types of cell surface receptors and coordinating intracellular signaling events. Receptors involved in AM phagocytic uptake of non-opsonized particles include: class A scavenger receptors and mannose receptors (81). Additionally, bacterial pathogens opsonized by serum proteins, such as IgG or complement, are phagocytosed via FcγRs and complement receptors respectively on phagocytes (60, 82). TLRs expressed on AMs are also important for induction of proinflammatory mediator production to enhance immune responses (3, 5). Once pathogens are recognized and phagocytosed by AMs, various enzymatic and oxidative microbial killing mechanisms are activated to begin pathogen degradation (80).

Neutrophils

Neutrophils are polymorphonuclear phagocytes and the primary granulocytes in the blood stream (83). Neutrophils are the first immune cells recruited to sites of infection and injury in response to chemokine production and the presence of complement proteins, such as C5a (5, 6, 84). Once at the site of infection, neutrophils are activated by the proinflammatory milieu to phagocytose and kill ingested bacterial pathogens (85). Neutrophils are also capable of producing cytokines and chemokines for further recruitment and activation of other innate immune cells (3). The neutrophil lifespan is short, and following clearance of bacterial pathogens, neutrophils must undergo apoptosis. This process helps to initiate anti-inflammatory responses for

immune resolution and tissue repair (3, 6). Therefore, excessive neutrophil recruitment combined with any delay in neutrophil death can lead to increased production of neutrophil-derived antimicrobial factors that may damage tissues. In sites such as the lung, this can lead to conditions such as pulmonary edema and can increase the risk for bacteremia and sepsis (3, 6).

AECs

AECs provide a physical barrier to inhaled pathogens and are the main structural cells involved in primary host defense against inhaled pathogens. AECs express a number of TLRs, and in response to TLR and cytokine signaling, AECs are capable of producing β-defensin; proinflammatory cytokines, such as IL-6 and IL-1β; and chemokines, such as IL-8 for neutrophil recruitment (3, 86-88). Ultimately, production of these mediators is thought to activate lung phagocytes and assist in the coordination of immune responses. It is reported that conditioned medium from type II AECs enhances phagocytosis and bactericidal ROS production in AMs (89).

Lymphocytes

Innate immune responses are effective in clearing acute bacterial lung infections. However, B- and T-cell antigen-specific immune responses in the lung are necessary for clearing chronic infections as well as infections caused by certain encapsulated bacteria, viruses, and intracellular pathogens surviving in AMs (4). For example, complement activation and FcγR-mediated phagocytosis of IgG-opsonized pathogens employ antigen-specific Ig from B cells for pathogen recognition and clearance (59, 82). Furthermore,

roles for T cells expressing NKG2D receptors and CD1d-restricted T cells have also been examined in murine models of *P. aeruginosa* pneumonia. Deficient expression of CD1d or blockade of NKG2D inhibits neutrophil recruitment and bacterial clearance during *P. aeruginosa* lung infection (90, 91). Additionally, AM production of IL-12 is known to promote T helper-1 cell responses, which in turn induce IFN-γ production for AM activation and promotion of B cell antibody-mediated immune responses (4).

Phagocytosis and Intracellular Bacterial Killing

Opsonic Phagocytosis

During infection, various proteins have been identified in the opsonization of *P. aeruginosa* for phagocytic uptake by AMs, monocytes, and recruited lung neutrophils. These opsonins primarily include IgG and complement proteins. Complement opsonization plays an important role in phagocytosis of *P. aeruginosa* during acute infections, while chronic or repeated infection with *P. aeruginosa* may involve opsonization with IgG.

IgG opsonization of specific-antigen generates an immune complex that is recognized by various FcγR on the surface of phagocytes (92). FcγR can have stimulatory (FcγRI, FcγRIII, and FcγRIV) and inhibitory (FcγRII) functions, and the net effect on phagocytosis and cytokine induction is determined by the relative expression of these receptors (93). Engagement of stimulatory FcγR with IgG-opsonized pathogen initiates intracellular signaling events that drive cytoskeletal rearrangement, phagocytic uptake, phagosome formation, and nicotinamide adenine dinucleotide phosphate

(NADPH) oxidase assembly. On the other hand, such signaling events are inhibited following engagement of inhibitory FcγR (94, 95).

Activation of stimulatory FcγR induces phosphorylation of tyrosine residues on the cytoplasmic tail of the receptor. This phosphorylation event allows for clustering of Src tyrosine kinases, Lyn and Hck, which phosphorylate other tyrosine residues that are bound by Syk kinase (94, 95). Syk kinase is critical for phosphorylation and activation of phosphatidylinositol 3-kinase (PI3K). Once activated PI3K converts PI-4,5-P2 (PIP_2) into the key second messenger PI-3,4,5-P3 (PIP_3) (95). PIP_3 can then bind to the pleckstrin homology domain of effectors downstream of the PI3K signaling pathway and mediate their activation. PIP_3 interactions with Vav, Rac, and Arf are reported to mediate NADPH oxidase assembly and phagocytic uptake (94, 95). PI3K-mediated activation of other effectors, such as AKT, leads to phosphorylation of caspases and transcription factors which regulate cell proliferation, metabolic changes, and survival (96).

During lung infection, complement-mediated phagocytosis of *P. aeruginosa* is reported to occur via activation of the alternative pathway and engagement of C3 opsonized *P. aeruginosa* with CR3 (82). To mediate phagocytosis of complement-opsonized pathogens, phagocytes must be activated by IFNγ, C5a, or FcγR engagement/signaling (82). Once activated, Src family kinases are recruited to the cytoplasmic tail of the CR and phosphorylate proteins which activate downstream effectors (such as PI3K, Vav, and AKT) and mediate cytoskeletal rearrangement, phagocytosis, and cytokine gene transcription. CR3 may play additional roles in phagocytosis of *P. aeruginosa* (82). CR3 has been reported to work together with TLR4-CD14 complexes to mediate phagocytosis of trappin-2 opsonized *P. aeruginosa*

(97). It is also speculated that SP-D opsonized *P. aeruginosa* may also bind CD14/TLR4 to mediate phagocytosis (82).

Non-opsonic Phagocytosis

AM phagocytosis of non-opsonized particles is critical for maintaining the sterility of the lower airways and clearance of bacterial pathogens early in infection. Several groups of receptors have been identified in the recognition of non-opsonized pathogens including: scavenger receptors, mannose receptor, asialoglycoprotein receptor, vitronectin receptor, CD48, CD14, CD44, and β-glucan receptor (81, 98). Although accompanying signaling mechanisms have not been fully described, it has been reported that inhibition of PI3K, tyrosine kinases, and ERK diminishes phagocytosis of non-opsonized particles (99).

Specific receptors involved in non-opsonized phagocytic clearance of *P. aeruginosa* by macrophages in the lung have not been identified. In monocytes, however, the mannose receptor is known to bind the mannose-rich cell surface of *P. aeruginosa* to facilitate its phagocytic uptake as well as synergize with TLR2 to induce TNF-α production (100). CD44-mediated phagocytosis has recently been shown to occur via interaction with hyaluronan coated-particles. It is speculated that phagocytosis of *P. aeruginosa* may be mediated via CD44 receptor binding, given that the cell surface of *P. aeruginosa* is rich in hyaluronic acid (98). Class A scavenger receptors, such as SRA-I/II and macrophage receptor with collagenous structure (MARCO), are additional cell surface receptors on AMs that facilitate non-opsonized phagocytosis of bacterial

pathogens (81); however, no scavenger receptors have been identified in the specific-binding and uptake of *P. aeruginosa*.

Intracellular Bacterial Killing

Bacterial killing is a well described host defense mechanism in lung phagocytes. Following phagocytic uptake, pathogens are enclosed in an intracellular compartment called the phagosome. The phagosome then undergoes maturation whereby the cellular machinery used to mediate killing of microbial cargo is activated and assembled (85). The first stage in maturation involves fusion of the phagosome with an early endosome, which is relatively acidic at pH 6.0. This is followed by fusion with late endosomes at pH 5.5 and finally the lysosome at pH 5 (and lower) to form the phagolysosome (85). In neutrophils, an additional fusion event also occurs with secretory vesicles and granules which are rich in antimicrobial peptides such as lactoferrin. Following phagosome maturation, proteolytic enzymes and hydrolases become activated by the low pH to degrade pathogens (101, 102). Additionally, ROS, which degrade pathogen surfaces, are formed by NADPH oxidase during respiratory burst. NADPH generation of ROS involves the transfer of one electron to molecular oxygen, generating a superoxide anion that is converted to various reactive oxygen metabolites (85).

Defective Pulmonary Host Defense Post-HSCT

During the post-engraftment phase, the risk for infectious complications significantly decreases primarily due to reestablishment of the immune system with donor-derived cells (9). However, functional defects in specific immune compartments

can persist for many patients, despite immune reconstitution (8, 10, 30). In particular, impaired pulmonary innate immune function has been reported in patients up to a year or more post-HSCT and is associated with increased risk for infectious pneumonia (17, 27, 103).

Multiple defects have been reported in the host defense function of AMs from BMT patients. Winston and colleagues conducted a study among twelve healthy individuals and seven patients who had successfully undergone allogeneic BMT for the treatment of either acute leukemia or aplastic anemia. All BMT patients were no longer on immunosuppressive drug therapy (104). Compared to healthy subjects, BMT patients displayed normal leukocyte composition in BAL fluid, and AMs constituted the majority of leukocytes counted. Chemotaxis, phagocytosis and killing in AMs were also assessed in patients two, three, six and fourteen months post-BMT. Two to four months post-BMT, patients showed substantial decreases in all host defense mechanisms tested. At six and twelve months post-BMT, AM phagocytosis and killing were improved but still significantly decreased relative to control subjects. AM chemotaxis at these later time points remained significantly impaired in BMT patients relative to controls. Interestingly, peripheral blood monocytes from these BMT patients displayed normal host defense functions, suggesting the possibly of a lung or cell-type specific defect (104).

Similarly, allogeneic BMT patients display impaired neutrophil chemotaxis, ROS production, phagocytosis, and killing. One study reported defects within the first few months of BMT that persisted in the majority of patients up to a year post-BMT (105). Ultimately, these studies suggest ongoing impairment of innate immune function, despite

complete immune reconstitution in HSCT patients. However, the presence of GVHD and use of cyclosporine A immunosuppressive therapy may have had a significant impact on immune function in these patients, aside from possible transplant-related immune defects. Therefore, studies employing syngeneic and autologous HSCT are necessary to assess the impact of HSCT alone on immune function, without the confounding effect of GVHD or use of exogenous immunosuppressive therapies.

Murine Model of *P. aeruginosa* Pneumonia Post-BMT

Our laboratory developed a mouse model of syngeneic BMT to determine the effect of conditioning and reconstitution alone on pulmonary innate immune function and the susceptibility of the host to *P. aeruginosa* pneumonia (106). In our model, C57BL/6 (B6) mice were given a fractionated dose of 13 Gy TBI and infused by tail vein injection with 5×10^6 whole bone marrow cells and 1×10^6 purified splenic T cells from B6 donors. 1 week following BMT, 99% of neutrophils were donor-derived and by week 3 post-BMT, mice displayed normal numbers of all immune cells and approximately 90% of AMs were of donor origin (106).

Despite complete immune reconstitution, BMT mice displayed increased susceptibility to *P. aeruginosa* pneumonia (106). Between BMT and non-transplant control mice given a sublethal dose of *P. aeruginosa*, BMT mice displayed increased bacterial burden in the lung and increased dissemination of *P. aeruginosa* to the blood relative to control mice. The increase in bacterial load in the lungs and blood of BMT mice was associated with decreased host defense function of both AMs and recruited lung neutrophils. BMT AMs treated with either IgG or non-IgG opsonized particles

showed reduced phagocytosis and bacterial killing. Recruited lung neutrophils phagocytosed both opsonized and non-opsonized particles at levels similar to control neutrophils; however, bacterial killing in BMT lung neutrophils was markedly reduced relative to control. Furthermore, diminished production of key proinflammatory mediators, such as TNF-α and cys-LTs, was observed in BMT AMs. These data suggest that the procedure of transplant can have a profound effect on pulmonary immune function and host defense against opportunistic pathogens, despite complete immune reconstitution (106). Although the cause may be multifactorial, we have since identified the immunosuppressive lipid mediator, PGE_2, as a factor induced following transplant that mediates this impairment in pulmonary host defense (68).

PGE_2

PGE_2 is a member of the eicosanoid family of immune active lipid mediators which primarily includes PGs and LTs (72). Eicosanoids are generated from cytosolic phospholipase A2 cleavage of cell membrane-derived phospholipids into arachidonic acid (Fig. 1.1). Cyclooxygenase (COX)-1 and -2 proteins in the cytoplasm are the sentinel enzymes involved in the conversion of arachidonic acid into the PG intermediate, PGH_2, from which the prostaglandins PGA_2, PGD_2, PGE_2, PGF_2, and PGI_2 are derived. As with all eicosanoids, PGs bind to GPCRs on the cell surface to modulate a both pro- and anti-inflammatory responses (72).

PGE_2, in particular, is produced by both leukocytes and structural cells and binds to the PG receptors, E prostanoid (EP) 1 through EP4 (107). PGE_2 binding to EP2 and EP4 is known to specifically modulate anti-inflammatory responses via the second

messenger cyclic adenosine monophosphate (cAMP). EP2 and EP4 are coupled to cAMP stimulatory Gs proteins. Engagement of these GPCRs activates the membrane-bound enzyme adenylyl cylcase (AC), which converts adenosine triphosphate (ATP) into cAMP (108). Increases in intracellular cAMP activates downstream effectors that inhibit proinflammatory cytokine production, bacterial killing, and phagocytosis (109). The remaining PGE_2 receptors, EP1 and EP3, have different functions. EP1 is coupled to Gq, which increases intracellular calcium levels for induction of proinflammatory responses, and EP3 is coupled to the AC inhibitor Gi (72).

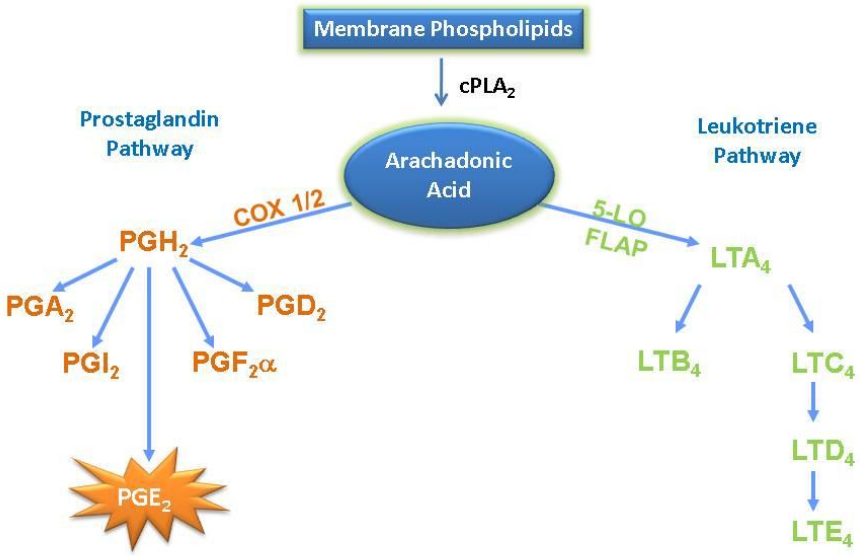

Figure 1.1: The eicosanoid signaling pathway
Eicosanoids are generated from cytosolic phospholipase A2 cleavage of cell membrane-derived phospholipids into arachadonic acid. Cyclooxygenase (COX)-1 and -2 proteins in the cytoplasm are the sentinel enzymes involved in the conversion of arachidonic acid into the PG intermediate, PGH_2, from which the prostaglandins PGA_2, PGD_2, PGE_2, PGF_2, and PGI_2 are derived. 5-lipoxygenase and 5-lipoxygenase activating protein (FLAP) synthesize the LT intermediate LTA_4 from which LTB_4 and cys-LTs are derived.

PGE_2-Mediated Suppression of Pulmonary Host Defense Post-BMT

In the setting of HSCT, PGE_2 levels are significantly elevated and play a major role in reduced immune function and host defense against opportunistic pathogens (68, 110). Overproduction of PGE_2 following HSCT was first reported by Cayeux and colleagues (110). In the study, 14 patients with acute myeloid leukemia, Hodgkin's lymphoma, or non-Hodgkin's lymphoma were given an autologous BMT following a conditioning regimen of either high-dose chemotherapy alone or combined TBI/chemotherapy. Following BMT, plasma PGE_2 levels were increased in all patients between 3- and 10-fold relative to control subjects. This increase was observed irrespective of the type of conditioning regimen used to ablate the host bone marrow. The increase in plasma PGE_2 levels lasted between 30 to 60 days post-BMT before returning to baseline levels (110). The cause of elevated PGE_2 levels post-BMT has yet to be determined but may involve release of PGs from damaged epithelial cell barriers following conditioning.

In our murine model of syngeneic BMT, we have demonstrated that PGE_2 is elevated in the lung (68). When mice were fully donor-cell reconstituted 3 weeks post-BMT, PGE_2 production was increased approximately 2.8-fold in lung homogenates, 2.7-fold in AECs, 125-fold in AMs, and 10-fold in recruited lung neutrophils. Furthermore, EP2 and EP4 gene expression was increased approximately 2- and 4-fold respectively in recruited lung neutrophils from BMT mice relative to control mice. EP2 was elevated approximately 3-fold in BMT AMs relative to control AMs; however, expression of EP4 was slightly downregulated in BMT AMs relative to control (68).

In our BMT model, elevated production of PGE_2 in the lung directly contributes to the impairment of innate immune function and host defense against *P. aeruginosa* pneumonia (68). Pharmacologic inhibition of prostaglandins, using the COX-2 inhibitor indomethacin, restored BMT AM phagocytosis and killing of IgG and non-IgG opsonized targets and enhanced phagocytosis in control AMs. Similarly, using COX-2 +/- mice as bone marrow donors also restored BMT AM phagocytosis. The EP2 antagonist, AH-6809, also conferred a similar effect suggesting a role for EP2 signaling in the inhibition of BMT AM phagocytosis. Interestingly, indomethacin also had a profound impact on host defense against *P. aeruginosa* lung infection. In BMT mice, *in vivo* treatment with indomethacin restored clearance of *P. aeruginosa*, prevented dissemination of *P. aeruginosa* to the blood, and restored production of cys-LTs in the lung (68). These data suggest a critical role for PGE_2 and potentially other prostaglandins in impaired pulmonary host defense post-BMT. However, the mechanism for this inhibition remains unclear. Moreover, it is important to determine whether the elevation we observe in PGE_2 production and its effect on immune function in our model would also occur following the use of more clinically relevant conditioning regimens, such as high dose chemotherapy.

Mechanisms of PGE_2-Mediated Immunosuppression

The mechanism of PGE_2-mediated inhibition of host defense in leukocytes generally involves binding of PGE_2 to EP2/EP4 and activation of AC to induce cAMP production (107, 109). cAMP is then able to interact with two downstream effectors, protein kinase A (PKA) and exchange protein activated by cAMP (EPAC)-1, which

inhibit inflammatory mediator production, microbial killing, and phagocytosis (Fig. 1.2) (109).

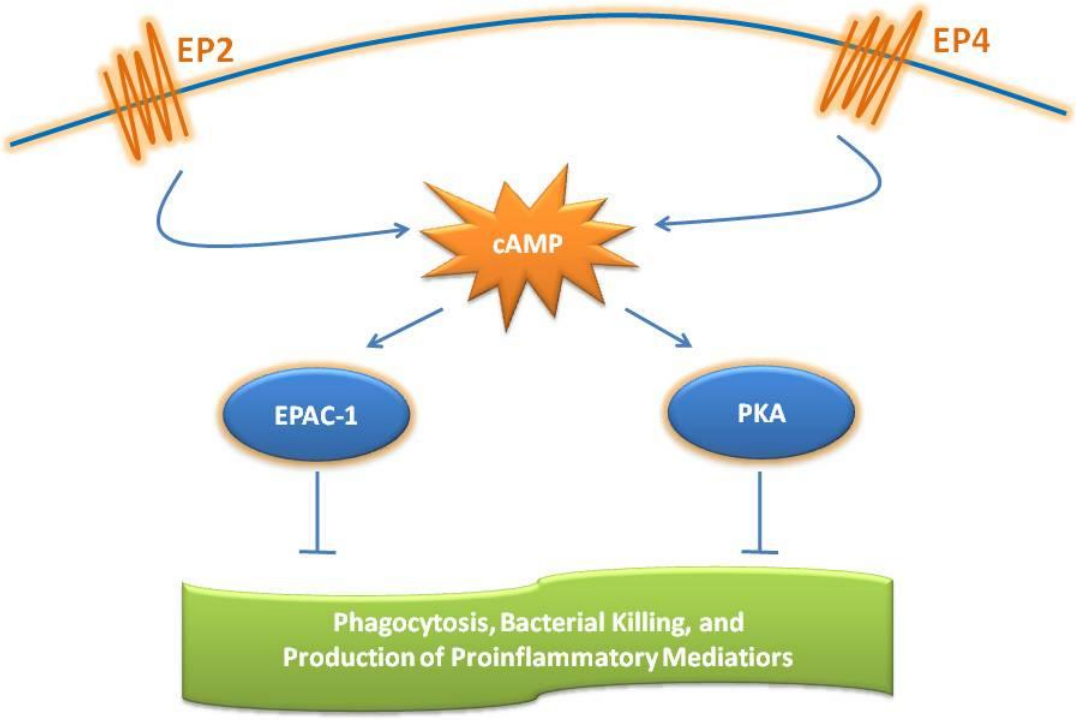

Figure 1.2: PGE_2-mediated inhibition of host defense
PGE_2 engagement of either EP2 or EP4 activates AC to induce cAMP production. cAMP is then able to interact with two downstream effectors, PKA and EPAC-1, which inhibit inflammatory mediator production, microbial killing, and phagocytosis.

cAMP modulates production of a variety of inflammatory mediators via interactions with PKA (111). Although a precise mechanism has not been determined, cAMP interaction with PKA is thought to attenuate activation of NFκ-B and inhibit NF-κB-mediated transcription of proinflammatory mediators (112, 113). Key mediators inhibited by PGE_2 in this manner include: TNF-α, IL-12, LTB_4, and MIP-1 (114-116). Elevated cAMP levels can also impair proinflammatory cytokine responses by inducing

production of the immunosuppressive cytokine, IL-10, and expression of the negative regulator of cytokine signaling, suppressor of cytokine signaling (SOCS)-3 (114, 117).

Elevated cAMP production can also inhibit opsonized and non-opsonized phagocytosis. Phagocytic clearance of myelin, the protein which surrounds axons, is inhibited by increased cAMP production in a PKA- and EPAC-1-dependent manner (118). FcγR-mediated phagocytosis in monocyte-derived macrophages is also regulated by PKA and EPAC-1 (119); however, recent work has also shown that PGE_2-mediated inhibition of FcγR phagocytosis in AMs is specifically regulated by EPAC-1 (120). It was shown that EPAC-1-specific inhibition of AM FcγR-mediated phagocytosis involves EPAC-1 activation of the tyrosine phosphatase src homology 2-containing inositol phosphatase (SHIP)-1. SHIP-1 in turn activates phosphatase and tensin homolog on chromosome 10 (PTEN), which negatively regulates FcγR signaling (120).

Intracellular microbial killing mechanisms are also suppressed by PGE_2-mediated increases in cAMP. cAMP activation of PKA can inhibit phagocyte release of ROS following stimulation with IgG-coated particles (121-123). In AMs, activation of either PKA or EPAC-1 inhibits hydrogen peroxide production following infection with *K. pneumoniae* (124). The mechanism for this suppression of ROS production has not been defined but is associated with reduced activation NADPH oxidase (109, 123). Roles remain conflicting for PGE_2 activation of the cAMP signaling cascade in the inhibition of nitric oxide (NO) production and phagolysosome maturation. In hepatic macrophages, increased cAMP levels inhibit activation of NO synthase (125); however, several studies have shown that cAMP is critical for NO synthase activation and stability (126-128).

Similarly, cAMP has been shown to inhibit phagolysosome maturation and acidification in some studies (129) and promote phagolysosomal acidification in others (130).

Taken together, PGE_2-mediated increases in cAMP have a number of effects on host defense in phagocytes. Although roles for PKA and EPAC-1 have been established in PGE_2-mediated suppression of host defense, specific roles for molecules downstream of PKA and EPAC-1 have yet to be delineated. Elucidation of these signaling pathways would provide greater insight into the mechanism behind impaired host defense in settings where PGE_2 is elevated, such as HSCT. Currently, our laboratory has focused on potential roles for the negative regulator of IL-1R/TLR signaling, IRAK-M, and the negative regulator of FcγR-mediated phagocytosis, PTEN, in impaired AM host defense post-BMT.

IRAK-M

In our model of BMT, AMs display impaired production of the proinflammatory cytokine, TNF-α (106). Numerous molecules have been identified in the negative regulation of pathways, such as TLR/IL-1R signaling, that induce production of proinflammatory mediators. A list of these molecules and their known inhibitory functions are reviewed extensively by Wang and colleagues (131). Of particular interest is the potential role for the negative regulator of TLR signaling, IRAK-M, in impaired AM host defense post-BMT.

Human IRAK-M was first characterized in 1999 by Wesche and colleagues (132). The IRAK-M gene is located on human chromosome 12 at position 12q14.1 – 12q15 and encodes a 68 kDa protein (132). All IRAKs (IRAK-1, -2, -M, and -4) have a conserved

N-terminal death domain for MyD88 TIR-TIR interaction and a central serine/threonine kinase domain consisting of 12 subdomains. However, only IRAK-1 and -4 contain a functional catalytic site with a critical aspartate residue in kinase subdomain VI (61). This residue is an asparagine and serine in IRAK-2 and -M respectively, rendering these kinases inactive. The murine IRAK-M homologue shows 71% sequence identity to human IRAK-M and has similar limitations in kinase activity (133). It is now known that IRAK-M negatively regulates TLR/IL-1R signaling by binding the MyD88 adaptor complex (61).

Human IRAK-M expression is generally confined to monocytes and macrophages, and its expression is induced during macrophage maturation and TLR/IL-1R signaling (132, 134). Tissue-specific mRNA expression in human brain, liver, thymus and small intestine has not been detected. However, recent studies by Balaci and colleagues show IRAK-M protein expression in lung epithelial cells of asthmatic patients (135). This is consistent with murine studies examining IRAK-M expression in the lung, where AECs express IRAK-M under both basal and inflammatory conditions (136, 137).

Murine IRAK-M has a broader expression pattern compared to human IRAK-M. Murine IRAK-M has been detected not only in monocytes and macrophages but also in neutrophils (138), the NIH 3T3 fibroblast cell line (133), B cells (139), and epithelial cells of the intestine, lung, and intrahepatic bilary ducts (136, 137, 140, 141). Furthermore, IRAK-M mRNA has been detected in various tissues, with highest expression in the thymus and liver and relatively lower expression levels in the heart, brain, spleen, and kidney (133). It is not clear whether this is related to the presence of tissue-specific macrophages or epithelial cells that are known to express IRAK-M.

IRAK-M Regulation of TLR/IL-1R Signaling Pathways

IRAK-M can heterodimerize with IRAK-1 or -2 and bind both MyD88 and tumor necrosis factor receptor associated factor 6 (TRAF6) (61). Upon TLR/IL-1R ligand engagement and formation of the MyD88 adaptor complex, IRAK-M is thought to bind MyD88/IRAK-4 and inhibit IRAK-4 phosphorylation of IRAK-1 (Fig 1.3). This prevents formation of TRAF6/IRAK-1 complexes, which initiate IκB kinase (IKK) and mitogen activated protein (MAP) kinase signaling pathways for activation of nuclear factor κ B (NF-κB) and activator protein (AP-1) transcription factors (134). This role for IRAK-M in the negative regulation of TLR/IL-1R signaling was established in experiments demonstrating that transfection of MyD88 and IRAK-1 into 293T cells increases IRAK-1 phosphorylation (134). However, transfection of IRAK-M along with MyD88 and IRAK-1 increases the amount of unphosphorylated IRAK-1 relative to phosphorylated IRAK-1. Furthermore, overexpression of IRAK-M diminishes formation of IRAK-1/TRAF6 complexes (134). Exactly how IRAK-M inhibits NF-κB and AP-1 activation likely depends on the particular stimulus.

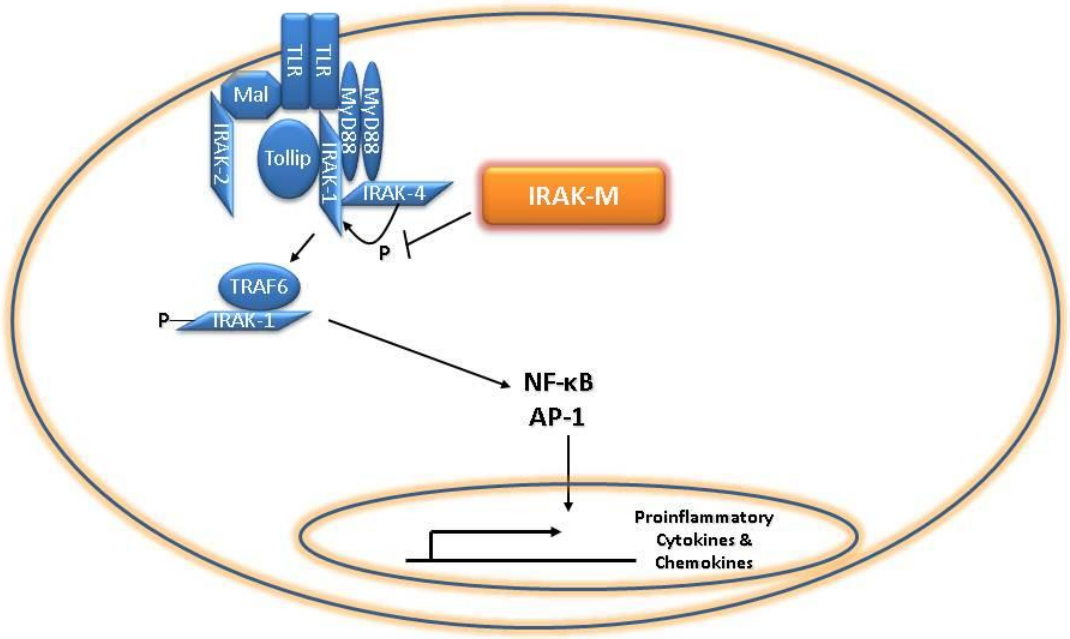

Figure 1.3: IRAK-M-mediated inhibition of TLR signaling
Upon TLR ligand engagement and formation of the MyD88 adaptor complex, IRAK-M binds MyD88/IRAK-4 to inhibit IRAK-4 phosphorylation of IRAK-1. This prevents formation of TRAF6/IRAK-1 complexes, which initiate IKK and MAP kinase signaling pathways for activation of the transcription factors NF-κB and AP-1.

IRAK-M in Impaired Pulmonary Host Defense Against *P. aeruginosa*

IRAK-M expression is regulated by a number of molecules, including endogenous and exogenous soluble factors as well as cell surface and intracellular signaling molecules. A well characterized group of cell surface molecules known to induce IRAK-M expression is TLRs. Induction of IRAK-M expression via TLR activation serves as a means to induce tolerance to subsequent TLR stimulus and macrophage deactivation (61, 131, 134). In macrophages, IRAK-M mRNA and protein

are induced within 3 and 6 hours respectively following stimulation with LPS, a component of bacterial cell walls that activates TLR4 (134, 142). This is a key feature of the induction of endotoxin tolerance in monocytes and macrophages during sepsis to prevent lethal cytokine storm. Deactivation of macrophages during sepsis, however, generates a state of immunosuppression that renders patients susceptible to secondary infections such as pneumonia (143).

One particular study demonstrated that in mice, the absence of IRAK-M expression can overcome the immunosuppressive stage of sepsis and promote protective immune responses against secondary challenge with *P. aeruginosa* (144). Deng and colleagues found that following sepsis, AMs had increased IRAK-M mRNA expression and decreased TNF-α production relative to sham controls (144). In the absence of IRAK-M, however, AMs from septic mice displayed enhanced TNF-α production. Following secondary challenge with *P. aeruginosa* lung infection, IRAK-M -/- septic mice displayed decreased mortality, improved bacterial clearance, elevated production of proinflammatory cytokines and chemokines, and increased neutrophil recruitment to the lung (144). Thus, deficiency in IRAK-M can improve the ability of septic mice to resolve secondary infection with *P. aeruginosa*. Similar enhancement in bacterial clearance was also observed in IRAK-M -/- mice compared to WT mice 72 h following oral challenge with *Salmonella typhimurium* (134). Taken together, these data suggest possible roles for IRAK-M in the suppression of lung phagocyte host defense against *P. aeruginosa* following BMT. Whether overproduction of the immunosuppressive lipid mediator, PGE_2, can regulate this process will be a focus of investigation in this dissertation.

PTEN

Following BMT, AMs display reduced ability to phagocytose both opsonized and non-opsonized targets (68, 106). PTEN is a known inhibitor of signaling pathways that facilitate both opsonized and non-opsonized phagocytosis (145), and PGE_2 activation of the cAMP signaling cascade has previously been shown to induce PTEN phosphatase activity (120). Therefore, PGE_2-mediated activation of PTEN may be a potential mechanism for the inhibition of phagocytosis and other host defense mechanisms in BMT AMs.

The PTEN gene is located on human chromosome 10q23 and encodes a 403 amino acid protein. PTEN is composed of four major domains: a catalytic phosphatase domain, a C2 domain, and an N- and C-terminal domain (146). The C2 domain and the N-terminal sequence are important for PTEN binding to membrane lipids, while the C-terminal domain is necessary for protein-protein interaction with scaffolding proteins (147, 148). The catalytic domain of PTEN is similar in structure to protein tyrosine phosphatases and may act on protein substrates including FAK and PTEN itself (149, 150). However, PTEN is best characterized for its dephosphorylation of the lipid second messenger, PIP_3 (146).

PTEN phosphatase activity is regulated by a number of post-translational modifications which contribute to its subcellular localization, protein-protein interactions, stability, and phosphatase activity (146). PTEN stability depends on interaction of various proteins with its C-terminus. Binding to casein kinase in this manner suppresses

PTEN cleavage by caspase 3 (151). PTEN also contains two PEST motifs which subject PTEN to ubiquitin-regulated proteasomal degradation (146). Oxidation of specific residues is also known to reversibly inactivate PTEN and promote PI3K signaling (152). Lastly, phosphorylation of various tyrosine and serine/threonine residues regulates PTEN activity (146). The serine/threonine residues involved in the regulation of PTEN are well described; however, the specific tyrosine residues involved in PTEN inhibition have yet to be identified.

PTEN Inhibition of PI3K/PIP_3 Signaling

PTEN dephosphorylation of PIP_3 inhibits activation of several effector molecules in the PI3K/PIP_3 signaling pathway (Fig. 1.4). PI3K/PIP_3 signaling mediates key cellular functions such as growth, survival, cell migration, cell differentiation, and metabolism (153). The primary immune functions regulated by PI3K/PIP_3 signaling include: phagocytosis, phagosome maturation, and NADPH oxidase assembly and activation. PI3K is activated in response to engagement of receptor tyrosine kinases at the cell surface, including FcγR (95). PI3K is then able to phosphorylate PIP_2 at the cell membrane, converting it into PIP_3. PTEN then antagonizes PI3K activity by dephosphorylating PIP_3 and converting the lipid substrate back to PIP_2 (146, 153).

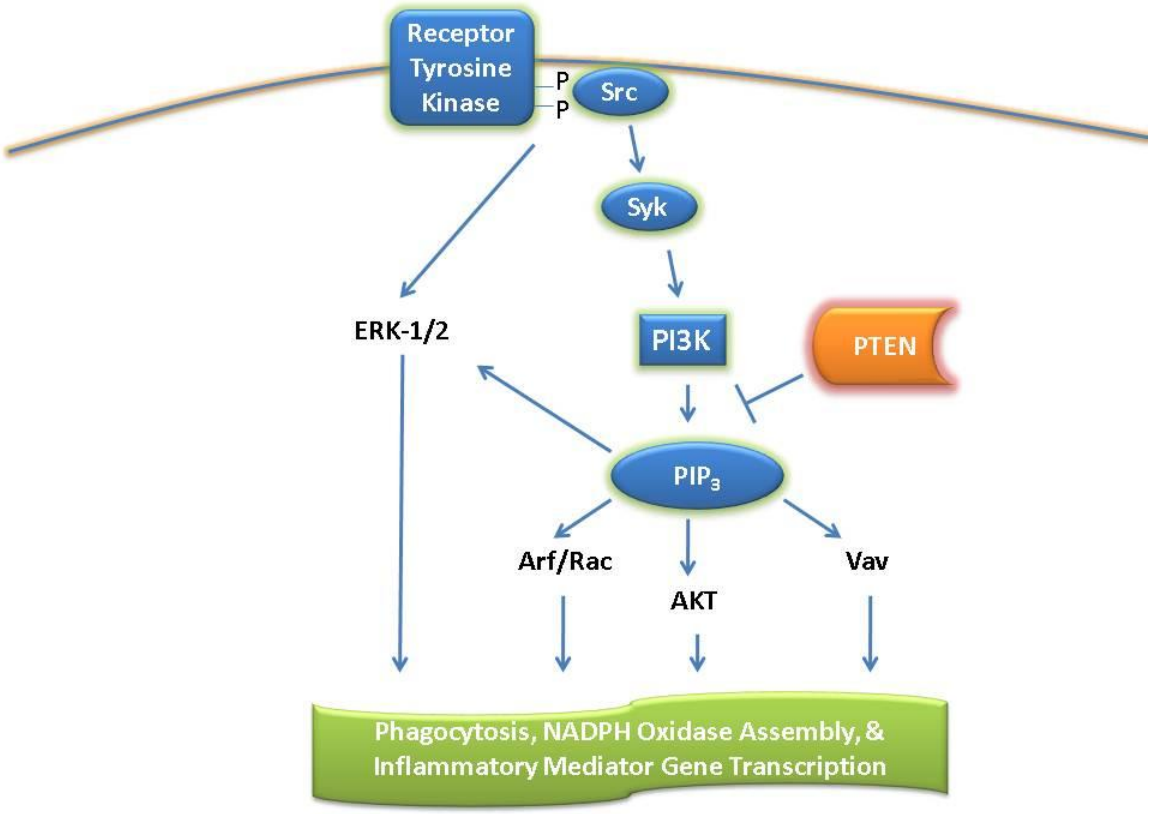

Figure 1.4: PTEN-mediated inhibition of PI3K/PIP_3 signaling.
PI3K is activated in response to engagement of receptor tyrosine kinases at the cell surface, including FcγR. PI3K is then able to phosphorylate PIP_2 at the cell membrane, converting it into PIP_3. PTEN then antagonizes PI3K activity by dephosphorylating PIP_3 and converting the lipid substrate back to PIP_2. PIP_3 activates multiple downstream effectors that modulate host defense in leukocytes.

PTEN and Impaired Host Defense in Phagocytes

PTEN expression is ubiquitous and can be detected early in development. However, examining the role of PTEN in the regulation of immune responses requires targeted or conditional ablation of PTEN expression, due to the embryonic lethality of PTEN -/- mice (154). As a result, several studies have employed conditional knockout systems to generate mice that are deficient in PTEN expression in specific tissues or

immune cell compartments. Use of myeloid-specific PTEN knockout mice, in particular, has provided much insight in the role of PTEN in the negative regulation of host defense functions in macrophages and neutrophils.

A role for PTEN in the regulation of FcγR-mediated phagocytosis and TLR signaling was first described in 2004 by Cao and colleagues (145). In this study, myeloid-specific ablation of PTEN in mice enhanced peritoneal macrophage FcγR-mediated phagocytosis and production of proinflammatory cytokines such as TNF-α and IL-6. Furthermore, their results suggested that PI3K activity was enhanced in the absence of PTEN, leading to AKT-mediated suppression of TLR4 signaling (145). Results of this study have been corroborated by several reports demonstrating enhanced tissue-specific macrophage and neutrophil function in myeloid-specific PTEN -/- models (155-158). Taken together, these data demonstrate that the negative regulation of PI3K by PTEN profound has effects on multiple host defense functions in macrophages and neutrophils. Furthermore, since PGE_2-mediated activation of the cAMP signaling cascade is known to increase PTEN activity (120), it is plausible that overproduction of PGE_2 in the lung following BMT may lead to increased PTEN activity in lung phagocytes, ultimately resulting in impaired phagocytosis, bacterial killing, and proinflammatory cytokine production.

Concluding Remarks

HSCT provides significant therapeutic benefit to patients suffering from various malignant and non-malignant disorders of the immune system. Unfortunately, much of

the success of this therapy is hampered by infectious complications arising from delayed immune reconstitution and ongoing impairment in immune function. Our studies have identified overproduction of PGE_2 in the lung as a major contributor to impaired pulmonary host defense following BMT. However, it is not known whether PGE_2-mediated suppression of pulmonary host defense that is observed in our model is specific to the type of conditioning regimen used to ablate the host HSC. Therefore, it is critical to establish whether use of chemotherapy, which is a more commonly used conditioning regimen for bone marrow ablation in patients, would induce similar suppression of pulmonary host defense. Moreover, it will also be critical to establish a mechanism for PGE_2-mediated inhibition of pulmonary host defense in BMT mice and to identify potential roles for inhibitory molecules, such as IRAK-M and PTEN, in this pathway.

CHAPTER 2: Materials and Methods

Animals

Wild type C57BL/6 (B6Ly5.1; CD45.2) mice were obtained from Jackson Laboratory (Bar Harbor, ME). B6Ly5.2 (CD45.1) mice were purchased from the Fredrick Cancer Research Facility (Fredrick, MD). IRAK-M-deficient mice bred on a B6 background have previously been described (134) and were bred at the University of Michigan. Conditional PTEN KO ($PTEN^{loxP/loxP}$) and myeloid-specific Cre mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). Myeloid-specific PTEN KO mice were generated by breeding as previously described (158). For all experiments involving myeloid-specific PTEN KO mice, $PTEN^{wt/wt}$;$Cre^{+/-}$ mice were used as WT bone marrow donors and $PTEN^{loxP/loxP}$;$Cre^{+/-}$ mice were used as PTEN conditional knockout (CKO) bone marrow donors. Mice were housed under specific pathogen-free conditions and were monitored daily by veterinary staff. All mice were euthanized by CO_2 asphyxiation. The University of Michigan Committee on the Use and Care of Animals approved these experiments

Bone marrow transplantation

Mice were given a syngeneic BMT. For our conditioning regimen studies, mice were treated as follows: 1) *TBI + T* mice received 5 x 10^6 whole bone marrow (BM) cells + 1 x 10^6 splenic T cells following 13 Gy TBI; 2) *TBI* mice received 5 x 10^6 bone

marrow cells following 13 Gy TBI; 3) *Dual chemo* mice received 5 x 10^6 bone marrow cells following conditioning with 25 mg/kg bulsufan x 4 days + 100 mg/kg cyclophosphamide x 2 days + 1 day rest; 4) *Cyclo* mice received 5 x 10^6 bone marrow cells following conditioning with 100 mg/kg cyclophosphamide x 2 days + 1 day rest; 5) *Bus Lo* mice received 5 x 10^6 bone marrow cells following conditioning with 50 mg/kg busulfan x 1 day + 1 day rest; 6) *Bus Hi* mice received 5 x 10^6 bone marrow cells following conditioning with 25 mg/kg bulsulfan x 4 days + 1 day rest. Mice given TBI in our conditioning regimen studies were exposed to a ^{137}Cs source. For our studies examining roles for IRAK-M and PTEN post-BMT, recipient mice received 13 Gy of TBI (orthovoltage x-ray source) split in two fractions, three hours apart. For our conditioning regimen studies, B6Ly5.2 mice (CD45.1) were used as bone marrow donors for B6Ly5.1 (CD45.2) recipients. For all studies, bone marrow cells and splenic T cells were harvested from donor mice and resuspended in serum-free media (SFM; DMEM, 0.1% BSA, 1% penicillin-streptomycin, 1% L-glutamine, 0.1% amphotericin B). Donor cells were administered by tail vein injection into recipient mice. All experiments were performed 3-6 weeks post-BMT when mice were fully donor-cell reconstituted (68, 159). Mice were maintained on acidified water (pH 3.5) for the first 21 days post-BMT.

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

P. aeruginosa PAO1 stock was grown in tryptic soy broth (Difco; BD, Sparks, MD), and the culture concentration was determined via absorbance measurements as previously described (106). For FITC-labeling, a *P. aeruginosa* culture was centrifuged and washed two times by resuspending cell pellet in 1 mL sterile PBS and transferring

into a sterile tube. *P. aeruginosa* was heat-killed by autoclaving for 20 minutes and resuspended at 10^9 - 10^{10} CFU/mL in 0.1 M $NaHCO_3$ (pH 9.2). 0.2 mg/mL FITC (Sigma, St. Louis, MO) in DMSO was added to heat-killed *P. aeruginosa* and allowed to incubate in dark for 1 hour on rocker at room temperature. Following FITC-labeling, heat-killed *P. aeurginosa* were washed three times and resuspended in 1 mL sterile PBS. Aliquots were prepared and stored at -80ºC until use.

Immune serum preparation and opsonization

P. aeruginosa-specific immune serum was prepared from Wistar rats immunized with *P. aeruginosa* as previously described (160). Immune serum was aliquoted and stored at -80°C until use. For serum opsonization of FITC-*P. aeruginosa*, 1 x 10^8 FITC-*P. aeruginosa* were resuspended in 5% immune serum in PBS and incubated on a rotator for 1h at 37°C. For serum opsonization of live *P. aeruginosa*, 1 x 10^8 *P. aeruginosa* was resuspended in 5% immune serum in PBS and incubated on a rotator for 15 mins at 37°C. No complement heat-inactivation of immune serum was performed prior to opsonization of heat-killed or live bacteria.

i.t. infection with *P. aeruginosa*

A culture of *P. aeruginosa* 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 CFU or a lethal dose of 1.5 x 10^6 CFU as previously described (68, 106).

Quantification of bacterial burden in lung and blood

Mice were euthanized 24 hours following i.t. infection with *P. aeruginosa*. As previously described (68), whole lung and blood samples were collected from each mouse and bacterial burden of each specimen was assessed by performing a CFU assay.

AM isolation and adherence purification

AMs were harvested by bronchoalveolar lavage (BAL), counted, and adherence purified as previously described (159). Where indicated, AMs were cultured overnight on 24-well tissue culture plates in the presence or absence of PGE_2 (Cayman Chemicals, Ann Arbor, MI), forskolin (Sigma), indomethacin (Sigma) or vehicle (0.25% ethanol) in SFM or complete media (CM; DMEM, 10% FCS, 1% penicillin-streptomycin, 1% L-glutamine, 0.1% amphotericin B). Following treatment, samples were prepared as described below for real-time RT-PCR or Western blot analysis.

IgG-sheep red blood cell FcγR stimulation assay

AMs cultured overnight in CM were washed once with PBS and incubated at 37°C in SFM for 20 mins. Culture media in wells was then replaced with indomethacin (5 μM, 2h) or PGE_2 (100 nM, 15 min) in SFM. Subsequently, freshly IgG-opsonized or non-opsonized sheep red blood cells (SRBCs; MP Biomedicals, Solon, OH) were prepared as described previously (13) and incubated with AMs (1:10 ratio) for 15 min at

37°C. Wells were then washed once with PBS on ice, and whole-cell lysates were prepared for Western blot analysis as described below.

PTEN phosphatase activity assay

PTEN was immunoprecipitated from control and BMT AM whole-cell lysates to assess *in vitro* lipid phosphastase activity as previously described (161). Briefly, 200μM D-*myo*PIP_3 (Echelon Biosciences) was incubated with PTEN immunoprecipitates for 30 min to allow for dephosphorylation of PIP_3. The amount of PIP_3-derived free phosphate released in each sample was determined by adding BIOMOL Green reagent to each sample and measuring the absorbance using a colormetric plate reader at 630 nm. PTEN lipid phosphatase activity was quantified in each sample as relative absorbance to control samples.

Neutrophil recruitment to lung and isolation

As previously described, neutrophils were recruited to the lung via i.t. injection of a 25 μg dose of *P. aeruginosa*-derived LPS (Sigma) and isolated by BAL 24 hours later (68)**.** Neutrophils comprised approximately 80% of all BAL cells at this time point in all groups.

***In vivo* inhibition of endogenous PGE_2 production**

As reported previously (68, 162), BMT mice were injected intraperitoneally (i.p.) with a 100 μL dose of either 1.2 mg/kg indomethacin (Sigma) or 1% DMSO (vehicle) in sterile PBS. 24 hours following i.p. injection, AMs were harvested from mice by BAL and purified as described above.

Total lung leukocyte preparation

Whole lung samples were harvested from mice and collagenase digested as previously described (144) to isolate lung leukocytes. For each sample, total viable cell number was counted on a hemacytometer by trypan blue exclusion. Where indicated, remaining cells were either stained for flow cytometry (as described below) to identify lymphocyte populations, or stained with modified Wright-Giemsa stain to determine the percentage of neutrophils, monocytes/macrophages, lymphocytes and eosinophils in each sample.

Flow cytometry

Donor vs. host reconstitution of AMs was determined by flow cytometry using CD45.1-PE and CD45.2-FITC directly conjugated Abs purchased from BD/Pharmingen (San Diego, CA). Following total lung leukocyte preparation described above, 1 $x10^6$ cells were stained using fluorochrome-conjugated antibodies against the cell surface markers CD45, CD4, CD8, CD19, NK1.1, TCRβ, (BD Pharmingen, San Jose, CA) following incubation with anti-CD16/CD32 (FcBlock, BD Pharmingen). To enumerate lymphocyte subsets, gates were first set on CD45-expressing cells followed by gating on

the lymphocyte-sized subset. CD4 T cells were identified as $TCR\beta^+CD4^+CD8^-$. CD8 T cells were identified as $TCR\beta^+CD8^+CD4^-$. NKT cells were identified as $TCR\beta^+NK1.1^+$. B cells were identified as $CD19^+$. NK cells were identified as $TCR\beta^-NK1.1^+$.

***In vitro* phagocytosis assay**

AMs isolated by BAL were plated at 2 x 10^5 cells per well and cultured overnight in CM on a 96-well, flat-bottomed, half-area tissue culture plate (Costar, Corning, NY). The following day, wells were aspirated and replaced with 50 μL of SFM. Where indicated, AMs were pretreated with or without 10 nM PGE_2 for 15 mins (Cayman Chemicals, Ann Arbor, MI). In some experiments, AMs were pretreated with or without 100 nM bpV(pic) in SFM for 30 mins (EMD Chemicals, Gibbstown, NJ) to inhibit PTEN activity. AMs were then incubated with either FITC-labeled *E. coli* using the Vybrant Phagocytosis Assay Kit according to manufacturers' instructions (Invitrogen, Carlsbad, CA) or FITC-labeled heat-killed *P. aeruginosa* (prepared as described above) at 300:1 MOI. 2 hours 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-phagocytosed FITC-labeled bacteria. AM phagocytosis of FITC-labeled bacteria was measured using a microplate fluorimeter and expressed in arbitrary fluorescence intensity units as described previously (106, 163). For possible differences in AM adherence to tissue culture plate, data were normalized for cell number using a LDH Cytotoxicity Detection Kit (Roche Diagnostics, Indianapolis, IN) as previously described (164).

Bacterial killing tetrazolium dye reduction assay

AMs or neutrophils isolated by BAL were plated at 2 x 10^5 cells per well in replicate on two 96-well tissue culture plates (one control and one experimental plate). Killing of *P. aeruginosa* was quantified using a tetrazolium dye reduction assay as described previously (165). Briefly, phagocytes on the control plate were allowed only to phagocytose bacteria, while phagocytes on the experimental plate were permitted to both phagocytose and kill ingested bacteria. After 5 hours incubation, the amount of surviving ingested bacteria was quantified using an MTT assay according to manufacturer's instructions (Sigma). Results were expressed as % surviving ingested bacteria = (A_{595} control/A_{595} experimental)/ x 100%.

Real-time RT-PCR

Real-time RT-PCR was performed on an ABI Prism 7000 thermocycler (Applied Biosystems, Foster City, CA). Gene-specific primers and probes were designed using Primer Express software (PE Biosystems, Foster City, CA) as previously published (68, 144). Sequences for all primers and probes used can be found in Table 2.1. Each AM sample was pooled from 2-3 mice and was run in duplicate. Average cycle threshold (C_T) was determined for each sample and was normalized to β-actin. Relative gene expression was calculated as previously described (159).

Table 2.1 Primers and probes used in real-time RT-PCR analysis

EP2 forward	TGCGCTCAGTCCTCTGTTGT
EP2 reverse	TGGCACTGGACTGGGTAGAAC
EP2 probe	CACTGAGAACACAAGAAGCTCAGCAAACAT
EP4 forward	ACGTCCCAGACCCTCCTGTA
EP4 reverse	CGAACCTGGAAGCAAATTCC
EP4 probe	CTGCCAGACCTGACTGAAAGCAGCCTC
IRAK-M forward	TGAGCAACGGGACGCTTT
IRAK-M reverse	GATTCGAACGTGCCAGGAA
IRAK-M probe	TTACAGTGCACAAATGGCACAACCCC
TREM-1 forward	CCAGAAGGCTTGGCAGAGACT
TREM-1 reverse	GAACTTCCCCATGTGGACTTCA
TREM-1 probe	CGGGAAGGAACCCTTGACCCTGGT
MARCO forward	CCTGGACGAGTCGGTCAGAA
MARCO reverse	CCTCAGCTCGGCCTCTGTT
MARCO probe	CCAACGCGTCCGGATCATGGGT
β-actin forward	CTGCCTGACGGCCAAGTC
β-actin reverse	CAAGAAGGAAGGCTGGAAAAGAG
β-actin probe	AACGAGCGGTTCCGATGCCCTG
TLR2 forward	ATGGGCTCGGCGATTTC
TLR2 reverse	ATGCAACCTCCGGATAGTGACT
TLR2 probe	CGGAGTCAGACGTAGTGAGCGAG
TLR4 forward	AAGGAGTGCCCCGCTTTC
TLR4 reverse	CACAATAACCTTCCGGCTCTTG
TLR4 probe	GCCAACATCATCCAGGAAGGCT
TLR9 forward	GAGTACTTGATGTGGGTGGGAATT
TLR9 reverse	GCCACATTCTATACAGGGATTGG
TLR9 probe	CCGTCGCTGCGACCATGCC

Western blot analysis

Whole-cell lysates of AMs or neutrophils were obtained by treating cells with RIPA buffer as previously described (144). Protein concentration of lysates was determined by the Bio-Rad DC Protein Assay (Hercules, CA). 10 μg protein samples were electrophoresed in a 4-20% Tris-Glycine Novex Pre-Cast Gel (Invitrogen; Carlsbad, CA) and transferred to a nitrocellulose membrane. Membranes were blocked for 1 hour at room temperature and probed with one of the following primary antibodies: rabbit anti-IRAK-M (Stressgen Bioreagents, Ann Arbor, MI); mouse anti-β-actin (Pierce, Rockford,

IL); rabbit anti-cPLA$_2$, rabbit anti-FLAP (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-COX-2, rabbit anti-PGES1**,** rabbit anti-PGES2, and rabbit anti-5-LO (Cayman Chemicals, Ann Arbor, MI); or mouse anti-phosho-Akt (Ser^{473}) and rabbit anti-PTEN (Cell Signaling, Danvers, MA). Primary antibody incubations were followed by goat anti-rabbit or anti-mouse IgG-HRP (Pierce) secondary antibody incubations. Bands were visualized using chemiluminescence (SuperSignal West Pico Substrate; Pierce). To assess total AKT protein levels relative to pAKT levels, membranes probed for pAKT were stripped, reblocked, and probed with mouse anti-AKT antibodies (Cell Signaling) according to manufacturer's instructions. To quantify protein expression, band intensity was measured using Image J Software available for download at http://rsbweb.nih.gov/ij/download.html.

ELISA/enzyme-linked immunoassay (EIA)

AMs were cultured overnight at 2 x 10^6 cells per mL in a 96-well tissue culture plate, and supernatants were collected the following day for enzyme immunoassay (EIA). For cys-LT determination, AMs were cultured in SFM instead of CM, and lung homogenates were prepared using the SepPak procedure to isolate lipids prior to EIA analysis as previously described (166). TNF-α production was measured using a DuoSet enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN). Production of PGE_2,6-keto-$PGF_{1\alpha}$ (a stable metabolite of PGI_2), and total cys-LTs (LTC_4, LTD_4, and LTE_4) was measured by EIA (Cayman Chemical) according to manufacturer's instructions.

cAMP measurement

For measurement of cAMP production, AMs were plated 5 x 10^5 cells per well in CM overnight. The following day, AMs were treated with 1 μM PGE_2 in SFM for 15 min, and cell lysates were prepared for cAMP measurement using the cAMP Direct EIA Kit (Assay Designs) according to the manufacturer's instructions.

Statistical analysis

Statistical significance was analyzed using the Prism 5.01 statistical program (GraphPad Software, San Diego, CA). The Kaplan Meier and log-rank methods were used to analyze survival rates. Comparisons between two experimental groups were performed using the Student's *t* test. Comparisons among three or more experimental groups were performed with ANOVA and a post hoc Bonferroni test. A value of $p<0.05$ was considered statistically significant.

CHAPTER 3: Comparison of Conditioning Regimens for AM Reconstitution and Innate Immune Function Post-BMT

Introduction

The lung has the largest interface with the outside environment of any internal organ in the body. As such, the lung is constantly bombarded with foreign particles and pathogens. Active host defense requires the orchestration of innate immune cells and immune mediators such as cytokines, chemokines and eicosanoids to aid in the recognition, containment and clearance of microorganisms (167). Unfortunately, patients who have undergone HSCT are at risk for pulmonary infections (7, 8, 168, 169) and pneumonia remains the leading infectious cause of death despite the implementation of numerous prophylactic strategies and advances in diagnosis and treatment (170). These transplant patients manifest increased susceptibility to infections for periods of time (months to years) post-transplantation extending long after engraftment of leukocytes from donor sources (8, 171).

We previously developed a murine model of syngeneic BMT and have demonstrated that these mice are more susceptible to *P. aeruginosa* pneumonia due to defects in pulmonary innate immune function. We found that by week 3 post-BMT, AMs from these mice are >87% donor-derived (168) but are defective in their ability to both phagocytose and kill bacteria, despite complete donor-cell reconstitution (168, 172).

Interestingly, we found that this defect in host defense post-BMT is related to elevated AM production of PGE_2, a known inhibitor of host defense (163, 168, 172). However, one caveat to the extrapolation of these findings is that the experimental conditioning regimen utilized in our previous studies does not mimic human clinical practice. The conditioning regimen employed in these studies involved 13 Gy of TBI followed by reconstitution with 5×10^6 whole bone marrow cells + 1×10^6 syngeneic splenic T cells (TBI + T). Thus, to determine the applicability of our findings to more clinically relevant conditioning regimens, we compared the level of AM reconstitution and PG production following multiple TBI and chemotherapy preparative regimens. Overall, our results demonstrate that significant reconstitution of AMs from donor bone marrow following either TBI or dual busulfan/cyclophosphamide chemotherapy conditioning results in elevated PGE_2 and PGI_2 production and impaired AM function.

Results

Optimal repopulation of AMs occurs at week 5 post-BMT using 13 Gy TBI

We first wanted to characterize a model for transplantation following irradiation conditioning that would result in significant reconstitution of AMs from donor bone marrow without the inoculation of splenic T cells. In order to determine the optimal timing and dose of TBI for AM reconstitution post-BMT, we transplanted CD45.2 mice with 5 x 10^6 whole bone marrow cells from CD45.1 mice following conditioning with 6.5, 8, 13 or 17 Gy TBI. The 17 Gy dose of TBI was lethal to over 60% of mice despite bone marrow transfer and surviving mice were not used for analysis. However, all mice survived conditioning with 6.5, 8 and 13 Gy TBI. There was a dose-dependent increase in the percentage of donor-derived AMs and peripheral (spleen) cells, with the highest repopulation seen at 13 Gy (Table 3.1). Figure 3.1 shows representative flow cytometry characterization of the donor vs. host reconstitution seen in the BAL of mice 5 weeks following 8 and 13 Gy TBI. Ultimately, week 5 following 13 Gy TBI was chosen as the optimal TBI regimen for further study.

Table 3.1: Percent chimerism of AMs and spleen 5 weeks post-BMT

Irradiation dose	Percent donor-derived AMs	Percent donor-derived spleen cells
6.5 Gy	0.7 ± 0.1	Not tested
8 Gy	36.2 ± 4.2	87.6 ± 0.2
13 Gy	82.3 ± 2.4*	94.9 ± 1.1*

*$P < .005$ between 8 and 13 Gy treatments.

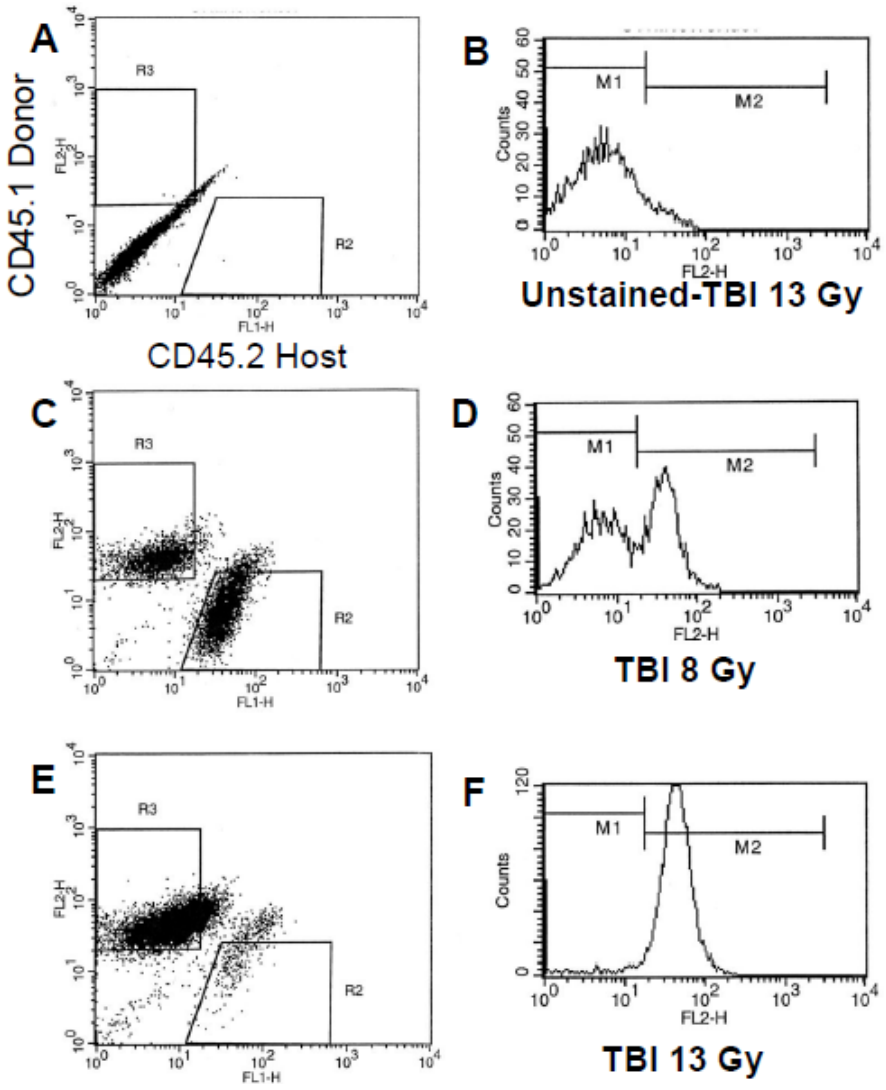

Figure 3.1: Donor vs. host reconstitution of BAL post-TBI conditioning
Mice were conditioned with either 8 Gy or 13 Gy of TBI (delivered in a split dose separated by 3 h) and were transplanted with 5 x 10^6 bone marrow cells. BAL was performed 5 weeks post-transplant, and cells were analyzed for expression of CD45.1 (PE-FL2) vs. CD45.2 (FITC-FL1) to differentiate donor vs. host reconstitution. *A* and *B,* Dot plot and histogram for CD45.1 PE stain showing unstained cells post-13Gy TBI. Background staining is approximately 7% with this gating strategy; *C* and *D,* Dot plot and histogram for CD45.1 stain of BAL from mice post-8 Gy TBI. In this example, 48% of the cells are donor-derived; *E* and *F,* Dot plot and histogram for CD45.1 stain of BAL from mice post-13 Gy TBI. In this example, 93% of the cells are donor-derived.

TBI conditioning is more effective in reconstitution of AMs from donor bone marrow

We next wanted to compare the effectiveness of chemotherapy conditioning regimens to irradiation conditioning regimens. CD45.1 mice were used as bone marrow donors for CD45.2 recipients following the conditioning regimens noted in Table 3.2. 5 weeks post-BMT, AMs were harvested, and the frequency of donor cell reconstitution was determined by calculating the percentage of AMs expressing either the CD45.1 or CD45.2 allele using flow cytometry. At this time point, we found that significant AM and splenocyte donor reconstitution was only observed following TBI, TBI + T, or dual chemotherapy conditioning (Table 3.2). Ultimately, TBI conditioning was more efficient than dual chemotherapy conditioning in the reconstitution of AMs from donor bone marrow. Additionally, we characterized the total cellularity of BAL from 13 Gy TBI and dual chemo mice 5 weeks post-BMT. By morphological assessment, we found that approximately 85% of the cells were AMs at this time point in both groups (Fig. 3.2).

Table 3.2: Reconstitution of AMs following various conditioning regimens

Abbreviation	Conditioning regimen	Donor inoculum	% donor-derived AMs (week 5)	% donor-derived spleen cells	Total number of AMs in BAL ($\times 10^4$)
TBI + T	6.5 Gy × 2 (3 h separation)	5×10^6 whole BM cells + 1×10^6 splenic T cells	88 ± 4.5	98.3 ± 0.4	164 ± 14
TBI	6.5 Gy × 2 (3 h separation)	5×10^6 whole BM cells	80.5 ± 5.8	94.9 ± 1.1	144 ± 45
Dual chemo	25 mg/kg busulfan x 4 days + 100 mg/kg cyclophosphamide × 2 days + 1 day rest	5×10^6 whole BM cells	56 ± 6.2	72.3 ± 2.1	136 ± 43
Cyclo	100 mg/kg cyclophosphamide × 2 days + 1 day rest	5×10^6 whole BM cells	<1%	4.64 ± 0.5	76 ± 12
Bus Lo	50 mg/kg busulfan × 1 day + 1 day rest	5×10^6 whole BM cells	<1%	6.17 ± 0.70	64 ± 15
Bus Hi	25 mg/kg busulfan × 4 days + 1 day rest	5×10^6 whole BM cells	2.2 ± 0.8	48.5 ± 2.3	32 ± 7

Note. By ANOVA for AM percent reconstitution: TBI + T versus TBI alone P = nonsignificant; TBI versus Dual chemo $P < .05$; TBI versus cyclo $P < .001$; TBI versus Bus Lo $P < .001$; TBI versus Bus Hi $P < .001$.

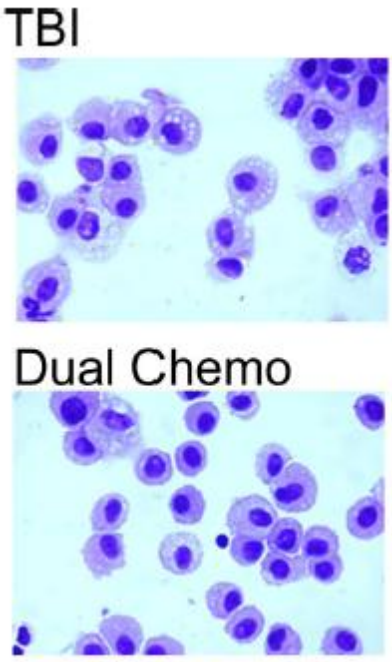

Figure 3.2: Differential analysis of BAL cells post-BMT
BAL cells were collected from TBI or dual chemo BMT mice at week 5 post-transplant. BAL cells were cytospun and stained with modified Wright-Geimsa stain. Greater than 85% of the cells collected from the lung lavage had the characteristic morphology of AMs at this time point.

Significant reconstitution of AMs from donor bone marrow is associated with elevated production of PGE_2

We previously demonstrated that AMs from TBI + T BMT mice display elevated PGE_2 production relative to non-transplant control AMs (68). Therefore, we wanted to determine whether this increase in AM PGE_2 production from irradiated BMT mice also characterizes AMs from chemotherapy conditioned BMT mice. We found that the conditioning regimens associated with significant turnover of AMs from donor bone marrow (TBI +T, TBI or dual chemotherapy) were also associated with elevated AM

PGE_2 production 5 weeks post-BMT, as compared to AMs from control mice (Fig 3.3, panel A). To determine whether this elevation was specific to PGE_2, we measured the levels of a stable metabolite of PGI_2 (6-keto $PGF_{1\alpha}$) in supernatants of AMs from control, TBI BMT, and dual chemo BMT mice. The levels of 6-keto $PGF_{1\alpha}$ were elevated in mice transplanted by both regimens; however these levels only reached statistical significance in the cells from the TBI mice (Figure 3.3, panel B). Thus, it is likely that following BMT there is a general upregulation of PGs in the lung.

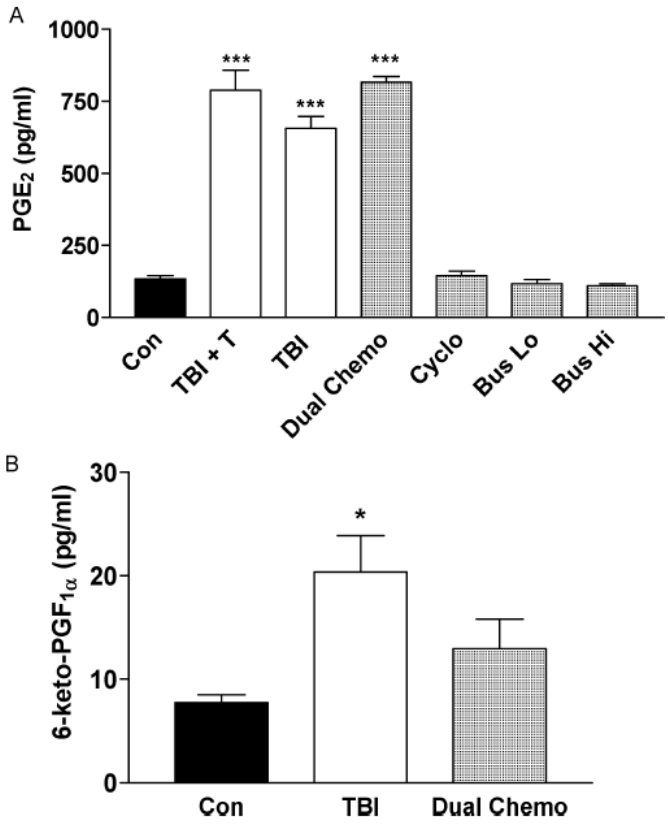

Figure 3.3: Prostaglandin secretion by AMs post-BMT
AMs were harvested from control, TBI + T, TBI, dual chemo, cyclo, bus lo, or bus hi conditioned BMT mice. Cells were cultured at 5 x 10^5/ml for 24 h, and PGE_2 (panel A) and 6-keto $PGF_{1\alpha}$ (panel B) were measured in overnight culture supernatants by EIA (n=5; ***$p<0.001$ compared to control AMs, *$p<0.05$).

Impaired AM phagocytosis characterizes both TBI BMT and dual chemo BMT mice

We next wanted to determine whether AM phagocytosis would be similarly impaired following either a TBI BMT or dual chemo BMT protocol. AMs were harvested from control, TBI BMT, and dual chemo BMT mice at week 5 post-BMT, and AM phagocytosis of non-opsonized bacteria was assessed in the presence or absence indomethacin. AMs from both TBI BMT and dual chemo BMT mice were similarly defective in their ability to phagocytose bacteria (Figure 3.4, panel A). Furthermore, inhibition of PG synthesis by the AMs improved phagocytosis to control levels in both groups of BMT mice (Figure 3.4, panel A). Thus, overproduction of PGE_2 (and possibly PGI_2) likely contributes to impaired AM phagocytosis observed in these mice, regardless of the conditioning regimen employed.

Both TBI and dual chemo BMT mice display impaired pulmonary host defense

To determine whether host defense was impaired following either conditioning regimen, control, TBI BMT, and dual chemo BMT mice were i.t. injected with a sublethal dose *P. aeruginosa*, and bacterial burden was assessed in lung samples 24 h later. Whereas control mice showed evidence of bacterial clearance, both the TBI BMT and dual chemo BMT mice were similarly deficient in their ability to clear the bacterial challenge (Figure 3.4, panel B). These results mirror our previous findings using the TBI + T conditioning regimen at week 3 post-BMT (168, 172).

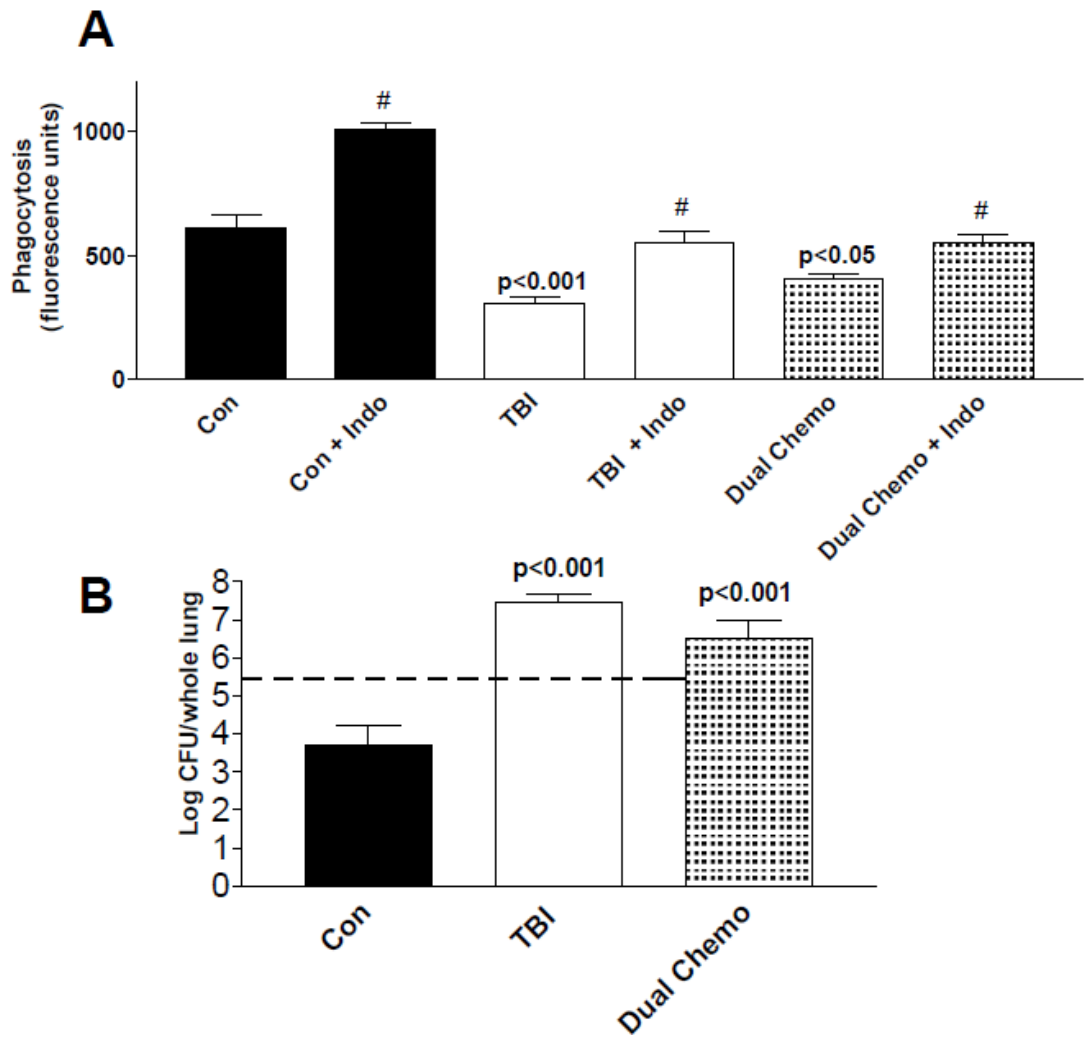

Figure 3.4 AM function and host-defense post-BMT
A, AMs were harvested from control, TBI BMT, or dual chemo BMT mice, and AM phagocytosis was assessed as described in *Materials and Methods*, n=6. *B*, Control, TBI BMT, and dual chemo BMT mice were given a sublethal dose of *P. aeruginosa* by i.t. injection 5 weeks post-BMT. Lungs were harvested 24 h later, and bacterial burden was assessed by CFU assay. Dotted line represents inoculated dose (n=5).

Discussion

Pulmonary complications, particularly infections, remain the leading cause of morbidity and mortality in patients undergoing HSCT, despite advances in prophylaxis (7, 8, 171). This is likely due to defects observed in pulmonary innate immunity post-transplant (173). Our previous work established a murine model of BMT (TBI + T) which was characterized by overproduction of PGE_2, impaired AM phagocytosis, and diminished pulmonary host defense (168, 172). However, the TBI + T conditioning regimen used in our earlier studies does not mimic clinical practice (8, 174-176). Additionally, the TBI + T conditioning regimen results in substantial reconstitution of AMs at an earlier time point (week 3) (168) than occurs when T cells are not added to the donor inoculum (177). Thus, we wanted to assess the effect of other conditioning regimens on AM reconstitution and function.

TBI conditioning was superior to all tested methods of chemotherapy conditioning in the reconstitution of AMs from donor bone marrow. Only the dual chemotherapy regimen (25 mg/kg busulfan x 4 days followed by 100 mg/kg cyclophosphamide x 2 days) resulted in significant AM reconstitution from donor bone marrow (Table 3.2). Furthermore, AM reconstitution was only seen in situations where there was also extensive peripheral (splenocyte) reconstitution (Table 3.2).

Previous studies have suggested that approximately 20% of AMs are capable of extensive replication and colony formation *in vitro*, (178). This population of cells, known as AM colony forming cells (AL-CFCs), are moderately resistant to ionizing irradiation (179) and likely represents a local lung progenitor cell for AMs. Using fractionated low dose irradiation schemes, Tarling et al. demonstrated that when the AL-

CFC population was not ablated, AMs were repopulated by host cells (presumably the lung-resident AL-CFC) (180). However, high dose irradiation schemes (>9Gy) ablated the AL-CFC population and resulted in AM repopulation by donor bone marrow cells (180). Our present results agree with these observations and confirm that 8 Gy TBI results in approximately 45% AM reconstitution from donor bone marrow, whereas high dose irradiation (13 Gy) results in approximately 80% AM repopulation from donor marrow (Fig. 3.1). Furthermore, these data suggest that of the chemotherapy regimens tested, only the dual chemotherapy regimen results in AM repopulation from donor bone marrow. We speculate that the 13 Gy TBI and dual chemo regimens likely ablate the AL-CFC local lung progenitors. Alternatively, these results may suggest that AM reconstitution from donor bone marrow requires significant lung injury/inflammation induced by either TBI or chemotherapy. A previous report by Maus et al. suggests that lung inflammation is required for efficient exchange of AM populations from circulating bone marrow-derived progenitors (181). In support of this idea, the regimens which result in significant AM repopulation are also associated with greater cellularity in the BAL (Fig. 3.2).

Like the TBI regimen, the dual chemotherapy protocol was also the only chemotherapy-based protocol to result in significant secretion of PGE_2 by AMs post-BMT (Fig. 3.3, panel A). Similarly, metabolites of PGI_2 were also elevated in AMs from the TBI regimen (and to a lesser extent in the dual chemo regimen), although the levels of this metabolite were expressed at lower levels than PGE_2 (Fig. 3.3, panel B). This finding suggests that conditioning for BMT may induce an upregulation in synthesis of

all PGs. Given that PGI_2 and PGE_2 both induce cAMP elevation, the downstream effects of either PGE_2 or PGI_2 signaling on host defense are presumably similar (163, 182).

Elevated PGE_2 synthesis is not restricted to donor-derived AMs post-BMT. We previously reported that PGE_2 levels are also elevated in AECs, neutrophils (172), and within the peritoneal cavity (data not shown) following the TBI + T regimen. Furthermore, we found in a preliminary experiment that production of PGE_2 is significantly elevated in both donor- and host-derived AMs compared to AMs from control mice; however, PGE_2 production by donor-derived AMs is significantly higher than host-derived AMs (data not shown). Thus, BMT likely results in global increases in PG production from cells of both donor and host origin. While it is not clear exactly what the trigger for elevated PG production is post-BMT, we speculate that oxidative stress or reactive oxygen species, a known inducer of PGE_2 (183), may play a role following the TBI and dual chemotherapy regimens.

We next wanted to determine whether the elevated levels of PGE_2 following BMT with either the TBI or the dual chemotherapy regimen would result in similar alterations in AM phagocytosis and host defense. We chose the TBI and dual chemotherapy regimens for analysis, because these conditioning regimens mimic human transplantation protocols and provide optimal donor cell reconstitution (174-176). 5 weeks post-BMT, AMs from BMT mice conditioned with either regimen were similarly defective in phagocytosis when compared to control AMs, and this inhibition could be reversed by the addition of indomethacin (Fig. 3.4, panel A). Impaired AM function in both the TBI and dual chemotherapy regimens likely led to similar defects in pulmonary clearance following *P. aeruginosa* infection (Fig. 3.4, panel B). Thus, we conclude that lung

myeloablative conditioning regimens (whether TBI or chemotherapy based) result in significant turnover of AMs from donor bone marrow and are associated with elevated AM production of PGs, impaired AM function, and diminished host defense. Interestingly, elevated levels of PGE_2 have been noted in the plasma of patients undergoing autologous BMT (184). The conditioning regimens differed between the patients (TBI/chemotherapy v. chemotherapy alone), but PGE_2 levels were elevated in all cases (184). Thus, our results may be widely applicable to HSCT in patients.

We were pleased to discover that our dual chemotherapy model of conditioning closely mimicked the hallmark features of our experimental TBI model. Both models show increases in AM PGE_2 production and similar defects in AM host defense. Our next goal will be to uncover the downstream signaling events which mediate the PGE_2-induced immunosuppression post-BMT. For these studies, we plan to focus on the TBI model of conditioning, given that this regimen provides the greatest level of donor-cell reconstitution in the lung. This will be an important feature for our model in future studies, which will use genetically modified mice as bone marrow donors.

CHAPTER 4: The Role of IRAK-M in Impaired Pulmonary Host Defense Post-BMT

Introduction

HSCT is an effective treatment for certain forms of cancer and genetic diseases. However, delays in immune reconstitution render HSCT patients susceptible to infection (7, 31). Even after immune reconstitution, donor-derived cells are often functionally immature, and patients remain at risk for infectious complications months to years post-transplant. This susceptibility is noted in both allogeneic and autologous transplant recipients (8, 10).

Bacterial pneumonia is a common infectious complication post-HSCT and remains one of the leading causes of infection-related patient mortality (31, 185). This incidence is likely related to impaired pulmonary innate immunity. AMs comprise up to 95% of the immune cells in the alveolar space and are essential in mediating innate immune responses in the lung (3-5). Post-allogeneic HSCT, AMs have a decreased ability to phagocytose and kill bacteria (104). Furthermore, neutrophils recruited to sites of infection display impaired chemotaxis and killing ability (105). Allogeneic transplants, however, have multiple confounding factors, such as immunosuppressive drug therapy and graft-versus-host disease, which can impair immune function (7, 8). Therefore, autologous or syngeneic transplants, which are not confounded by such

factors, are useful for investigating the effect of conditioning and reconstitution alone on pulmonary host defense.

We previously developed a mouse model of syngeneic bone marrow transplant (BMT) to determine how HSCT alone impacts host defense in the lung. Compared to non-transplant controls, BMT mice are more susceptible to pneumonia following i.t. challenge with *P. aeruginosa* (106). Furthermore, BMT AMs display impaired host defense mechanisms in *ex vivo* assays, including decreased phagocytosis, bacterial killing, and TNF-α production. Impaired bacterial killing is also observed in recruited lung neutrophils (68). These defects in host defense were directly related to overproduction of prostaglandin E_2 (PGE_2) in the lung post-BMT (68, 185, 186). PGE_2 is a lipid mediator with a variety of immunosuppressive properties. Several studies have shown that PGE_2 inhibits bacterial killing, phagocytosis (124, 163), chemotaxis (187), and production of proinflammatory mediators in leukocytes (188-190). However, a mechanism for this is not well described, and it remains unclear how overproduction of PGE_2 post-BMT impairs pulmonary host defense.

In this study, we suggest a role for IRAK-M as a mediator of PGE_2-induced immunosuppression post-BMT. IRAK-M is a member of the IRAK family of serine/threonine kinases involved in MyD88-dependent IL-1R/TLR signaling. In mice, IRAK-M is expressed mainly in monocytic cells in response to repeated endotoxin exposure (61, 133, 134). To prevent excessive inflammatory responses to endotoxin, IRAK-M limits proinflammatory cytokine production by inhibiting MyD88-dependent IL-1R/TLR signaling (61, 134). This inhibitory response may also be induced during sepsis, where increased IRAK-M expression in macrophages downregulates excessive

proinflammatory cytokine production (144, 191). Given the role for IRAK-M in immunosuppression, we hypothesize that overproduction of PGE_2 post-BMT upregulates IRAK-M expression in AMs, thus inhibiting host defense.

To address this hypothesis, expression of IRAK-M was measured in BMT and non-transplant control AMs. In addition, experiments were setup using IRAK-M deficient mice to determine the role of IRAK-M in pulmonary host defense post-BMT. We show for the first time that PGE_2 signaling can upregulate IRAK-M expression in AMs, and hematopoietic expression of IRAK-M post-BMT impairs pulmonary host defense.

Results

BMT AMs have elevated IRAK-M expression

IRAK-M can limit proinflammatory cytokine production in macrophages (61, 134). Given that AMs have impaired TNF-α production post-BMT (106), we wanted to determine whether IRAK-M expression was elevated in BMT AMs. AMs from BMT and non-transplant control mice were harvested and analyzed for IRAK-M mRNA and protein expression. Relative to control AMs, BMT AMs had approximately a 3.5-fold increase in both IRAK-M mRNA (Fig. 4.1, panel A) and protein (Figs. 4.1, panels B and C) expression. IRAK-M protein expression was also measured in neutrophils recruited to the lungs of control and BMT mice following LPS i.t. injection. No appreciable differences were observed in IRAK-M protein expression between control and BMT neutrophils (Fig. 4.1, panel D).

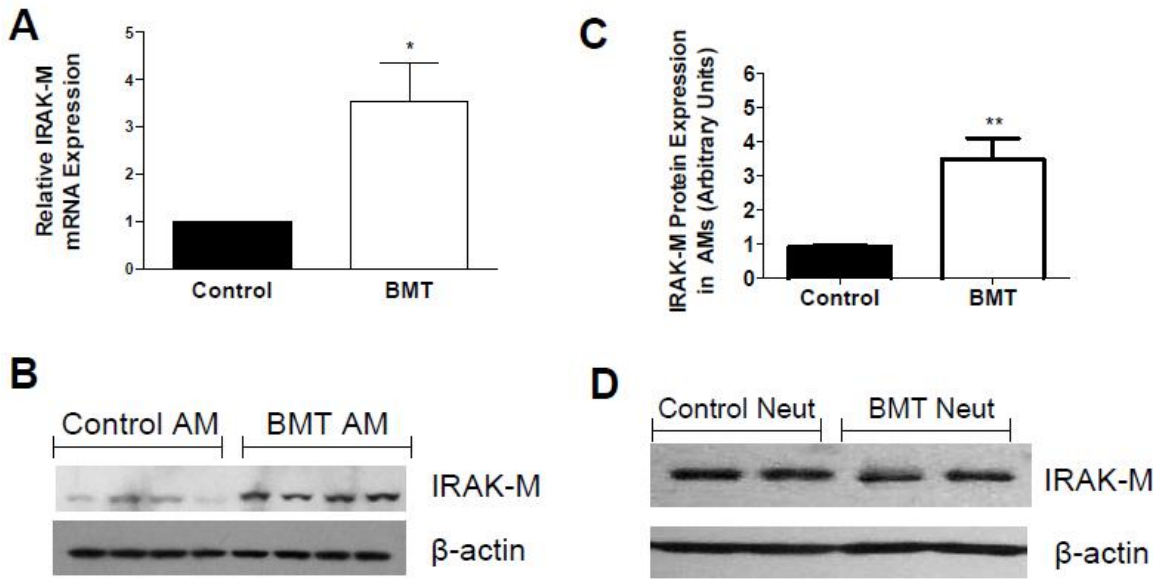

Figure 4.1: IRAK-M mRNA and protein is elevated in BMT AMs.
Mice were given a syngeneic BMT and harvested 5-6 weeks post-BMT. AMs and recruited lung neutrophils were harvested by BAL as described in *Materials and Methods* from BMT and control mice. *A,* mRNA was prepared and analyzed by semiquantitative real-time RT-PCR for expression of IRAK-M in AMs. For each sample, expression of IRAK-M was normalized to β-actin. The average of the normalized control samples was set to 1, and relative IRAK-M expression in BMT samples was measured by the ΔΔCT method (n=3 combined from 2 experiments). *B,* Protein lysates of control and BMT AMs were prepared, and expression of both IRAK-M and β-actin was measured by Western blot analysis. Blot shown contains samples from independent experiments. *C,* Protein expression was quantified by densitometry. For each sample, expression of IRAK-M was normalized to β-actin. A single control sample was then set to 1 and all other samples were graphed relative to that control value (**, $p < 0.01$; n=4). *D,* IRAK-M protein expression was measured by Western blot analysis in control and BMT lung neutrophils recruited via i.t. LPS injection as described in the *Materials and Methods* (n=2).

Elevated IRAK-M expression in BMT AMs is related to overproduction of PGE_2

Our previous work has demonstrated that BMT AMs display increased PGE_2 production relative to control AMs (68), and this elevation in PGE_2 production is accompanied by increased expression of PG synthetic enzymes and decreased expression

of LT synthetic enzymes in BMT AMs (Fig. 4.2). To determine whether elevated IRAK-M expression in AMs is related to increased PGE_2 signaling, WT non-transplant AMs were cultured overnight with or without 10 nM PGE_2. PGE_2 treatment increased IRAK-M protein expression 2.5-fold over untreated AMs (Figs. 4.3, panels A and B). Similar results were observed by treating AMs with 10 nM butaprost free acid (data not shown), an agonist selective for the E prostanoid 2 (EP2) receptor which is known to mediate the suppressive effect of PGE_2 on AM phagocytosis and killing (68, 123, 163).

We next sought to determine whether overproduction of PGE_2 was elevating IRAK-M expression in BMT AMs. AMs from BMT and control mice were cultured overnight in the presence or absence of 5 μM indomethacin (or vehicle control) to inhibit endogenous PGE_2 production. Relative to untreated control AMs, vehicle-treated BMT AMs expressed 3.1-fold more IRAK-M protein; however, treatment with indomethacin reduced elevation of IRAK-M protein in BMT AMs by 40% (Figs. 4.3, panels C and D). Inhibition of endogenous PGE_2 production was verified by EIA using overnight culture supernatants (data not shown). We then wanted to determine whether *in vivo* inhibition of endogenous PGE_2 production could also reduce elevation of IRAK-M in AMs post-BMT. 24 hours following i.p. injection of either indomethacin or vehicle control into BMT mice, we found that AMs from vehicle-treated BMT mice had a 3.2-fold increase in IRAK-M expression relative to AMs from untreated control mice (Figs. 4.3, panels E and F). *In vivo* administration of indomethacin reduced IRAK-M elevation in BMT AMs by 51%, relative to BMT AMs from vehicle-treated mice (Figs. 4.3, panels E and F). We previously demonstrated that *in vivo* treatment with indomethacin improved host defense of BMT mice when infected with *P. aeruginosa* (68). Collectively, these data indicate

that AM overproduction of PGE_2 may contribute to elevated IRAK-M expression in AMs and decreased host-defense post-BMT.

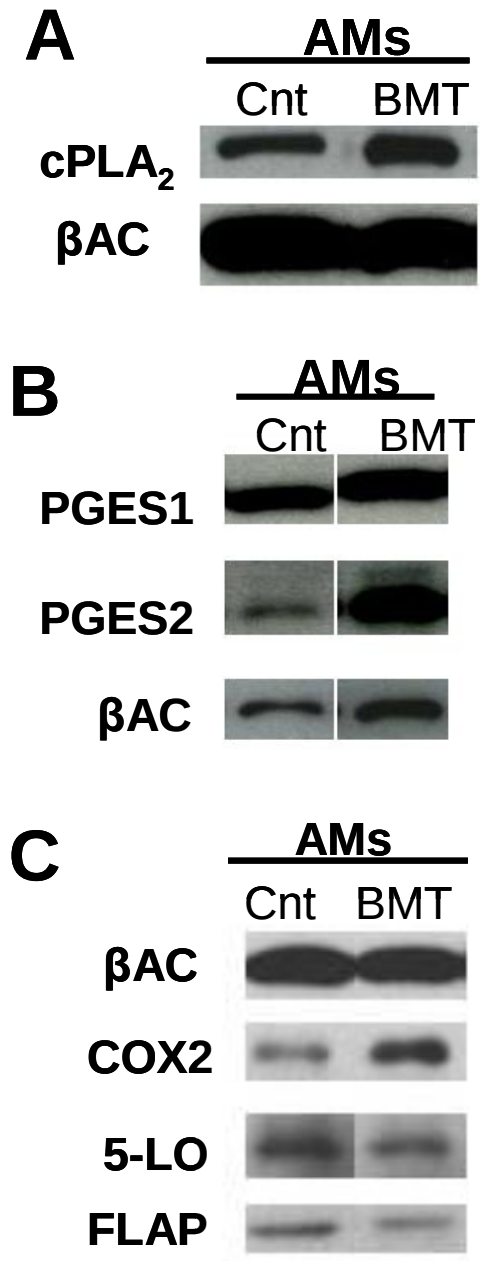

Figure 4.2: Elevated BMT AM expression of PGE_2 synthetic enzymes
AMs were harvested by BAL as described in *Materials and Methods* from BMT and control mice. Protein lysates of control and BMT AMs were prepared, and expression of $cPLA_2$, COX-2, 5-LO, FLAP, PGES1, PGES2, and β-actin was measured by Western blot analysis. Blots are representative of an n=3 per group.

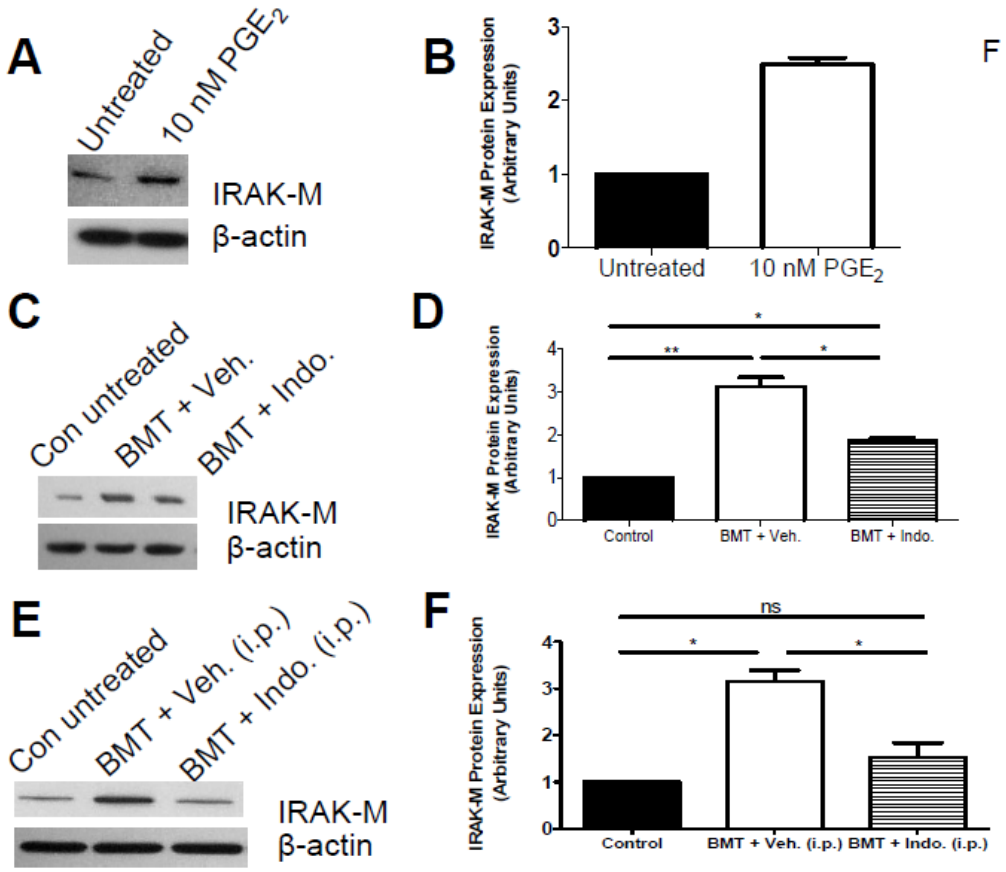

Figure 4.3: PGE_2 increases IRAK-M expression in BMT AMs.
A, Protein lysates were prepared from WT non-transplant AMs cultured overnight at 5 x 10^5 cells/well in the presence or absence of 10 nM PGE_2 in CM. IRAK-M and β-actin protein expression was analyzed by Western blot. Blot shown is from a single experiment representative of two. *B*, Densitometry quantification of blots from two experiments as shown in part *A*. IRAK-M expression was normalized to β-actin for each sample and expression was graphed as fold change above control. *C*, Protein lysates were prepared from control and BMT AMs cultured overnight at 5 x 10^5 cells/well in the presence or absence of 5 μM indomethacin (to inhibit PGE_2) or vehicle (0.25 % ethanol) in CM. IRAK-M and β-actin protein expression was analyzed by Western blot. Blot shown is from a single experiment representative of two. *D*, Densitometry quantification of blots from two experiments as shown in part *C*. *E*, BMT mice were i.p. injected with a 100 μL dose of either 1.2 mg/kg indomethacin (Sigma) or 1% DMSO (vehicle) in sterile PBS. 24 hours following i.p. injection, AMs were harvested from control and BMT mice, and protein lysates of AM samples were prepared. IRAK-M and β-actin protein expression was analyzed by Western blot. Blot shown is from one experiment representative of two. *F*, Densitometry quantification of blots from two experiments as shown in part *E*. For panels D and F,*, $p < 0.05$; **, $p < 0.01$.

IRAK-M -/- BMT mice are protected from pneumonia following P. aeruginosa infection

Expression of IRAK-M is associated with reduced host defense following *in vivo* bacterial challenge (134, 144). Since IRAK-M is elevated in BMT AMs (Fig. 4.1), we wanted to determine whether the absence of IRAK-M in the hematopoietic cell compartment could improve pulmonary host defense post-BMT. Control, WT BMT (WT > WT), and IRAK-M -/- BMT (IRAK-M -/- > WT) chimeric mice were challenged with a 24 hour, acute *P. aeruginosa* lung infection. As previously published, WT BMT mice had increased bacterial burden in the lung (Fig. 4.4, panel A) and blood (Fig. 4.4, panel B) relative to control mice. However, IRAK-M -/- BMT mice, had enhanced clearance of *P. aeruginosa* from the lung and reduced dissemination to the blood relative to WT BMT mice. In fact, the host defense capabilities of IRAK-M-/- BMT mice were not significantly different from control mice. Overall, these results suggest that hematopoietic IRAK-M expression post-BMT impairs host defense against *P. aeruginosa* infection, and genetic ablation of IRAK-M in the bone marrow donor can be protective.

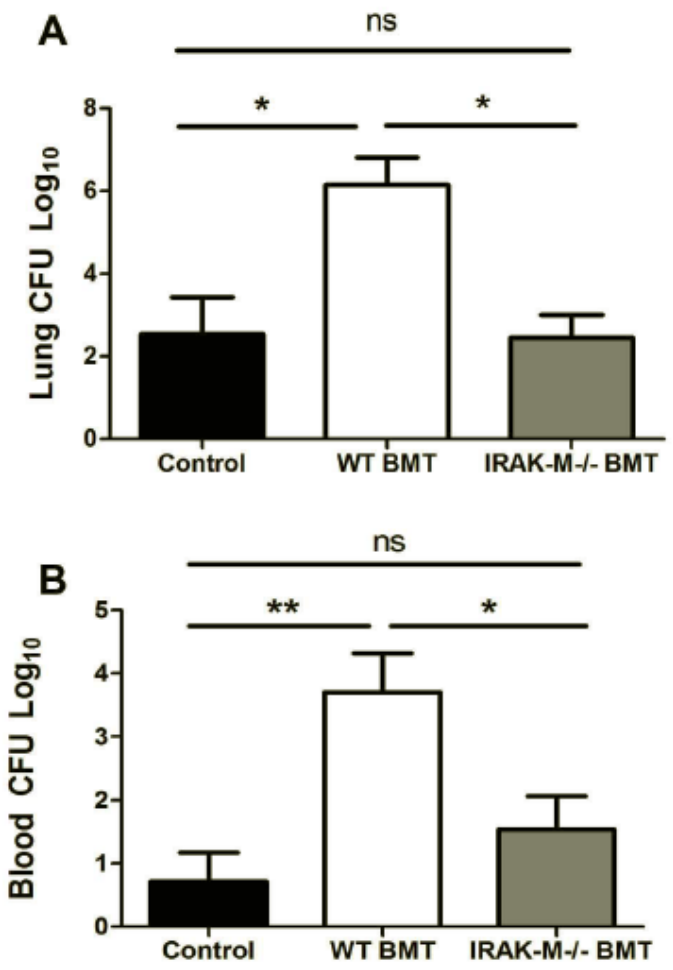

Figure 4.4: Restored host defense in IRAK-M -/- BMT mice following acute *P. aeruginosa* lung infection

Bone marrow from WT or IRAK-M -/- mice was transplanted into lethally-irradiated WT recipients. Control, WT BMT (WT > WT), and IRAK-M -/- BMT (IRAK-M -/- > WT) mice were i.t. injected with a 50 μL dose of 5 x 10^5 CFU *P. aeruginosa* 5-6 weeks post-BMT. 24 hours following infection, bacterial burden of whole lung (*A*) and blood (*B*) samples from each mouse was assessed by CFU assay (*, $p < 0.05$; **, $p< 0.01$; combined data from two independent experiments, n=6-10 mice per group).

IRAK-M -/- BMT mice have a survival advantage following lethal P. aeruginosa infection

IRAK-M -/- BMT mice show restored bacterial clearance relative to WT BMT mice following acute *P. aeruginosa* lung infection (Fig. 4.4). To determine whether

enhanced bacterial clearance in IRAK-M -/- BMT mice would provide a survival advantage following a lethal *P. aeruginosa* lung infection, we i.t. injected control, WT BMT, and IRAK-M -/- BMT mice with 1.5 x 10^6 CFU *P. aeruginosa*. As predicted, the rate of survival of IRAK-M -/- BMT and control mice was not statistically different (p= 0.274), but WT BMT mice had a significantly increased mortality rate compared to both control and IRAK-M -/- BMT mice (p > 0.05; Fig 4.5).

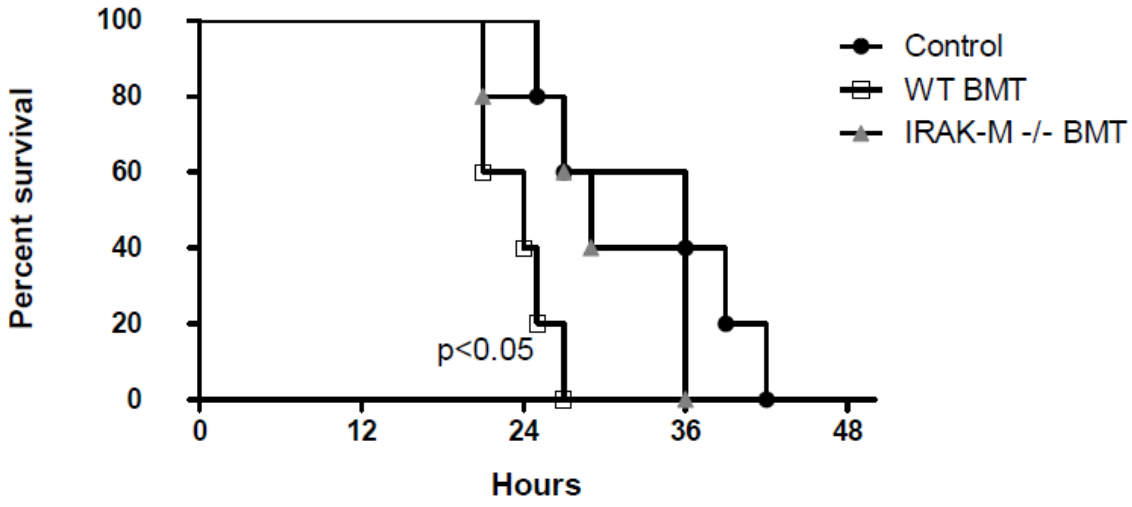

Figure 4.5: IRAK-M -/- BMT mice have survival advantage following lethal *P. aeruginosa* infection.
Control, WT BMT, and IRAK-M -/- BMT mice were i.t. injected with a 50 μL lethal dose of 1.5 x 10^6 CFU *P. aeruginosa* and rate of mortality was monitored for 2 days. Kaplan-Meier and log-rank methods were used to analyze survival rates (n=5 mice per group).

Inflammatory response during early P. aeruginosa infection

We next wanted to determine whether the improvement in host defense in IRAK-M-/- BMT mice was a result of differential accumulation of leukocytes in the lung during early infection. Control, WT BMT, and IRAK-M -/- BMT mice were injected with *P. aeruginosa* and 4 h later, lungs were harvested to assess leukocyte populations. Total lung leukocyte number was similar between all three groups (Fig. 4.6, panel A). The percentage of lung macrophages, lymphocytes, neutrophils, and eosinophils was not statistically different between WT BMT and IRAK-M -/- BMT mice 4 h post-infection (Fig. 4.6, panel B). Percent neutrophil accumulation ranged between 8-25% among control, WT BMT, and IRAK-M -/- BMT mice at 4 h post-infection (Fig. 4.6, panel B), but increased to 67-78% 24 hours post-infection (data not shown). Frequency of lung lymphocyte populations was also determined among uninfected mice, and no significant differences were observed between WT BMT and IRAK-M -/- BMT mice (Fig. 4.7).

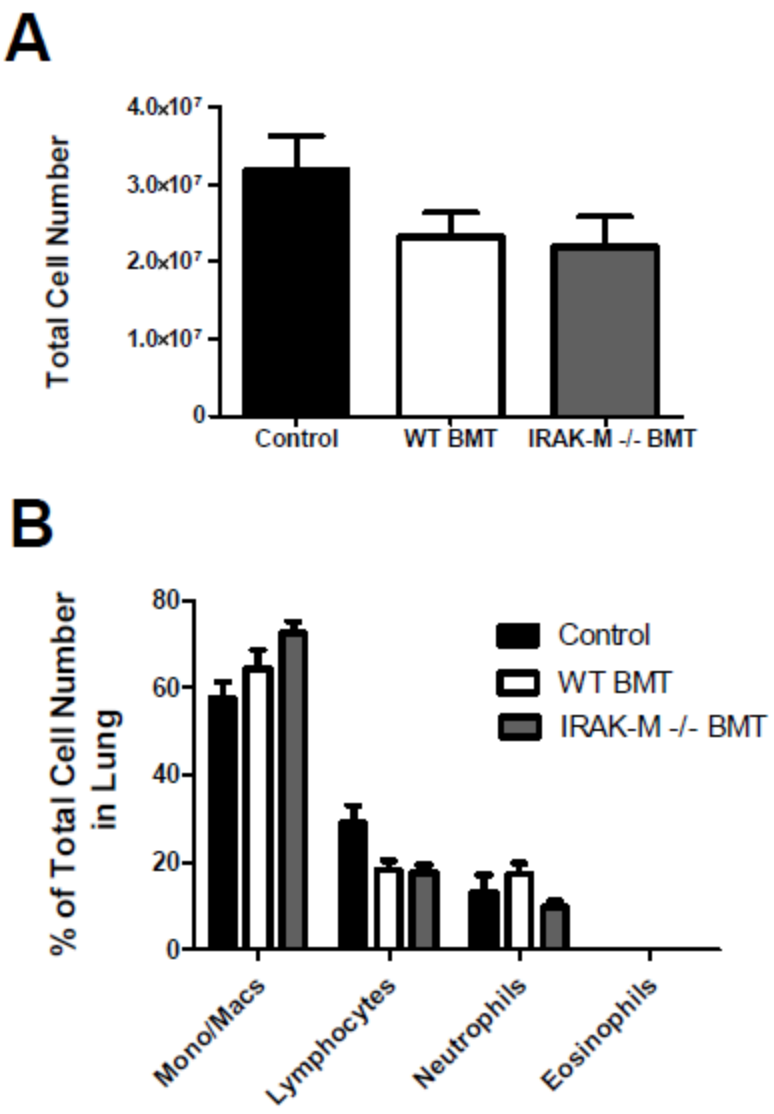

Figure 4.6: Inflammatory response during early *P. aeruginosa* infection
Control, WT BMT, and IRAK-M -/- BMT mice were i.t. injected with a 50 μL dose of 5 x 10^5 CFU *P. aeruginosa*. 4 hours following infection, whole lung samples were harvested from mice, and lung leukocytes were isolated, counted, and stained for differential cell analysis as described in the *Materials and Methods*. *A,* Percentage of macrophages (MΦ), lymphocytes, neutrophils, and eosinophils, and *B,* total lung leukocyte number during early infection (n= 4 mice per group).

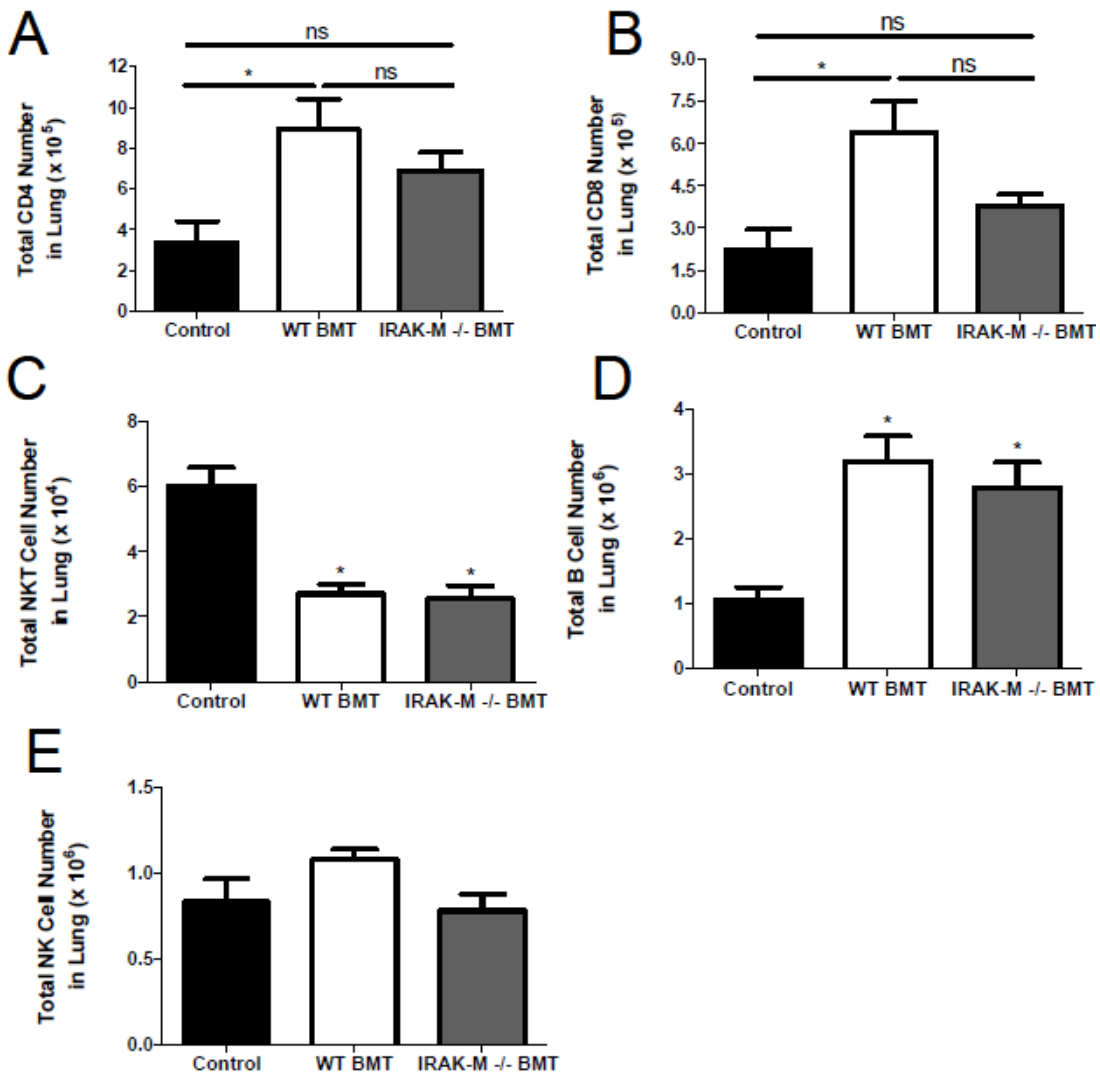

Figure 4.7: Lung lymphocyte populations in control and BMT mice
Lungs were harvested from uninfected control, WT BMT, and IRAK-M -/- BMT mice 5-6 weeks post-BMT. Lung leukocytes were isolated and analyzed by flow cytometry for specific cell surface markers as described in *Materials and Methods* to determine the total number of CD4 T cells (A), CD8 T cells (B), NK-T cells (C), B cells (D), and NK cells (E) in each sample (*, $p < 0.05$ compared to the conrol group; n=4-5 per group).

IRAK-M -/- BMT AMs display enhanced phagocytosis and killing of P. aeruginosa

Restored pulmonary host defense in IRAK-M -/- BMT mice may be explained by enhanced AM function in the absence of IRAK-M. To verify this hypothesis, *ex vivo* phagocytosis of FITC-*P. aeruginosa* was compared among AMs harvested from control, WT BMT, and IRAK-M -/- BMT mice. Similar to previous reports, WT BMT AMs displayed a 35% decrease in phagocytosis relative to control AMs; however, IRAK-M -/- BMT AMs had restored phagocytic ability (Fig. 4.8, panel A). Bacterial killing was also assessed in control and BMT AMs *ex vivo*. Relative to WT BMT AMs, survival of ingested bacteria was significantly diminished in the IRAK-M -/- BMT AMs (Fig. 4.8, panel B). The amount of surviving bacteria ingested by IRAK-M -/- BMT AMs was not statistically different from control AMs, indicating restoration of bacterial killing by BMT AMs in the absence of IRAK-M.

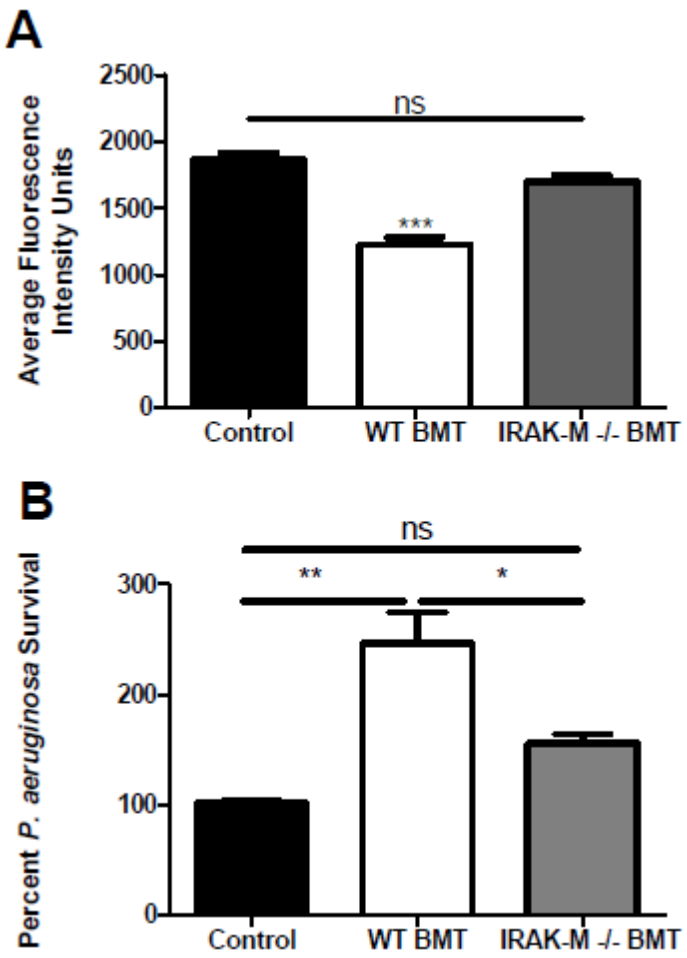

Figure 4.8: Restored phagocytosis and killing in IRAK-M -/- BMT AMs
AMs were harvested from control, WT BMT, and IRAK-M -/- BMT mice and cultured at 2 x 10^5 cells/well overnight. *A*, Phagocytosis of unopsonized FITC-*P. aeruginosa* and *B*, killing of *P. aeruginosa* was measured as described in *Materials and Methods.* Note impaired AM killing correlates with increased percentage of surviving bacteria relative to control in *B* (*, $p < 0.05$; **, $p< 0.001$; ***; n=10-11 per group).

IRAK-M -/- BMT AMs have restored TNF-α and cys-LT production

Macrophage production of cys-LTs enhances phagocytosis, microbial killing, and production of proinflammatory cytokines such as TNF-α (70, 72, 165, 192). We have previously demonstrated that AMs from BMT mice are defective in production of both of these mediators (68, 106). We therefore wanted to determine whether improved host defense mechanisms in IRAK-M -/- BMT AMs were also marked by restored AM cys-LT and TNF-α production. AMs harvested from control, WT BMT, and IRAK-M -/- BMT mice were cultured unstimulated overnight and supernatants were analyzed for cys-LT and TNF-α production. WT BMT AMs had significantly reduced levels of TNF-α (Fig. 4.9, panel A) and cys-LTs (Fig. 4.9, panel B) relative to control AMs. In contrast, levels of both TNF-α and cys-LTs were restored in IRAK-M -/- BMT AMs to levels seen in control AMs. Interestingly, this same trend was observed in whole lung homogenates from control, WT BMT, and IRAK-M -/- BMT mice given *P. aeruginosa* at 4 hours post-infection. TNF-α and cys-LT production was significantly reduced in WT BMT lung samples relative to control; however, IRAK-M -/- BMT mice had restored production of lung TNF- α and cys-LTs (Figs. 4.9, panels C and D).

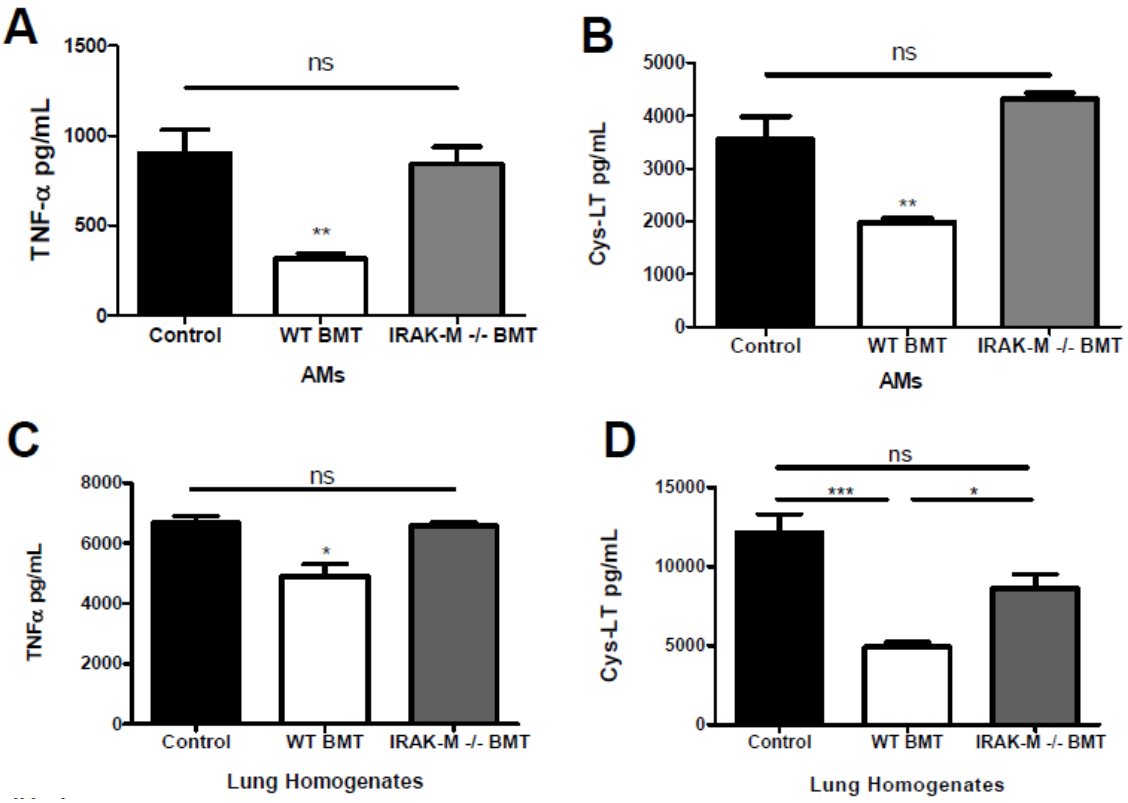

Figure 4.9: IRAK-M -/- BMT mice have restored TNF-α and cys-LT production
AM TNF-α (*A*) and cys-LT (*B*) production was measured by EIA/ELISA as described in *Materials and Methods* using overnight culture supernatants in complete media and serum-free media respectively from unstimulated AMs plated at 2 x 10^6 cells/mL (**, p< 0.01; n=4-7 per group). TNF-α (*C*) and cys-LT (*D*) levels in lung homogenates 4 hours post-i.t. infection with 50 μL dose of 5 x 10^5 CFU *P. aeruginosa* (*, p<0.05; ***, p< 0.001; n=3-4 per group).

Altered BMT AM TLR, MARCO, and TREM-1 expression

Enhanced macrophage production of proinflammatory cytokines and phagocytosis can be related to altered/elevated expression of cell surface receptors such as TLRs or scavenger receptors (193, 194). We wondered whether such alterations could explain the differences we observe between WT BMT and IRAK-M -/- BMT AMs in their host defense function. Expression of mRNA for TLR-2, -4, and -9, was measured (Fig. 4.10, panels A-C). The mRNA expression for TLR2 was downregulated in both BMT AM groups relative to control AMs. TLR4 mRNA expression was similar between control, WT BMT, and IRAK-M -/- BMT AMs. TLR9 mRNA expression was upregulated approximately 1.7-fold and 1.9-fold in WT BMT and IRAK-M -/- BMT AMs respectively. Additionally, we measured mRNA expression of the scavenger macrophage receptor with collagenous structure (MARCO, Fig. 4.10, panel D). Interestingly, we found that MARCO mRNA expression was downregulated not only in WT BMT AMs relative to control AMs, but MARCO was also downregulated in IRAK-M -/- BMT AMs. Finally, we looked at expression of the cell surface molecule, triggering receptor expressed on myeloid cells (TREM)-1. However, TREM-1 mRNA expression was reduced nearly 50% in both WT BMT and IRAK-M -/- BMT AMs relative to controls (Fig. 4.10, panel E).

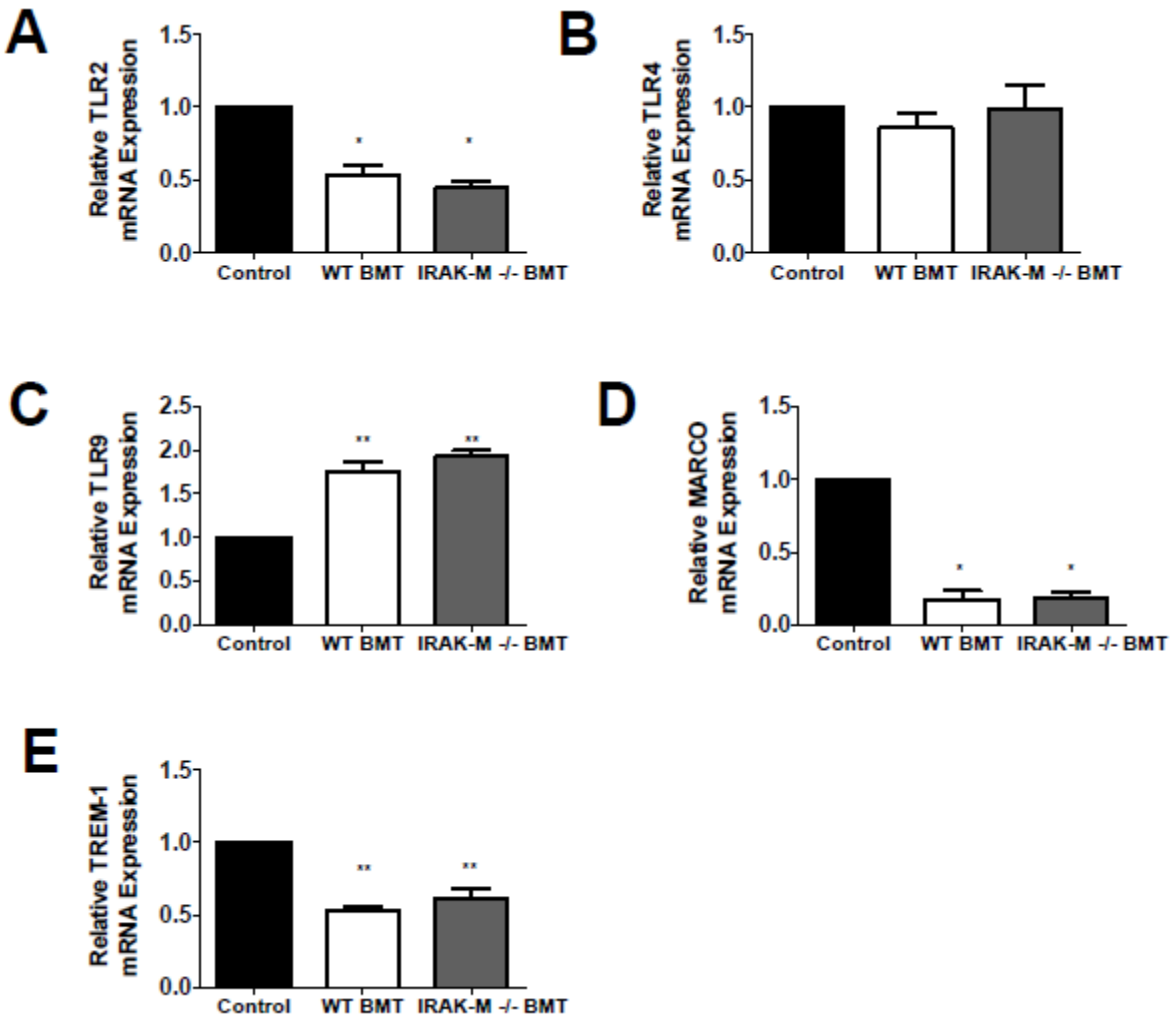

Figure 4.10: BMT AM TLR, MARCO, and TREM-1 expression
mRNA from AMs was prepared and analyzed by semiquantitative real-time PCR for expression of TLR2 (A), TLR4 (B), TLR9 (C), MARCO (D), and TREM-1 (E). For each sample, mRNA expression of the gene of interest was normalized to β-actin. The average of a single control sample was set to 1, and relative mRNA expression in other samples was measured by the ΔΔCT method (*, $p < 0.05$; samples were generated in two independent experiments, n=3-4 per group).

Both WT and IRAK-M -/- BMT AMs have elevated PGE_2 production and EP2 receptor expression

Post-BMT, elevated PGE_2 production in the lung significantly impairs AM phagocytosis and killing (68, 185, 186), and this is associated with elevated EP2 receptor expression in BMT AMs (68, 159). Therefore, we wanted to determine whether improved pulmonary host defense in IRAK-M -/- BMT mice was a result of reduced PGE_2 production and/or altered EP2 receptor expression in AMs. AMs were harvested from control, WT BMT, and IRAK-M-/- BMT mice, and AM EP2 mRNA expression was measured by real-time RT-PCR. It should be noted that reliable Abs to detect EP2 in the mouse are not available. Expression of EP2 in AMs was elevated in both transplant groups relative to control (Fig. 4.11, panel A). As noted previously (68), analysis of overnight culture supernatants revealed an 8-fold increase in WT BMT AM PGE_2 production over control AMs (Fig. 4.11, panel B). Interestingly, we found that IRAK-M -/- BMT AMs overproduced PGE_2 by 32-fold relative to control AMs. This phenotype of PGE_2 overproduction was unique to the transplant environment, given that PGE_2 production by AMs isolated from IRAK-M -/- non-transplant mice was comparable to WT control AMs (data not shown). Thus, AMs from IRAK-M -/- BMT mice display similar PGE_2 and EP2 receptor profiles that characterize AMs from WT BMT mice.

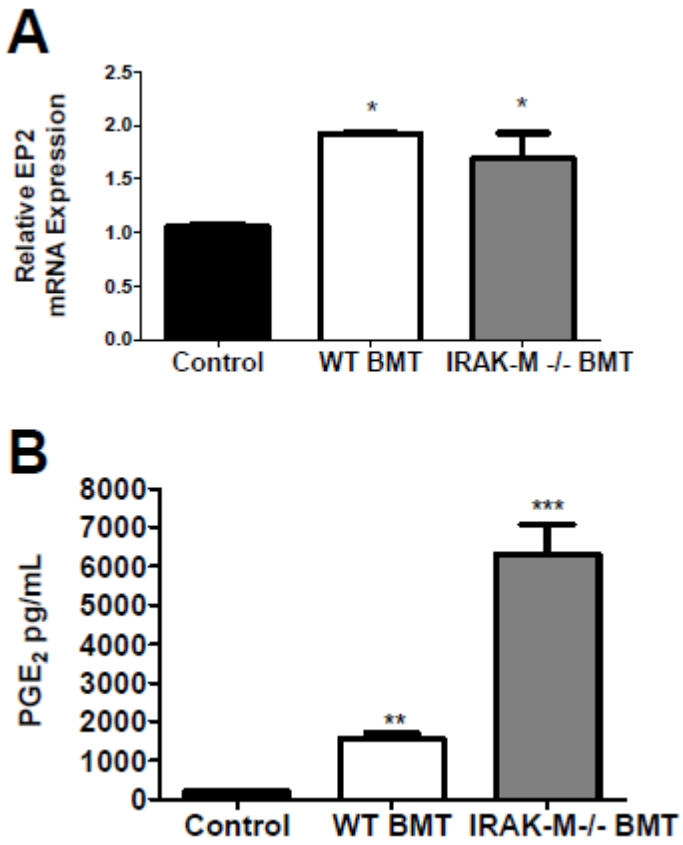

Figure 4.11: WT BMT and IRAK-M -/- BMT AMs display increased PGE_2 production and EP2 receptor expression

A, mRNA from AMs was prepared and analyzed by semiquantitative real-time PCR for expression of EP2. For each sample, EP2 expression was normalized to β-actin. The average of a single control sample was set to 1, and relative EP2 expression in other samples was measured by the ΔΔCT method (*, $p < 0.05$; samples were generated in two independent experiments, n=3-4 per group). *B*, AMs were harvested and cultured overnight at 2 x 10^6 cells/mL. The following day, supernatants were collected and analyzed for PGE_2 concentration by EIA as described in *Materials and Methods* (** $p < 0.01$, *** $p<0.001$; n=3-4 per group).

IRAK-M is necessary for PGE_2-mediated inhibition of AM phagocytosis

IRAK-M -/- BMT AMs have restored host defense (Figs. 4.8 and 4.9), despite overproduction of PGE_2 and elevated EP2 receptor expression (Fig. 4.11). Therefore, we wanted to determine whether IRAK-M was required for PGE_2 to mediate inhibition of AM host defense. *Ex vivo* phagocytosis of FITC-labeled bacteria was assessed in non-transplant WT and IRAK-M -/- AMs pretreated in the presence or absence of 10 nM PGE_2 . PGE_2 significantly inhibited WT AM phagocytosis but did not inhibit IRAK-M -/- AM phagocytosis (Fig. 4.12). Thus, in the absence of IRAK-M, AMs were not sensitive to the inhibitory effects of PGE_2.

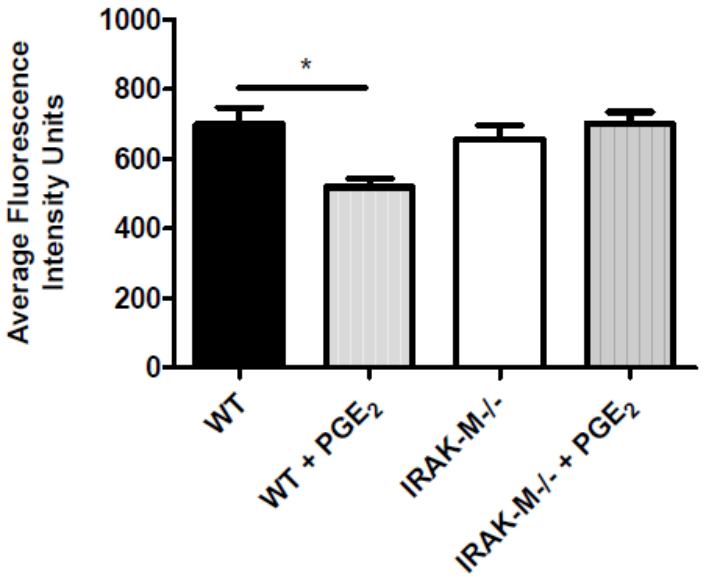

Figure 4.12: IRAK-M mediates PGE_2 inhibition of AM phagocytosis.
AMs were harvested from non-transplanted WT and IRAK-M -/- mice and cultured at 2 x 10^5 cells/well overnight. Phagocytosis of unopsonized FITC-*E. coli* was measured following 15 minute pretreatment with 10 nM PGE_2 as described in *Materials and Methods* (*, $p < 0.05$; n=6 per group).

Discussion

Overproduction of PGE_2 in the lung post-BMT significantly diminishes AM phagocytosis, killing, and TNF-α production (68, 159, 185, 186). Although a mechanism has not been defined, our current study suggests a role for IRAK-M in PGE_2-induced immunosuppression post-BMT. We are the first to show that IRAK-M is elevated in BMT AMs, and this phenotype is related to increased PGE_2 signaling. Furthermore, we demonstrate a suppressive role for IRAK-M in host defense post-BMT, as genetic ablation of IRAK-M in the bone marrow restores AM function and host defense against *P. aeruginosa* lung infection.

Our results demonstrate that IRAK-M expression is elevated in AMs but not elicited lung neutrophils post-BMT (Fig.4.1). IRAK-M expression has been associated mainly with monocytic cells; however, expression has also been detected in tissues as well as fibroblasts, B cells, and alveolar epithelial cells (61, 133, 134, 136). IRAK-M expression is rapidly induced by MyD88-dependent IL-1R/TLR signaling as part of a negative feedback mechanism. As a result, IRAK-M stably binds the MyD88 adaptor complex and prevents activation of pathways that initiate NFκ-B-induced proinflammatory cytokine production (61, 134). This negative regulation of MyD88-dependent IL-1R/TLR signaling induces endotoxin tolerance as well as macrophage deactivation in sepsis and cancer (144, 191, 195). In our studies, we show that post-BMT, elevated IRAK-M expression in AMs is related to increased PGE_2 signaling (Fig. 4.3). It is interesting to speculate that this may also be a mechanism involved in macrophage deactivation in cancer, as certain tumors are known to overproduce PGE_2 (196).

PGE_2 is a part of the eicosanoid family of lipid mediators with potent immunomodulatory functions (72). PGE_2 in particular has a number of immunosuppressive properties. By elevating intracellular cAMP, PGE_2 signaling activates two key effectors: protein kinase A and Epac-1 (124, 163). In undefined pathways, activation of these two molecules collectively induces production of IL-10 and inhibits NFκ-B induced proinflammatory cytokine production, Fc receptor-mediated phagocytosis, and microbial killing pathways. Our results suggest that upregulation of IRAK-M may be one mechanistic pathway by which PGE_2 signaling limits AM host defense. PGE_2 can increase the transcription of IRAK-M within 3 hours of stimulation (unpublished observation), and protein levels are elevated by 24 hours post-treatment (Fig. 4.3). However, our results cannot determine whether IRAK-M has direct inhibitory effects on phagocytosis and killing, or whether the improved anti-bacterial host defense seen in the absence of IRAK-M post-BMT is secondary to the restoration of proinflammatory cytokine and/or eicosanoid production.

Although exogenous and endogenous PGE_2 can elevate IRAK-M protein expression in AMs as much 3.5-fold above controls (Fig. 4.3), it is possible that factors aside from PGE_2 may also contribute to elevation of IRAK-M in AMs post-BMT. We previously reported that BMT AMs overproduce GM-CSF relative to control AMs (159). Recent studies have shown that GM-CSF regulates expression of IRAK-M in addition to other components of the TLR4 signaling pathway in primary mouse AMs (197). However, we did not detect increases in IRAK-M protein expression in non-transplant AMs following overnight treatment with 50 ng/mL GM-CSF (data not shown); whereas we could demonstrate augmentation of IRAK-M expression by exogenous PGE_2

treatment. In addition, it is possible that IRAK-M is upregulated due to increased endogenous endotoxin levels following BMT. Epithelial cell damage caused by TBI and/or chemotherapy conditioning regimens can result in loss of gut integrity (198). This can increase gut bacterial leakage and raise levels of circulating endotoxin. However, IRAK-M levels in BMT AMs remain elevated to a similar degree if the AMs are cultured overnight in CM or if they are assayed directly from mice. This suggests to us that the elevation in IRAK-M is related to autocrine production of PGE_2 in those cultures, rather than gut-derived LPS which would presumably have been washed out of the culture.

We next assessed whether the alteration in AM function between the WT BMT and IRAK-M-/- BMT mice could be related to increased TLR and/or scavenger receptor expression post-BMT (Fig. 4.10) We found that expression of TLR2 was increased and TLR9 was decreased in AMs from both WT BMT and IRAK-M-/-BMT mice relative to control. Additionally, expression of TLR4 was not different among the three groups. MARCO is the major receptor on AMs for binding of non-opsonized particles (199) and MARCO-deficient mice are more susceptible to pneumococcal infection (200). Expression of the scavenger receptor MARCO, was downregulated in both BMT groups relative to control AMs. The same trend was observed in regard to BMT AM mRNA expression of TREM-1, which has been shown to improve bacterial clearance from the lung following *Streptococcus pneumoniae* lung infection via negative regulation of IRAK-M (136). Thus, while there are some reductions in these surface molecules between the control and BMT groups, the differences we observe in WT BMT and IRAK-M -/- BMT AM host defense functions are likely not related to altered expression of TLRs, MARCO or TREM-1.

Our data demonstrate that IRAK-M -/- BMT mice display enhanced clearance of *P. aeruginosa* from the lung and diminished dissemination of *P. aeruginosa* to the blood (Fig. 4.4). Furthermore, IRAK-M -/- BMT mice have a significantly reduced mortality rate relative to WT BMT mice following lethal *P. aeruginosa* lung infection (Fig. 4.5). These data suggest that hematopoietic expression of IRAK-M increases susceptibility to *P. aeruginosa* lung infection post-BMT, and ablation of hematopoietic IRAK-M expression provides more effective clearance of *P. aeruginosa* to improve survival rates in BMT mice following infection. Other studies have also demonstrated a protective role for IRAK-M deficiency in improved host defense. In both septic patients and mice, IRAK-M expression is elevated in monocytic cells and is associated with increased susceptibility to secondary infections, particularly bacterial pneumonia (144, 191). Interestingly, impaired pulmonary host defense following sepsis is improved in IRAK-M -/- mice (144). In addition, IRAK-M -/- mice display enhanced ability to clear infection, following challenge with *S. typhimurium*. This was associated with enhanced macrophage TLR signaling and pro-inflammatory cytokine production in response to TLR ligand and pathogen stimulus respectively (134). Therefore, targeting IRAK-M may serve a number of therapeutic roles in reducing bacterial infections in immunosuppressed patients.

We also found that the absence of IRAK-M in BMT AMs enhances phagocytosis and killing of non-opsonized *P. aeruginosa* (Fig. 4.8). Whether TLR signaling regulates phagocytosis is unclear. It has been argued that phagocytosis is a mechanical process independent of the TLR signals produced from endocytosed cargo (201-203). However, other studies indicate the possibility for TLR signaling to enhance phagocytosis via

increases in phagosome maturation or upregulation of gene transcription programs associated with phagocytosis (204-206). Interestingly, cys-LTs are known to enhance a number of host defense mechanisms in AMs, such as phagocytosis, killing, and proinflammatory cytokine production (70, 72, 165, 192). Therefore, restored cys-LT production in IRAK-M -/- BMT AMs (Fig. 4.9) likely contributes to enhanced phagocytosis, killing, and TNF-α production post-BMT. It is important to note, however, that AMs from non-transplanted IRAK-M -/- mice display similar phagocytic ability as WT non-transplant mice (Fig. 4.12), and TNF-α production is comparable between groups (data not shown). Thus, these differences may not be apparent under basal conditions where expression of IRAK-M is low.

Given that IRAK-M has been identified in other cell types, we questioned whether IRAK-M could also be detected in neutrophils and if so, whether expression of IRAK-M in these cells was altered in the setting of BMT. After eliciting neutrophils to the lungs of control or BMT mice via i.t. injection of LPS as described (68, 106), we found no difference in total numbers of recruited neutrophils (data not shown). In these recruited lung neutrophils, we could detect IRAK-M by Western blot, but there was no increase in BMT neutrophil IRAK-M expression relative to control neutrophils (Fig. 4.1). This may reflect the fact that IRAK-M is maximally elevated in these cells in response to the LPS recruitment signal. However, there was no clear evidence that neutrophils upregulated IRAK-M in response to BMT. This is consistent with our earlier findings which demonstrated no defect in the ability of neutrophils from BMT mice to phagocytose bacteria, despite elevations in neutrophil PGE_2 production post-BMT (68, 106, 159). Additionally, we wondered whether there was a defect in neutrophil

recruitment to the lung in response to *P. aeruginosa* challenge between WT BMT and IRAK-M-/- BMT mice. Both WT BMT and IRAK-M-/- BMT mice were effectively able to recruit neutrophils in response to bacterial challenge and no differences were noted between the groups (Fig. 4.6). Taken together, our results suggest that the suppressive actions of IRAK-M post-BMT may be restricted to alveolar macrophages. These results are also consistent with a previous report which demonstrated impaired host-defense against *P. aerugionsa* following clodronate liposome depletion of AMs (207).

Our earlier studies demonstrated that impaired host defense in AMs post-BMT requires elevation in PGE_2 production and concomitant increases in expression of the inhibitory EP2 receptor. It is not known for certain why expression of PGE_2 is elevated post-BMT, but it is likely to be the result of a general upregulation in the transcription or stability of all key prostaglandin synthetic enzymes (Figure 4.2). Interestingly, improved host defense in IRAK-M -/- BMT mice is observed despite elevated PGE_2 production and EP2 receptor expression in IRAK-M -/- BMT AMs (Fig. 4.11). Furthermore, IRAK-M -/- AMs were insensitive to PGE_2-mediated inhibition of non Fc-mediated phagocytosis (Fig. 4.12). This suggests that PGE_2 may require IRAK-M to inhibit AM function, and therefore, IRAK-M -/- BMT AMs may not be responsive to PGE_2-induced immunosuppression despite the upregulation of the inhibitory receptor. PGE_2 is also known to signal via another inhibitory receptor, EP4 (108). However, EP4 mRNA expression is slightly downregulated in both WT BMT and IRAK-M -/- BMT AMs relative to control AMs (data not shown). We suspect that the high levels of PGE_2 secreted by AMs from IRAK-M-/- BMT mice relative to WT BMT mice represent a

compensation that occurs in the absence of appropriate EP2-induced IRAK-M feedback inhibition. It is likely that TLR-mediated NF-κB activation of COX-2 is unchecked in the absence of IRAK-M. In total, our results demonstrate that IRAK-M is a critical mediator of the inhibitory signaling mediated by EP2 activation.

HSCT patients are at risk for infections with many organisms, and it would be interesting to know whether PGE_2-induced IRAK-M expression could limit host defense against most pathogens. We speculate that the elevations in IRAK-M will only impair innate immune function in AMs, and thus will likely only inhibit host defense against pathogens (e.g. bacteria) that are primarily cleared by AMs. Consistent with this hypothesis, treatment of BMT mice with indomethacin did not improve the clearance of murine gammaherpesvirus-68, a pathogen known to require CD4 T cell adaptive immunity for immune control (208).

Overall, our data provide further insight into the mechanism by which the procedure of transplant (without immunosuppressive therapy or GVHD) can impair immune function following complete hematopoietic reconstitution. While immune suppression in humans post-HSCT is likely to be complex, our data suggest that IRAK-M likely plays an important role in PGE_2-induced immunosuppression post-BMT, and that genetic ablation of IRAK-M in bone marrow improves pulmonary host defense post-BMT. This presents the possibility for therapeutic targeting of IRAK-M post-BMT to improve patient outcomes in response to infection. An important aspect for our future studies will be to find ways to target IRAK-M and to determine the level of suppression necessary to improve AM function.

CHAPTER 5: The Role of PTEN in Impaired Pulmonary Host Defense Post-BMT

Introduction

AMs are the primary immune cell type in the alveolar space and are critical for initiating immune responses against inhaled pathogens (3-5). However, AMs are reported to have decreased host defense capability following HSCT. Furthermore, neutrophils recruited to sites of infection display impaired chemotaxis and killing ability post-HSCT (105). These studies using allogeneic transplant patients, however, involve various confounding factors (such as immunosuppressive drug therapy and graft-versus-host disease) which are known to impair immune function (7, 8). Thus, the specific impact of conditioning and reconstitution alone on pulmonary innate immune function remains unclear.

To determine the impact of HSCT on pulmonary host defense, we previously developed a mouse model of syngeneic BMT. Compared to non-transplant controls, BMT mice were more susceptible to *P. aeruginosa* pneumonia following i.t. infection, despite full hematopoietic reconstitution in the lung and periphery (106). Furthermore, donor-derived BMT AMs and recruited lung neutrophils displayed impaired host defense functions (68). We found that this reduction in innate immune function was induced by elevated production of the immunosuppressive lipid mediator, PGE_2, in the lung post-BMT (68, 185, 186).

PGE_2 is known to inhibit bacterial killing, phagocytosis (124, 163), chemotaxis (187), and production of proinflammatory mediators in leukocytes (188-190). At least one consequence of increased PGE_2 production after transplant is the upregulation of IRAK-M which limits AM function (Fig. 4.3). However, it is not known what downstream signaling pathways PGE_2 activates to upregulate IRAK-M. One potential candidate in the PGE_2 signaling pathway that may regulate IRAK-M is PTEN, which has also been reported to negatively regulate phagocytosis and bacterial killing in AMs (120). However, it remains unknown whether PTEN regulates AM function in the BMT setting.

PTEN is a dual-specificity phosphatase that can dephosphorylate both protein and lipid targets; however, PTEN is best characterized by its ability to dephosphorylate the lipid second messenger PIP_3, a key mediator of the PI3K/AKT signaling pathway (209). This dephosphorylation event results in the inhibition of Fcγ-R-mediated phagocytosis of IgG-opsonized particles (94, 95). PTEN phosphatase activity is negatively regulated via a number of post-translational mechanisms, including tyrosine phosphorylation (146). Interestingly, PGE_2 activation of the cAMP signaling cascade has been shown to induce PTEN activity via tyrosine dephosphorylation in a SHIP-1 dependent manner (120, 210). Furthermore, these studies showed that PGE_2-mediated increases in PTEN activity diminish AM phagocytosis and killing of IgG-opsonized particles (120). Additional roles for PTEN may also exist in the inhibition of non-Fcγ-R mediated phagocytosis, given that PI3K activity has also been reported to contribute to phagocytosis of non-opsonized particles (98, 99, 211). Therefore, we hypothesize that in our model of syngeneic BMT, increased PGE_2 signaling in the lung inhibits host defense against acute *P. aeruginosa* infection by elevating PTEN activity in AMs. Additionally, we wish to determine the

influence of PTEN in both opsonized and non-opsonized phagocytosis pathways and whether PTEN signaling is related to IRAK-M elevation post-BMT.

To address our hypothesis, we measured PTEN activity and AKT phosphorylation levels in BMT and non-transplant control AMs in the presence or absence of an inhibitor of endogenous PGE_2 production. Additionally, we transplanted WT mice with bone marrow from myeloid-specific PTEN KO mice to determine whether PTEN plays a role in impaired pulmonary host defense post-BMT. We demonstrate that increased PGE_2 signaling augments PTEN activity in BMT AMs and diminishes pAKT levels. Furthermore, we show that myeloid-specific ablation of PTEN in the bone marrow of transplant mice can restore AM phagocytosis of serum opsonized bacteria and improve bacterial clearance following *P. aeruginosa* infection. Finally, we demonstrate that the effect of PTEN on AM host defense post-BMT likely is independent of IRAK-M.

Results

Increased PTEN activity and diminished pAKT levels in BMT AMs

Following syngeneic BMT, AMs display elevated production of PGE_2, which directly impairs AM phagocytosis and killing of IgG-opsonized and non-opsonized particles (68). Given that PGE_2 is known to inhibit FcγR-mediated phagocytosis and killing in AMs by upregulating PTEN activity (120), we wanted to determine whether overproduction of PGE_2 in BMT AMs resulted in elevated PTEN phosphatase activity. PTEN was immunoprecipitated from BMT and non-transplant control AMs, and the lipid phosphatase activity of the purified PTEN protein was assessed following incubation with PIP_3. Relative to control AMs, BMT AMs had approximately a 50% increase in PTEN activity (Fig.5.1, panel A). This increase was blocked in BMT AMs following overnight treatment with indomethacin, an inhibitor of endogenous PGE_2 production (Fig. 5.1, panel A).

Increased PTEN activity inhibits the activation of a number of downstream targets in the FcγR-signaling pathway, such as AKT (146, 209). To determine whether increased PTEN activity in BMT AMs translates into functional AKT suppression, AMs from control and BMT mice were stimulated with IgG-opsonized SRBCs to induce phosphorylation of AKT. FcγR stimulation of control AMs induced phosphorylation of AKT (Fig. 5.1B, lane 2); however, as previously reported (120), pretreatment with 100 nM PGE_2 blocked FcγR-induced AKT phosphorylation (Fig. 5.1B, lane 3). As expected, BMT AMs had no detectable levels of pAKT following FcγR-stimulation (Fig 5.1B, lane 5). pAKT levels were restored in FcγR-stimulated BMT AMs following pretreatment with indomethacin (Fig. 5.1B, lane 6). Taken together, these data suggest that elevated

PTEN activity and diminished pAKT levels in BMTs AM are related to increased PGE_2 signaling.

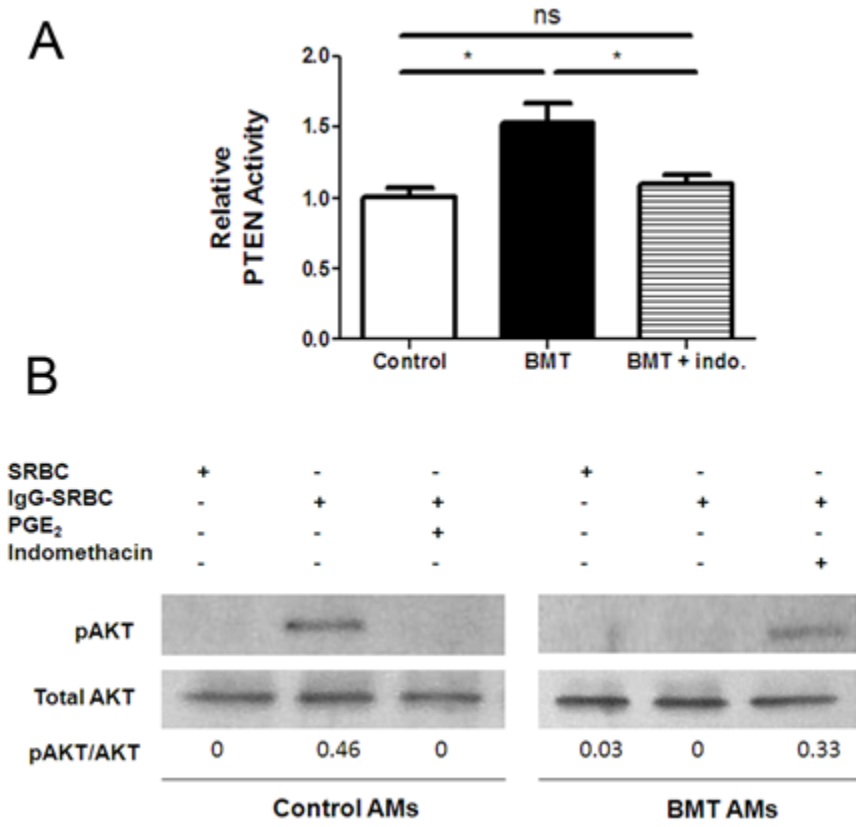

Figure 5.1: Overproduction of PGE_2 elevates PTEN activity and diminishes pAKT levels in BMT AMs.
Mice were given a syngeneic BMT and harvested 5-6 weeks post-transplant. AMs were isolated by BAL as described in *Materials and Methods* from BMT and non-transplanted control mice. *A*, PTEN protein was immunoprecipitated from whole-cell lysates of AMs cultured at 5 x 10^5 per well in the presence of 5 μM indomethacin or 0.05% vehicle control overnight. PTEN phosphatase activity was determined as described in *Materials and Methods*. Results are represented as percent PTEN phosphatase activity relative to untreated control group (*, $p<0.05$; n=3-4 per group combined from 2 experiments). *B*, AMs were pretreated with either 5 μM indomethacin for 2h or 100 nM PGE_2 for 15 mins at 37°C. AMs were then stimulated with IgG-SRBCs (1:10 ratio) for 15 mins at 37°C. Following IgG-SRBC stimulation, AMs were prepared for Western blot analysis as described in *Materials and Methods*. Average relative band densitometry data are indicated under each lane (Data shown is representative of 2 experiments).

Inhibition of PTEN activity restores phagocytosis in BMT AMs

Since PTEN activity is known to inhibit FcγR-mediated phagocytosis in macrophages (145), we wanted to determine whether pharmacologic inhibition of PTEN activity could restore phagocytosis in BMT AMs. The phosphatase inhibitor, bpV(pic), is known to specifically inhibit PTEN phosphatase activity at concentrations within the nanomolar range (6). We found that pretreating BMT AMs with 100 nM bpV(pic) restored phagocytosis of serum opsonized *P. aeruginosa* to that of control AM levels (Fig. 5.2, panel A). In experiments using non-opsonized *P. aeruginosa*, bpV(pic) treatment was also able to significantly improve the phagocytic function of BMT AMs, although not to control AM levels (Fig. 5.2, panel B). These data suggest that PTEN negatively regulates phagocytosis but may play a more important role in the inhibition of serum opsonized phagocytosis as opposed to non-opsonized phagocytosis.

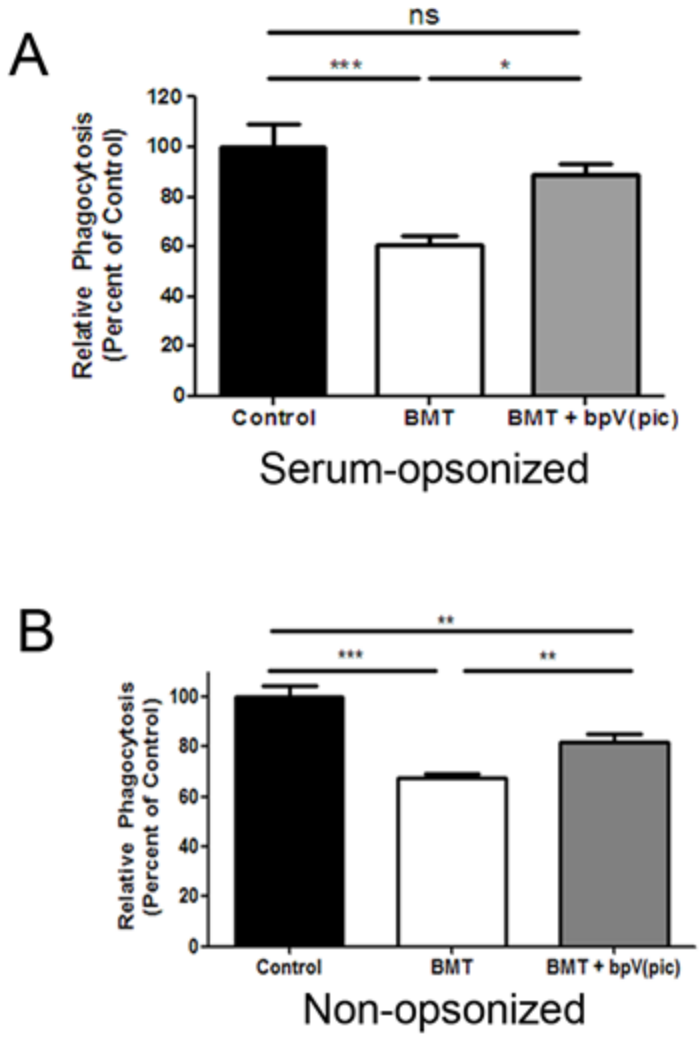

Figure 5.2: Inhibiting PTEN activity improves BMT AM phagocytic ability. AMs were harvested from control and BMT mice and cultured at 2 x 10^5 cells/well overnight. Phagocytosis of serum-opsonized (A) and non-serum-opsosonized (B) FITC-*P. aeruginosa* was assessed as described in *Materials and Methods* (* $p<0.05$, ** $p<0.01$, *** $p<0.001$; Panel A n=10 per group, panel B n=5 per group).

Myeloid-specific ablation of PTEN expression in BMT mice

Myeloid-specific ablation of PTEN enhances lung phagocyte function and pulmonary host defense against Gram-negative bacterial infections (1, 2); however, it is not known whether similar mechanisms are relevant in the setting of BMT. Since our data demonstrate that pharmacologic inhibition of PTEN activity improves BMT AM

phagocytosis *in vitro* (Fig. 5.2), we generated myeloid-specific PTEN KO mice to assess whether reconstituting PTEN deficient bone marrow into irradiated WT mice would restore host defense function in AMs. To generate myeloid-specific PTEN KO mice, we obtained mice homozygous for a "floxed" PTEN mutant allele containing *loxP* sites on either side of exon 5 encoding the phosphatase domain. The PTEN floxed mice were bred to myeloid-specific Cre mice, which express the Cre recombinase gene under the control of the lysozyme M (LysM) promoter. As previously described, mice bred to express two copies of the floxed PTEN allele and at least one copy of LysM-Cre recombinase are deficient in PTEN expression in cells of the myeloid lineage (2, 9). Furthermore, these mice do not display an abnormal phenotype and are viable, fertile, and normal sized (2, 9).

To verify PTEN ablation in our BMT model, AMs were harvested from non-transplanted WT control mice and lethally TBI WT mice transplanted with either WT bone marrow (WT BMT) or myeloid-specific (conditional) PTEN KO bone marrow (PTEN CKO BMT). PTEN protein expression was comparable in both control and WT BMT AMs (Fig. 5.3). As expected, PTEN protein expression was ablated in AMs from PTEN CKO BMT mice (Fig. 5.3). Similar results were observed in recruited lung neutrophils (data not shown).

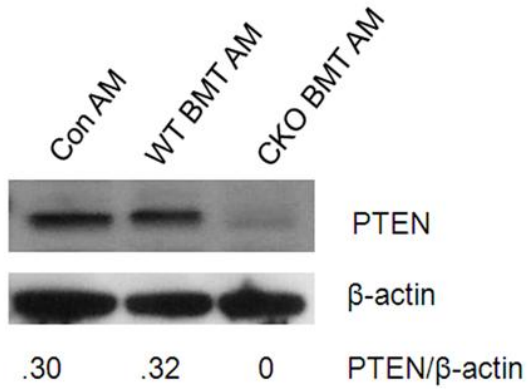

Figure 5.3: PTEN expression is ablated in AMs from PTEN CKO BMT mice. Whole-cell lysates were prepared from control, WT BMT, and PTEN CKO BMT AMs following 1h SFM adherence. PTEN and β-actin protein expression were analyzed by Western blot. Ablation of PTEN was verified in this manner for all experiments using PTEN CKO BMT mice.

AMs from PTEN CKO BMT mice display enhanced opsonized phagocytosis and killing of P. aeruginosa, *despite overproduction of* PGE_2

We next performed experiments to determine whether myeloid-specific disruption of PTEN could improve opsonized phagocytosis and killing of *P. aeruginosa* in BMT AMs. We found that AMs from PTEN CKO BMT mice displayed restored phagocytosis of serum-opsonized FITC-*P. aeruginosa in vitro* relative to WT BMT AMs (Fig. 5.4, panel A). Furthermore, WT BMT AMs had increased survival of ingested *P. aeruginosa* relative to control AMs, indicating defective bacterial killing ability (Fig. 5.4, panel B). However, the amount of surviving bacteria was significantly lower in PTEN CKO BMT AMs compared to control AMs, suggesting bacterial killing is not only restored in AMs from PTEN CKO BMT mice but enhanced (Fig. 5.4, panel B). Interestingly, AMs from PTEN CKO BMT mice produce significantly more PGE_2 than both control and WT BMT AMs, as measured from overnight culture supernatants (Fig. 5.4, panel C). These data

suggest that PTEN ablation in AMs restores phagocytosis of serum opsonized bacteria post-BMT, despite AM overproduction of PGE_2.

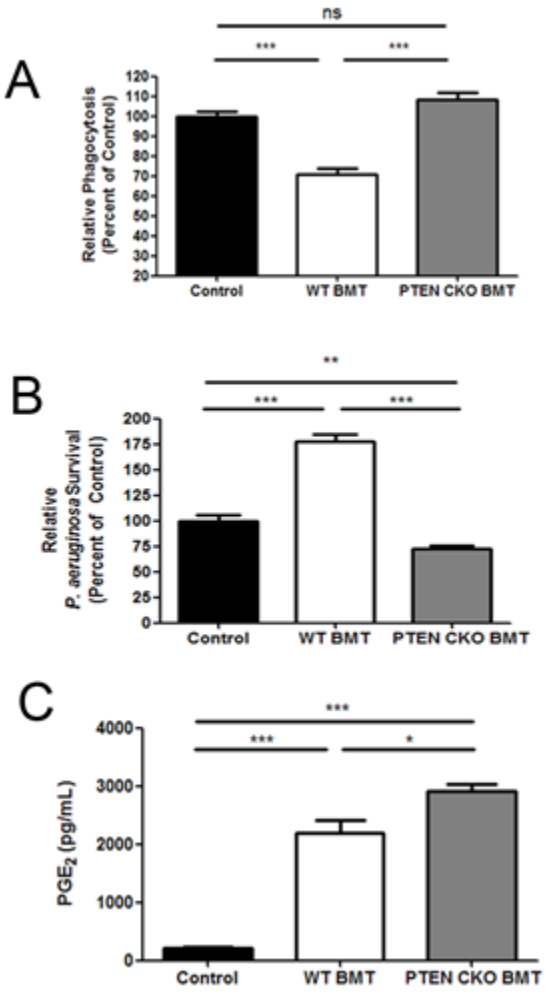

Figure 5.4: Restored AM phagocytosis and killing of *P. aeruginosa* in PTEN CKO BMT mice, despite AM overproduction of PGE_2

Bone marrow from WT or myeloid-specific PTEN CKO mice was transplanted into lethally irradiated WT recipients. WT control, WT BMT (WT>WT), and PTEN CKO BMT (PTEN CKO>WT) AMs were harvested and cultured at 2 x 10^5 cells/well overnight. Phagocytosis of serum-opsonized FITC-*P. aeruginosa* (A) and killing of serum opsonized *P. aeruginosa* (B) was assessed as described in *Materials and Methods* (** $p<0.01$, *** $p<0.001$; n=7-10 per group). PGE_2 levels were also measured in overnight culture supernatants by EIA as described in *Materials and Methods* (C) (* $p<0.05$, *** $p<0.001$; n=4-5 per group).

PTEN ablation improves but does not fully restore non-opsonized phagocytosis in AMs post-BMT

Our results with the PTEN inhibitor, bpV(pic), suggest that PTEN function may be less important for non-opsonized phagocytosis of bacteria. To verify this with the genetic ablation, AMs were harvested from control, WT BMT and PTEN CKO BMT mice, and phagocytosis of non-opsonized bacteria was tested *in vitro* (Figure 5.5). Similar to our findings in Figure 5.2, PTEN CKO BMT AMs showed improved phagocytosis when compared to WT BMT AMs. However, this improvement in phagocytic ability was not restored to control AM levels. Thus, BMT-mediated increases in PTEN may play a lesser role in non-opsonized phagocytosis as compared to opsonized phagocytosis.

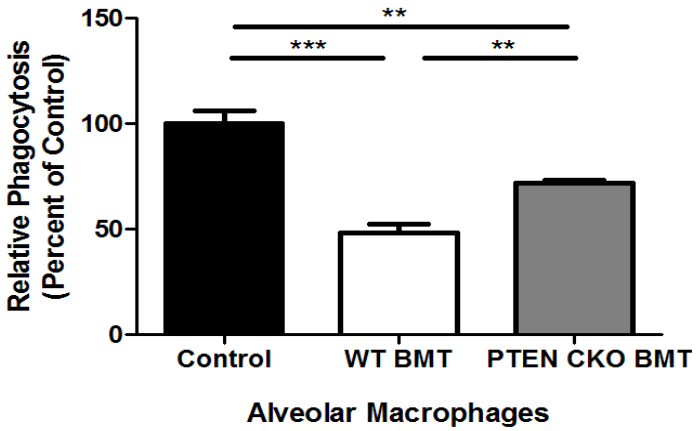

Figure 5.5: Improved phagocytosis of non-opsonized *P. aeruginosa* in PTEN CKO BMT AMs
AMs were harvested from control, WT BMT, and PTEN CKO BMT mice and cultured at 2 x 10^5 cells/well overnight. Phagocytosis of non-serum-opsosonized FITC-*P. aeruginosa* was assessed as described in *Materials and Methods* (** $p<0.01$, *** $p<0.001$; n=7-10 per group).

Restored AM and lung TNF-α levels in PTEN CKO BMT mice

AMs from neutropenic myeloid-specific PTEN KO mice display enhanced proinflammatory cytokine profiles, including increased production of TNF-α, relative to neutropenic WT mice (155). Because TNF-α is a key proinflammatory mediator in pulmonary host defense against *P. aeruginosa* and can activate bacterial killing mechanisms in macrophages (4, 11, 12), we measured TNF-α levels in AM overnight culture supernatants and lung homogenates from mice challenged with an acute *P. aeruginosa* lung infection. As previously reported (106, 159), defective TNF-α production was observed in WT BMT AMs and lung homogenates relative to control samples (Figs. 5.6, panels A and B). However, PTEN CKO BMT mice displayed improved AM TNF-α production (Fig. 5.6, panel A), and lung homogenates from infected mice had fully restored TNF-α levels (Fig. 5.6, panel B).

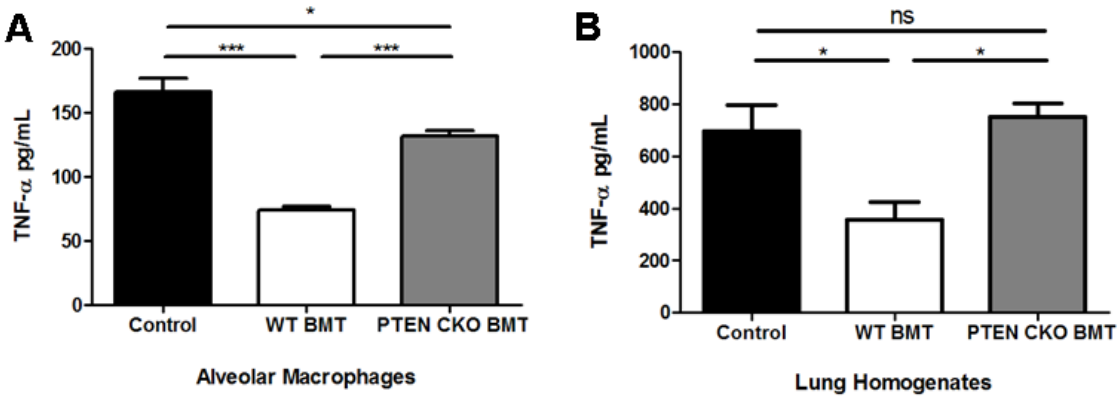

Figure 5.6: PTEN CKO BMT mice display improved TNF-α production.
TNF-α production was measured by ELISA as described in *Materials and* Methods using AM overnight culture supernatants (A) and lung homogenates from mice 24h post-i.t. *P. aeruginosa* infection (B; * $p<0.05$, *** $p<0.001$; n=3-5 per group).

Improved bacterial clearance in PTEN CKO BMT mice following P. aeruginosa *infection*

To determine whether PTEN CKO BMT mice display improved bacterial clearance post-BMT, we challenged control, WT BMT, and PTEN CKO BMT mice with a sublethal *P. aeruginosa* lung infection. 24 hours post-infection, bacterial burden was assessed in lung and blood samples. In WT BMT mice, bacterial burden was significantly higher in lung and blood samples relative to control (Fig. 5.7, panels A and B). In PTEN CKO BMT mice, bacterial burden was significantly reduced in both lung and blood samples relative to WT BMT mice, although not fully restored to control levels (Fig. 5.6, panels, A and B). We speculate that the reason PTEN CKO BMT mice have an intermediate phenotype is that the acute *in vivo* infection model relies heavily on AM clearance of non-opsonized bacteria— a pathway in which PTEN may be less important.

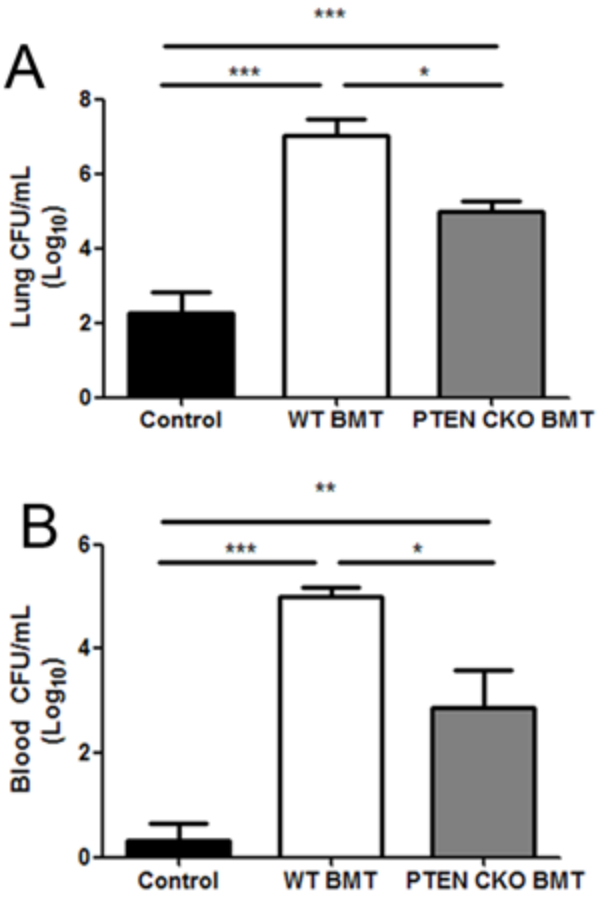

Figure 5.7: Improved bacterial clearance in PTEN CKO BMT mice following *P. aeruginosa* pneumonia
Mice were i.t. injected with a 50 µL dose of 5 x 10^5 CFU *P. aeruginosa* 5-6 weeks post-BMT. 24h following infection, bacterial burden of whole lung (A) and blood (B) samples from each mouse was assessed by CFU assay (* $p<0.05$, ** $p<0.01$, *** $p<0.001$; n=9-10 mice per group).

IRAK-M expression is elevated in both WT BMT and PTEN CKO BMT AMs

We previously demonstrated that in response to increased PGE_2 signaling, BMT AMs display elevated expression of IRAK-M, a well described negative regulator of TLR/IL-1R signaling, (Fig. 4.1). Furthermore, we found that despite overproduction of PGE_2, ablation of IRAK-M in the hematopoietic compartment of BMT mice fully restores BMT AM phagocytosis of non-opsonized bacteria, killing, and production of proinflammatory mediators (Figs. 4.8 and 4.9). Therefore, we wondered whether the

partial restoration of non-opsonized phagocytosis in PTEN CKO BMT AMs was the result of elevated IRAK-M expression. IRAK-M protein expression was assessed in AMs from control, WT BMT, and PTEN CKO BMT mice. IRAK-M protein expression was significantly elevated in both WT BMT and PTEN CKO BMT AMs compared to control AMs (Fig. 5.8). These data highlight the idea that following BMT, IRAK-M may play a critical role in the negative regulation of non-opsonized phagocytosis, whereas PTEN primarily inhibits pathways of opsonized phagocytosis.

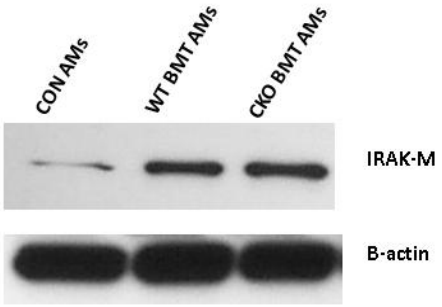

Figure 5.8: Elevated IRAK-M expression in both WT BMT and PTEN CKO BMT AMs
Whole-cell lysates were prepared from control, WT BMT, and PTEN CKO BMT AMs following 1h SFM adherence. IRAK-M and β-actin protein expression were analyzed by Western blot as described in *Materials and Methods*. Blot is representative data from 2 experiments.

Lung leukocyte composition in PTEN CKO BMT mice

We next wanted to assess the total number of leukocytes and the frequency of leukocyte populations in the lungs of control, WT BMT, and PTEN CKO BMT mice at baseline and 24 hours following *P. aeruginosa* pneumonia. Total lung leukocyte numbers were increased in uninfected PTEN CKO BMT mice relative to uninfected WT BMT mice (Fig. 5.9, panel A). However, 24 hours post-infection, total leukocyte

numbers were comparable between groups (Fig. 5.9, panel B). Interestingly, a shift was observed in whole lung macrophage and neutrophil counts in uninfected PTEN CKO BMT mice (Fig. 5.9, panel C). We found that the percentage of macrophages was significantly reduced and neutrophils increased relative to both control and WT BMT lungs (Fig 5.9, panel C). Following infection, however, the percentages of neutrophils, macrophages, and lymphocytes were comparable between groups (Fig. 5.9, panel D).

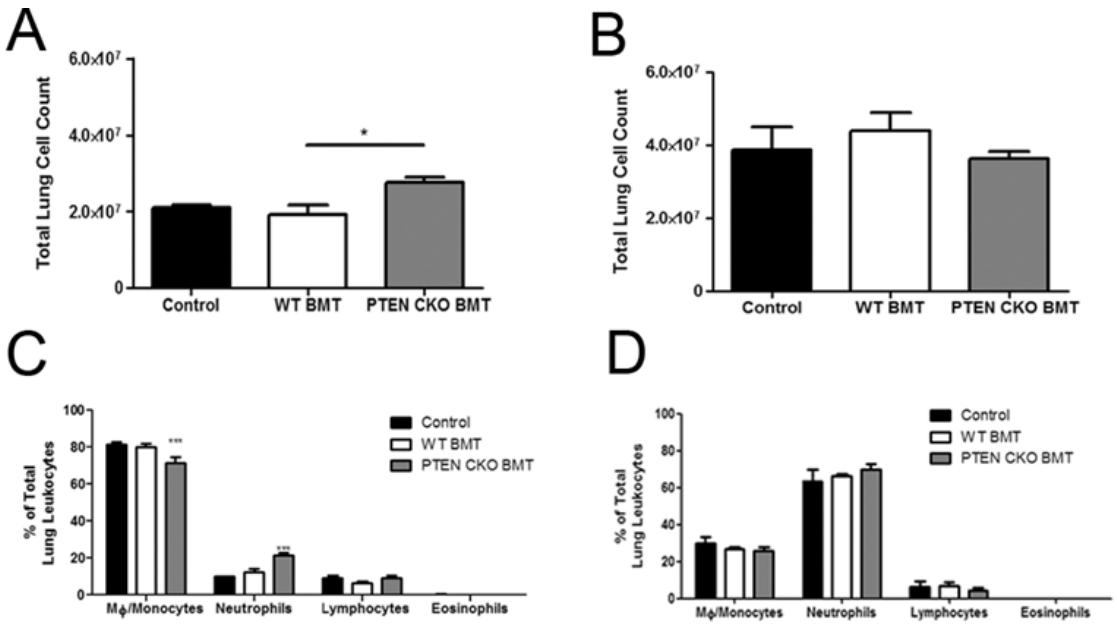

Figure 5.9: Lung leukocyte composition pre- and post-*P. aeruginosa* infection
Control, WT BMT, and PTEN CKO BMT mice were i.t. injected with a 50 µL dose of 5 x 10^5 CFU *P. aeruginosa*. Lung samples were harvested from both uninfected and infected mice 24h later, and lung leukocytes were isolated, counted and stained for differential cell analysis as described in *Materials and Methods*. Total lung leukocyte count in naïve (A) and infected (B) mice. Percentage of macrophages/monocytes, neutrophils, lymphocytes, and eosinophils in the lungs of naïve (C) and infected (D) mice (* $p<0.05$, *** $p<0.001$; n=4 per group).

Neutrophil function is improved in PTEN CKO BMT mice

Previous studies have suggested that PTEN-deficient neutrophils have improved host defense functions (155). Given that PTEN CKO BMT mice have increased

neutrophil numbers in the lung at baseline, we wanted to assess the functional status of these phagocytes with regard to bacterial killing. Relative to control neutrophils, WT BMT neutrophils displayed impaired bacterial killing of serum opsonized *P. aeruginosa,* and this defect was restored in the absence of PTEN post-BMT (Fig. 5.10). Thus, despite the fact that PTEN CKO BMT mice have increased neutrophils in the lung at baseline and display normal killing of serum-opsonized *P. aeruginosa*, the *in vivo* clearance of bacteria in these mice is still not completely restored. In total, these data highlight the idea that impaired mechanisms of non-opsonized phagocytosis are likely contributing to the partial restoration of host defense against acute of *P. aeruginosa* lung infection in PTEN CKO BMT mice.

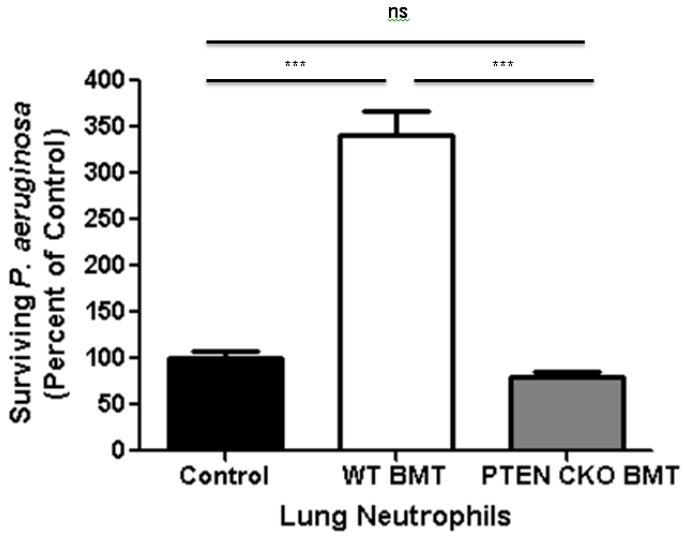

Figure 5.10: Restored neutrophil killing of *P. aeruginosa* in PTEN CKO BMT mice
Neutrophils were elicited to the lung and harvested by BAL from control, WT BMT, and PTEN CKO BMT mice as described in *Materials and Methods.* Killing of serum opsonized *P. aeruginosa* was assessed in neutrophils as described in *Materials and Methods* (*** $p<0.001$; n=8-10 per group).

Discussion

AMs are the primary immune cell type in the alveolar space and serve as the first line of cellular defense against inhaled pathogens (3-5). Therefore, any defects in AM phagocytosis/killing and production of inflammatory signals hinder the ability of the host to clear pathogens entering the lung and inhibit effector immune responses. In the case of HSCT, donor-derived AMs reconstituting the lung airspaces have a significantly impaired ability to ingest and kill bacterial pathogens (104, 106). Thus, this deficiency in immune function creates a lung environment that is highly susceptible to pathogenic organisms, such as *P. aeruginosa*.

Our previous work has shown that overproduction of PGE_2 in lung and AMs post-BMT directly impairs AM host defense (68). One particular effector in the PGE_2 signaling cascade, PTEN, has recently been shown to inhibit AM phagocytosis and killing of IgG-opsonized particles by negatively regulating the FcγR signaling pathway in control mice (120). In this paper, we show a similar mechanism may be involved post-BMT, where PGE_2 is overproduced by AMs and signals an increase in the lipid phosphatase activity of PTEN. Furthermore, we show that PTEN activity inhibits BMT AM phagocytosis of both serum and non-serum opsonized *P. aeruginosa,* although it appears that PTEN may play a more critical role in the negative regulation of opsonized phagocytosis. In addition, we show that myeloid-specific ablation of PTEN restores bacterial killing functions in both AMs and neutrophils post-BMT.

Our data demonstrate that BMT AMs have increased PTEN activity relative to non-transplant control AMs (Fig 5.1, panel A). As PGE_2 has been shown to increase PTEN activity via activation of the cAMP signaling cascade, we examined whether BMT

AM overproduction of PGE_2 was mediating this increase in PTEN activity. Indomethacin treatment, which inhibits COX-2 synthesis of prostaglandins, effectively abrogated the increase we observed in PTEN activity (Fig. 5.1, panel A). Enhanced PTEN activity translated into the functional consequence of suppressed AKT activity following FcγR stimulation in BMT AMs, but we observed that AKT function could be restored with indomethacin pretreatment (Fig 5.1, panel B). Thus, these data suggest that PGE_2 may be increasing BMT AM PTEN activity and diminishing AKT activation following FcγR activation.

During acute bacterial lung infections, AM phagocytosis likely involves uptake of bacteria that is either non-opsonized or opsonized by serum-derived complement (5, 106). Interestingly, it has previously been shown that PI3K activity, which is tightly regulated by PTEN, is involved in both complement-mediated and non-opsonized phagocytosis (98, 99, 211) in non-transplant settings. Therefore, we examined whether inhibiting PTEN activity could restore BMT AM phagocytosis of both serum and non-serum opsonized FITC-*P. aeruginosa*. Pharmacologic PTEN inhibition significantly increased phagocytosis of non-opsonized *P. aeruginosa,* but did not restore levels to those seen in control AMs. In contrast, pharmacologic inhibition of PTEN fully restored serum-opsonized phagocytosis to control levels (Fig. 5.2). It is likely that the presence of IgG (in addition to complement) in our immune serum contributed to the greater magnitude of improvement we observed in phagocytosis of serum-opsonized bacteria in bpV(pic)-treated BMT AMs compared to non-opsonized bacteria. As discussed below, these pharmacologic results were also corroborated by studies using genetic deletion of PTEN.

Previous studies have shown that in murine models of neutropenia, myeloid-specific ablation of PTEN can enhance opsonized bacterial clearance as well as proinflammatory cytokine production in AMs (155). Our data demonstrate a similar enhancement of host defense following transplantation of myeloid-specific PTEN KO bone marrow into lethally irradiated WT recipients. Despite overproduction of PGE_2 (Fig. 5.4, panel C), we found that AM phagocytosis and killing of serum opsonized *P. aeruginosa* is restored (Fig. 5.4), and that AM TNF-α production is improved in the absence of PTEN expression post-BMT (Fig. 5.6, panel A). Interestingly however, phagocytosis of non-opsonized bacteria is not fully restored in the PTEN CKO BMT AMs (Fig. 5.5). Furthermore, bacterial clearance in mice challenged with *P. aeruginosa* lung infection is only partially recovered in PTEN CKO BMT mice (Fig. 5.7), despite the fact that lung TNF-α levels are restored (Fig. 5.6, panel B). Taken together, these data suggest that PTEN may be necessary for PGE_2 to mediate its suppressive effects on BMT AM function against opsonized targets; however, BMT AMs retain defects in non-opsonized phagocytosis that are likely PTEN-independent.

Following infection, total lung leukocyte numbers and neutrophil recruitment are similar in both WT BMT and PTEN CKO BMT mice (Fig. 5.9, panels B and D). However, uninfected PTEN CKO BMT mice display an increase in total leukocyte number relative to WT BMT mice (Fig. 5.9, panel A). We also found that significantly more neutrophils and fewer macrophages are present in the lungs of uninfected PTEN CKO BMT mice compared to both uninfected control and WT BMT mice (Fig. 5.9, panel C). In figure 5.10, we demonstrate that neutrophil killing of serum opsonized *P. aeruginosa* is restored to control levels in PTEN CKO BMT mice. Taken together, our

results indicate that PTEN CKO BMT mice have fully restored AM phagocytosis of serum opsonized bacteria, more neutrophils at baseline, and normal neutrophil killing ability.

Despite these improvements in host defense, *in vivo* clearance of *P. aeruginosa* in PTEN CKO BMT mice is not fully restored to control levels. We believe the reason for this is best explained by the observation that non-opsonized bacterial phagocytosis is still impaired in PTEN CKO BMT AMs relative to control AMs (Figure 5.5) . This is likely due to the fact that IRAK-M is elevated in the AMs from PTEN CKO BMT mice (Fig. 5.8). We have previously shown that increased PGE_2 production post-BMT elevates IRAK-M expression in AMs (Fig. 4.3). Transplantation of IRAK-M-/- bone marrow into WT mice fully restored *in vivo* clearance of *P. aeruginosa* to control levels following an acute (24 hour) infection. Putting our two studies together, we conclude that non-opsonized phagocytosis of *P. aeruginosa* plays a critical role in clearance of acute infection and is negatively regulated by PGE_2-induced IRAK-M. While we believe the partial restoration of bacterial clearance in our PTEN CKO BMT mice is best explained by our observation that non-opsonized phagocytosis by AMs is not fully recovered, another possibility is that neutrophils in the PTEN CKO BMT may be displaying prolonged survival (2, 158) resulting in increased neutrophil damage to the lung, delayed immune resolution, and diminished tissue repair. Indeed, since PTEN is a critical regulator of cellular apoptosis, this may well be occurring.

As mentioned, IRAK-M protein expression is elevated in both WT BMT and PTEN CKO BMT AMs relative to control AMs (Fig. 5.8). These data suggest PGE_2-mediated upregulation of IRAK-M may occur independently of PTEN to regulate AM

host defense functions. An alternative hypothesis could be that IRAK-M is an upstream regulator of PTEN function. Overall, our data support a model where PGE_2 signaling induces two unique pathways which regulate host defense. The upregulation of PTEN by PGE_2 primarily causes inhibition of opsonized AM phagocytosis and PMN killing post-BMT. Simultaneously, PGE_2-mediated upregulation of IRAK-M inhibits non-opsonized phagocytosis in AMs post-BMT.

Our data have several important implications. First, susceptibility to *P. aeruginosa* infection is widely considered to be a consequence of neutropenia (207). While it is clear that neutropenia can increase host susceptibility to infection, our data clearly demonstrate that isolated defects in AM phagocytosis of non-opsonized bacteria can also render the host susceptible to this infection. Our findings are supported by previous observations demonstrating that clodronate liposome depletion of AMs negatively impacted lung host defense against *P. aeruginosa in vivo* (207). Our results also highlight the fact that PGE_2 signaling initiates unique pathways for the inhibition of opsonized versus non-opsonized bacteria. While PTEN activation negatively regulates phagocytosis of serum opsonized bacteria and has modest effects on non-opsonized phagocytosis in our model, it appears that IRAK-M elevation mediates more pronounced inhibition of phagocytosis of non-opsonized particles. Thus, these data are the first to highlight divergent pathways by which PGE_2 can inhibit opsonized and non-opsonized phagocytosis.

Overall, our data suggest that PGE_2 may inhibit multiple mechanisms of phagocytosis in BMT AMs via the upregulation of PTEN activity and the induction of IRAK-M. Blocking PTEN activity mitigates the suppressive effects of PGE_2 on

opsonized bacterial phagocytosis in AMs and also restores neutrophil killing post-BMT. However, ablation of PTEN may not be sufficient to fully restore host defense against acute *P. aeruginosa* infection, given that non-opsonized phagocytosis by PTEN CKO BMT AMs appears to be inhibited by elevated IRAK-M expression. As such, our results suggest that a better strategy to restore host defense post-BMT may be to target the production of PGE_2 or EP2 receptor signaling. In fact, we have already demonstrated that pharmacologic blockade of PGE_2 production by indomethacin post-BMT can fully restore host defense (68). An important future goal will be to determine whether these same PGE_2-induced alterations are present in human AMs post-HSCT and if therapeutic approaches to block PGE_2 production or signaling can improve host defense by reversing the activation of PTEN and the upregulation of IRAK-M.

CHAPTER 6: Conclusion

Summary of Results

Conditioning Regimen Studies

Our previous work demonstrated that pulmonary host defense against acute *P. aeruginosa* infection is impaired post-BMT (68). However, our TBI + T conditioning regimen used to ablate host bone marrow in our studies did not mimic clinical practice (8, 174-176). Our current work demonstrates that following 13 Gy TBI conditioning and infusion of whole bone marrow alone (no addition of splenic T cells), mice are fully donor cell reconstituted at 5 weeks post-BMT (Table 3.1). Furthermore, use of busulfan/cyclophosphamide dual chemotherapy regimens, as opposed to individual chemotherapy regimens, was the only chemotherapy regimen that provides significant reconstitution of donor-type AM and peripheral cells (Table 3.2). Similarly, use of dual chemotherapy was the only chemotherapy-based protocol that induces overproduction of PGE_2 and PGI_2 in AMs post-BMT (Fig. 3.3).

AMs from BMT mice conditioned with either TBI or dual chemotherapy were similarly defective in phagocytosis relative to control AMs, and this inhibition was directly related to overproduction of PGE_2 (Fig. 3.4, panel B). Reduced clearance of *P. aeruginosa* following acute lung infection was also observed in both TBI and dual chemotherapy conditioned mice (Fig. 3.4, panel B). Taken together, these data demonstrate that use of either TBI or dual chemotherapy conditioning regimens results in

impaired pulmonary host defense mediated by overproduction of PGE_2. Ultimately, these results offer further clinical relevance to our murine model of BMT and suggest that our findings are applicable to multiple conditioning protocols and result in significant donor-cell reconstitution.

IRAK-M Studies

Our work suggests a role for IRAK-M in PGE_2-induced immunosuppression post-BMT. We are the first to show that IRAK-M expression is upregulated in BMT AMs, and this phenotype is related to increased PGE_2 signaling (Fig. 4.1 and 4.3). Furthermore, we demonstrate an inhibitory role for IRAK-M in pulmonary host defense post-BMT, given that genetic ablation of IRAK-M in the bone marrow restores AM function and pulmonary host defense against acute *P. aeruginosa* infection.

Restored bacterial clearance and improved survival rate following *P. aeruginosa* lung infection in IRAK-M -/- BMT mice was not explained by enhanced neutrophil recruitment to the lung during early infection (Fig. 4.6) or alterations in the frequency of lymphocyte populations in the lungs before infection (Fig. 4.7). Additionally, although neutrophils expressed IRAK-M, there was no clear evidence that recruited lung neutrophils upregulated IRAK-M in response to BMT (Fig. 4.1). Moreover, our data do not suggest a role for altered AM expression of TLRs or scavenger receptors in enhanced host defense displayed by IRAK-M -/- BMT mice (Fig. 4.10). Our data do indicate, however, that IRAK-M -/- BMT AMs displayed restored non-opsonized phagocytosis, killing, and production of cys-LTs and TNF-α (Fig. 4.8 and 4.9). Moreover, restored host

defense in IRAK-M -/- BMT AMs was observed despite elevated EP2 receptor expression and overproduction of PGE_2 in AMs (Fig. 4.11). Overall, these data suggest that PGE_2 may require IRAK-M to inhibit AM function against non-opsonized pathogens, and therefore, IRAK-M -/- BMT AMs may not be responsive to PGE_2-induced immunosuppression despite AM upregulation of the inhibitory EP2 receptor. Our work in the PTEN studies, described below, suggests that IRAK-M may be a unique inhibitor of non-opsonized phagocytosis pathways.

PTEN Studies

Similar to our IRAK-M studies, our data demonstrate that PTEN also plays a role in PGE_2-mediated suppression of AM host defense post-BMT. Relative to control AMs, BMT AMs had increased PTEN activity in response to increased PGE_2 signaling (5.1). Furthermore, pharmacologic inhibition of PTEN activity using bpV(pic) fully restored phagocytosis of serum-opsonized *P. aeruginosa* but only moderately improved non-opsonized phagocytosis (Fig. 5.2). In the absence of PTEN expression post-BMT, AM phagocytosis and killing of serum opsonized *P. aeruginosa* was restored (Fig. 5.4), and AM TNF-α production was improved (Fig. 5.6), despite overproduction of PGE_2. Similar to our bpV(pic)-treated WT BMT AMs, non-opsonized phagocytosis of *P. aeruginosa* was only partially restored in PTEN CKO BMT AMs (Fig. 5.5). Additionally, we found that bacterial clearance was partially restored (Fig. 5.7) and lung TNF-α levels were improved in PTEN CKO BMT mice following *P. aeruginosa* lung infection (Fig. 5.6).

Enhanced neutrophil recruitment following *P. aeruginosa* pneumonia was not observed in PTEN CKO BMT mice; however, increased numbers of neutrophils were found in the lungs of PTEN CKO BMT mice prior to infection (Fig. 5.9), and bacterial killing of serum-opsonized *P. aeruginosa* was fully restored in these cells (Fig. 5.10). The increased percentage of neutrophils is likely due to the increased production of MIP-2 secreted by AMs in PTEN CKO BMT mice relative to both control and WT BMT mice (data not shown).

Finally, a role for PTEN in PGE_2-mediated elevation of IRAK-M was also assessed, and we found that IRAK-M was elevated in both WT BMT and PTEN CKO BMT AMs relative to control AMs (Fig. 5.8). It is possible that IRAK-M may specifically inhibit non-opsonized phagocytosis pathways, and thus, non-opsonized phagocytosis in PTEN CKO BMT AMs remains impaired while serum-opsonized phagocytosis is restored. It is also interesting to speculate that this defect in non-opsonized phagocytosis likely accounts for the intermediate improvement in host defense noted in the PTEN CKO BMT mice post-acute *P. aeruginosa* lung infection. Overall our data suggest that upregulation of PTEN activity may be necessary for PGE_2 to mediate much of its suppressive effects on BMT AM function towards opsonized pathogens and overall pulmonary host defense. Furthermore, IRAK-M expression may be upregulated independently of PTEN in response to PGE_2 signaling, given that ablation of either PTEN or IRAK-M has differential effects on BMT AM phagocytosis and clearance of *P. aeruginosa* lung infection.

Critical Review and Future Directions

Conditioning Regimen Studies

Conditioning regimens used for HSCT in the United States normally employ either high dose chemotherapy or a combination of TBI and chemotherapy (7). Thus, our model of TBI BMT does not accurately reflect how patients are conditioned prior to HSC infusion. Therefore, comparing the effect of TBI and chemotherapy conditioning on immune reconstitution and host defense function was critical in determining the clinical relevance of our TBI conditioning regimen.

Our results indicate that compared to TBI, dual chemotherapy conditioning yields a significantly lower frequency of donor-derived cells 5 weeks post-BMT. Despite this altered level of chimerism in our chemotherapy conditioned mice, the effect of TBI and chemotherapy on immune function is similar in that PGE_2-mediated impairment of AM host defense was observed in both TBI and chemotherapy conditioned mice. Interestingly, preliminary data suggest that following BMT, host-derived AMs display increased PGE_2 production (Fig. 6.1A) and impaired phagocytosis of non-opsonized bacteria (Fig. 6.1B). These results suggest that even if the conditioning regimen does not result in maximal donor cell reconstitution, the process of BMT still induces significant impairment of both donor and host cell function. Therefore, our decision to use TBI as the main conditioning protocol for our studies was based primarily on our ability to examine the effect of transplant largely on donor-derived cells as opposed to an evenly mixed chimera of donor-to-host cells.

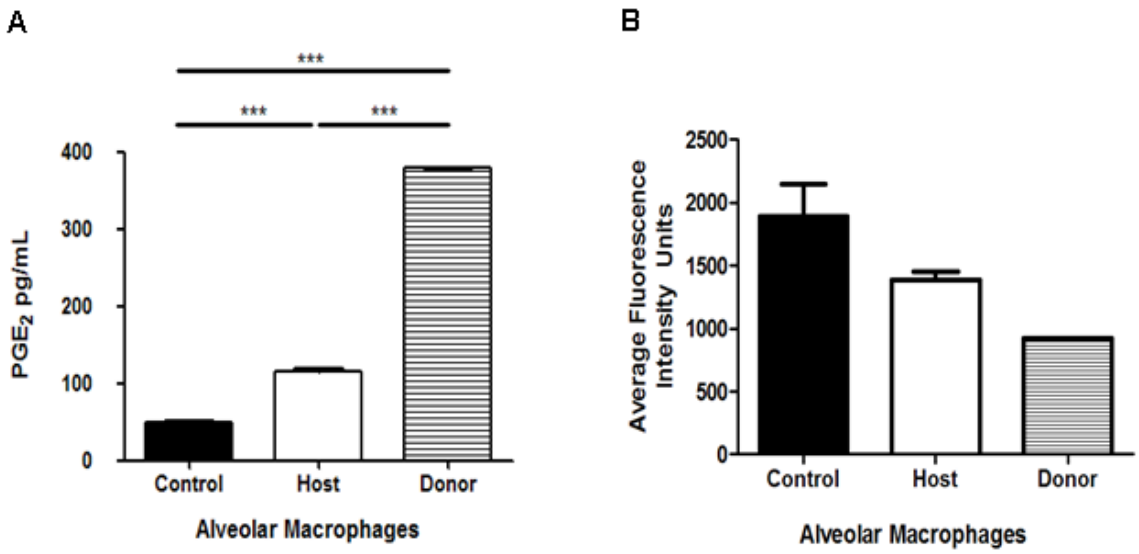

Figure 6.1: Elevated PGE_2 production and impaired phagocytosis in host-derived and donor-derived AMs post-BMT
5 weeks post-BMT, AMs from B6 (CD45.1) > B6 (CD45.2) BMT mice conditioned with only 8 Gy of TBI and non-transplant B6 (CD45.2) control mice were harvested. Donor CD45.1 AMs were positively selected by MACS. Control, donor, and host AMs were plated 2 x 10^5 cells/well. Overnight supernatants were collected and analyzed for PGE_2 levels by EIA (A). Phagocytosis of AMs was then assessed following incubation with FITC-*E. coli* as described in *Materials and Methods* (B) (*** $p<0.001$; n=2-3 per group).

Our conditioning regimen studies provide much needed insight into the effect of chemotherapy conditioning on pulmonary host defense in BMT mice. However, multiple aspects of PGE_2-mediated impairment of pulmonary host defense must still be addressed. In our previous studies, indomethacin treatment restored bacterial clearance in TBI BMT mice challenged with *P. aeruginosa* lung infection (68). The effect of indomethacin treatment on dual chemo BMT mice, however, was not determined in our present studies. Future work will need to address this point in order to fully assess the effect of overproduction of PGE_2 on pulmonary host defense in both TBI and chemotherapy conditioned mice. Furthermore, our current studies do not determine the effect of both conditioning regimens on lung neutrophil function or AM bacterial killing and

phagocytosis of non-opsonized bacteria. These experiments will be required to fully support our claim that PGE_2 mediates similar impairment of pulmonary host defense in both TBI and chemotherapy conditioned BMT mice.

Additionally, mechanisms of PGE_2-mediated suppression of pulmonary host defense need to be determined in both conditioning groups. Future studies should address roles for known effectors in the PGE_2 signaling pathway, such as cAMP, EPAC-1, and PKA. Also roles for IRAK-M and PTEN in AM and neutrophil host defense should be considered in both models. Ultimately, these results would give us a more accurate picture of the similarities and differences in pulmonary host defense following TBI and dual chemotherapy conditioning.

Some limitations to our conditioning regimen studies remain. Primarily, we have not identified why PGE_2 is overproduced following BMT and whether this induction is lung specific. The literature indicates that PGE_2 production is induced following TBI or chemotherapy/TBI combination treatment in HSCT patients (110) and following radiation treatment in cancer patients (212). It is speculated that this induction is related to epithelial cell damage which occurs during TBI and myeloablative therapy. Currently, we have only demonstrated that PGE_2 synthetic enzymes are elevated in AMs from mice conditioned with TBI (Fig. 4.2). Future studies will need to explore whether irradiation can induce production of PGE_2 in cultured AECs. Furthermore, we will need to examine whether irradiated AECs or AECs from TBI or dual chemotherapy conditioned BMT mice are capable of inducing PGE_2 production in co-cultured phagocytes. If positive results are produced from these experiments, we would then compare host defense function among phagocytes co-cultured with AECs. To determine whether this

induction is lung-specific, serum PGE_2 levels would be measured in control and BMT mice, and EP receptor expression profiles would be assessed among various tissue specific macrophages to ascertain whether phagocytes outside of the lung are also be responsive to increased PGE_2 production.

In addition, it remains unknown whether increased PGE_2 production is induced in the lungs of HSCT patients and whether this may mediate impaired pulmonary innate immunity. Thus, future work will specifically examine AM and BAL PGE_2 levels in normal subjects and patients given either an allogeneic or autologous HSCT. If PGE_2 levels are elevated in the lungs of HSCT patients, we would then assess AM host defense function in the presence or absence of indomethacin.

IRAK-M Studies

Our current work describes a critical role for IRAK-M in AM host defense following BMT. However, multiple areas of this work require further study. Primarily, our future work must incorporate experiments that would: 1) provide insight into the mechanism of PGE_2-mediated upregulation of IRAK-M expression and 2) identify additional factors induced during BMT that may also play a role in the upregulation of IRAK-M.

PGE_2 signaling through the inhibitory EP2 and EP4 receptors activates the cAMP signaling cascade. Effectors of this signaling pathway mainly include EPAC-1 and PKA. However, we have not established roles for any of these molecules in the upregulation of IRAK-M. Preliminary data suggest that cAMP increases IRAK-M expression in non-transplant AMs (Fig. 6.2). This was demonstrated following treatment of AMs with the

AC activator forskolin. Interestingly, we have found in other preliminary experiments that following PGE_2 treatment, BMTs AMs display diminished cAMP production in the absence of IRAK-M (Fig. 6.3). These data suggest to us that IRAK-M may be upregulated via the cAMP signaling cascade to negatively regulate factors that inhibit cAMP. Future studies will verify whether cAMP can elevate IRAK-M expression and if so, determine whether this cAMP-mediated upregulation involves PKA or EPAC-1. Additionally, we would like to assess whether cAMP degradative phosphodiesterases are upregulated in IRAK-M -/- deficient AMs as a possible explanation for diminished cAMP production in IRAK-M -/- BMT AMs.

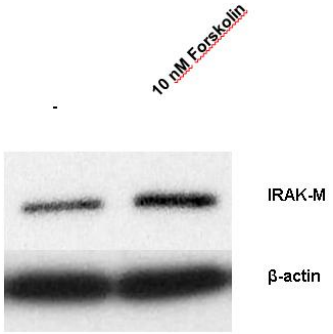

Figure 6.2: AC-mediated increases in cAMP elevate IRAK-M expression in non-transplant AMs.
AMs from non-transplant mice were plated 5 x 10^5 cells/well overnight in the presence or absence of 10 nM forskolin in SFM. Protein lysates of AMs were prepared for Western blot analysis, and expression of IRAK-M and β-actin was assessed (n=1).

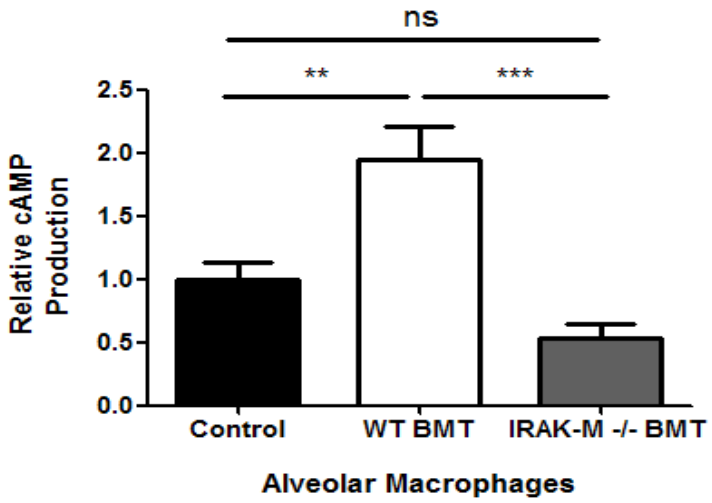

Figure 6.3: Diminished cAMP production in IRAK-M -/- BMT AMs following stimulation with PGE_2
AMs from control, WT BMT, and IRAK-M -/- BMT mice were plated 5 x 10^5 cells/well in CM overnight, and the following, day AMs were treated with 1 μM PGE_2 for 15 mins. AMs lysates were then prepared for cAMP EIA as described in *Materials and Methods* (** $p< 0.01$; *** $p<0.001$; n= 7-8 per group).

Our data also indicate that WT BMT AMs display defective phagocytosis of non-opsonized *P. aeruginosa* and that this impairment is restored in the absence of IRAK-M (Fig. 4.8). We did not identify, however, what phagocytic receptors may be differentially expressed among WT BMT and IRAK-M -/- BMT AMs. Future experiments will need to identify the receptors involved in AM phagocytosis of non-opsonized *P. aeruginosa* and whether they are altered in the setting of BMT. Moreover, pulmonary host defense against acute *P. aeruginosa* lung infection involves multiple humoral opsonins that were not addressed in any of our studies. Therefore, it will be important to consider roles for complement and collectins in phagocytosis of *P. aeruginosa in vitro*, since these humoral opsonins may play an important role in clearance of *P. aeruginosa* from the lung during acute infection (82). Lastly, mechanisms for defective bacterial killing in WT BMT AMs

that are restored in IRAK-M -/- BMT AMs must be examined. Future experiments can employ measurement of ROS, tracking of phagosome maturation and lysosomal fusion, and quantification of antimicrobial factors produced in the lung during infection (such as lactoferrin, defensins, and lysozyme).

Finally, some experimental limitations to our studies impeded our ability to fully assess the role of IRAK-M in lung phagocyte host defense post-BMT. Neutrophil function is severely impaired in BMT mice (68); however, due to our neutrophil recruitment protocol, we were unable to meaningfully assess whether this defect was related to PGE_2-mediated elevation of IRAK-M. In our studies, neutrophil recruitment involves i.t. injection of LPS, and it appeared that the LPS recruitment signal maximally upregulated IRAK-M in both control and WT BMT neutrophils (Fig. 4.1). Other neutrophil recruiting agents, such as chemokines or thioglycollate, may be tested in the future to overcome this problem. Additionally, ablation of IRAK-M in our model was not myeloid-specific and ignores the effect of IRAK-M deficiency on lymphoid-derived cells and the interaction of these cell types with lung phagocytes during infection. In order to address this concern, future studies would require the use of conditional IRAK-M knockout systems that have not yet been developed.

PTEN Studies

Previous work demonstrates that PGE_2 signaling through the EP2 receptor elevates PTEN activity and inhibits FcγR-mediated phagocytosis and bacterial killing (120). Our work suggests a similar mechanism of inhibition of AM host defense post-BMT; however, details of this mechanism remain unexplored. Our data show that PTEN

lipid phosphatase activity is elevated in BMT AMs as a result of increased PGE_2 production (Fig. 5.1, panel A). Previous work identifies a role for EPAC-1 and SHIP-1 in PGE_2-mediated increases in PTEN activity (120). It remains unknown whether these molecules are also upregulating PTEN activity in BMT AMs or if alternative effectors are involved, such as PKA or even IRAK-M (120). Therefore, future experiments will need to examine the effect of PKA and EPAC-1antagonists on PTEN activity in BMT AMs. Furthermore, future experiments must assess whether IRAK-M is acting upstream in the PGE_2 signaling pathway to regulate PTEN activity or if IRAK-M is regulated independently of PTEN to inhibit AM host defense. To verify this, we would need to confirm that PTEN activity is not elevated in IRAK-M -/- BMT AMs as well as IRAK-M -/- non-transplant AMs treated with PGE_2.

SHIP-1 phophosphatase activity has also been shown to contribute to PTEN activation by dephosphorylating PTEN on tyrosine residues. It remains to be explored what other post-translational modifications of PTEN are regulated by PGE_2 signaling and if these modifications are applicable to the setting of transplant. Future experiments will confirm whether alterations in the phosphorylation state of PTEN are also present in BMT AMs.

Previous work has demonstrated that diminished activation of downstream effectors in the PI3K signaling pathway, such as AKT and ERK-1/2, is observed in AMs following treatment with PGE_2 (120). Our studies demonstrate that AKT activation is also diminished in BMT AMs due to increased PGE_2 production (Fig. 5.1, panel B). It is currently assumed that diminished AKT activation is due to elevated PTEN activity in BMT AMs; however, we have yet to confirm this experimentally. Moreover, we have

not identified whether overproduction of PGE_2 in BMT AMs reduces activation of other downstream effectors in the PI3K signaling pathway, such as Vav, Rac, Cdc, and ERK-1/2. Therefore, we plan to perform experiments where BMT AMs are treated with the PTEN inhibitor, bpV(pic), and phosphorylation of AKT is assessed. Finally, we will assess whether activation of other effectors in the PI3K signaling pathway is reduced in BMT AMs and if treatment with indomethacin can restore activation of these molecules.

Aside from identifying signaling molecules associated with PGE_2-mediated increases in PTEN activity, our future studies must also focus on whether PTEN can regulate pathways associated with non-opsonized or complement-mediated phagocytosis. Our data show that myeloid-specific ablation of PTEN enhances BMT AM phagocytosis of serum-opsonized *P. aeruginosa* (Fig. 5.4, panel A). Furthermore, our data also demonstrate that non-opsonized phagocytosis is improved but not fully restored in PTEN CKO BMT AMs (Fig. 5.5). This finding is important given that PTEN has not previously been shown to regulate non-opsonized phagocytosis. Future experiments will confirm this finding and also determine whether PTEN can specifically regulate complement-mediated phagocytosis. The latter experiments will involve use of complement opsonized bacteria in *in vitro* phagocytosis assays for control, WT BMT, and PTEN CKO BMT AMs.

Although PTEN appears to have some contribution to impaired non-opsonized phagocytosis in AMs post-BMT, we believe that IRAK-M may play a more critical role in the inhibition of this phagocytic pathway. To verify this hypothesis, we plan to assess PTEN activity and serum opsonized phagocytosis in IRAK-M -/- BMT AMs. Furthermore, assessing non-opsonized phagocytosis in PTEN CKO BMT AMs treated

with IRAK-M-specific siRNAs may also provide further insight into the role of IRAK-M in the regulation of non-opsonized phagocytosis post-BMT.

Lastly, the role of neutrophils needs to be further clarified in improved pulmonary host defense in PTEN CKO BMT mice. Our data demonstrate that PTEN CKO BMT mice have improved bacterial clearance and reduced bacteremia following *P. aeruginosa* lung infection (Fig. 5.7). Although this improvement in host defense is quite significant, bacterial load in the lung and blood of PTEN CKO BMT mice is not restored to control levels. This difference could be due to impaired non-opsonized phagocytosis in AMs. Alternatively, this difference may be related to increased neutrophil survival and damage in the lungs of PTEN CKO BMT mice. PTEN is a critical negative regulator of cell survival and migration signals mediated by PI3K/AKT signaling (146, 153). Therefore, future experiments will need to assess the level of neutrophil apoptosis in control, WT BMT, and PTEN CKO BMT mice following infection. Furthermore, lung histology will also be performed to identify signs of neutrophil damage to lung tissue. These experiments should help determine whether neutrophil damage is contributing to reduced bacterial clearance in the lung.

A few limitations were encountered in our PTEN studies. Of major concern was the effect of lysozyme M disruption in the myeloid compartment of our WT BMT and PTEN CKO BMT mice. Although the effect of lysozyme M disruption was minimized by using Cre heterozygous mice, it is not clear whether defects in pulmonary host defense in these mice were further exacerbated by reduced expression of this important antimicrobial factor. Furthermore, residual expression of functional PTEN protein in our PTEN CKO BMT AMs may have also contributed to the partial restoration of host

defense in these mice. Lastly, it would also be interesting to measure IRAK-M expression in PTEN CKO BMT lung neutrophils to confirm the results we found in PTEN CKO BMT AMs. However, we were again limited by the LPS recruitment signaling in the proper assessment of whether IRAK-M is elevated in neutrophils in response to BMT.

Final Thoughts

Our model of syngeneic BMT has provided extensive insight into the various factors involved in impaired pulmonary innate immunity following transplant (Fig. 6.4). Moreover, we have been able to identify defects in host defense that are related specifically to the procedure of transplant as opposed to exogenous immunosuppressive factors or GVHD. Future work will help to identify additional signaling molecules in the PGE_2 signaling pathway that mediate the upregulation of IRAK-M and PTEN in BMT AMs. Additionally, identifying a role for PGE_2 in impaired pulmonary host defense in both autologous and allogeneic HSCT patients will be a major focus.

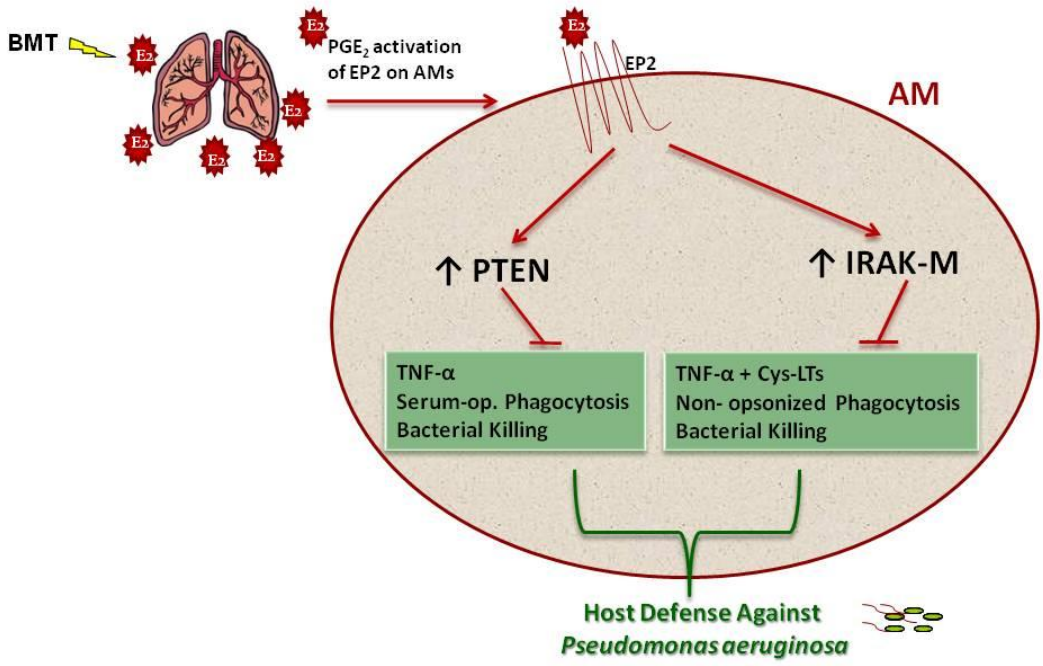

Figure 6.4: Roles for PTEN and IRAK-M in PGE_2-mediated suppression of AM host defense

Following BMT, AMs display increased PGE_2 production and signaling via the EP2 receptor. This increase in PGE_2 signaling results in upregulation of PTEN activity and IRAK-M expression in BMT AMs. Ultimately, upregulation of these two effectors in the PGE_2 signaling pathway inhibits host defense against *P. aeruginosa* via the suppression of AM proinflammatory mediator production, phagocytosis, and bacterial killing.

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