Understanding Impaired Immunity to Bacterial Pathogens Post-Bone Marrow Transplantation

by

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To my family and friends

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Abstract

Respiratory tract infections (RTI) are major causes of morbidity and mortality, with lower respiratory infections alone being the fourth major cause of death in the world, taking the life of over 3 million individuals in 2016 (World Health Organization (WHO), 2018). Bacterial infections are a major cause of RTI. In this dissertation we focused on a subject group who is at high risk of developing severe illness due to opportunistic bacterial infections, hematopoietic stem cell transplant (HSCT) patients. HSCT utilizes stem cells derived from bone marrow, umbilical cord blood, or peripheral blood of patients themselves (autologous) or matched donors (allogeneic) to treat or cure a variety of hematological and inherited disorders. This procedure has become standard of care with more than 18,000 HSCTs performed every year in the United States alone. Unfortunately, patients that undergo HSCT (both autologous and allogeneic) are immunosuppressed and remain so even after stem cell engraftment, making them susceptible to infections by a wide array of opportunistic pathogens. *Pseudomonas aeruginosa* is an opportunistic Gram-negative bacterium that can cause life-threatening complications in HSCT patients and has recently been identified by the WHO as a critical pathogen for which new therapeutic strategies are needed. In the lung of immunosuppressed individuals, *P. aeruginosa* can cause lethal organ injury mainly by stimulating alveolar macrophages to secrete high levels of Interleukin-1β (IL-1β). IL-1β is a potent pro-inflammatory cytokine that is mainly activated by the serine protease caspase-1, but can be activated by caspase-8. Here, we aimed to understand

the reasons behind the success of *P. aeruginosa* infections in HSCT subjects with the use of murine bone marrow transplantation (BMT) model. We identified that high levels of prostaglandin E2 (PGE_2), a cyclooxygenase (COX) lipid metabolite with hormone-like characteristics and found at elevated levels in HSCT patients, induces exacerbated levels of IL-1β in HSCT subjects leading to severe lung injury post-*P. aeruginosa* infection. We identified that the PGE_2-mediated increase in IL-1β is dependent on adenyl cyclase (AC) activation by EP2 and/or EP4 receptor stimulation which leads to activation of the transcription factor CREB. We hypothesized that reducing the levels of PGE_2 in BMT mice can reduce IL-1β-mediated acute lung injury and improve outcome. In accordance with our hypothesis, we were able to decrease IL-1β levels, improve bacterial killing, and reduce lung injury by treating HSCT mice with indomethacin, a non-selective inhibitor of the two isoforms of COX (COX1 & COX2), post-*P. aeruginosa* infection. Additionally, we showed how PGE_2 production impaired neutrophil extracellular trap (NETs), an important antimicrobial pathway, release post-HSCT in human and mouse neutrophils which could implicate immunosuppression to multiple microbes including bacterial pathogens. Our findings suggest new therapeutic strategies aimed at blocking PGE_2 production or signaling may have positive impacts against bacterial infections in HSCT subjects. The appendix of this dissertation contains an additional chapter looking at influenza-induced immunosuppression of lung innate immunity and describes a novel role for toll like receptor 9 signaling in regulating secondary bacterial infection post-influenza.

Chapter 1. Introduction

1.1 Hematopoietic stem cell transplant (HSCT)

HSCT utilizes stem cells from bone marrow, umbilical cord blood, or peripheral blood to treat or cure a variety of malignant disorders such as acute myeloid leukemia as well as non-malignant disorders including autoimmune diseases, and sickle cell anemia[1, 2]. Patients that undergo HSCT are first exposed to myeloablative or non-myeloablative conditioning followed by infusion of stem cells which can come from autologous (same individual), allogeneic (different individual, but compatible human leukocyte antigen), or syngeneic (genetically identical individual such as a twin sibling) sources. The conditioning regimen is usually high dose chemotherapy, total body irradiation and/or a combination of the two. This procedure has become a standard of care with more than 60,000 HSCT performed in 2012 worldwide, in which 47% were allogeneic and 53% of them where autologous[3-5]. Although HSCT can be a cure for multiple disorders, unfortunately HSCT patients are at high risk of post-transplant complications due to immunosuppression and treatment-related toxicity[4, 6, 7]. Thus, better therapeutic strategies are needed to supplement HSCT to improve the outcome for patients. Apart from disease relapse, the major post-transplant clinical problems are infectious and non-infectious pulmonary complications.

1.2 Non-infectious complications post-HSCT

Pulmonary complications are common post-HSCT and affect around 40%-60% of recipients and follow a predictable timeline[7]. The timeline is divided in three phases. Phase 1 is the pre-engraftment period (0-30 days post-HSCT), and it is characterized by neutropenia. Phase 2 is the post-engraftment period (30-100 days post-HSCT), and it is characterized by impaired cellular immunity. Phase 3 is the late phase (≥100 days post-HSCT) and it is characterized by impaired humoral and cellular immunity[8]. Non-infectious complications can be present in any of the phases and are a significant cause of morbidity and mortality post-HSCT. The most common non-infectious early pulmonary complication is Idiopathic Pneumonia Syndrome (IPS) which occurs in the pre-engraftment or early post-engraftment period. IPS is characterized by pulmonary edema, alveolar injury, acute pulmonary dysfunction, and cardiac or renal disease, and is believed to be caused by the toxicity of chemotherapy and radiation[7, 9]. Another early complication present in HSCT patients is acute graft versus host disease (GVHD) which can manifest in the pre-engraftment and post-engraftment period and it is described as an immune reaction of donor cells to the host tissue. GVHD is the result of HLA mismatch and thus is a complication just seen in allogeneic HSCT and not in autologous HSCT patients. Chronic GVHD is the most common late complication and can lead to the development of Bronchiolitis Obliterans (BOS), defined by airway obstruction, and characterized by bronchiolar fibrosis. Mortality caused by BOS post-HSCT is around 12%-27%[7, 10, 11]. The study of non-infectious complications has been well described elsewhere[7, 11-14]. In this dissertation, I will focus on infectious

complications post-HSCT which are also a significant cause of morbidity and mortality post-HSCT.

1.3 Infectious complications post-HSCT

The incidence of infectious complications post-HSCT can be influenced by multiple variables including type of transplant (allogeneic or autologous) as well as intensity of conditioning [8]. Despite the use of antibiotics and other prophylactic strategies, pneumonia remains the leading infectious cause of mortality post-HSCT. Infectious complications are generally found to be more common in allogeneic transplantation rather than autologous procedures as patients are administered immunosuppressive drugs to prevent or treat GVHD leading to susceptibility to opportunistic pathogens. However, infections are also problematic for autologous HSCT patients. For example, one study reported that infectious complications contributed to 21% of deaths in autologous transplants compared to 17% of deaths in allogeneic transplant patients [6]. In another study, autologous patients showed a14% incidence for infection[15]. Thus, both autologous and allogeneic HSCT transplant patients are at risk of infectious complications. Infections can occur with bacterial, viral, and fungal pathogens; but bacterial pathogens have the higher cumulative incidence[15].

Bacterial infections can occur at any time in the post-transplantation period, but they are detected with more frequency in the early phase of neutropenia with a reported 20% to 50% incidence[16, 17]. Success in infection by opportunistic bacterial pathogens is attributed to damage of the mucosal barrier by conditioning, neutropenia, and compromise of the immune system. Gram negative bacteria such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* infections predominate early post-transplant,

whereas Gram positive bacteria including *Streptococcus pneumoniae* predominates in the late phase[18]. Reports have shown that bacterial infections post-transplant can be seen in over 51% of patients with 61% of infections coming from Gram negative bacteria and 39% from Gram positive[6]. Antibiotics including ciprofloxacin, penicillin, and aminoglycosides are routinely given to HSCT patients. However, many of the successful colonizers, including *P.aeruginosa* and *Staphylococcus aureus,* are known to be resistant to a wide array of antibiotics. Thus, better therapeutic strategies that are different than antibiotic usage are needed.

Viral and fungal infections are also a main cause of post-HSCT infectious complications. Cytomegalovirus (CMV) and community acquired respiratory viruses including respiratory syncytial virus (RSV), influenza A and B, and parainfluenza are among the main viral opportunistic pathogens post-HSCT[8]. RSV is the most common isolated viral pathogen[19]. Around 11% of HSCT patients develop viral pneumonia. *Aspergillus spp* are the most common fungal pathogens isolated from HSCT patients, with invasive pulmonary aspergillosis being the most common invasive fungal infection. Allogeneic recipients have a reported incidence of 5%-30%, whereas autologous recipients have a 1%-5% incidence of pulmonary aspergillosis [20]. Other fungal pathogens from *Fusarium* and *Scedosporium* genera can also cause pulmonary infections post-HSCT[8]. Additionally, patients treated with immunosuppressive therapy due to chronic GVHD are at high risk of developing pneumonia mediated by *Pneumocystis jiroveci*[8]. Thus, infectious complications post-HSCT can occur from bacterial, viral, and fungal pathogens. Herein, I will focus on bacterial infections post-HSCT, and will use a mouse model to experimentally describe post-transplant infections.

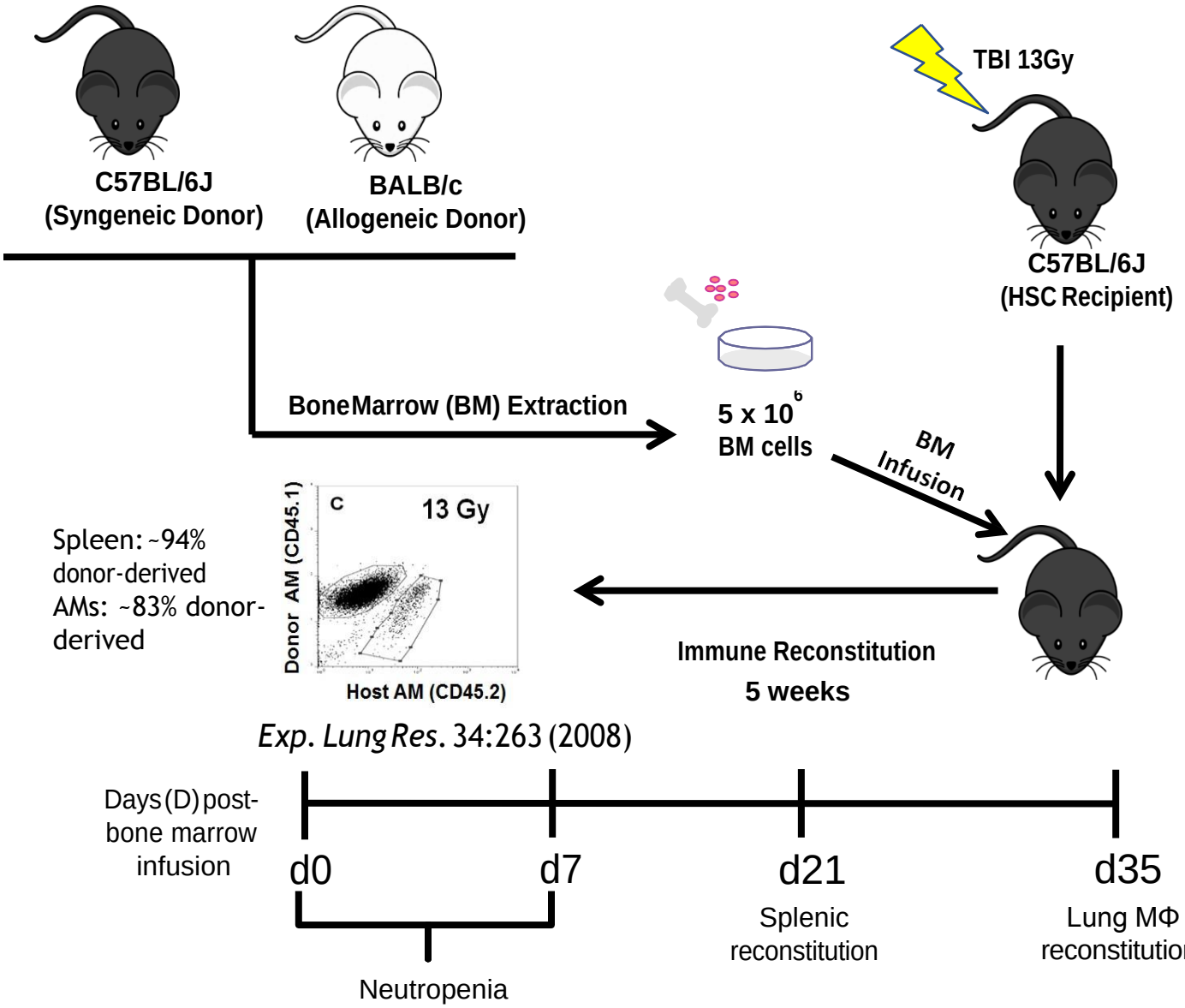

Figure 1-1: Murine Bone Marrow Transplantation Model. C57BL/6J mice are irradiated with a split dose of 13Gy. Irradiated mice are then infused with $5x10^6$ bone marrow cells from a genetically identical (C57BL/6J) or unrelated (BALB/c) donor. Mice are then housed for 5 weeks after bone marrow infusion to achieve full reconstitution of their immune system.

1.4 Animal model to study post-HSCT infections

A murine bone marrow transplantation (BMT) model (Figure 1-1) is used to study infectious complications post-HSCT. In this model, mice are exposed to myeloablative conditioning before infusion with bone marrow stem cells from a genetically identical (syngeneic) or an unlike donor (allogeneic). Total body irradiation with a split dose of

13Gy is often used as a conditioning regimen, followed by infusion of $5x10^6$ bone marrow stem cells. Mice are then housed for up to 5 weeks for immune cell reconstitution. After immune reconstitution around 83% of the alveolar macrophages and 94% spleen cells in the recipient mice are donor derived in syngeneic transplants [21]. This mouse model is used to mimic pulmonary immunosuppression to bacterial infections as seen in humans post-HSCT[22-24].

1.5 Prostaglandin E2 (PGE_2) in HSCT patients

As mentioned earlier, immunosuppressant drugs given to HSCT patients to prevent or treat pathologies such as GVHD can increase susceptibility to infections[8]. However, even in the absence of immunosuppressant drugs and in the late phase of transplantation, patients remain susceptible to pathogens. In search for an explanation, in the early 90's, an article was published noting that HSCT patients had elevated levels of prostaglandin E_2 (PGE_2)[25]. This was seen in 100% of the patients and it was independent of their conditioning regimen[25]. PGE_2 is a lipid molecule secreted by all cells and has been known to have multiple immunosuppressant roles against bacterial, viral, and fungal pathogens[26]. Thus, this article opened the door to explore a possible role for an endogenous molecule that might be inducing susceptibility against a wide array of opportunistic pathogens post-HSCT. Interestingly, my mouse model of BMT has previously been shown to also have elevated levels of PGE_2 in the bronchoalveolar lavage fluid (BALF) and in whole lung homogenates when compared to non-BMT mice[27]. Elevated levels of PGE_2 were shown to have a negative effect in bacterial clearance and morbidity, and suppression of PGE_2 was shown to improve *P. aeruginosa* infection[28, 29]. Reasons behind elevation of PGE_2 post-HSCT are unknown

but, in one study, were attributed to hypomethylation of the COX-2 gene responsible for processing of PGE_2[30]. Thus, in the absence of immunosuppressant drugs, elevated levels of PGE_2 in HSCT patients may be inducing susceptibility to infections.

1.6 PGE_2 synthesis

PGE_2 belongs to the family of eicosanoids which are lipid hormone-like signaling molecules that have important roles in inflammatory and physiological functions [31]. Homeostatic levels of eicosanoids are always present in mammalian tissue and dysregulation of these levels can have different physiological outcomes[31, 32]. All eicosanoid molecules are produced from the enzymatic modification of arachidonic acid (AA), a polyunsaturated fatty acid esterified in membrane phospholipids of mammalian cells[33]. Metabolism of AA is mediated by a cascade of events starting by being released from cellular membranes by phospholipase A_2 (PLA_2) enzymes[31, 32]. Three PLA_2 enzymes can mediate this process, cytosolic calcium-dependent PLA_2 ($cPLA_2$), cytosolic calcium-independent ($iPLA_2$), and secreted PLA_2 ($sPLA_2$)[34]. The $iPLA_2$ enzyme is in charge of maintaining homeostatic levels of free AA, whereas $cPLA_2$ and $sPLA_2$ are activated by various stimuli (e.g. microbial recognition)[31, 35-37]. Membrane liberated AA can then be further modified to produce prostaglandins or leukotrienes[32] (Figure 1-2). For prostaglandin synthesis, free AA is oxidized by one of the cyclooxygenase (COX) enzymes, COX-1 or COX-2.[37] The COX enzymes are structurally equivalent and are around 60% homologous at the amino acid level[38]. Yet, while COX-1 is constitutively expressed in most mammalian cells producing homeostatic levels of prostaglandins, COX-2 is inducibly expressed by different stimuli and its expression is highly restricted [39]. The enzymatic action of COX on AA is twofold; first it oxidizes AA to form

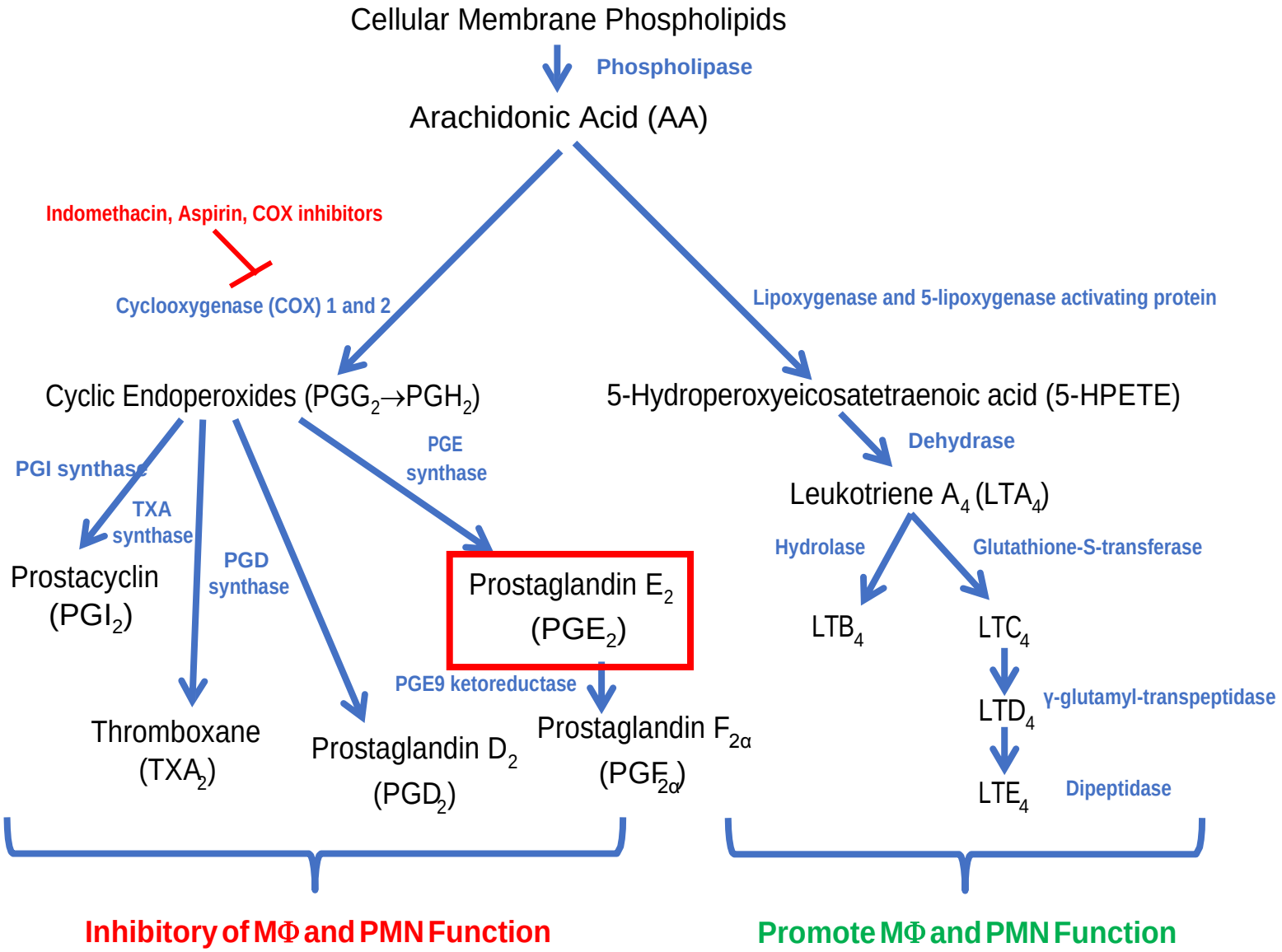

Figure 1-2: Prostaglandin and leukotriene synthesis. Phospholipases release arachidonic acid (AA) from the phospholipid membrane. Free AA can then be modified by the cyclooxygenase or the lipoxygenase pathway to produce prostaglandins and leukotrienes, respectively. During the cyclooxygenase pathway, to the left, COX enzymes modify AA to produce PGH2, which can then be modified by specific enzymes to produce PGI2, TXA2, PGD2, PGE2, and PGF2α. COX enzymes can be blocked by the nonsteroidal anti-inflammatory drugs, indomethacin and aspirin. Prostaglandins are known inhibitors of the antimicrobial function of both macrophages (Mϕ) and polymorphonuclear leukocytes (PMN). The lipoxygenase pathway, to the right, leads to the production of LTA4 which can then be modified by specific enzymes to produce LTB4 or LTE4. Leukotrienes are known to promote Mϕ and PMN antimicrobial functions.

prostaglandin G_2 (PGG_2), secondly COX reduces PGG_2 to form prostaglandin H_2 (PGH_2). Dependent on specific enzymes, PGH_2 can be modified to produce 5 known different prostaglandins known as prostaglandin D_2, prostaglandin I_2, thromboxane A_2, prostaglandin $F_{2\alpha}$, and PGE_2 [31, 40, 41]. Alternatively, if AA is metabolized via the actions

of 5-lipoxygenase and 5-lipoxygenase activating protein, ultimately leukotriene synthesis can occur[42]. In terms of innate immunity there is an intricate “yin and yang” in regulation of innate immune cell function via the actions of prostaglandins versus leukotrienes with prostaglandins generally inhibiting the functions of macrophages and neutrophils whereas leukotrienes can promote function of these cells [43].

PGE_2 synthesis is mediated by 3 different enzymes termed: cytosolic PGE synthase (cPGES), microsomal PGE synthase-1 (mPGES-1), and microsomal PGES-2 (mPGES-2). It is known that physiological levels of PGE_2 are maintained by the enzymatic action of cPGES and mPGES-2, whereas mPGES-1 is activated by a wide array of stimuli including pathogen recognition. Upon intracellular production, PGE_2 is secreted to the extracellular compartment by the multidrug resistance protein 4 channel[44]. Extracellularly, PGE_2 can signal through 4 different G-protein coupled receptors (GPCRs) or it can be rapidly degraded by 15-hydroxyprostaglandin dehydrogenase to an inactive metabolite termed 15-keto prostaglandin E_2 [45] (Figure 1-3). Interestingly, dysregulation of PGE_2 has been implicated to play a role in many disease outcomes, including diabetes and cancer[32, 45-47]. The full spectrum of its role as an inflammatory mediator is still unknown as both pro- and anti-inflammatory roles have been shown. For example, PGE_2 can mediate endothelial permeability to promote inflammatory cell recruitment[48]; yet actions on inflammatory cells themselves are often inhibitory as described below. PGE_2 can signal through 4 different PGE_2 receptors which are known as E type prostanoid or EP receptors, which might explain the multiple reported functions.

1.7 PGE_2 signaling

PGE_2 effector functions have been shown to modulate many biological processes including cell proliferation, apoptosis, angiogenesis, inflammation, and immune surveillance. This is due to the ability of PGE_2 to signal in both autocrine and paracrine manners through four different types of EP GPCRs, namely EP1, EP2, EP3, and EP4 [31, 38, 40, 45, 49]. EP receptors differ in their intracellular signaling pathways, affinity to PGE_2, as well as tissue and cellular localization. EP signal transduction pathways have been an important topic of study in host defense against microbial pathogens [34, 50].

In humans and rats, the EP1 receptor has the lowest affinity for PGE_2 among all EP receptors [51], whereas in mice, it is the second lowest affinity. It is coupled to a Gq alpha subunit (Gαq), which activates the phospholipase C pathway leading to an increase in intracellular Ca^{2+} levels by activation of Ca^{2+} protein channels and subsequently activation of protein kinase C (PKC)[52]. Signal transduction mediated by PKC leads to the activation of mitogen-activated protein kinases (MAPK) which are essential in inducing an immune response against viral infections[53, 54]. PKC also leads to the activation of the transcription factors NFAT and NFκB. Dysfunction of the EP1 receptor results in decreased colon tumorigenesis in models of chemically-induced colon cancer in mice, suggesting an important role of this receptor and PGE_2 in inflammation-induced cancer [45]. EP1 has not been shown to play major roles in innate immunity, but it can be an important facilitator of naïve T cell differentiation, particularly to suboptimal stimuli[55, 56].

The EP2 and EP4 receptors mediate most of the immunomodulatory effects of PGE_2 in innate and adaptive immune cells. EP2 and EP4 are coupled to the Gs alpha subunit (Gαs) and activation of these receptors leads to increased cyclic adenosine monophosphate (cAMP) mediated by adenylate cyclase activation [40]. EP4 has a higher affinity for PGE_2 compared to EP2. However, EP2 can increase cAMP levels to a higher degree than EP4 stimulation; which is believed to be caused by a faster desensitization of the EP4 receptor [40]. Increased intracellular cAMP leads to activation of protein kinase A (PKA) and its sister molecule, the exchange protein directly activated by cAMP (EPAC), which activates the transcription factor CREB [57]. In addition to CREB activation, EP2 and EP4 signaling can lead to inhibition of glycogen synthase kinase-3 (GSK-3) which inhibits the translocation of β-catenin into the nucleus. Interestingly, inhibition of GSK-3 by EP2 is dependent on increased levels of cAMP, whereas EP4-mediated inhibition of GSK-3 is independent of cAMP and dependent on activation of phosphatidylinositol 3-kinase (PI3K)[40]. The ability of EP4 to directly activate PI3K upon PGE_2 binding also leads to the activation of the extracellular signal-regulated kinases (ERKs) that activate early growth response factor-1 which can control the expression of many genes including tumor necrosis factor (TNF)-α, COX-2, and mPGES1[40]. EP2 and EP4 signaling have also been shown to increase the levels of interleukin-10 (IL-10) while decreasing the levels of TNF-α [58, 59]. The fact that EP2 and EP4 can both activate cAMP, but that they also differ in downstream signaling likely explains why these receptors have been shown to have both similar and divergent roles depending on the infection as discussed in greater detail below.

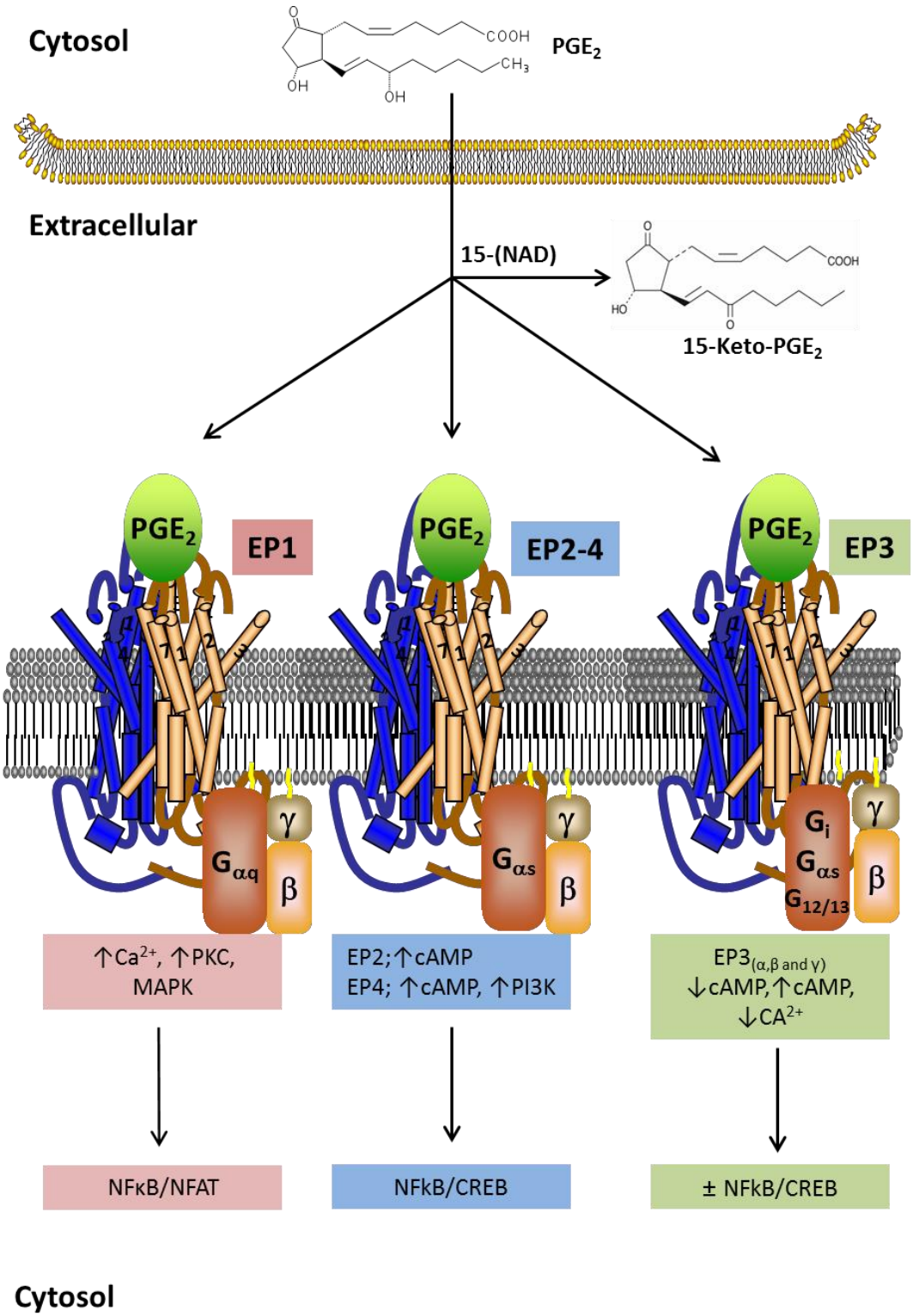

Figure 1-3: Prostaglandin E_2 signaling. PGE_2 can signal through 4 different GPCR subtypes termed EP1-4 or can get degraded to its inactive metabolite, 14-Keto-PGE_2. The EP1 receptor is coupled to the G_{aq} subunit which leads to the increase of intracellular Ca^{2+} levels. EP2 and EP4 are coupled to G_{as} while EP3 can couple to G_i, $G_{\alpha s}$ or $G_{12/13}$. EP1 is known to induce NFkB and NFAT transcription factors, while EP2 and EP4 can induce NFkB and CREB. When EP3 is coupled to G_{as}, it can induce NFkB/CREB, but when coupled to G_i, it can inhibit these factors. Figure created using ScienceSlides

EP3 is the only EP receptor that exists in multiple variants (α, β, and γ) that are generated by alternative splicing of the carboxyl terminal tail[38, 60]. These variants of the EP3 receptor have equal affinity for PGE_2, yet they differ in their signal transduction, desensitization, and relative expression. The three isoforms of EP3 can inhibit the activation of cAMP when coupled to the Gi protein subunit, which can also increase the levels of intracellular Ca^{2+} and can result in the antagonism of PGE_2 signaling via EP2 and/or EP4. Alternatively, EP3 can also bind to Gαs and G12/13, meaning that in some instances EP3 signaling can mimic EP2 or EP4 elevations of cAMP [61, 62]. Figure 1-3 summarizes the downstream signaling associated with each of the EP receptors.

1.8 The role of PGE_2 in bacterial infection

PGE_2 signaling can also impair immunity to viral and fungal pathogens, yet, for the purpose of this dissertation work, I will focus just on the role of PGE_2 in bacterial infections and its implication in HSCT individuals. The role of PGE_2 in host defense against multiple bacterial infections has been well described elsewhere [34]. Thus, in this dissertation, I will restrict my focus on how PGE_2 regulates infection to some of the microbes that present complications to HSCT patients and that are also on the World Health Organization's (WHO, 2017) list of the most critical bacterial pathogens impacting human health which includes *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, and *Staphylococcus aureus*. As noted below, the impact of PGE_2 on these infections is not uniform.

Mycobacterium tuberculosis (MTb) is an intracellular bacterium and the causative agent of tuberculosis, which killed 1.8 million infected individuals in 2015 and according to the WHO has surpassed human immunodeficiency virus (HIV) as the leading cause of

death due to a single infectious agent. MTb, in any phase post-transplant, can cause complications post-HSCT[6]. In response to MTb infection, macrophages produce high levels of PGE_2 partially mediated by activation of the TLR2/p38 MAPK signaling pathway and the nuclear receptor PPARγ [63-65]. Despite the fact that PGE_2 is known to inhibit phagocytosis and killing of many bacterial pathogens by macrophages [24, 66], increased production of PGE_2 and signaling through the EP2 receptor is protective against MTb and $EP2^{-/-}$ mice are more susceptible to infection, compared to wildtype mice [67]. However, these protective effects are more closely aligned with adaptive immune alterations than innate immune signaling which is not surprising given that MTb replicates intracellularly. EP2 signaling was necessary to mount antigen-specific immunity and to downregulate the number of T regulatory cells which can dampen effector T cell responses[67]. PGE_2 inhibition by indomethacin reduces MTb specific TH1 cells upon MTb infection, suggesting that PGE_2 signaling is necessary to generate MTb specific adaptive immunity[68]. In addition, indomethacin treatment has been shown to activate the DP2 receptor in Th2 cells leading to higher levels of IL-2 and IL-5, which could also influence Mtb responses independent of PGE_2 levels[69]. Furthermore, genetic polymorphism of EP2 in humans is correlated with susceptibility to MTb infection, as shown by a study of healthy and infected individuals from a large Chinese Han population [70]. Infection with a virulent strain of MTb leads to decreased PGE_2 and high lipoxin A_4 leading to successful spread of the pathogen by induction of macrophage necrosis, whereas high levels of PGE_2 leads to control of infection by induction of apoptosis [71-73]. A shift towards necrosis within alveolar macrophages as a result of decreased levels of PGE_2 post-MTb was dependent on the virulence factor,

phospholipase C [74]. Interestingly, the role of PGE_2 in MTb infections also seems to be dependent on the stage of infection. During acute infection, COX-2 inhibition leads to a successful pathogen colonization of the lung, whereas an inhibition of COX-2 during chronic infection leads to improved clearance of MTb [34, 75].

Pseudomonas aeruginosa is an extracellular Gram-negative, multi-drug resistant, opportunistic bacterial pathogen that infects immunocompromised individuals including cystic fibrosis patients and HSCT patients [22, 76]. In contrast to the effects of PGE_2 to promote clearance of intracellular MTb, elevated levels of PGE_2 in the lungs of HSCT patients or bone marrow transplant (BMT) mice are known to enhance susceptibility of the host to *P. aeruginosa* infection [22]. Conditioning of the host by either total body irradiation or chemotherapy can cause elevations of PGE_2 [21, 25]. In the mouse, one mechanism that has been identified is that lung epithelial cells release transforming growth factor beta (TGFβ) in response to total body irradiation. In turn, the TGFβ binds to receptors on alveolar macrophages to upregulate a miRNA known as miR29b which then destabilizes the mRNA for DNA methyltransferase enzymes [77]. In the absence of DNA methyltransferases, the COX-2 gene becomes hypomethylated and overexpressed promoting the production of PGE_2 post-BMT [77]. In addition to the elevations of PGE_2 by BMT alone, *P. aeruginosa* infection can cause macrophages to increase PGE_2 secretion by activation of phospholipase A2α (PA2α) [78]. PGE_2 signaling via the cAMP-coupled EP2 or EP4 receptors leads to activation of IRAK-M and phosphatase and tensin homolog on chromosome 10 (PTEN) impairing phagocytosis and *P. aeruginosa* killing, as well as suppressing production of TNF-α and cysteinyl leukotrienes [79, 80]. BMT mice are more susceptible to *P. aeruginosa* infection unless

transplantation has been done with stem cells from either IRAK-M$^{-/-}$ and/or PTEN$^{-/-}$ mice, suggesting that inhibition of IRAK-M and PTEN are potential therapeutic targets to improve *P. aeruginosa* clearance post-BMT. In addition, improved clearance of *P. aeruginosa* in the lungs of BMT mice has been achieved by indomethacin treatment which blocks COX function and reduces PGE_2 levels post-transplant [81]. PGE_2 has also been shown to downregulate the scavenger macrophage receptor MARCO, which is the receptor necessary for recognition of non-opsonized *P. aeruginosa* [24]. Thus, *P. aeruginosa*-induced PA2α activation with subsequent secretion of PGE_2 is a mechanism that confers successful colonization with *P. aeruginosa* in normal hosts. Moreover, in BMT hosts which have elevations of PGE_2 in the lungs constitutively, infection with *P. aeruginosa* is particularly deadly[82].

Streptococcus pneumoniae is a Gram-positive facultative anaerobic bacterium that is responsible for the majority of community-acquired pneumonia and is the main bacterial pathogen inducing mortality in influenza-associated secondary infections[83]. *S. pneumoniae* causes complications post-HSCT in the late phase post-transplant[18]. Upon *S. pneumoniae* lung infection, alveolar type II cells upregulate COX-2, secreting high levels of PGE_2 that signal mainly through the EP4 receptors on lung macrophages [84]. Additionally, neutrophils secrete high amounts of PGE_2 and LTB_4 upon exposure to *S. pneumoniae*'s virulence factor, pneumolysin [85]. Signaling through EP4 receptors in alveolar macrophages post-*S. pneumoniae* infection was shown to decrease the levels of *S. pneumoniae*-induced cytokines such as TNF-α [86]. Moreover, PGE_2 has been shown to play a pivotal role in efferocytosis (apoptotic cell removal) by alveolar macrophages. High levels of PGE_2-mediated by uptake of apoptotic bodies compromise

the immune response to *S. pneumoniae* by decreasing phagocytosis, bacterial killing, and H_2O_2 production dependent on EP2/EP4 activation of cAMP [87, 88]. Mice deficient in EP2 or treated with indomethacin have improved killing and survival post-*S. pneumoniae* lung infection [89, 90]. Interestingly, EP3-deficient mice also have improved bacterial clearance, phagocytosis and survival post-*S. pneumoniae* infection [91]. The reason for EP3 to influence *S. pneumoniae* killing was linked to higher production of nitric oxide (NO) by the $EP3^{-/-}$ alveolar macrophages and one possible explanation for this was that the unchecked signaling of PGE_2 via the remaining EP2 and EP4 receptors might explain this phenomenon [91]. Additionally, since the EP3γ receptor isoform has been shown to also activate adenylate cyclase in overexpression studies [61, 62, 92], this may be the reason EP3-deficient mice experience improved survival and bacterial clearance post-*S. pneumoniae* lung infection, compared to wildtype mice even though their febrile response was blunted [91].

In contrast to these studies which suggest PGE_2 limits host defense against *S. pneumoniae*, one study evaluating genetic ablation of mPGES-1 with subsequent reduction of PGE_2 showed higher bacterial burden, decreased mouse survival, lower expression of NADPH oxidase and lower NO production post- *S. pneumoniae* infection [93]. It is not immediately clear how to reconcile these differences, but one possibility is that selective targeting of mPGES-1 caused a redistribution of eicosanoid synthesis that is not fully characterized that may have impacted outcomes. Therefore, the role of PGE_2 in host defense against *S. pneumoniae* is complex, yet the majority of evidence suggests a negative role of PGE_2 on *S. pneumoniae* clearance.

Staphylococcus aureus is a Gram positive, biofilm-producing bacterium that has acquired antibiotic resistance to multiple drugs making it one of the leaders of nosocomial infections. Like other Gram positive bacteria, *S. aureus* causes complications to HSCT patients in the post-engraftment and late phase periods[18]. *S. aureus*'s lipoteichoic acid is a known inducer of COX-2 expression and PGE_2 signaling [94-97]. *S. aureus*-mediated activation of COX-2/PGE_2 pathway in oral epithelial cells facilitates *S. aureus*'s growth and biofilm production improving bacterial attachment to fibronectin [94]. In addition, the synergistic interaction between the fungal pathogen *Candida albicans* and *S. aureus* is mediated by increased PGE_2 caused by *C. albicans* infections, inducing improved biofilm formation of *S. aureus* [98]. Thus, *S. aureus* induction of PGE_2 leads to improved growth and survival of the bacteria at the early stages of infection. However, PGE_2 is also a well-known inducer of TH17 immunity, which is necessary to clear *S. aureus* infections. One study showed that diabetic mice have low levels of PGE_2 leading to low TH17 immunity and high susceptibility to *S. aureus* infections [99]. Thus, inhibition of PGE_2 at the early stages of infection might improve *S. aureus* clearance, whereas at the late stages in might be beneficial to stimulate PGE_2 synthesis to increase IL-17 and improve survival in chronic infections.

In addition to the pathogens discussed here, virulence factors of enteric pathogens including *Salmonella* serotypes (*S.enterica*, *S.dublin*, and *S.typhimirium*), *Escherichia coli*, and *Vibrio cholerae* are well-known inducers of PGE_2 as a means of establishing infection and limiting host defense [34]. A successful infection of *Salmonella* depends on the induction of COX-2 expression by ERK1/2 activation and inhibition of COX-2 leads to better killing of intracellular *Salmonella* in infected macrophages [100]. Furthermore, in a

model of sepsis induced by lethal administration of *E.coli's* LPS, COX-$2^{-/-}$ mice have shown improved survival compared to wild-type mice [101]. Thus PGE_2 plays an essential regulatory role in bacterial defense to a wide array of pathogens. Overall, PGE_2 seems to promote growth of extracellular bacterial species by limiting host innate immune responses. In contrast, PGE_2 impairs adaptive immune responses needed to clear intracellular MTb.

1.9 Rationale for this dissertation

This dissertation research work is focused on the impact that PGE_2 has on immunity to bacterial pathogens in the context of HSCT. As mentioned before, there are more than 60,000 HSCT performed every year as a way to cure multiple diseases. However, HSCT patients are at high risk of post-transplant complications, many of them caused by opportunistic pathogens. These infections are a significant cause of morbidity and mortality post-transplant. This work focuses on the effect of PGE_2 in critical intracellular pathways known to play a role in immunity to bacterial pathogens, the inflammasome and neutrophil extracellular traps (NETs) release.

1.10 PGE_2 and inflammasome-dependent IL-1β secretion

One of the most critical cytokines needed to initiate inflammation and innate immune responses is interleukin 1 (IL-1)β which is proteolytically cleaved and activated from an immature precursor protein by caspase-1 as part of a multi-protein complex known as an inflammasome. Inflammasomes react to pathogenic stimuli leading to the activation and secretion of both IL-1β and IL-18. If not properly regulated, overzealous inflammasome activation can lead to a form of caspase-1-dependent cell death known as, pyroptosis [102]. The mechanism of IL-1β secretion by inflammasome activation is

known to be dependent primarily on two main signals. Signal 1 is mediated by activation of pattern-recognition receptors (PRRs) [e.g. toll like receptors (TLRs) and nod-like receptors (NLRs)] by pathogen-associated molecular patterns or PAMPs [e.g. lipopolysaccharide (LPS) found in Gram negative bacteria and lipoteichoic acid found in Gram positive bacteria)]. PAMP activation of the PRRs increases the levels of mRNA and ultimately protein for the intracellular immature IL-1β and IL-18 precursors. Signal two is delivered by the recognition of multiple agonists (e.g. adenosine triphosphate or bacterial Type III secretion system proteins) leading to inflammasome aggregation and caspase-1-dependent processing of IL-1β and IL-18 into their mature forms [103]. The activation of the inflammasome needs to be highly regulated as low activation might lead to a decrease in pathogen clearance and a high activation can lead to the development of IL-1β-driven auto-inflammatory syndromes [102]. Dysregulation of one of the members of the inflammasome family, the NLRP3 inflammasome, has been linked to the development of cryopyrin-associated periodic syndrome (CAPS), as well as to vascular diseases, non-alcoholic fatty liver diseases, obesity and type II diabetes [104, 105].

The role of PGE_2 on inflammasome and IL-1β activity stills needs to be fully elucidated as reports are conflicting. Two groups have reported that elevation of intracellular cAMP by PGE_2 inhibits the NLRP3 inflammasome, with subsequent decreased IL-1β secretion[104, 106]. Inhibition of the NLRP3 inflammasome by PGE_2 was demonstrated to be dependent on EP2/4-mediated activation of PKA which was found to phosphorylate the Ser295 residue in NLRP3 inhibiting its function [104]. Furthermore, this residue is found to be mutated in CAPS patients, explaining the over-activation of the NLRP3 inflammasome in these individuals[106]. These findings are specific to the NLRP3

inflammasome and not to other inflammasome scaffolds. Other studies have shown that PGE_2 stimulation exacerbates NLRP3 dependent IL-1β secretion in a mouse model of scorpion venom infection. These discrepancies in the association of PGE_2 with IL-1β levels provided the rationale for my studies in Chapter 3 looking more carefully at the regulation of IL-1β by PGE_2.

1.11 PGE_2 and Neutrophil Extracellular Traps (NETs) formation

Neutrophils are the most abundant leukocyte circulating in the blood and are the first recruited cells to the site of infection after tissue sentinel cells encounter pathogens [107-109]. Upon pathogen recognition, neutrophils can kill microbes by phagocytosis, degranulation (secretion of antimicrobial molecules), and the release of NETs [110]. NETs are web-like structures of decondensed chromatin containing the antimicrobial peptides neutrophil elastase (NE) and myeloperoxidase (MPO), which can trap and kill pathogens [111]. This recently described antimicrobial pathway has been shown to play a role in the clearance of pathogens that are too big to be engulfed by phagocytosis including *Candida albicans* hyphae and extracellular aggregates of *Mycobacterium bovis* [112]. Yet, they can also play a role in killing of smaller bacterial pathogens including *S. aureus*, *Salmonella typhimurium*, and *Shigella flexneri* [111]. NETs release has been shown to be dependent on the scavenger receptor dectin-1, superoxide generation and the intracellular pathway known as autophagy which allows for cellular homeostasis under periods of stress [113]. Inhibition of either autophagy or NADPH oxidase as well as dectin-1 deficiency can inhibit the formation of NETs [112, 113]. Given that PMNs and NETS are likely to play an important role in defending against numerous bacterial and fungal pathogens known to infect HSCT patients and knowing that HSCT patients are

more susceptible to such infections provided the rationale for why this dissertation addressed the question of how HSCT and PGE_2 regulate NETs in Chapter 4.

1.12 Conclusions

HSCT patients are at high risk of post-transplant complications due to immunosuppression and treatment-related toxicity. Pulmonary infections, particularly bacterial infections, are major complications in HSCT patients and contribute significantly to increased morbidity and mortality. HSCT patients have elevated levels of PGE_2 in the serum and in the BALF. PGE_2 has multiple functions on immune cells (Figure 1-4) and tends to lend the host more susceptible to bacterial, fungal, and viral infections. In this dissertation, the role of PGE_2 in regulating immunity to bacterial pathogens post-HSCT was studied in alveolar macrophages and PMNs, with a focus on the innate intracellular pathways, inflammasome and NETs.

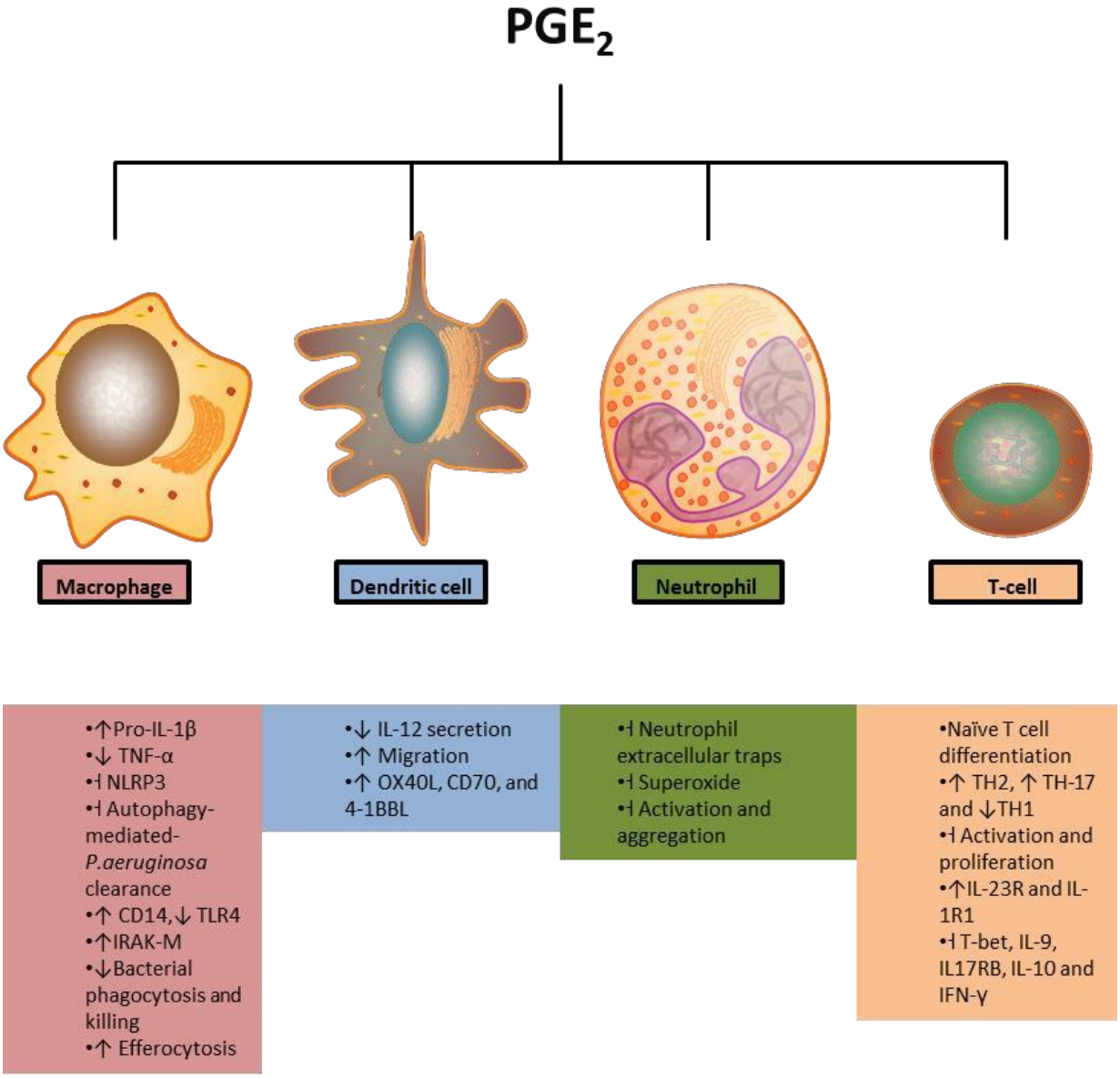

Figure 1-4: PGE_2 effects on immune cells. (From left to right) Macrophages, dendritic cells, neutrophils and T cells. The known effects of PGE_2 on these cells are summarized below each cell. Symbol meaning: ↑ increase/activation; ↓ decrease; ꟾ inhibits. Figure created using ScienceSlides.

1.13 References

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Chapter 2.

Material and Methods

2.1 Human Subjects

Experimental samples were from human subjects that received hematopoietic stem cell transplant; patients were from the age of 27-71 at the moment of blood collection. Control samples were from healthy volunteers from the age of 23-53 at the moment of blood collection. Written informed consent was received and all experiments were approved by the University of Michigan institutional review board.

2.2 Animal

C57BL/6J and BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME) and bred in the animal facilities at the University of Michigan (Ann Arbor, MI). Breeding colonies of $TLR9^{-/-}$ mice, on a BALB/c background, were kindly donated by Dr. Shizuo Akira and were also bred in the animal facilities of the University of Michigan (Ann Arbor, MI). All mice used were at least 6-7 weeks old by the time of infection and/or treatment. Experiments were approved by the University of Michigan Institutional Animal Care and Use Committee.

2.3 Cell culture media

Supplemented media (Complete media) (89% Dulbecco's Modified Eagle Medium (DMEM) (Lonza) without L-Glutamine, 10% Fetal bovine serum (FBS) (Gibco), 1% Penicillin-streptomycin (Pen-Strep) (Gibco), 2mM L-Glutamine (Gibco), and

250ng/ml Amphotericin (Gibco)); Serum Free media (SFM) (DMEM without L-Glutamine, 0.1% Bovine serum albumin (BSA) (Sigma-Aldrich), 1% Pen-Strep, 2mM L-Glutamine, and 250ng/ml Amphotericin (Gibco)); Bronchoalveolar lavage media (Complete media, and 5mM ethylenediaminetetraacetic acid (EDTA) (Lonza)); L-cell (L-929) media (10% FBS, 1% Pen-Strep, 89% Iscove's Modified Dulbecco's Medium with 4mM L-Glutamine (IMDM) (Hyclone)); Bone Marrow Derive Macrophages (BMDM) media (10% FBS, 1% Pen-Strep, 59% IMDM with 4mM L-Glutamine, and 30% L-cell supernatant).

2.4 Reagents

PGE_2 (1-1000nM; Cayman Chemicals, Ann Arbor, MI); Forskolin (25μM; Cayman Chemical, Ann Arbor, MI); *Pseudomonas aeruginosa* LPS (100ng/ml; Sigma-Aldrich, St. Louis, MO); Adenosine triphosphate (ATP) (1μM; Sigma-Aldrich, St. Louis, MO); Caspase-1 inhibitor (10nM Ac-YVAD-CHO; Enzo Life Sciences, Farmingdale, NY); Caspase-8 inhibitor (10nM Ac-IETD-CHO; BD Biosciences, San Jose, CA); CREB inhibitor (100μM Naphthol AS-E phosphate; Sigma-Aldrich, St. Louis, MO); EP2 agonist (1μM Butaprost; Cayman Chemical, Ann Arbor, MI), EP3 agonist (10nM Sulprostone; Cayman Chemical, Ann Arbor, MI) EP4 agonist (500nM ONO-AE1-329; Sigma-Aldrich, St. Louis, MO); Protein Kinase A (PKA) agonist (50μM 6-BNZ-cAMP; Biolog, Hayward, CA); Epac agonist (50μM 8-pcpt-2'-OM-cAMP; Biolog, Hayward, CA). Indomethacin (1.2mg/kg; Sigma-Aldrich, St. Louis, MO); Diclofenac (30mg/kg; Sigma-Aldrich, St. Louis, MO); PF-04418948 (EP2 antagonist) (10nM; Pfizer); AE3-208 (EP4 antagonist) (1μM; Cayman Chemical, Ann Arbor, MI); Rapamycin (1μM; Sigma-Aldrich, St. Louis, MO); H-89 dihdrochloride hydrate (PKA antagonist) (20μM; Sigma-Aldrich, St. Louis,

MO); ESI-09 (Epac antagonist) (35 μM; Sigma-Aldrich, St. Louis, MO); Wortmannin (100nM; Sigma-Aldrich, St. Louis, MO); Diphenyleneiodonium (DPI) (10μM; Sigma-Aldrich, St. Louis, MO); Anti-IFN-γ neutralizing antibody (XMG1.2) (200Mg; BioXcell; West Lebanon, NH); ODN 2088 and 2088 control (50ug; InvivoGen; San Diego, CA); Recombinant mouse IFN-γ (10ng/ml, RD systems; Minneapolis, MN).

2.5 Isolation of Human Neutrophils

Neutrophils were isolated from human blood by gradient centrifugation. Briefly, blood was overlaid gently in Ficoll-Paque (GE healthcare) medium prior to centrifuging at 1,400rpm with slow acceleration and no brake for 20min. Neutrophils were then separated from the plasma and PBMCs and mixed with 20% dextran for 15min. Solution was then diluted with PBS and left undisturbed for 30min. After incubation there are two layers, neutrophils are isolated from the top layer, the bottom layer contains the red blood cells-Dextran. Solution containing neutrophils was then centrifuged and pellet was resuspended in 20mL of 0.2% NaCl for 3min followed by 30mL of 1.8% NaCl to lyse red blood cells. Neutrophils were washed, counted, and differential staining was performed to detect purity of the assay.

2.6 Isolating murine neutrophils from lung

Neutrophils were recruited to the lung by administration of 50μl of lipopolysaccharide (LPS) (25μg/ml) diluted in saline solution. BAL was performed 18 hours post-LPS administration with RPMI with L-Glutamine without phenol red (Gibco). BALF fluid was centrifuged for 10min at 1,200rpm prior to RBC lysis. Pellets were reconstituted in complete media and cells were counted before platting. Differential staining was performed to detect purity of the assay.

2.7 Isolating murine bone marrow neutrophils

Neutrophils were isolated from the femur and tibia of healthy C57BL/6J mice by gradient centrifugation. Briefly, mice were euthanized by CO_2 asphyxiation and femur and tibia were collected in a sterile environment. Epiphyses of the bones were cut and 26Gx½ needles were used to flush 10mls of complete media containing 2mM EDTA onto a 50ml conical tube. Larger bone marrow tissue was disaggregated by aspirating up and down with a syringe containing an 18Gx½ needle. Samples were centrifuged at 1,400 rpm for 7 min at 4 °C. RBC was lysed with RBC lysis buffer. 1ml of sample was added onto a 15ml conical tube containing 3ml of warmed Histopaque 1119 (Sigma-Aldrich) below 3ml of Histopaque 1077 (Sigma-Aldrich). Samples were centrifuged for 30min at 2,000 rpm at 25^{o}C without brake. Neutrophils were collected from the interface of the Histopaque 1119 and Histopaque 1077 layers. Neutrophils were washed in PBS, counted, and differential staining was performed to detect purity.

2.8 Isolation of human peripheral monocyte-derived macrophages

Monocytes were isolated from peripheral blood mononuclear cells (PBMCs) of human blood from healthy volunteers, and then differentiated *in vitro* with complete media containing macrophage colony stimulation factor (M-CSF) (RD systems). Briefly, blood was gently overlaid into Ficoll-Paque (GE healthcare) prior to centrifugation at 1,440 rpm with slow acceleration, and no break at room temperature (25^{o}C) for 20min. PBMCs were isolated from the interface of the plasma and Ficoll-Paque. PBMCs were washed in PBS and incubated in 175cm^2 tissue culture flask in complete media for one hour at 37^{o}C with 5%CO_2. After an hour, media was aspirated and cells were washed in

PBS before incubating for 6-7 days at 37^{o}C with 5%CO_2 in complete media containing 2.5ng/ml of M-CSF.

2.9 Isolation of murine Alveolar Macrophages (AMs)

AMs are harvested by performing bronchoalveolar lavages (BAL). Briefly, mice were euthanized by CO_2 asphyxiation, and placed in a sterile foam board prior to exposing the chest cavity and trachea. Two washes with 10ml of BAL media were performed per mouse where BAL fluid (BALF) was collected in a 10ml sterile syringe and transferred onto a 50ml conical. BALF was centrifuged at 1,200rpms for 10mins prior to lysing the red blood cell with a RBC lysis buffer. AMs pellet was then dissolved in complete media. Each mouse provides around $3x10^5$-$5x10^5$ AMs. Thus, multiple mice have to be used dependent on the experiments.

2.10 Isolation of lung macrophages

Total lung cells were obtained by perfusing the lung with PBS followed by digesting the whole lung with Collagenase A (Sigma-Aldrich, St. Louis, MO). Lung macrophages were selected by adherence selection to a cell culture tissue plate from total lung cells that were added for 30min and then washed twice in PBS. Over 90% purity was confirmed by differential staining of attached cells.

2.11 Differentiation of Bone Marrow Derived Macrophages (BMDMs)

BMDMs were differentiated by isolating bone marrow stem cells and incubating them with L-cell supernatant. Briefly, mice were euthanized by CO_2 asphyxiation and femur and tibia were isolated. Epiphyses of the bones were cut and 26Gx½ needles were used to flush 10mls of complete media containing 2mM EDTA onto a 50ml conical

tube. Cells were centrifuged to remove media at 1,200rpm for 10 minutes in 4^{o}C. Red blood cells were then lysed, and bone marrow stem cells were incubated for 7 days on BMDMs differentiation media. Every two days media was changed.

2.12 Bone Marrow Transplantation

Healthy 6-8 week old C57BL/6J male mice were irradiated with a split dose of 13 gray (13-Gy) with the use of an X-rad 320 irradiator. Irradiated mice were infused with $5x10^{6}$ bone marrow cells from a genetically identical donor (C57BL/6J). Mice were housed for 5 weeks after infusion to achieve full reconstitution of their immune system. The percentage of donor-derived cells was 95 ± 1% in the spleen and 82 ± 2% in the lung at this time point, as assessed by transplanting $CD45.1^{+}$ bone marrow into C57BL/6J $CD45.2^{+}$ mice[1].

2.13 LPS-induced lung injury

Acute lung injury was induced by intratracheal inoculation of 50µg of *P.aeruginosa*-derived LPS (Sigma-Aldrich) in 40µl of saline solution. BALF and lung were harvested 24hours after treatment.

2.14 *In vivo* influenza infections

All influenza infections were done with the influenza A virus (H1N1) strain A/PR/8/34 (PR8) purchased from American Type Culture Collection (ATCC) (Manassas, VA). Briefly, intranasal instillation of 30µl of PBS containing 10-100 plaque forming units of PR8 were performed in healthy female or male mice that were anesthetized with a mixture of ketamine and xylazine. Mice were monitored daily for weight loss until harvest day, usually 5-10 days post-infection.

2.15 Viral load quantification in lung by plaque assay

Madin-Darby canine kidney (MDCK) cells were used for the viral titer quantification from whole lungs of infected mice. Briefly, $2x10^5$ MDCK cells were grown in 12-well plate until well-covered cell monolayer was achieved. Cells were then incubated with serial dilutions of homogenized lungs from infected mice. Cells were washed in 1x MEM-BSA (DMEM, 2mM L-Glutamine, 250ng/ml amphotericin, Pen-Strep, and 10% BSA) medium, then incubated with gentle agitation for an hour at 37^{o}C with virus containing sample prior to incubation with a MEM-BSA & 3% Carboxy Methyl Cellulose solution. Plates were incubated at 37^{o}C for 48-72 hours before adding a crystal violet solution for plaque quantification.

2.16 In vivo bacterial infections

Lung bacterial infections were done with a non-invasive oral instillation method. Briefly, mice were anesthetized with a mixture of ketamine and xylazine prior to be placed in a sterile foam board where sterile forceps were used to gently pull the tongue prior administrating 50µl of PBS-containing bacteria in their oral cavity; nasal air passage were close with forceps and mice aspirated the bacteria. *Pseudomonas aeruginosa* (PA01) infections were intended to be at $5x10^5$ CFUs per mouse but instillations through all experiments came in the range of $2x10^5$-$1x10^7$ CFUs. *Streptococcus pneumoniae* (SPS3)(Serotype 3, 6303) infections were intended to be at $1x10^5$ CFUs per mouse but instillations through all experiments came in the range of $1x10^5$-$4x10^5$ CFUs. Methicillin-resistant *Staphylococcus aureus* (MRSA) (NRS384; US300) infections were intended to be $7x10^7$CFUs per mouse but instillations through

all the experiments came in the range of $5x10^7$-$2x10^8$CFUs. Mice were monitored daily until harvest day, usually 24-48 hours post-infection.

2.17 Bacterial burden quantification

Total lung bacterial burden measurements were obtained by performing BAL in infected lung before homogenizing the lung in 1ml PBS with a tissue homogenizer (OMNI International; Kennesaw, GA). Serial dilutions of BALF and lung were performed and plating was done on nutrient agar plates. Plates were incubated for 24 hours at 37^{o}C prior to counting colonies. Absolute bacterial numbers in the BALF and lung were added together and were transformed to logarithmic values for statistical analysis.

2.18 Viral and bacterial co-infection

Initial influenza infections were performed by intranasal instillation of 30µl of PBS containing 100 PFUs of PR8 to mice that were anesthetized with a mixture of ketamine and xylazine. Secondary bacterial infection with MRSA $7x10^7$CFUs or SPS3 $1x10^5$CFUs were performed five days post initial viral infection. Mice were monitored daily during the course of infection and euthanized when reaching a weigh loss of 25%.

2.19 *In vivo* indomethacin treatments

Intraperitoneal treatments with indomethacin (1.2mg/kg) were given to anesthetized mice at the time of *P. aeruginosa* infection. Lung and BALF were harvested 24hours post-infection.

2.20 *In vivo* IFN-γ neutralization

Influenza infected mice were treated with 200µg of IFN-γ neutralizing antibody (XMG1.2; BioXcell; West Lebanon, NH) or its isotype control (HRPN; BioXcell; West

Lebanon,NH) intraperitoneally on day 5 post-influenza infection prior to a secondary bacterial infection with MRSA ($7x10^7$ CFUs). Successful neutralization was confirmed by measuring IFN-γ levels in BALF by ELISA.

2.21 In vivo TLR9 inhibition

TLR9 inhibition was achieved by treating mice with three doses (50µg/each) of TLR9 inhibitor (ODN 2088; InvivoGen; San Diego, CA) and/or its control (ODN 2088 Control; InvivoGen; San Diego, CA) on day 0, 2, and 5 post-influenza infection.

2.22 FITC-labeling bacteria

Bacteria were grown at a density of $6x10^6$ CFUs/µl and heat inactivated by incubating for 15min in 70^{o}C using a heat block. Heat killed bacteria were centrifuged at 13,000 rpm for 5 mins. Supernatant was removed and 1ml of 0.1M NaHCO3 was added to the pellet followed by 20µl of a 10mg/ml FITC (Sigma-Aldrich) DMSO solution. Bacteria stock was then incubated protected from light, rocking for an hour at room temperature. Bacterial stock was then washed 3 times with PBS by centrifuging at 13,000 rpm for 5mins. Bacteria-FITC stock was then stored on -80^{o}C.

2.23 Bacterial phagocytosis assay

Cells were plated at 200,000 cells/well in 100µl on a half-area black 96-well plate (black with clear bottom) (Corning 3882). Cells were then incubated for an hour, media was removed, and cells were washed with cold PBS prior to adding 100µl of complete medium containing FITC-labeled heat inactivated bacteria (10ul of FITC-bacteria stock per 100ul of media). Cells were then incubated at 37^{o}C for 2 hours before adding 50µl of trypan blue (Sigma-Aldrich) to quench extracellular fluorescence. Intracellular

fluorescence was measured at (485ex/535em) with a bottom read using the Spectra Max M3 (Molecular Devices, LLC). Media was discarded and cells were washed with PBS. We then added 110µl of PBS with 2% TritonX-100 per well and incubated plates on ice. We next used this media to measure intracellular lactate dehydrogenase (LDH) with the Cytotoxicity Detection Kit assay (Roche). Briefly, to do this, we mixed equal parts of LDH reagents and sample in a 96 well flat-bottom plate, incubated for 15 minutes at 37ºC and measured absorbance at 490nm using a plate reader.

2.24 Tetrazolium Dye Reduction Assay (MTT assay) of Bacterial Killing

AMs, lung macrophages, or BMDMs were placed into duplicate 96-well plates: one experimental plate and one control plate. Cells from both plates were infected with IgG-opsonized *P. aeruginosa, S. pnuemoniae,* or *S. aureus* (multiplicity of infection 50:1) for 30 min at 37°C. The cells on the experimental plate were washed and then incubated with or without treatments at 37°C for 120 min, whereas the cells on the control plate were washed and then lysed with 0.5% Saponin (Sigma-Aldrich) in growth medium (Tryptic soy broth, *P. aeruginosa*; Todd Hewitt broth*, S. pnuemoniae*; Nutrient broth, *S. aureus*) and placed at 4°C. After 2 hours, the cells from the experimental plate were lysed with 0.5% Saponin in growth medium. Both plates were then incubated in a shaker at 37°C for 4h for *P. aeruginosa* and *S. aureus* of at 37ºC with 5%CO_2 with no shaking for *S. pneumoniae*. Thiazolyl blue Tetrazolium Bromide (MTT) assay was performed as recommended by the company. Briefly, a total of 5 mg/ml MTT (Sigma) was added to each plate and incubated for 30 min. Solubilization solution was added to dissolve formazan salts, and the absorbance was read at 595 nm (A595). Results were expressed as percentage of survival of ingested bacteria normalized to the percentage

of control, where the A595 experiment values were divided by the average of the A595 control values. Survival of ingested bacteria = (A595 experimental plate/A595 control plate) × 100%.

2.25 H_2O_2 detection assay

Cellular H_2O_2 secretion was determined from LPS-recruited neutrophils via Amplex Red agent.

2.26 Sytox green fluorescence assay

Neutrophil extracellular traps (NETs) release was quantified using a cell-impermeable nucleic acid dye, Sytox Green, post bacterial challenge or PMA treatment. Briefly, $1x10^5$ neutrophils, from human or mice, were seeded in a black with clear bottom, non-treated, flat bottom 96-well plate (Costar 3631, Corning, NY). Sytox Green Nucleic Acid Stain (s7020; Invitrogen, Carlsbad, CA) was added at the time of treatments at a final concentration of 0.2 µM. Cells were incubated for 5 hours before adding 100 µL of 4% PFA to every well followed by storing at 4°C overnight protected from light. Next day fluorescence was measured at 485nm/525nm.

2.27 Immunofluorescence

To visualize NETs**,** $2x10^5$ neutrophils were seeded in poly-lysine-coated cover slips, and treatments were added directly for 5-7 hours (murine) or 3 hours (human), before cells were fixed and stained with anti-neutrophil elastase and Hoescht. For autophagy experiments, alveolar macrophages were harvested by BAL and $1.5\text{-}2x10^5$ cells were seeded in poly-lysine-coated cover slips cells were cell were nutrient starved and/or treated with PGE_2 (100nM) for 4 hours before fixation. Cells were permeabilized

with TBS- with 0.2% of Triton-X, and cover slips were blocked with 5% BSA. Primary antibody was incubated for 4 hours, and secondary antibody was incubated for 2 hours. After antibody incubations, prolong Gold anti fade reagent with DAPI (Invitrogen) was added to the cells. Immunofluorescence images were taken with the use of an Olympus 500 confocal microscope (Olympus Corporation, Center Valley, PA).

2.28 Western blotting and protein measurements

Cell lysates were obtained using RIPA buffer (Thermo Fisher; Waltham, MA) with protease inhibitor (Calbiochem; San Diego, CA) and samples were centrifuged at 15,000rpm to remove debris. Pierce BCA assay kit (Thermo Fisher; Waltham, MA) was used for quantification of total amount of protein in cell lysate. Around 20-50µg of total protein from samples were mixed with laemmli buffer (Bioland Scientific LLC; Paramount, CA) containing β-mercaptoethanol and heated at 95^{o}C for 10min. Protein samples were separated in a polyacrylamide gel using a mini gel tank (Invitrogen, Carlsbad, CA), following by transferring protein to a polyvinylidene fluoride (PVDF) membrane that was blocked with 5% non-fat milk. Incubation with primary antibody was done overnight at 4^{o}C. Multiple washes of TBS with 0.5% of Tween 20 were performed before incubation with secondary antibody for 1-2 hours at room temperature. Images were taken with the Amersham Imager 600 (GE Healthcare) and in some cases densitometry was obtained with Image Studio Lite version 5.2.

2.29 ELISA

Cytokine measurements were performed with the use of R&D duo set ELISA kits for murine IFN-γ, TNF-α, IL-1β, IL-6, IL-10, and IL-17. Murine albumin measurements

were performed with the use of the Bethyl laboratory (Montgomery, TX) albumin ELISA kit.

2.30 Histology

Hematoxylin and Eosin stain was performed after perfusion of the lung with PBS and tissue fixation with 10% formalin. Tissues were left in 10% formalin overnight before replacing with 70% ethanol. Lung samples were processed and stained by McClinchey Histology Services (Stockbridge, MI).

2.31 Real-time quantitative PCR

mRNA was isolated using TRIzol according to the manufacturer's instructions. Relative gene expression measurements were achieved with the use of a Step-one plus real-time PCR system from Applied Biosystems (Foster City, CA). Gene-specific primers and probes were designed with the Genscript Real-time PCR primer design software (Genscript Biotech Corporation, Piscataway, NJ).

Table 2-1 List of Primers and Probes used in qRT-PCR:

Name	Sequence (5'-3')	Strand	Modification
TLR2	ATGGGCTCGGCGATTTC	Forward	
	ATGCAACCTCCGGATAGTGACT	Reverse	
	CGGAGTCAGACGTAGTGAGCGAG	Probe	5'Fam - 3'Tamra
TLR3	GCCCTCCTCTTGAACAACGC	Forward	
	ACTTCAGCCCAGAGAAAGTGCT	Reverse	
	ACCAGCTGCTGGCCACCAGCGAG	Probe	5'Fam - 3'Tamra
TLR4	AAGGAGTGCCCCGGTTTC	Forward	
	CACAATAACCTTCCGGCTCTTG	Reverse	
	TGCCAACATCATCCAGGAAGGCT	Probe	5'Fam - 3'Tamra
TLR7	TCTGCAGGACCTCTGTCCTTG	Forward	

	TGATTGTCTGTGGTCAGGGCAT	Reverse	
	TGGCCTGCAAATCCACAGGCTCACCCA	Probe	5'Fam - 3'Tamra
TLR9	GAGTACTTGATGTGGGTGGGAAATT	Forward	
	GCCACATTCTATACAGGGATTGG	Reverse	
	CCGTCGCTGCGACCATGCC	Probe	5'Fam - 3'Tamra
iNOS	ACATCAGGTCGGCCATCACT	Forward	
	CGTACCGGATGAGGCTGTGAAT	Reverse	
	CCCCACCGGAGTGACGGCA	Probe	5'Fam - 3'Tamra
MARCO	CCTGGACGAGTCGGTCAGAA	Forward	
	CTTCAGCTCGGCCTCTGTT	Reverse	
	CCAACGCGTCCGGATCATGGGT	Probe	5'Fam - 3'Tamra
SRAI/II	TGAAGGACTGGGAACACTCACA	Forward	
	CAGTAAGCCCTCTGTCTCCCTTT	Reverse	
	TTCATTCAAGGGCCTCCTGGACCC	Probe	5'Fam - 3'Tamra
Influenza A virus	GGACTGCAGCGTAGACGCTT	Forward	
	CATCCTGTTGTATATGAGGCCCAT	Reverse	
	CTCAGTTATTCTGCTGGTGCACTTGCCA	Probe	5'Fam - 3'Tamra
IL-1β	GAGCCCATCCTCTGTGACTCA	Forward	
	GTTGTTCATCTCGGAGCCTGTAG	Reverse	
	AACCTGCTGGTGTGTGA	Probe	5'Fam - 3'Tamra
Human IL-1β	AAGCTGAGGAAGATGCTGGT	Forward	
	CGTTATCCCATGTGTCGAAG	Reverse	
	CCTGCCCACAGACCTTCCAGG	Probe	5'Fam - 3'Tamra
COX-1	CTTCTTAGGGAATCCCATCTG	Forward	
	CTTCAGTGAGGCTGTGTTGACAAG	Reverse	
	ACCCAGCACCTTCGGTGGT	Probe	5'Fam - 3'Tamra
COX-2	TGACCCCCAAGGCTCAAAT	Forward	
	GAACCCAGGTCCTCGCTTATG	Reverse	
	TTTGCCCAGCACTTCACCCACAGT	Probe	5'Fam - 3'Tamra
ATG5	TCAAGTTCAGTGGAGGCAAC	Forward	
	TGGAATGTTCTCCTCCTTGG	Reverse	
	CTTGCAGCAGCCCGGGTTTC	Probe	5'Fam - 3'Tamra
β-Actin	CCGTGAAAAGATGACCCAGATC	Forward	
	CACAGCCTGGATGGCTACGT	Reverse	
	TTTGAGACCTTCAACACCCCA	Probe	5'Fam - 3'Tamra

2.32 Flow cytometry

Lung immune cells were obtained after lung perfusion, and whole lung digestion with Collagenase A. Cells were then stimulated with PMA (20nM), ionomycin (1μM) and protein transport inhibitor (Golgi stop) for 4-6 hours in treated cell culture dishes. After incubation, cells were centrifuged and washed in FA buffer with 2% FCS. Staining with antibodies for myeloid or lymphocyte population were performed at 4^{o}C for 30min. Cells were the fixed and permeabilized overnight with Fix/perm solution (Foxp3 / Transcription Factor Staining Buffer Set, Invitrogen). Next day the cells were stained for intracellular markers for 30mins at 4^{o}C. Cells were washed and diluted in PBS before analyzing in a Fortessa cell analyzer (BD Biosciences; San Jose, CA).

2.33 Flow cytometry antibodies

TLR9 (J15A7; BD Biosciences; San Jose, CA), IgG1 (MOPC-21; BD Biosciences; San Jose, CA), CD45 (30-F11; BD Biosciences; San Jose, CA), CD11b (M1/70; BD Biosciences; San Jose, CA), CD11c (N418; Biolegend; San Diego, CA), Siglec F (E50-2440; BD Biosciences; San Jose, CA), MHC II (I-A/I-E) (M5/114.15.2; BD Biosciences; San Jose, CA), CD64 (X54-5/7.1; Biolegend; San Diego, CA), F4/80 (BM8 eBiosciences; SanDiego, CA), LY6G (1A8; BD Biosciences; San Jose, CA), CD3 (17A2, BD Biosciences; San Jose, CA), CD90.2 (53-2.1; BD Biosciences; San Jose, CA), CD4 (GK1.5; Biolegend; San Diego, CA) CD8 (53-6.7; BD Biosciences; San Jose, CA), NKP46 (29A1.4; Biolegend; San Diego, CA), CD19 (1D3; BD Biosciences; San Jose, CA), Fc Block(CD16/CD32) (2.4G2; BD Biosciences; San Jose, CA).

2.34 Statistical analysis

Graphpad Prism version 6 software (Graphpad Prism Software Inc., La Jolla, CA) was used to analyze experimental results. When groups of two were compared, student's T-test was used to determine statistical significance. Groups of ≥ 3 were compared using one-way analysis of variance with Bonferroni multiple mean comparisons. Fisher exact test was used to compare clearance of bacteria and albumin levels between *Pseudomonas aeruginosa* infected mice. In human studies, the following steps were used to normalize patient data according to patient-specific outcomes in the media treatment group. First the mean across an individual's media replicates was calculated. This individual-specific mean was then used to rescale outcomes from all experimental conditions for that individual, via division, giving a mean media outcome in each individual of 100. Other experimental conditions are then summarized as percentage of control units (above or below 100% with media group as reference). Mixed models with a random intercept for individual were used to estimate experimental condition means and corresponding 95% confidence intervals used in the figures; correlation between outcomes within individual are accounted for via the random intercept term. A *P* value less than 0.05 was considered statistically significant.

2.35 Reference

1. Hubbard LL, Ballinger MN, Wilke CA, Moore BB. Comparison of conditioning regimens for alveolar macrophage reconstitution and innate immune function post bone marrow transplant. *Exp Lung Res* 2008; **34**(5)**:** 263-275.

Chapter 3.

Elevated Prostaglandin E_2 Post-Bone Marrow Transplant Mediates Interleukin-1β Related-Lung Injury

3.1 Background

Hematopoietic stem cell transplant (HSCT) utilizes stem cells derived from bone marrow, umbilical cord blood, or peripheral blood to treat or cure a variety of hematological disorders[1]. This procedure has become a standard of care with more than 18,000 HSCT performed every year in the United States alone[1]. HSCT requires a conditioning regimen (e.g. total body irradiation and/or chemotherapy) to allow the patient's hematopoietic system to be replaced by donor cells. Depending on disease, the patient can undergo autologous (self-donation of stem cells) or allogeneic (stem cells from a Human Leukocyte Antigen (HLA)-matched donor) transplants. Unfortunately, patients that undergo HSCT become immunosuppressed and susceptible to infections by opportunistic pathogens[2]. Around 60% of HSCT patients develop pulmonary complications, many of them due to bacterial infections, which correlate with high mortality and morbidity[1-3]. Infections post-HSCT are independent of conditioning regimen and type of transplant as both autologous and allogenic patients are highly susceptible. Infections can take place in the pre-engraftment phase (before 30 days post-transplant) as well as after immune reconstitution (after 30 days post-transplant)[3]. Although infections can be caused by viral, bacterial, or fungal organisms, bacterial infections are increasingly problematic due to the rise in drug-resistant bacteria.

Bacterial infections post-HSCT can occur in 51.3% of HSCT patients[2]. These infections, including infections by the Gram negative bacterial pathogen, *Pseudomonas aeruginosa,* cause life-threatening complications[2].

P. aeruginosa is an opportunistic pathogen that normally infects immunocompromised individuals such as HSCT patients[2, 4-7]. It is a leading nosocomial pathogen, and it is the most frequently isolated Gram negative bacteria in the intensive care unit [8]. This pathogen causes urinary tract infection, hospital-acquired pneumonia, and bacteremia in burn patients[9]. It is also the predominant cause of morbidity and mortality in Cystic Fibrosis patients. There has been a significant increase in research effort studying *P. aeruginosa* due to the difficulty of treating infected patients, as 26% of its isolates are resistant to antibiotics and disinfectants [8]. Understanding how to modulate *P. aeruginosa* infections in an antibiotic-independent method is likely to have positive impacts on the outcome of infected patients. Although many antimicrobial pathways have been linked to *P. aeruginosa* clearance, induction of inflammasome activation and Interleukin 1β (IL-1β) secretion play pathogenic roles during *P. aeruginosa* infection[5].

The inflammasome is a multi-protein complex expressed mainly in immune cells and activated by pathogenic stimuli. Its activation leads to secretion of two potent inflammatory cytokines, IL-1β and IL-18. Their secretion pathways are complex and partially unknown, but have been well established to require two signals. Signal one leads to up-regulation of immature pro-cytokines and is mediated by Toll-like receptor (TLR) stimulation by pathogen-associated molecular patterns (PAMPs). Gram negative bacteria like *P. aeruginosa* can trigger signal one by stimulation of TLR4 and TLR5 via lipopolysaccharide (LPS) and flagella, respectively[10-12]. Signal two can be triggered by a

wide array of pathogenic stimuli that culminate in aggregation of inflammasome components (e.g. caspase-1 or NOD-like receptor proteins) and result in secretion of mature IL-1β and IL-18. *P. aeruginosa* can induce signal two via recognition of type III secretion proteins. Although a protective mechanism against many pathogens, overproduction of IL-1β has been associated with auto-inflammatory syndromes such as gout and periodic fever syndromes, such as Familial Mediterranean Fever and cryopyrin-associated periodic fever syndromes (CAPS)[13]. Therapies targeting IL-1β signaling have shown better outcomes in CAPS patients[14]. Moreover, asbestos and silica inhalation can cause IL-1β-dependent pulmonary fibrosis mediated by alveolar macrophage (AMs)[15]. In the lung, *P. aeruginosa* can induce IL-1β secretion by AMs[5]. Interestingly, depleting AMs prior to *P. aeruginosa* infection leads to significantly lower levels of IL-1β in the lung, improving survival[5]. Apart from IL-1β secretion, *P. aeruginosa* infection has also been shown to be regulated by prostaglandin E_2 (PGE_2)[6, 7, 16].

PGE_2 is a lipid mediator derived from arachidonic acid by the enzymatic activity of cyclooxygenase (COX) and PGE synthases that signals through 4 different G-protein-coupled plasma membrane receptors (GPCRs) of the E-prostanoid (EP) family termed EP1, EP2, EP3, and EP4. Each receptor activates different intracellular pathways. Stimulation of EP1 receptor increases intracellular calcium and activation of protein kinase C which stimulates the transcription factors, NFAT and NFκB [17]. EP2 and EP4 receptors are stimulators of adenylyl cyclase (AC) and phosphoinositide 3-kinase (PI3K) , respectively. AC mediates conversion of ATP to cyclic adenosine monophosphate (cAMP) leading to activation of protein kinase A (PKA) and the transcription factor CREB. The EP3 receptor is a regulator of the EP2-EP4 signaling pathway as its

activation leads to inhibition of AC. *P. aeruginosa* infection increases levels of inducible COX-2 leading to high levels of PGE_2 and inhibition of COX2, with subsequent diminished production of PGE_2, can lead to a better outcome in *P. aeruginosa*-infected mice [7]. Interestingly, HSCT patients possess higher levels of PGE_2 in blood and bronchoalveolar lavage (BAL) when compared to healthy non-transplanted individuals[18, 19].

PGE_2 is dysregulated in HSCT patients[18] and suppression of PGE_2 confers protection against *P. aeruginosa* infection in murine HSCT models [6]. However, other roles of PGE_2 in the context of HSCT and pulmonary *P. aeruginosa* infection remain elusive. To study bacterial lung infections post-HSCT, we use a mouse model of syngeneic bone marrow transplant (BMT). Total body irradiation is used as a conditioning regimen[6, 20-22]. Experiments are performed in the post-engraftment period, 5 weeks post-BMT when lung leukocytes are composed of 82% donor cells and splenic leukocytes are 95% donor cells[20]. We previously reported BMT mice are deficient in phagocytosis and killing of *P. aeruginosa*[6, 23], correlating with observations in humans[2]. In the present study, we compare the cytokine profile and lung tissue injury of control and BMT mice post *P. aeruginosa* infection. We also tested the direct relationship of PGE_2 with *P. aeruginosa*-induced IL-1β and examined the effect that PGE_2 stimulation had on autophagy, a main mechanism of *P. aeruginosa* clearance by AMs that has also been linked to inflammasome regulation[24, 25].

3.2 Results

Bone Marrow Transplant (BMT) mice are deficient in clearing *P. aeruginosa* infection and experience exacerbated lung tissue injury. BMT mice are deficient in clearing a *P. aeruginosa* PAO-1 infection compared to healthy control mice 24 hours post-infection (Fig.3-1A) confirming published data[6]. Deficiency in clearing bacteria is not due to low numbers of immune cells in the alveolar compartment as there are no differences in cell numbers or percentages of monocyte/macrophages and lymphocytes in the bronchoalveolar lavage (Fig.3-2A, B) between groups. We also noted higher levels of albumin in the bronchoalveolar lavage fluid (BALF) of BMT mice compared to control mice (Fig. 3-1b) suggesting more severe pulmonary injury (Fig.3-1c). Acute lung injury (ALI) is a leading cause of death in the intensive care unit, characterized by accumulation of leukocytes, protein leakage, and epithelial injury. ALI has been linked to high levels of IL-1β in the lung [26]. Thus, we measured levels of several pro-inflammatory cytokines. We found higher levels of IL-1β, but not other pro-inflammatory cytokines (IL-6, IL-12, TNF-α; data not shown) in BALF from infected BMT mice compared to control mice (Fig.3-3A). We tested levels of PGE_2 in BALF and detected higher levels of PGE_2 in BMT mice infected with PA01 compared to infected control mice. Moreover, we noticed mice expressing higher levels of PGE_2 have direct and significant correlations between levels of IL-1β and tissue injury, but not with other cytokines such as TNF-α (Fig.3-3C-F). Previous research has suggested a role for PGE_2 in IL-1β induction[27, 28]. Thus, exacerbated levels of IL-1β post-*P. aeruginosa* infection might mediate lung tissue injury and be dependent on PGE_2. Additionally, *P. aeruginosa*-mediated IL-1β release has

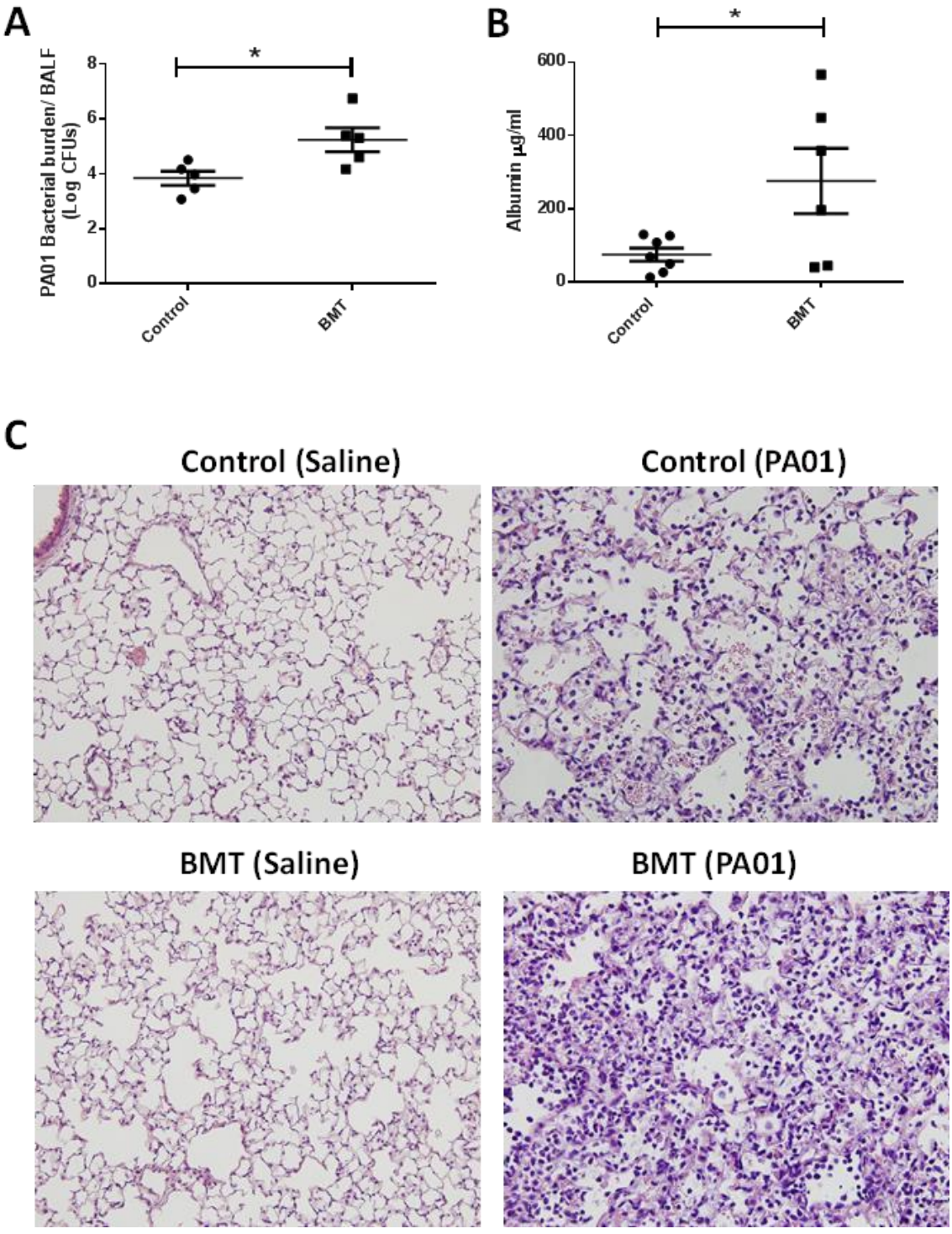

Figure 3-1: BMT Mice are Deficient in Clearing *P. aeruginosa* Infection and Experience Exacerbated Lung Tissue Injury. (a) Colony Forming Units (CFUs) were counted 24 hours after infecting non-transplanted control and BMT mice (C57BL/6J→C57BL/6J) with $5x10^5$ CFUs of *Pseudomonas aeruginosa* (PA01). (n=5 control; n=5 BMT). (b) Albumin measurements from bronchoalveolar lavage fluid (BALF) from PA01 infected control and BMT mice. (n=7 control; n=6 BMT) (c) Hematoxylin and Eosin stain (H&E) of lungs from saline or PA01 infected control and BMT mice; images taken at 40x magnification (representative of n=3 control; n=3 BMT). Statistics are student T test between comparative groups. *P<0.05. Data is representative of at least two independent experiments.

been shown to be dependent on alveolar macrophages (AMs) [5, 26]. Thus, we directed our attention to AMs in BMT mice.

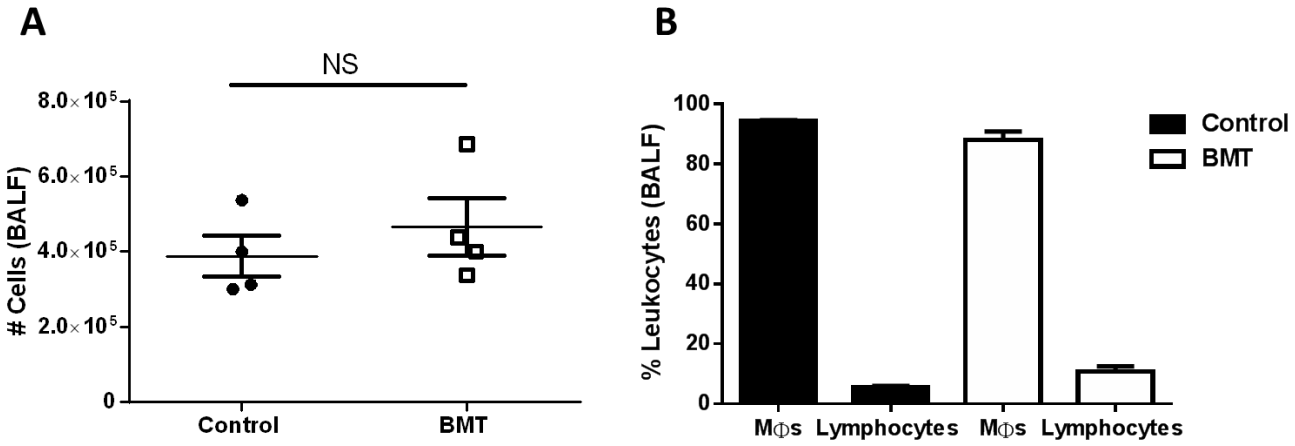

Figure 3-2: Leukocytes in BALF Post-BMT. (a) Absolute number of leukocytes in BALF, quantified with a hemocytometer, (n=3). (b) Percentage of leukocytes from BALF, taken via differential staining, (n=4). Statistics done by student T test. Not significant (NS).

Pro-inflammatory AMs in BMT mice account for higher levels of IL-1β in response to *P.aeruginosa* infection. To determine sources of IL-1β and PGE_2 in the lung, we compared mRNA levels from AMs obtained by BALF to lung interstitium. Transcripts for the rate limiting enzymes in PGE_2 synthesis, cyclooxygenase (COX) 1 and 2, were higher in AMs from BMT mice compared to control (Fig.3-4A). This was unique to AMs, but not interstitial samples. In addition, AMs from BMT mice have higher levels of IL-1β transcripts compared to control cells (Fig.3-4A). Consistent with our past studies, overnight culture of AMs from BMT mice secreted higher levels of PGE_2 compared to cells from control mice (Fig.3-4B). To assess whether PGE_2 can have an effect on IL-1β secretion, we pre-treated AMs with or without PGE_2 prior to PA01 infection, *in vitro*. We detected higher levels of IL-1β in supernatants of AMs pretreated with PGE_2 and

infected with PAO1 compared to AMs infected with PA01 alone (Fig.3-4C). Moreover, to determine whether higher bacterial burden in BMT mice was responsible for higher levels of IL-1β, we induced acute lung injury with lipopolysaccharide instillation in control and BMT mice. We detected higher IL-1β, but not IL-6 or TNF-α in BALF from BMT mice compared to control mice post-LPS instillation (Fig.3-4 D-F).

PGE_2 induces higher levels of IL-1β upon pathogenic stimuli. To assess whether PGE_2 could induce IL-1β in macrophages other than AMs, we prepared bone marrow derived macrophages (BMDMs) from healthy mice and pretreated them with PGE_2 prior to PA01 infection, *in vitro*. Similar to AMs, we detected higher IL-1β, but not IL-10, in supernatants of PGE_2- stimulated cells compared to non-treated cells post-PA01 (Fig.3-5A-B). In addition, to detect whether PGE_2-mediated higher levels of IL-1β with different stimuli; we pretreated BMDMs with and without PGE_2 and stimulated cells with LPS, and/or heat-killed PA01. In all cases, PGE_2 pretreatment increased levels of IL-1β compared to non-treated cells (Fig.3-5C). A canonical method to achieve IL-1β secretion is to activate the NLRP3 inflammasome by stimulation of the pannexin-1 channel and the purinergic P2X7 by LPS priming and adenosine triphosphate (ATP) treatment[25, 28-30]. We also detected higher IL-1β in BMDMs pretreated with PGE_2 and treated with LPS and ATP when compared to treatment with LPS and ATP alone (Fig.3-5D). Furthermore, we noted higher levels of IL-1β in BMDMs pre-treated with PGE_2 and infected with methicillin-resistant *Staphylococcus aureus* (MRSA) or *Streptococcus pneumoniae* compared to infected BMDMs not treated with PGE_2(Fig.6B). MRSA and *S.pneumoniae* are also main pathogens that infect immunosuppressed individuals[6, 19, 31, 32].

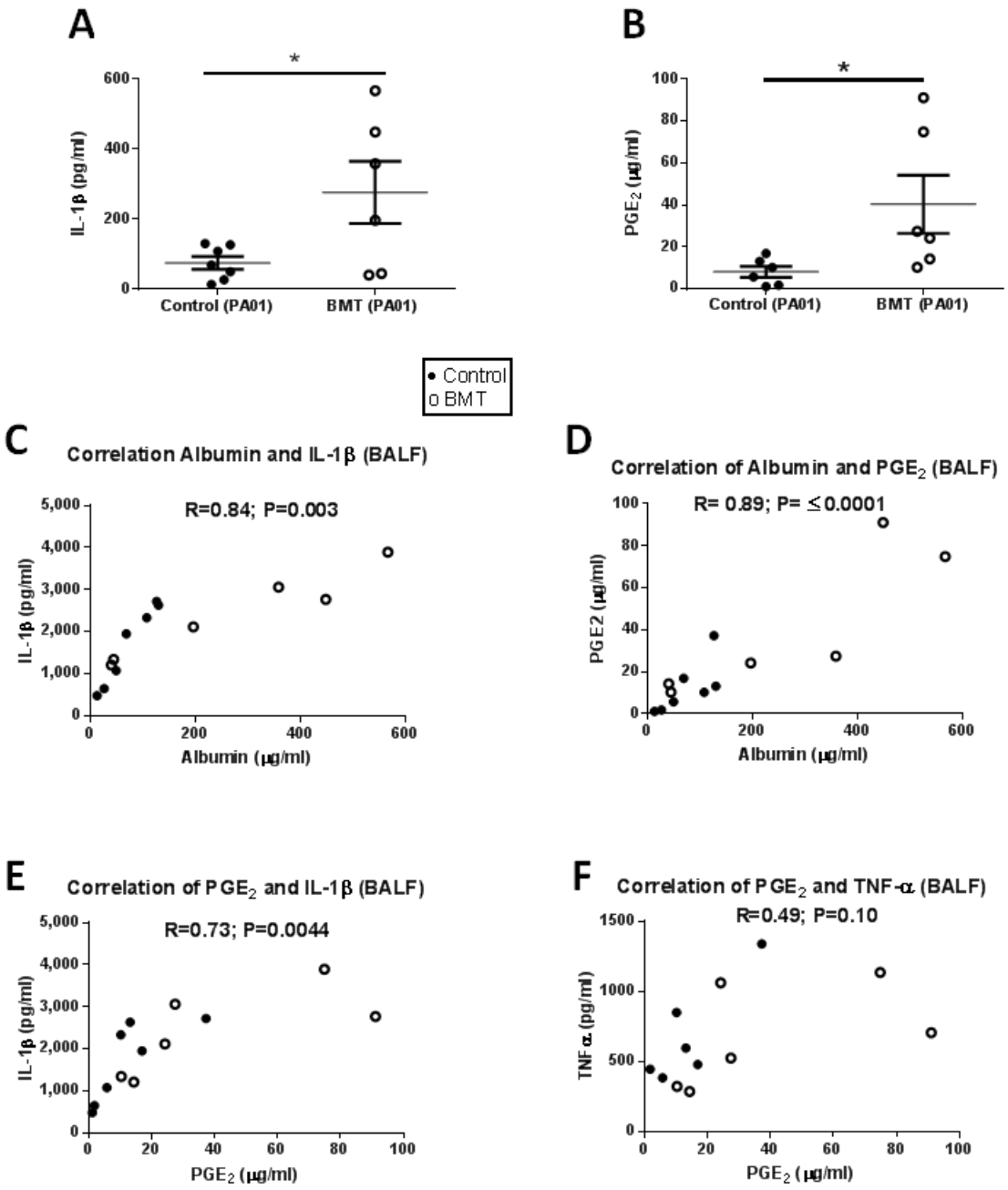

Figure 3-3: *P. aeruginosa* Infection Induces Higher Levels of IL-1β and PGE_2 post-BMT Correlating with Increased Lung Injury. (a) IL-1β and (b) PGE_2 measurements from the BALF of control and BMT mice 24 hours after infection with PA01; measurements done by ELISA. (n=7 control; n=6 BMT); (c) Correlation between albumin and IL-1β in BALF 24h post-PAO1; (d) Correlation between albumin and PGE_2 in BALF 24h post-PAO1; (e) Correlation between PGE_2 and IL-1β in BALF 24h post-PAO1 ; (f) Correlation between PGE_2 and TNF-α in BALF 24h post-PAO1. In all correlations, closed symbols represent control mice whereas open circles are BMT mice. R= Pearson correlation coefficient; *P<0.05.

PGE_2-mediated increase in IL-1β is dependent on activation of transcription factor CREB via increased levels of cAMP dependent on EP2 and EP4 signaling. We next

probed PGE_2 signaling pathways. We stimulated PGE_2 receptors using pharmacologic agonists for the EP2, EP3, and EP4 receptors[17]. A selective EP1 agonist is not available. We found pre-stimulating BMDMs with EP2 and EP4 agonists lead to higher levels of IL-1β post-*P.aeruginosa* infection (Fig.3-7A). EP3 stimulation did not lead to higher IL-1β (data not shown). EP2 and EP4 receptors activate adenylate cyclase (AC) and increase cyclic adenosine monophosphate (cAMP) from cytosolic ATP. We pretreated BMDMs with the AC stimulator forskolin prior to PA01 infection and observed higher levels of IL-1β in forskolin-pretreated BMDMs (Fig.3-7B). Moreover, as increased levels of cAMP lead to the activation of protein kinase A (PKA) and Epac (exchange protein directly activated by cAMP), we used agonists for the activation of these two proteins [33]. We found higher levels of IL-1β in supernatant of BMDMs pre-stimulated with PKA and Epac agonists prior to PA01 infection (Fig.3-7C). PKA and Epac activation lead to activation of the transcription factor CREB, thus, we used a CREB inhibitor during PGE_2 stimulation prior to PA01 infection. We decreased the levels of PGE_2 mediated-IL-1β using the CREB inhibitor (Fig.3-7D). Thus, PGE_2 increases IL-1β by activation of the transcription factor CREB via stimulation of EP2 and/or EP4 receptors.

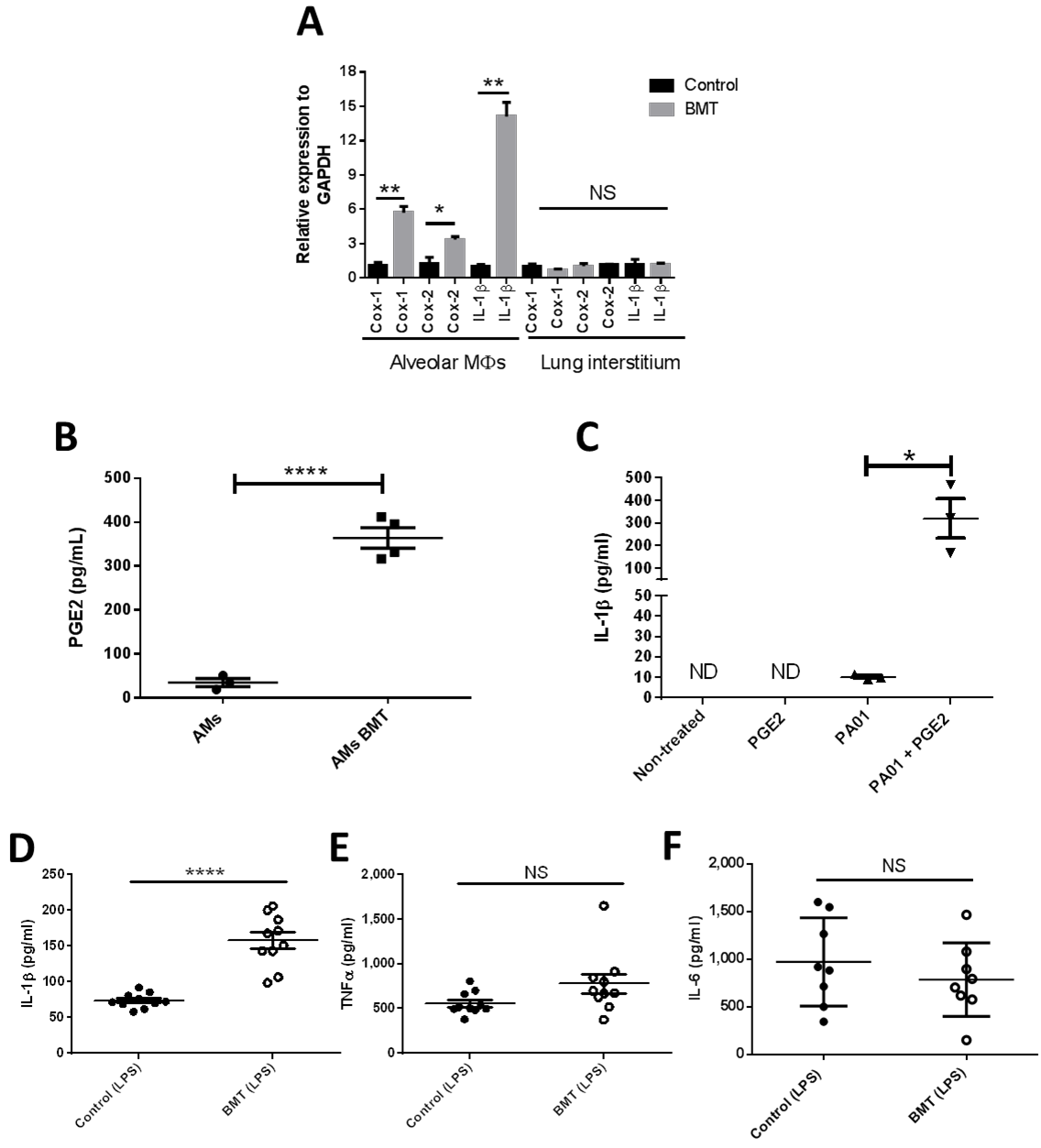

Figure 3-4: Alveolar Macrophages in BMT Mice Account for Higher IL-1β Release post-*P.aeruginosa* in Response to PGE_2. (a) RTqPCR measurement of relative gene expression of Cox-1, Cox-2, and IL-1β from AMs and whole lung cells from uninfected control and BMT mice normalized to GAPDH (n=3 control; n=3 BMT/group). (b) PGE_2 measurements by ELISA from overnight culture of untreated AMs from control and BMT mice (n=4 Control; n= 4 BMT). (c) IL-1β measurements by ELISA from AMs infected or not in vitro with PA01 (MOI:10), treated or not with 100nM of PGE_2 (n=3). (d) IL-1β, (e) TNF-α and (f) IL-6 measurements by ELISA from BALF of LPS (50ug)-treated control and BMT mice (n=10 control, n=10 BMT). Statistics are student T test between comparative groups. *P<0.05 ,**P<0.01, ***P<0.001, ****P<0.0001. Data is representative of at least two independent experiments.

PGE_2-dependent IL-1β release post-*P. aeruginosa* infection can be mediated by canonical or non-cannonical inflammasomes and is independent of autophagy inhibition. *Mycobacterium tuberculosis* and *Candida albicans* have been shown to induce IL-1β by a non-canonical inflammasome pathway dependent on caspase-8 in macrophages[34, 35]. Although a role of caspase-8-mediated IL-1β processing post-*P.aeruginosa* infection hasn't been proposed, research has shown that *P.aeruginosa*-infected caspase-1 deficient mice can secrete normal levels of IL-1β compared to control mice[36]. Thus, other proteases might play a role in processing pro-IL-1β post-*P.aeruginosa* infection. We stimulated macrophages with or without caspase-1 and caspase-8 inhibitors. Interestingly, in both cases, IL-1β secretion was inhibited with or without the effects of PGE_2 (Fig.3-8A-B). Stimulating macrophages with LPS and ATP in the presence or absence of caspase-1 and/or caspase-8 inhibitors confirmed the specificity of these inhibitors (Fig.3-9). AMs can clear *P.aeruginosa* infection by autophagy and IL-1β release can be negatively regulated by autophagy[24, 25]. Autophagy can be induced by serum starvation; thus, we tested effects of PGE_2 on autophagy-enhanced clearance of *P. aeruginosa* carried out under conditions of serum deprivation. Our results indicate PGE_2 can inhibit autophagy-dependent clearance in AMs (Fig.3-10). To detect whether there is a direct effect of PGE_2 on autophagy, we treated BMDMs with different concentrations of PGE_2 and detected levels of the autophagy-related proteins, LC3 and P62. We detected downregulation of LC3 and accumulation of P62 when we treated AMs with PGE_2. These changes are characteristic of autophagy inhibition (Fig.3-10B). In addition, we induced autophagy by serum starvation with or without PGE_2 stimulation and determined that PGE_2 inhibits levels of

the autophagy-related protein, ATG5, by western blot and RTqPCR (Fig.3-10C). Although we found PGE_2 inhibits autophagy, autophagy-deficient BMDMs were still able to upregulate IL-1β release when PGE_2 was present, suggesting PGE_2 inhibition of autophagy is not required for processing and upregulation of IL-1β (Fig.3-10D).

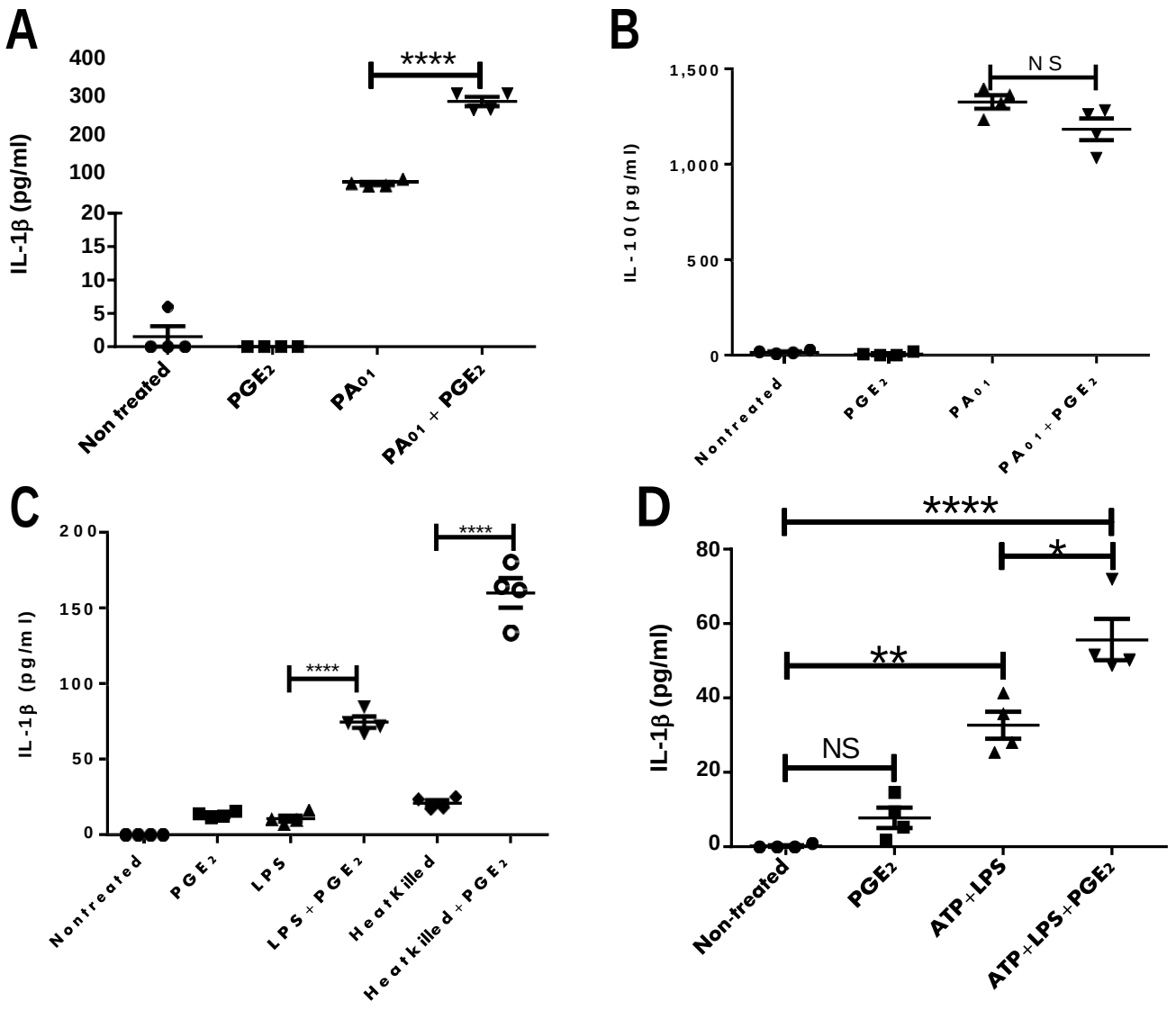

Figure 3-5: PGE_2 Increases IL-1β BMDMs Upon Pathogenic Stimulation. (a-b) IL-1β and IL-10 measurements from supernatants of PA01 infected (MOI:10) BMDMs treated or not with 100nM PGE_2. (c) IL-1β measurements from supernatant of BMDMs treated with lipopolysaccharide (LPS) derived from *P. aeruginosa* (500ng/ml), heat killed PA01 (MOI:10), and (c) ATP (1mM) with LPS with or without 100nM of PGE_2. Supernatant taken 2 hours post-stimulation. One-way ANOVA with Bonferroni's post-test. *P<0.05 ,**P<0.01, ***P<0.001, ****P<0.0001. Data is representative of three independent experiments.

PGE_2 elevated the levels of IL-1β transcripts by EP2 and EP4 stimulation in human and mouse cells. Considering that neither autophagy inhibition nor inflammasome activation explain why PGE_2 boosted IL-1β release; we next studied the transcriptional effects of PGE_2 on IL-1β. We treated BMDMs with PGE_2 in a dose and time dependent manner and detected significant increases in IL-1β transcripts as soon as 2 hours post-PGE_2 stimulation (Fig.3-11A-B). Furthermore, we confirmed elevated levels of IL-1β transcripts by PGE_2 stimulation are dependent on increasing levels of cAMP by stimulation of EP2 and/or EP4 receptors but not by EP3 stimulation (Fig.3-11C). Moreover, we confirmed our findings in human peripheral monocytes and human AMs (Fig.3-12).

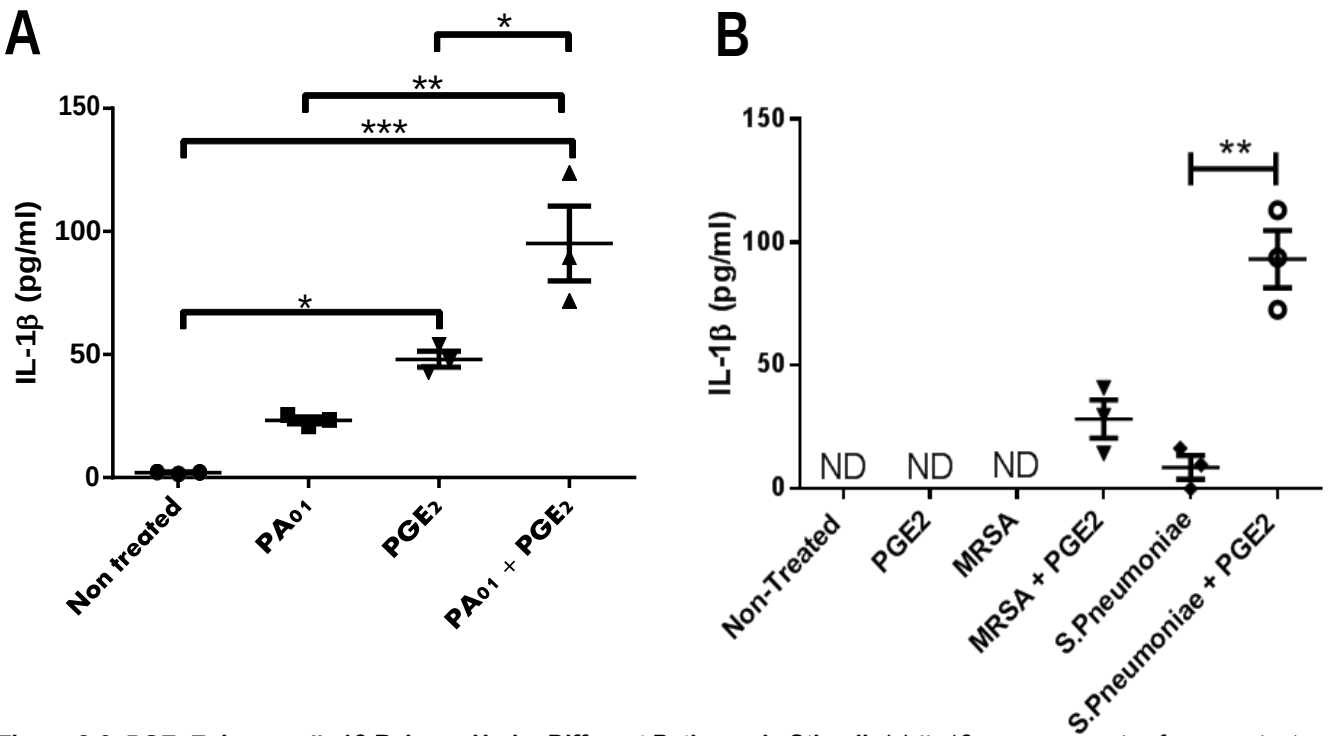

Figure 3-6: PGE_2 Enhances IL-1β Release Under Different Pathogenic Stimuli. (a) IL-1β measurements of supernatant from BMDMs treated or not with PGE_2 (100nM) and infected or not with PA01 (MOI:10) for 30 minutes after which supernatant was replaced with complete media containing 1% gentamycin. (b) IL-1β measurements of supernatant after MRSA (MOI:10) or *S. pneumoniae* (MOI:10) infection with or without PGE_2 (100nM). Samples were taken two hours after infection. Statistics used one-way ANOVA with Bonferroni's post-test. *P<0.05 ,**P<0.01, ***P<0.001. Data is representative of three independent experiments

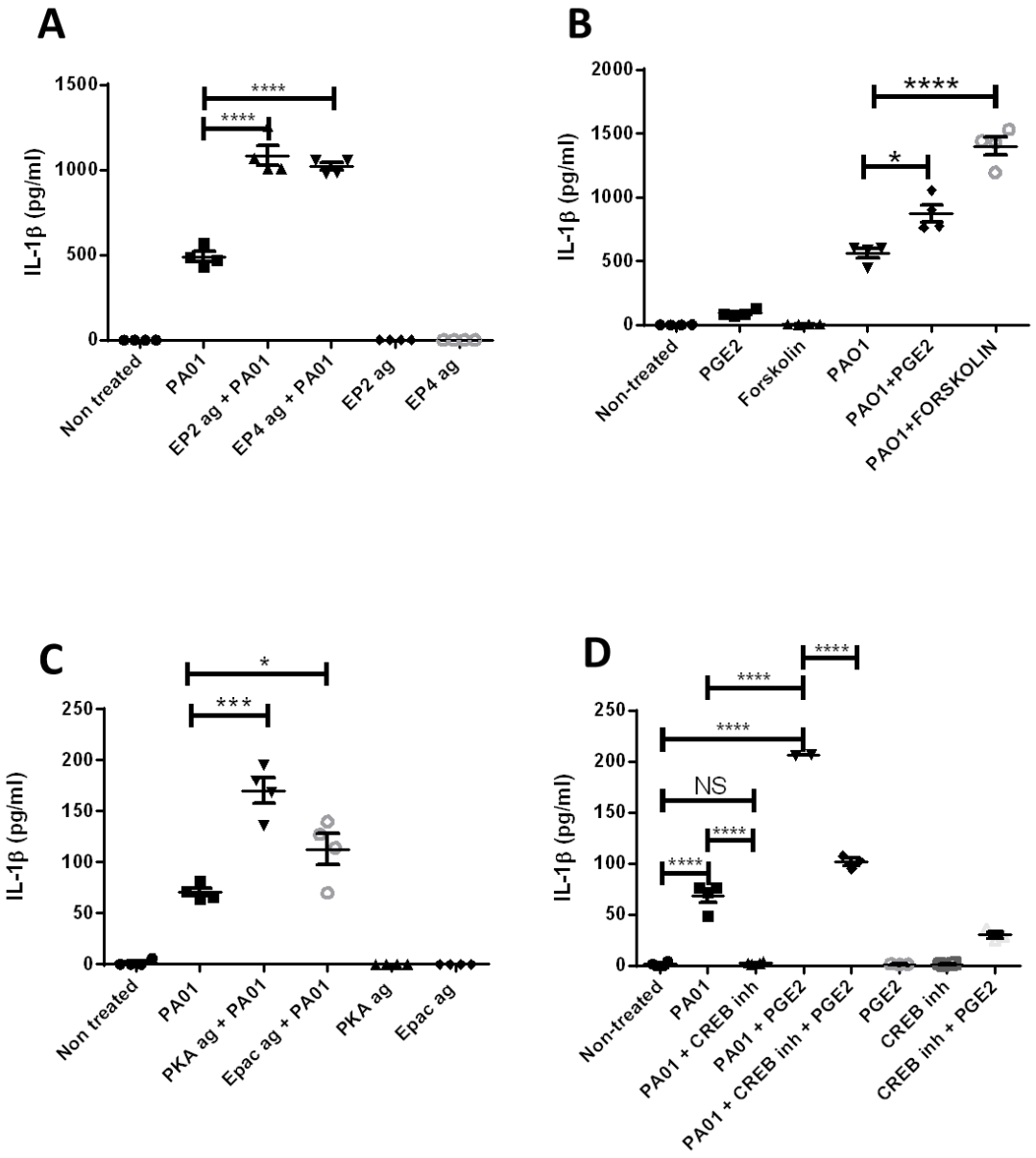

Figure 3-7: PGE_2 Mediated Increase in IL-1β is Dependent on Activation of Transcription Factor CREB by Increasing Levels of cAMP Dependent on EP2 and EP4 Signaling. IL-1β measurements from supernatant of BMDMs treated or not with PAO1 (MOI:10) with or without (a) EP2 agonist (1μM, Butaprost), EP4 agonist (500nM, ONO-AE1-329) (b) forskolin (25μM), PGE_2 (100nM), (c) PKA agonist (50μM, 6-BNZ-cAMP), Epac agonist (50μM, 8-pcpt-2'-OM-cAMP). (d) IL-1β protein measurement from supernatants of BMDMs treated or not with 100nM of PGE_2, CREB inhibitor (100μM, Naphthol AS-E phosphate) and infected or not with PA01 (MOI:10). In all cases, supernatant taken 2 hours after infection. One-way ANOVA with Bonferroni's post-test. *P<0.05 ,**P<0.01, ***P<0.001, ****P<0.0001. Data is representative of two independent experiments.

Decreasing levels of PGE_2 reduces *P. aeruginosa*-mediated lung tissue injury.

Increased levels of IL-1β post-*P.aeruginosa* infection aggravate lung tissue injury[4, 5]. Therapeutic strategies using caspase-1 inhibitors reduced severity of IL-1β pulmonary injury [26]. However, other research has shown that inhibiting IL-1β signaling has no impact on bacterial burden or immune cell recruitment, with minimal effects on lung injury[36]. As we have shown that PGE_2 elevation in BMT mice aggravates lung injury, we tested the effect that PGE_2 inhibition had on lung injury outcomes. We instilled *P. aeruginosa* to control and BMT mice with or without administration of the COX inhibitor, indomethacin. We noted reduced IL-1β in the BALF as well as lower levels of protein leakage, suggesting inhibition of PGE_2 can decrease levels of ALI post-*P.aeruginosa* infection (Fig.3-13).

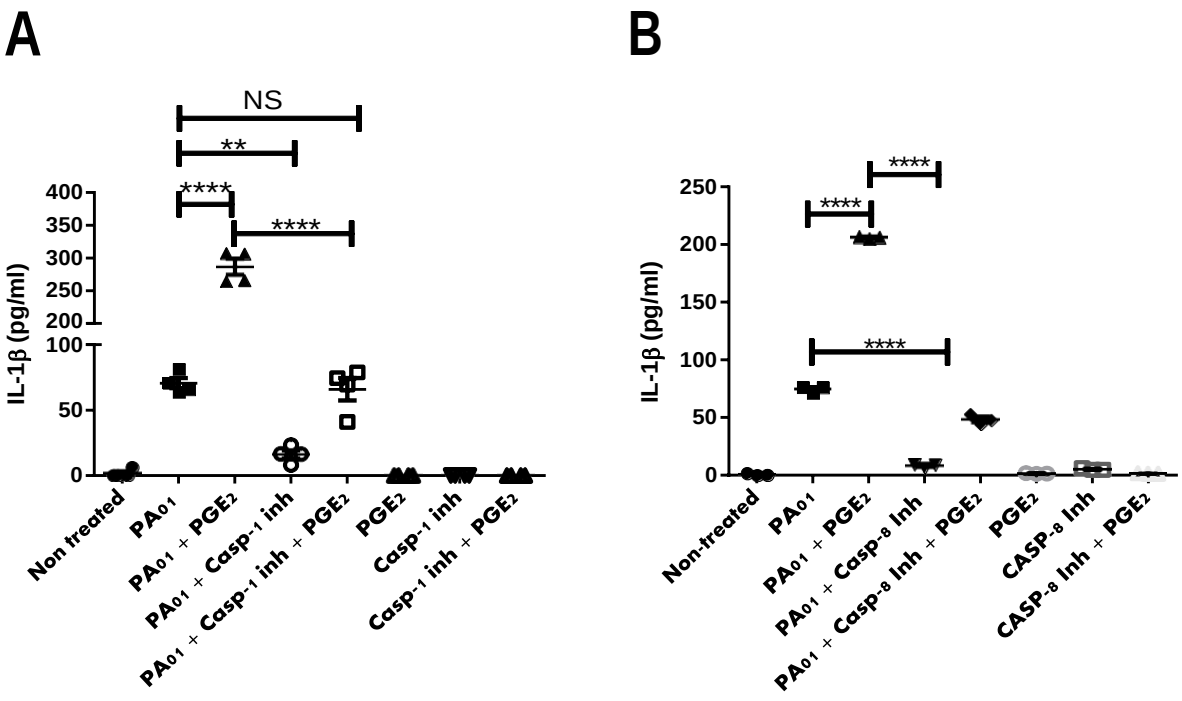

Figure 3-8: PAO1 can use cannonical or non-cannonical inflammasomes to make IL-1β. IL-1β measurements from supernatant of BMDMs treated or not with (a) Caspase 8 inhibitor (10nM) or Caspase 1 inhibitor (10nM) with or without PGE_2 (100nM). Supernatant taken 2 hours after infection. One-way ANOVA with Bonferroni's post-test. *P<0.05 ,**P<0.01, ***P<0.001, ****P<0.0001. Data is representative of two independent experiments

3.3 Discussion

In February 2017, the World Health Organization (WHO) published a report containing a list of 12 bacterial pathogens for which new therapeutic strategies are urgently needed. *Pseudomonas aeruginosa* was considered to be of critical importance. *P. aeruginosa* is an opportunistic pathogen which causes minimal pathogenicity in healthy individuals, but major mortality and morbidity in HSCT patients [2, 6, 19, 31, 37, 38]. Susceptibility to this pathogen has been reported to occur even after immune reconstitution following HSCT[2, 6, 31]. HSCT patients have high levels of PGE_2 in the blood and BALF when compared to healthy individuals[18-20]. We have previously reported that elevated PGE_2 is causally related to the impaired ability of AMs from BMT mice to phagocytize and kill bacteria [20, 23, 39]. The purpose of the current study was to determine whether PGE_2 production post-BMT was responsible for enhanced lung injury post-infection as well, and if so, by what mechanism. We now know that inflammasome-dependent IL-1β secretion is induced by *P. aeruginosa*, and better health outcomes have been reported by inhibiting IL-1β signaling[4, 5, 12, 26, 36]. In addition, recent articles have linked PGE_2 signaling with IL-1β regulation[27, 28, 40]. However, reports have shown contradictory effects of PGE_2 on IL-1β release[27, 28]. Thus, in this study we focused on the PGE_2-mediated effects on IL-1β response to *P. aeruginosa* infection by AMs from BMT mice in our quest to determine new therapeutic strategies.

A dose of 2 x 10^6 CFU *P. aeruginosa* instilled into un-transplanted mice causes a moderate infection [41]. Yet, BMT mice have difficulty clearing this bacterial dose and 100% of them die within 48 hours[23]. Interestingly, the susceptibility to *P. aeruginosa* in BMT mice is seen even after immune reconstitution[6, 23, 42]. Here, we show evidence that

BMT mice have severe vascular and epithelial leakage indicating a more severe ALI post-*P.aeruginosa* infection. ALI can be caused by exacerbated levels of pro-inflammatory cytokines. Thus, we searched for upregulated levels of different pro-inflammatory cytokines (IL-12, IL-6, TNF-α, and IL-1β) and found that only IL-1β was significantly higher in BMT mice when compared to un-transplanted mice post-infection. In addition, we noticed a direct correlation between IL-1β, PGE_2, and albumin levels in the BALF, but not a correlation with other pro-inflammatory cytokines such as TNF-α.

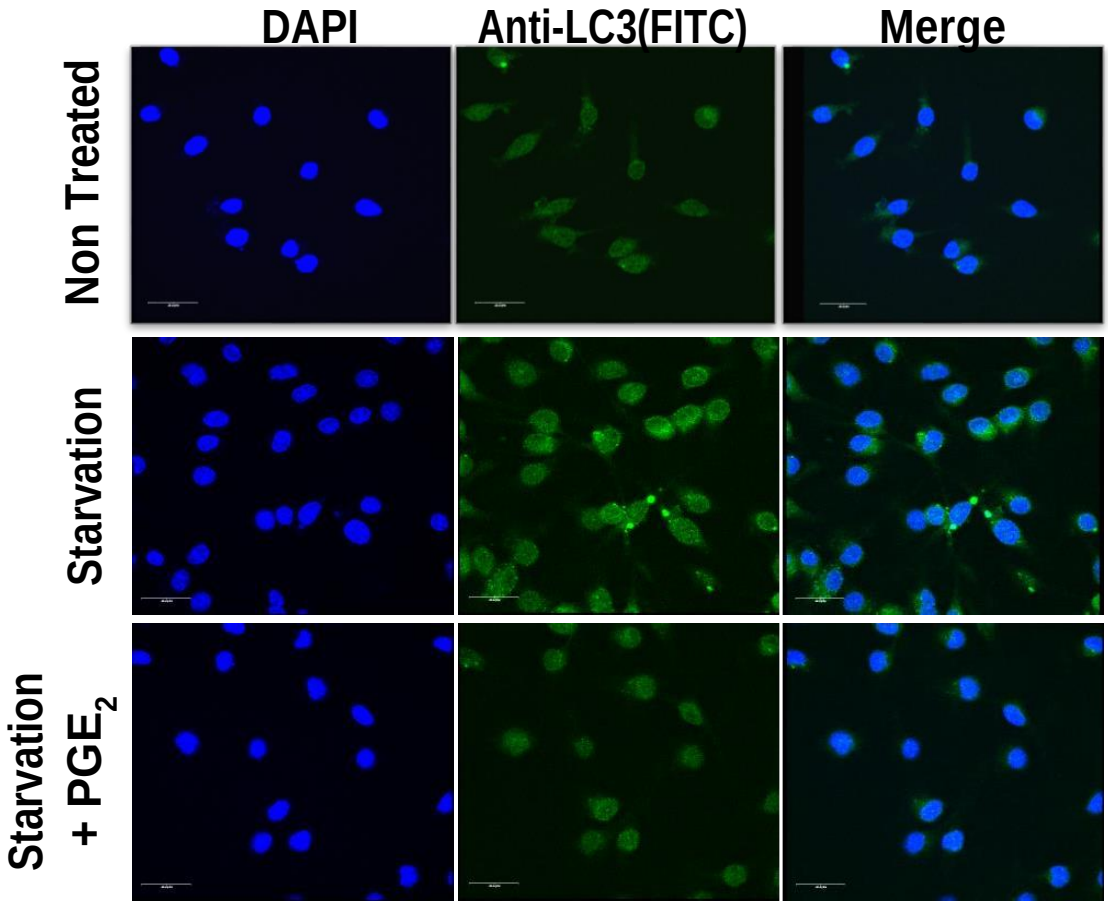

Figure 3-9: PGE_2 Limits Autophagosome Formation in Response to Serum Starvation. BMDMs were serum-starved for 1 h in the presence or absence of 100nM PGE_2. Cells were fixed and stained with FITC-labeled LC3 or DAPI to visualize autophagosomes and nuclei and immunofluorescence images were captured singly or were merged. Panels shown are representative of 2 different experiments.

Because we observed exacerbated levels of IL-1β post-*P.aeruginosa* infection in BMT mice, we analyzed the capability of AMs from BMT mice to secrete IL-1β. Interestingly,

AMs from BMT mice have higher levels of IL-1β transcripts compared to control mice, correlating with higher levels of cyclooxygenase 1 and 2 transcripts. Furthermore, stimulating AMs with PGE_2 prior to infection with *P. aeruginosa* also leads to higher levels of IL-1β compared to untreated AMs. These findings show that higher levels of PGE_2 in AMs from BMT mice might influence the higher levels of IL-1β post-*P.aeruginosa*.

As higher amounts of bacterial burden in BMT mice might influence the levels of IL-1β, we adopted a bacterial-free model of ALI dependent on lipopolysaccharide instillation [43]. We were able to get exacerbated levels of IL-1β, but not IL-6 and TNF-α, in BMT mice compared to un-transplanted mice. In addition, in an *in-vitro* model where we controlled the stimulants, we detected that PGE_2 could increase secretion of IL-1β in bone marrow derived macrophages by a wide array of pathogenic stimuli such as LPS, live and heat killed *P. aeruginosa*, MRSA infection, and *Streptococcus pneumoniae* infection. All stimuli induce increased IL-1β after previous exposure to PGE_2 in macrophages. Thus, PGE_2 signaling acts as signal 1 for IL-1β secretion. Interestingly, dual stimulation of macrophages with PGE_2 and another signal-1 stimulant, LPS, can lead to increased IL-1β secretion. This phenomenon is likely explained by the moderate activation of caspase-1 (signal 2) in macrophages by LPS stimulation as previously noted [44] .

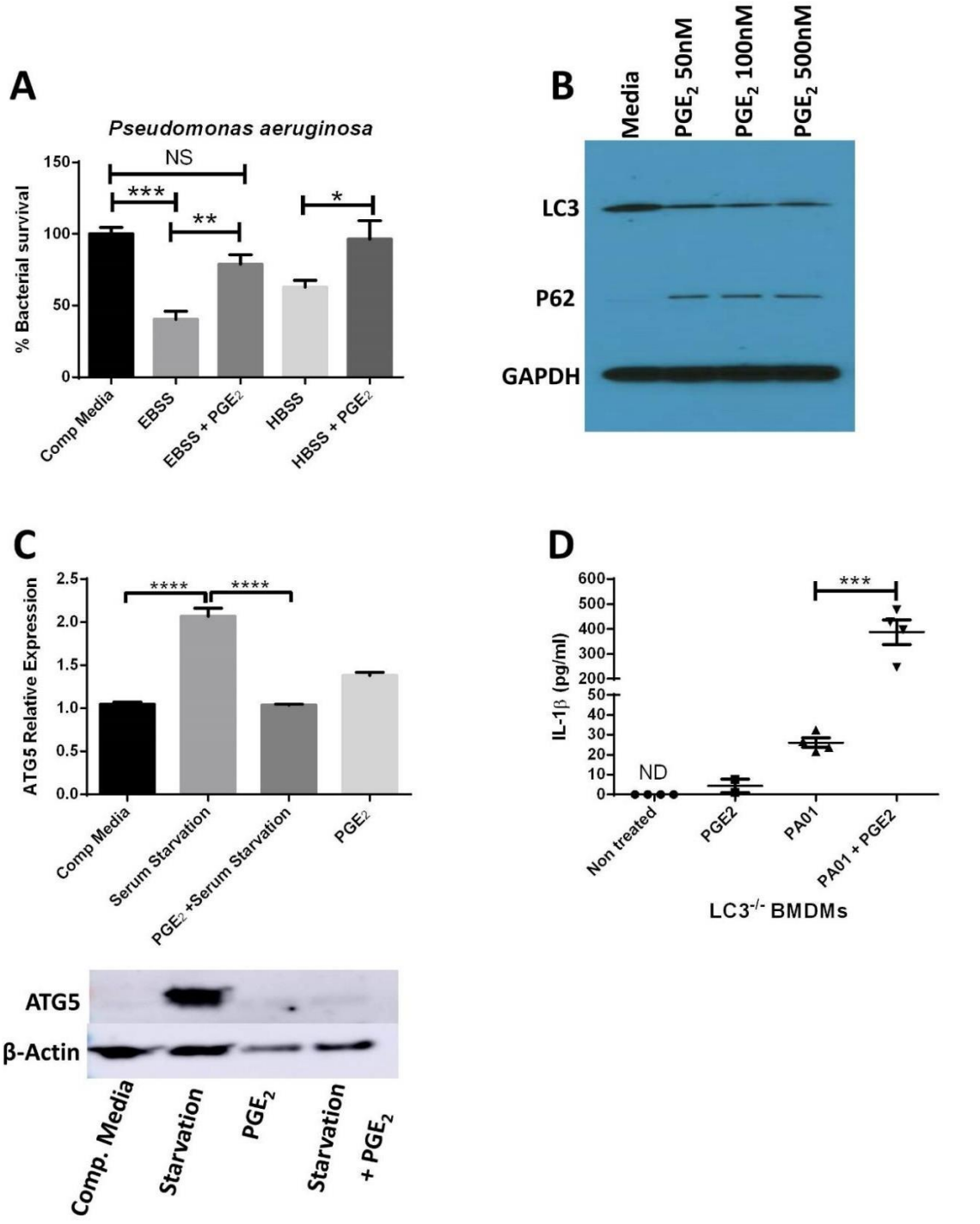

Figure 3-10. Inhibition of Autophagy Impacts Bacterial Killing but Not IL-1β Release. (a) AMs were cultured in complete media or were nutrient starved (EBSS or HBSS) in the presence or absence of PGE_2 (100nM) for 2 hours. (b) AMs were collected from control mice and stimulated for 1 hour in the presence of serum-free media with or without PGE_2 (50-500 nM) for 1h. LC3 and p62 was analyzed by Western blot (WB). (c) AMs were cultured in complete media or were serum-starved for 1 h with or without 100nM PGE_2 before RNA was prepared and analyzed for expression of ATG5 relative to β-actin by qRT-PCR; (bottom) ATG5 was analyzed by WB.(d) BMDMs from $LC3^{-/-}$ mice were stimulated with 100nM PGE_2, PAO1 (MOI:10) or the combination for 2 hours before supernatants were collected and measured for IL-1β by ELISA (n=4); Statistics were measured by one-way ANOVA with Bonferroni post-hoc test. *P<0.05 ,**P<0.01, ***P<0.001, ****P<0.0001.

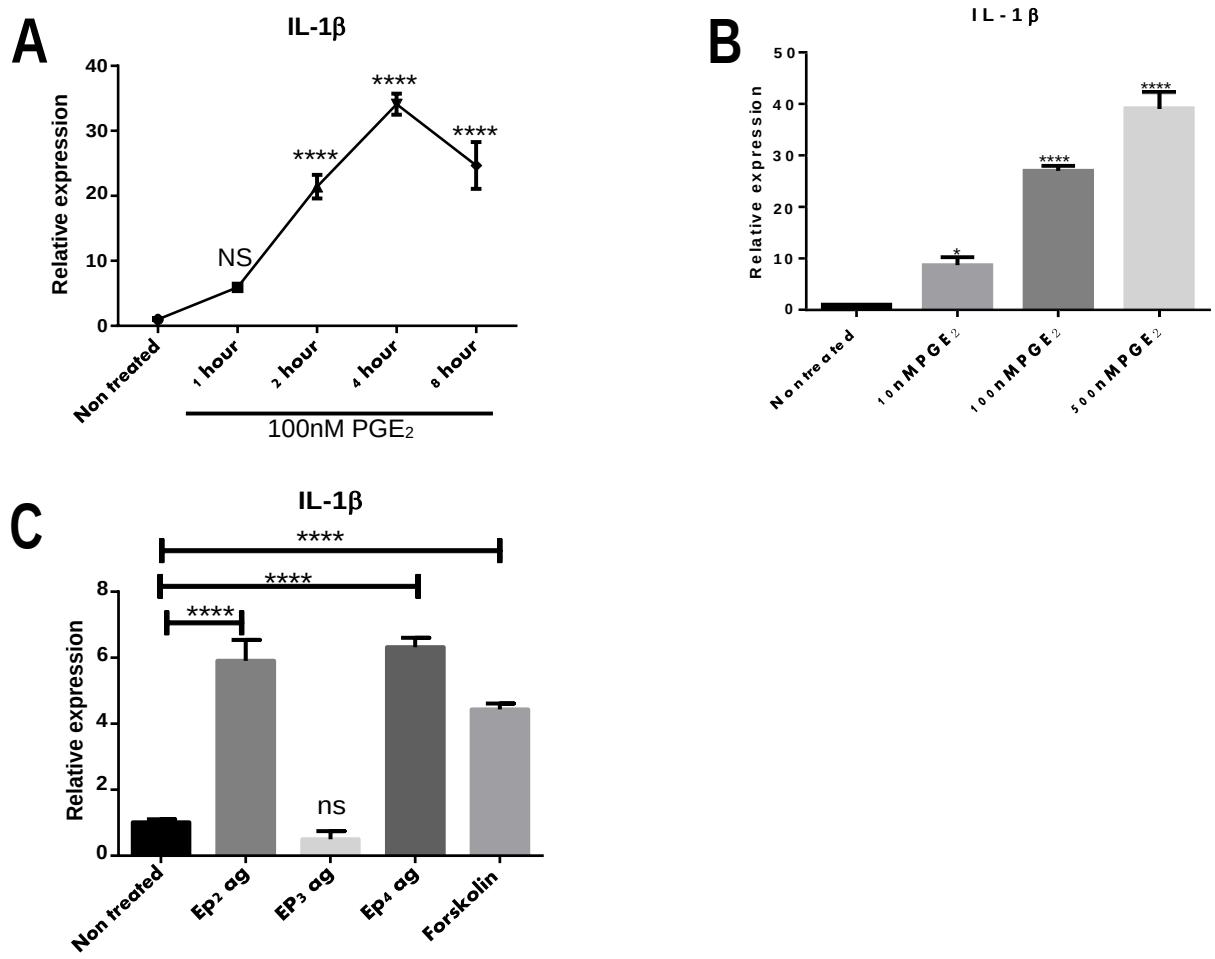

Figure 3-11: PGE_2 Elevates the Levels of IL-1β Transcripts by EP2 and EP4 Stimulation. (a) BMDMs were treated or not with 100nM PGE_2 for 1, 2, 4, and 8 hours before RNA was prepared and analyzed for expression of the IL-1β gene. (b) BMDMs were treated or not with 10nM, 100nM, and 500nM of PGE_2 during 4 hours before RNA was prepared and analyzed for expression of IL-1β. (c) BMDMs were treated with EP2 agonist (1μM, Butaprost), EP3 agonist (10nM, Sulprostone), EP4 agonist (500nM, ONO-AE1-329), and forskolin (25μM) before RNA was prepared and analyzed. All RNA data was normalized to expression levels of GAPDH. Data are representative of two independent experiments and statistics were measured by one-way ANOVA with Bonferroni post-hoc test. *P<0.05 ,**P<0.01, ***P<0.001, ****P<0.0001

Recent reports have shown an opposite effect of PGE_2 regulation on IL-1β[27] than our results. We compared their PGE_2 stimulation method, based on 5 minutes of stimulation by PGE_2, side by side with our stimulation method, based on 4 hours pre-stimulation with PGE_2. As expected, we were able to obtain exacerbated levels of IL-1β post-4 hours of PGE_2 stimulation whereas 5 minutes of stimulation had no effect on IL-1β regulation (Fig.3-14). Thus, these comparative results suggest that the kinetics of PGE_2

exposure is important. However, in the setting of HCST, it is important to remember that PGE_2 levels are chronically elevated[18, 19], and thus, in this setting PGE_2 is promoting IL-1β.

PGE_2 signaling is mediated by 4 members of the G-coupled protein receptor family termed EP1-EP4[17]. We determined that stimulating the EP2 and EP4 receptor in macrophages prior to *P. aeruginosa* infection leads to higher levels of IL-1β when compared to non-stimulated macrophages. Furthermore, as EP2/EP4 receptors share the ability to activate PKA and Epac, we activated PKA or Epac with the use of intracellular agonists prior to *P. aeruginosa* infection and detected higher levels of IL-1β when compared to non-stimulated macrophages. We were able to abolish IL-1β secretion in *P. aeruginosa*-infected mice by stimulating macrophages with an inhibitor for the CREB transcription factor. These data indicate that the PGE_2-mediated increase in IL-1β is due to CREB transcription factor activation by EP2/EP4-mediated stimulation of PKA and/or Epac. Although we present evidence that CREB is mediating PGE_2-dependent elevation of IL-1β, we do not discard the possibility that other transcription factors such as NF-κB [28] may also play a role. Furthermore, while both EP2 and EP4 can mediate the transcriptional effect of PGE_2 in macrophages from control mice, in the

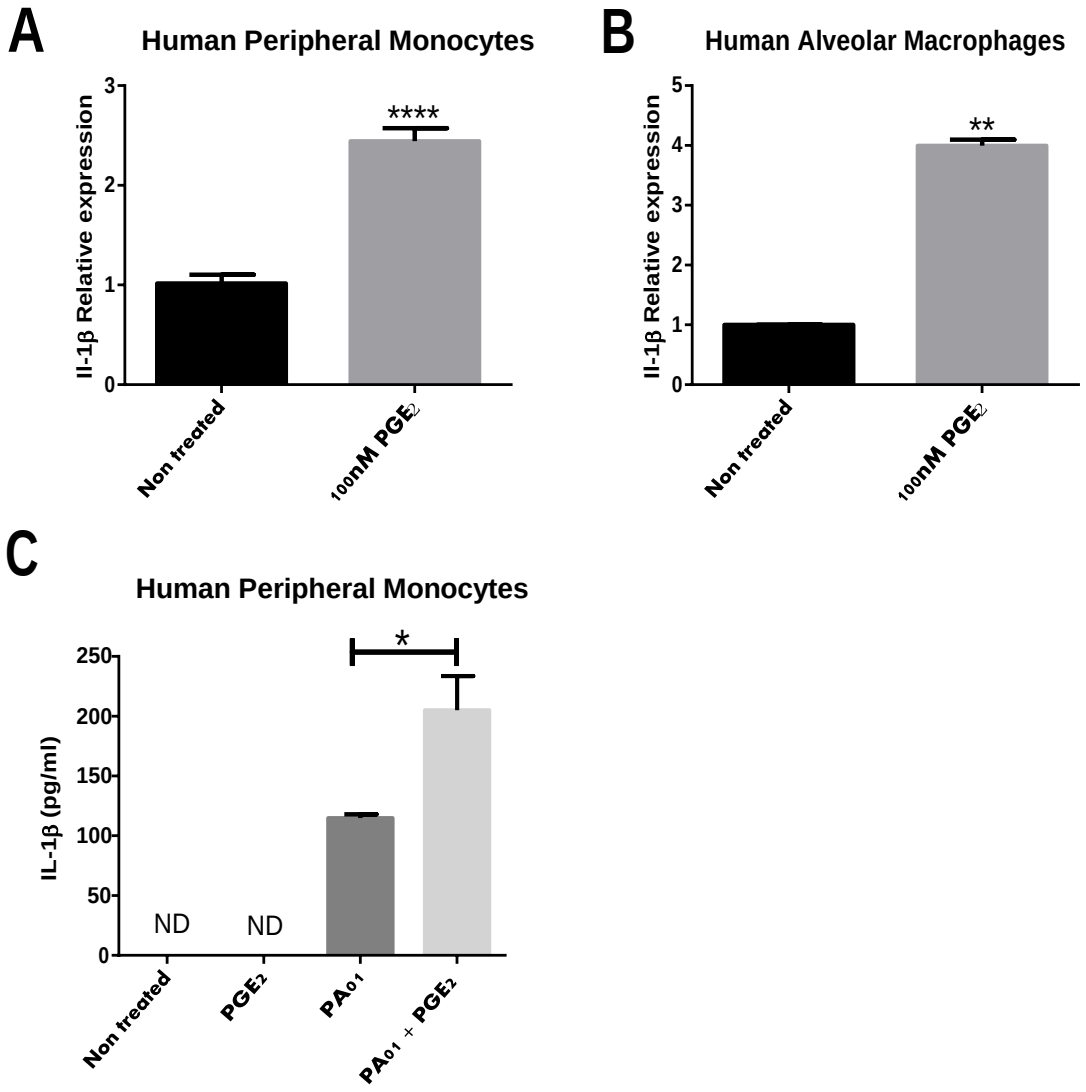

Figure 3-12: Human AMs and Peripheral Monocytes Upregulate IL-1β under PGE_2 stimulation. IL-1β relative expression from 100nM PGE_2 stimulated (a) Human peripheral monocytes and (b) human AMs normalized to GAPDH. (c) IL-1β protein measurements from supernatant of PGE_2-treated or not peripheral macrophages during or not PA01 infection. Statistics are student T test between comparative groups. *P<0.05,**P<0.01, ***P<0.001, ****P<0.0001.

setting of BMT, we have previously shown that levels of EP2 are upregulated, while EP4 is slightly downregulated on alveolar macrophages and an EP2 antagonist, AH6809 was able to mimic effects of indomethacin on alveolar macrophage phagocytosis[39].

Researchers have established autophagy as a main mechanism of *P. aeruginosa* clearance [24] as well as IL-1β regulation[10, 25]. We tested the effects of PGE_2 in the regulation of autophagy and determined that PGE_2 can inhibit autophagy-induced clearance of *P. aeruginosa*. Therefore, these data suggested that PGE_2-mediated IL-1β increase might be due to autophagy inhibition. However, we detected high levels of IL-1β protein in autophagy-deficient macrophages after PGE_2 administration and infection. Thus, the mechanism of PGE_2-mediated increase in IL-1β production cannot be attributed to impaired clearance of inflammasome components as a result of defective autophagy [25]. While we previously demonstrated that PGE_2 was associated with impaired autophagy in neutrophils[19], this is the first description of its ability to limit autophagy in macrophages. Thus, PGE_2 may be a common negative regulator of autophagic flux in other cell types as well. Future work will be needed to understand what impact this negative regulation may have on processes such as epithelial repair. In macrophages, IL-1β secretion can be mediated by the cysteine proteases, caspase-1 and caspase-8[35]. Thus, we tested the role of these two caspases during *P. aeruginosa* infection. We determined that we could abolish the secretion of IL-1β prior to *P. aeruginosa* infection with the use of inhibitors to caspase-1 or caspase-8. To our knowledge, these are the first data to link IL-1β secretion post-*P. aeruginosa* to caspase-8; this pathway has been previously established for *Mycobacterium*

tuberculosis and fungal infection [34].These results suggested that *P. aeruginosa* infection can induce the activation of IL-1β release using either caspase-1 and/or caspase-8. If true, this suggests that *P. aeruginosa* may be able to stimulate non-cannonical

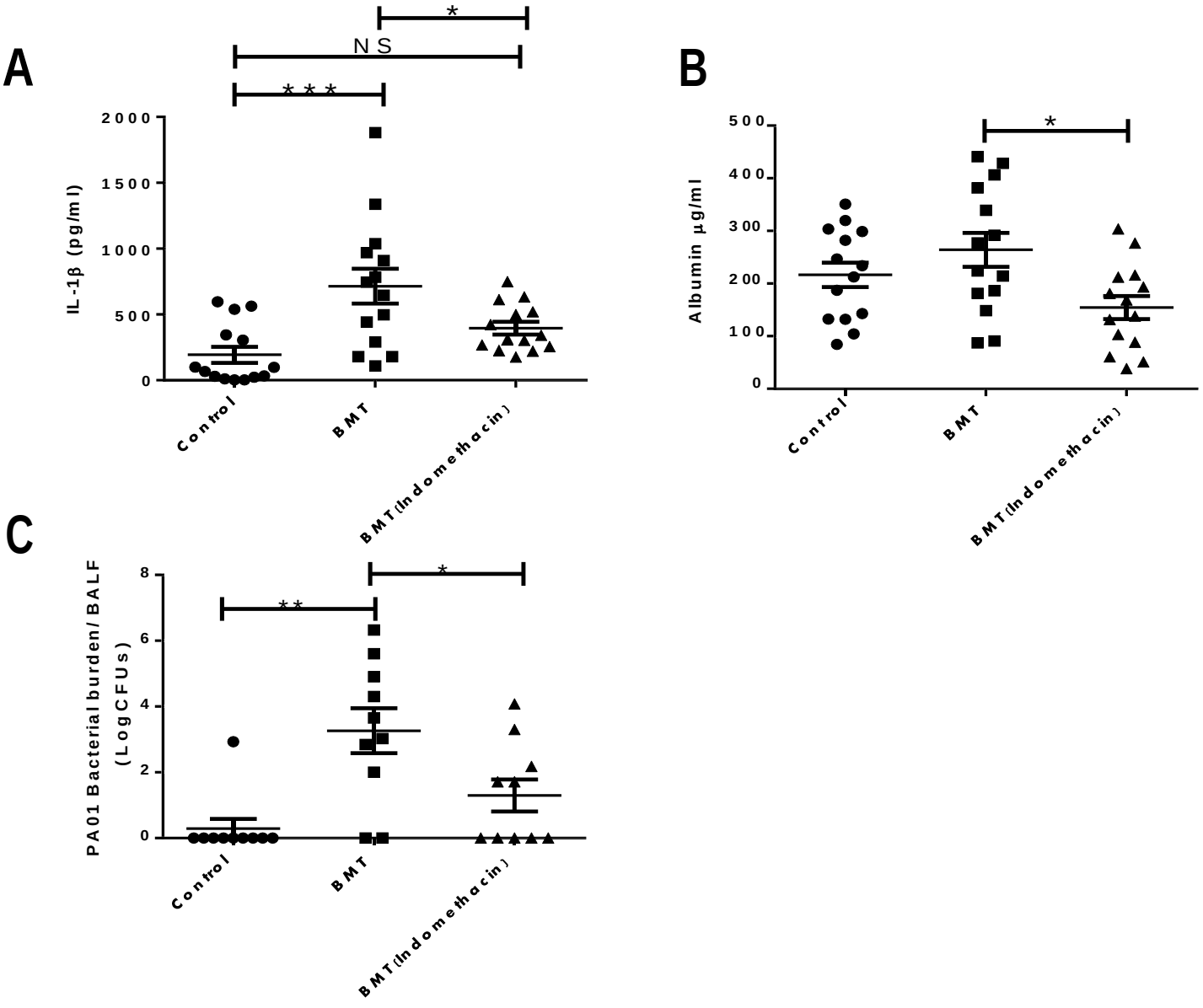

Figure 3-13 Decreasing Levels of PGE_2 by Indomethacin Treatment Leads to Decreased IL-1β in the Lung after *P. aeruginosa* Infection in BMT mice. (a) IL-1β and (b) albumin measurements from the BALF of control, and BMT mice treated or not with Indomethacin (1.2mg/kg) for 24 hours after PA01 infection; measurements done by ELISA. (n=14 control; n=14 BMT; n=14 BMT treated with Indomethacin); (C) PA01 CFU measurement in BAL from infected mice, 24 hours. (n=10 control; n=10 BMT; n=10 BMT treated with Indomethacin). Statistics were measured by one-way ANOVA with Bonferroni post-hoc test. *P<0.05 ,**P<0.01, ***P<0.001, ****P<0.0001; non-significant (ns).

inflammasome activation, possibly via cross-reactivity caused by recognition of *P. aeruginosa* by dectin receptors [45] which are known to be linked to caspase 8 activation[34] .

Our data suggest that PGE_2 can influence a pro-inflammatory environment by exacerbating levels of IL-1β, in macrophages. We detected that PGE_2 can mediate a massive increase in IL-1β transcripts. We detected increased IL-1β transcripts in macrophages within an hour post-stimulation with PGE_2. Increases in IL-1β transcripts were dependent on stimulation of EP2 and EP4 receptors but not by EP3. While we did not have access to an EP1-selective agonist, we wouldn't anticipate this receptor to regulate cAMP levels[17]. Additionally, we were able to detect an increase in IL-1β transcripts following activation of cAMP with forskolin. Moreover, PGE_2 stimulation increased transcription of IL-1β in human alveolar macrophages and human monocyte-derived macrophages. Altogether, we conclude that PGE_2 can strongly prime macrophages for IL-1β, but not other cytokines, and upon pathogenic stimulation will lead to exacerbated levels of IL-1β causing IL-1β-mediated injury. Thus, we expected to decrease the IL-1β-mediated lung injury post-BMT with the use of COX inhibitors. When we tested the effects of PGE_2 inhibition in BMT mice, we were able decrease bacterial load as previously reported[7], but also reduce protein leakage and IL-1β in the lung. When our results are taken together, we identified new mechanisms by which *P. aeruginosa* causes life threatening effects in HSCT patients. These findings can help in the development of new therapeutic strategies that can improve outcome of HSCT patients with pulmonary complications due to *P. aeruginosa*, and possibly other

pathogens. We speculate that COX inhibitors or possibly Anakinra may offer therapeutic benefit at limiting lung injury caused by bacterial infection post-HSCT.

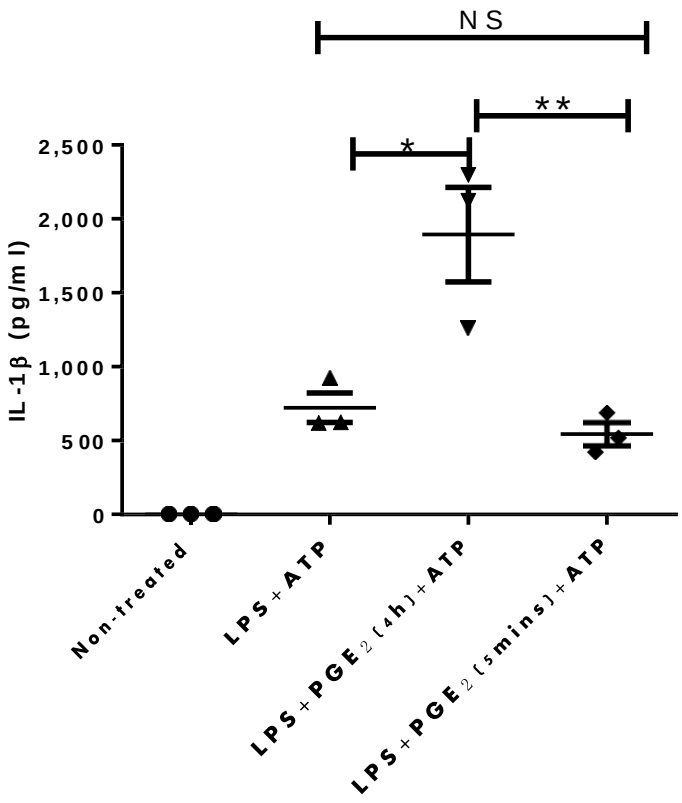

Figure 3-14 PGE_2 Enhances IL-1β Release after 4h, but not 5' Stimulation. IL-1β measurements by ELISA of supernatant samples from BMDMs treated for 3.5 hours or 5 minutes with LPS (100ng/ml) and/or PGE_2 (500nM) followed by 30 minutes of ATP (5mM). Statistics used one-way ANOVA with Bonferroni's post-test. *P<0.05,**P<0.01, ***P<0.001.

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Chapter 4.

Prostaglandin E_2 inhibits neutrophil extracellular trap formation post-stem cell transplant

4.1 Background

Approximately 50,000 hematopoietic stem cell transplants (HSCTs) are performed annually (57% autologous; 43% allogeneic) [1]. HSCT patients exhibit susceptibility to pulmonary infections even late post-engraftment [2-4]. Neutrophil recruitment and function is important for innate immunity. In response to inhaled pathogens, neutrophils are recruited by chemotactic signals released from activated alveolar macrophages and epithelial cells [5]. Chronic granulomatous disease (CGD) patients with mutations in the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex exhibit functional defects in neutrophils [e.g. reduced formation of neutrophil extracellular traps (NETs)] that render them more susceptible to pathogens also afflicting HSCT patients, such as *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, and *Aspergillus species* [6, 7]. Interestingly, CGD patients receiving gene therapy complementing NADPH oxidase function restore anti-*Aspergillus* responses via restored NETs [7, 8].

Long term defects in neutrophil functions have previously been noted in HSCT patients [6]. Neutrophils from autologous HSCT patients exhibit a diminished capacity to produce respiratory burst [6, 9, 10] while allogeneic HSCT patients exhibit defects in neutrophil chemotaxis in addition to impaired respiratory burst [6, 11, 12]. However, the cause for

neutrophil dysfunction has remained unclear. Furthermore, the ability of neutrophils from HSCT patients to undergo NETosis is unknown.

NETosis is a cell death pathway characterized by release of extracellular web-like structures composed of chromatin, histones, and granular proteins [13-15]. NETs serve as antimicrobial defenses against extracellular pathogens including bacteria [16]. Takei *et al*. described this as a novel form of cell death, distinct from apoptosis and necrosis, due to its dependence on chromatin decondensation, increase in membrane permeability and its independence from necrosis-inducing or apoptosis-inducing stimuli [17, 18]. Studies have shown NETosis may be dependent on NADPH oxidase or myeloperoxidase-generated reactive oxygen species, autophagy, neutrophil elastase, and histone citrullination by peptidylarginine deiminase 4 [19-21]. Live cells can also participate in a process called “vital NETosis” where neutrophils maintain their membrane integrity while rapidly releasing NETS and continuing to chemotax and phagocytize bacteria [22, 23].

We previously demonstrated that host defense against both *Pseudomonas aeruginosa* and *Staphylococcus aureus* is impaired following BMT in mice [24-26]. As NETs can effectively kill both *S. aureus* and *P. aeruginosa* [14, 18, 27], it is unclear whether the bactericidal defects relate to impaired NETosis post-transplant. We showed defective neutrophil function is attributable to overproduction of PGE_2 [25]. PGE_2 is generated using cyclooxygenase (COX) enzymes (basal COX-1 or inducible COX-2) [28]. Inhibition of COX with indomethacin rescued the functional bactericidal defects *in vivo* [25]. Similar pathways can be involved in both intracellular killing and NETosis. NADPH oxidase activity and autophagy [29] can promote NETosis and killing, but regulation is poorly

understood. While much is known about inducers of NETosis (e.g. PMA, bacterial components, and IL-8), nothing is known of physiological inhibitors or negative regulators. Here, we propose a novel role for PGE_2 as an inhibitor of NETosis.

4.2 Results

NETosis is impaired post-syngeneic (syn) and allogeneic (allo) BMT: To determine whether the capacity to undergo NETosis was deficient or intact following BMT, neutrophils were recruited into the lungs of untransplanted control or syn or allo BMT mice. We next examined NET formation following treatment with PMA or infection with *S. pneumoniae*. PMA, a known inducer of NETosis, stimulated NETs in untransplanted control neutrophils; however, PMA-treated syn and allo BMT neutrophils exhibited impaired extracellular DNA release as measured by extracellular Sytox fluorescence vs. controls (Fig.4-1A-B). These observations were supported by immunofluorescence that showed few visible NETs in untreated groups (Fig.4-1C top row), but extensive, intact NETs upon PMA stimulation in controls. In contrast, NETs from both syn and allo BMT cells were significantly decreased and appeared structurally less intact (Fig.4-1C bottom row). Defective NETosis was also noted in neutrophils from BMT mice exposed to *S. pneumoniae*, a relevant HSCT pathogen (Fig.4-1A). We confirmed that neutrophils from syn BMT mice exhibited basal defects in production of hydrogen peroxide (Fig.4-1D).

COX inhibitors restore NET formation post-BMT: To determine whether NETosis was negatively regulated by increased COX-2 activity, syn BMT mice were intraperitoneally injected with indomethacin, a non-selective COX inhibitor prior to the

recruitment of neutrophils. Following *in vivo* inhibition of COX, NETosis was restored in neutrophils from syn BMT mice (Fig.4-2A). *In vivo* treatment with indomethacin did not

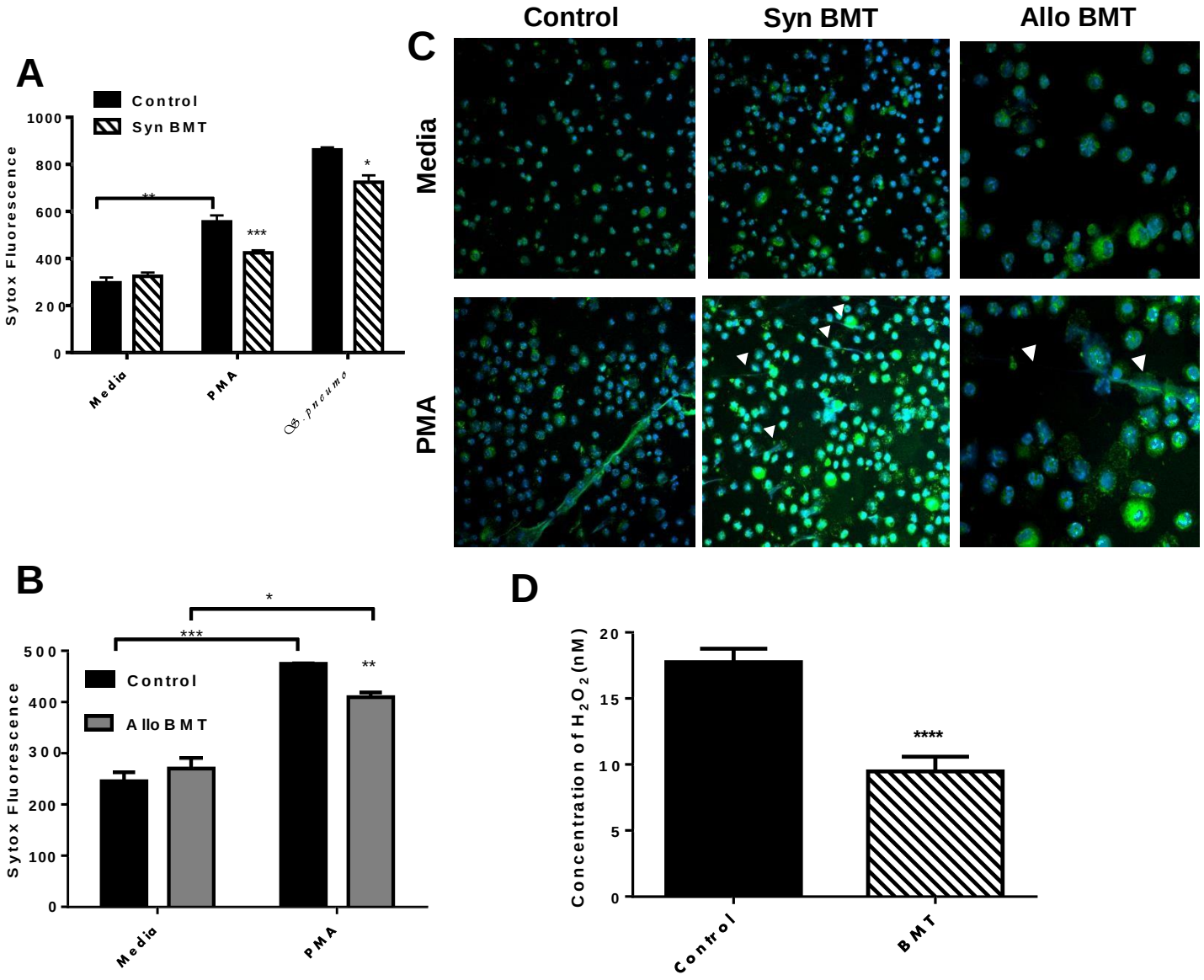

Figure 4-1: NETosis is impaired post-syn and allo BMT. LPS-recruited neutrophils from the lung of (A) syn BMT or (B) allo BMT and untransplanted control mice were stimulated for 5 h with PMA (100 nM), *S. pneumoniae* (MOI: 5), or left untreated and NETosis was measured by Sytox Green fluorescence. (C) NETosis following 5 h PMA treatment in LPS-recruited neutrophils from untransplanted control, syn BMT, and allo BMT mice was visualized by immunofluorescence via staining of DNA (Hoechst, blue) and neutrophil elastase (green, control and syn BMT 20x; allo BMT 40x magnification; arrowheads denote NETs in BMT groups). (D) Lung neutrophils were harvested 18-20h post-LPS intratracheal injection and H_2O_2 production was measured colorimetricly via the Amplex Red reagent; *P<0.05, **P <0.01, ***P <0.001, ****P <0.0001 relative to control. In panel A, n=6 /group for media and PMA stimulations and n=3/group for S. pneumoniae stimulation; in panel B, n=3/group; in panel D, the n=15/group

affect neutrophil recruitment as LPS exposure recruited 89±1.77% neutrophils in syn BMT receiving indomethacin which was comparable to the 88.7±1.34% neutrophils recruited in untreated control and 87.1±1.32% in vehicle-treated syn BMT mice. Immunofluorescence studies again confirmed the stimulatory effects of indomethacin on NETosis, as *in vivo* inhibition of COX visibly enhanced NET production from syn BMT neutrophils compared to untransplanted control neutrophils (Fig.4-2C). NETosis was increased in allo BMT mice upon *in vitro* COX inhibition with indomethacin (Fig.4-2B). The ability of PMA to induce NETs in syn BMT mice was also restored by diclofenac, an inhibitor with higher affinity for COX-2 than COX-1, or by addition of anti-PGE_2 (Fig.4-3A-B). Similarly, neutrophils from mice deficient in PGE synthase showed enhanced PMA-induced NETosis compared to cells from wild-type mice (Fig.4-3C).

PGE_2 inhibits PMA-induced NETs from murine and human neutrophils: Neutrophils from BMT mice have elevated levels of cytosolic phospholipase A_2 and COX-2 (Fig.4-4). PGE_2 causes functional defects in alveolar macrophages and neutrophils that occur post-BMT [25, 26, 30]. Thus, we focused on the effects of PGE_2 on NETosis. Interestingly, *in vitro* treatment with PGE_2 was able to decrease NET release from control murine neutrophils despite stimulation with PMA (Fig.4-5A). This effect was reversed in the presence of anti-PGE_2 antibodies (Fig.4-5B). PGE_2 was also able to inhibit NETosis in cells from normal volunteers (Fig.4-5C). Activation of the COX pathway in syn BMT mice results in ~4 ng/ml (11.34 nM) PGE_2 in the BALF[25] and elevated levels are also seen in HSCT BALF (Fig.4-4B). Figure 3D demonstrates that physiologic levels of

PGE_2 (10 nM) are sufficient to limit PMA-induced NETosis in a control subject. Immunofluorescence studies showed a significant absence of intact NET formation upon the simultaneous treatment of human or murine neutrophils with PMA and PGE_2 (Fig.4-3E).

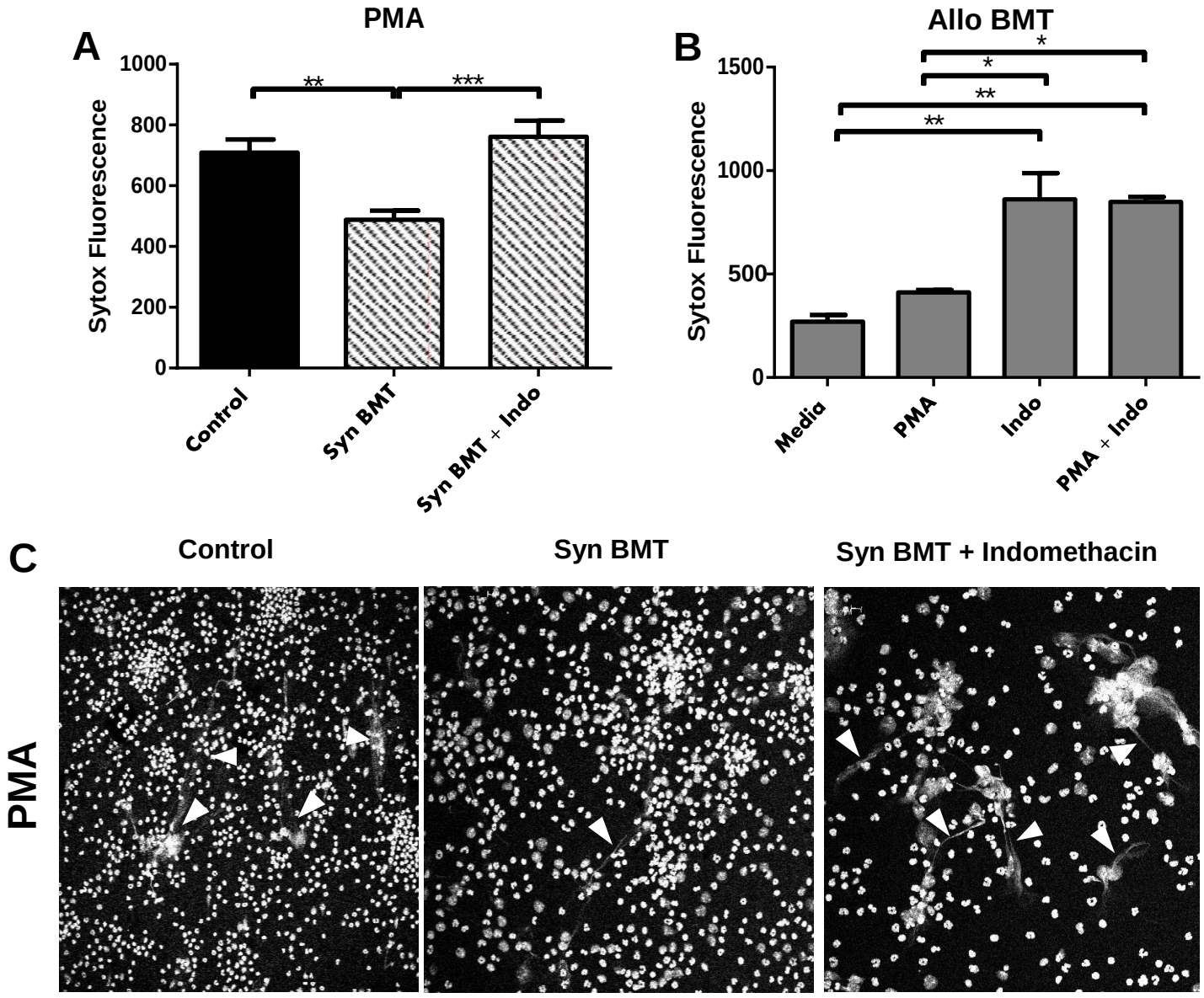

Figure 4-2: Indomethacin rescues impaired NETosis post-BMT. LPS-recruited pulmonary neutrophils from untransplanted control, syn BMT, or syn BMT mice following i.p. injection with indomethacin (1.2 mg/kg) were treated with PMA (100 nM) or left untreated for 5 h and NETosis was measured via (A) Sytox fluorescence or (C) immunofluorescence studies (gray scale of Hoechst DNA staining at 20x magnification; arrowheads denote NETs). (B) Sytox fluorescence was performed in LPS-recruited allo BMT neutrophils to measure NETosis following 5 h in vitro treatment with indomethacin (10 µM), PMA (100 nM), PMA and indomethacin, or media alone; *P <.05, **P <.01, ***P <.001. In panels A and B, n=4/group.

PGE_2 signaling inhibits NETosis via both PKA- and Epac-mediated pathways: PGE_2 signals through EP receptors 1-4 and we previously showed that both EP2 and EP4 were enhanced on BMT neutrophils when compared to their respective levels on control cells [[25] and inset in Fig.4-6A]; however, it should be noted that EP2 levels are still higher than EP4 levels on BMT neutrophils when compared to each other (Fig.6A, large graph). To determine whether EP2 signaling regulated NETosis, BMT neutrophils were treated with an EP2 receptor antagonist (PF-04418948). Treatment with the EP2 antagonist enhanced NETosis in both syn and allo murine BMT neutrophils (Fig.4-6B-C). As EP2 and EP4 signal through PKA and/or Epac [31-33], the effects of PKA- or Epac-agonists on NETosis were analyzed. Activation of either the PKA or Epac pathway was able to effectively block NET production (Fig.4-6D).

Blocking PGE_2 signaling rescues NETosis in allogeneic HSCT patients: We next investigated the effects of EP receptor antagonism on NETosis in neutrophils from the peripheral blood (Fig.4-7A-C) or bronchoalveolar lavage (Fig.4-7B) of allogeneic HSCT patients. Blocking EP2 or EP4 signaling enhanced NET formation in HSCT samples. Healthy human peripheral blood neutrophils were treated with PMA, PKA agonist, Epac agonist, or a combination of these treatments. Similar to our murine data, PMA was able to induce NETs and this function was decreased by concurrent treatment with PKA agonist or the Epac agonist (Fig.4-8A). In HSCT patients, as expected PMA did not significantly induce NETs alone, but was able to in the presence of a PKA antagonist (Fig.4-8B). The Epac antagonist was toxic in the HSCT neutrophils (data not shown).

PGE_2 inhibits autophagy-induced NET release: The mechanism(s) by which NETs are released into the extracellular space may involve autophagy as well as reactive oxygen species, myeloperoxidase (MPO), and neutrophil elastase expression [29]. In support of this, PMA treatment can stimulate NADPH oxidase activity and autophagy [34]. Autophagy has been shown to play an important role in promoting clearance of intracellular pathogens [35]. In the presence of nutrients, *m*ammalian *t*arget *o*f *r*apamycin (mTOR) functions to inhibit autophagy [36]. Rapamycin, an mTOR inhibitor, promotes

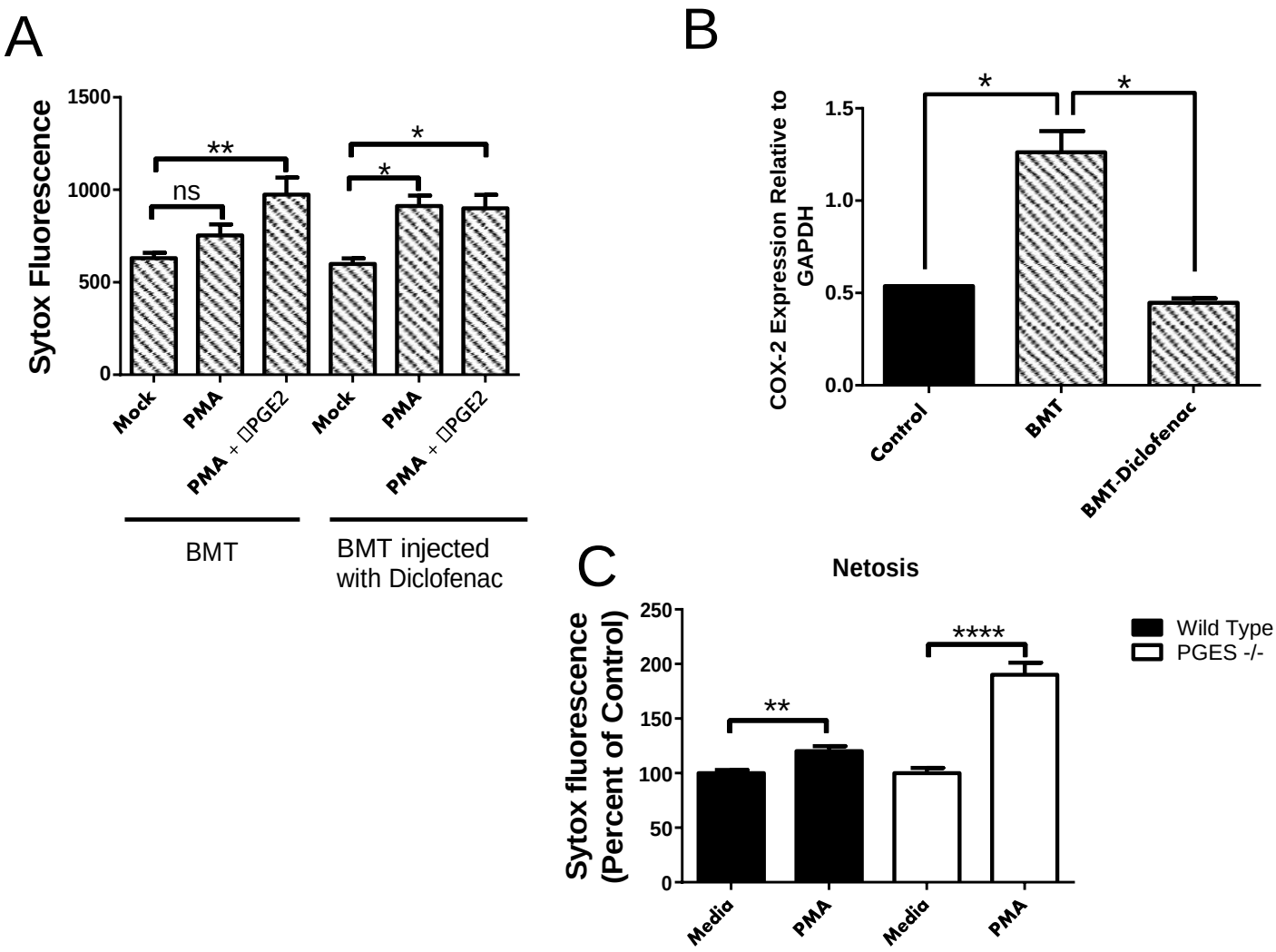

Figure 4-3: Diclofenac or anti-PGE_2 can rescue NETosis post-BMT and mPGES $^{-/-}$ mice have enhanced NETosis. A) LPS-recruited pulmonary neutrophils were isolated from syn BMT mice or syn BMT mice treated with diclofenac (30 mg/kg in saline) and were then cultured in the presence of media alone (mock), PMA (100 nM) or PMA + anti-PGE_2 for 5 h and NETosis was measured via Sytox fluorescence (SF). B) LPS-recruited neutrophils isolated from control, syn BMT or syn BMT mice treated with diclofenac were collected and total RNA was isolated. qRT-PCR was used to analyze expression of COX-2. C) LPS-recruited neutrophils from WT and PGE synthase $(mPGES)^{-/-}$ mice were treated with media or PMA as above and NETosis was measured by SF; *P <.05, **P <.01, ****P<0.0001, ns=not significant.

NET release in neutrophils from control mice but not in cells from syn BMT mice (Fig.4-9A), suggesting impaired autophagy-mediated NETosis post-transplant. Treatment with PGE_2 conferred defective NET release in control murine neutrophils despite concomitant stimulation with rapamycin (Fig.4-9B). This inhibition was corroborated by western blot analysis as less MPO was detected in the supernatant of rapamycin plus PGE_2 –treated neutrophils than was released from cells treated with rapamycin alone (Fig.4-9C). Furthermore, EP2 receptor antagonism restored rapamycin-induced NET formation in syn BMT lung neutrophils (Fig.4-9D).

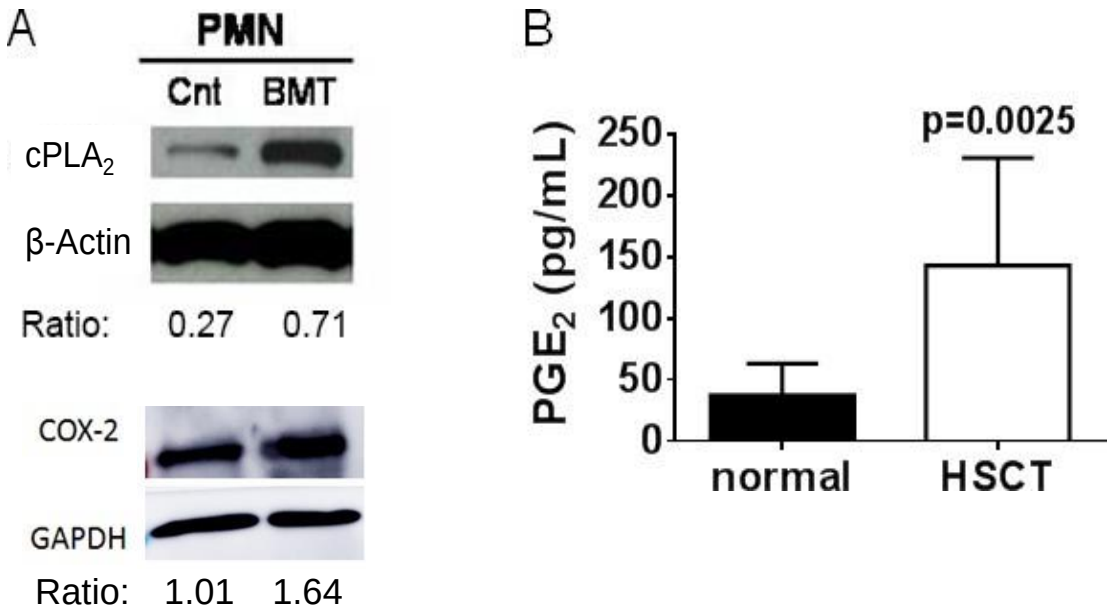

Figure 4-4: BMT neutrophils have elevated cPLA2 and COX-2 levels and human BALF post-HSCT has increased PGE_2 levels. A) LPS-recruited neutrophils were collected from untransplanted control mice or syn BMT mice and Western lysates were prepared for analysis of cPLA2 and COX-2. Expression was compared to levels of β-actin protein in cells. Shown are representative blots from one mouse, representative of data from 3 animals. B) BALF that had been collected from n=22 HSCT patients and n=8 normal volunteers as part of a long term follow up study on HSCT patients were analyzed for levels of PGE_2 by ELISA, p=0.0025.

NETosis is dependent on both NADPH oxidase and autophagy [29], so we next modulated these pathways in neutrophils collected from the bone marrow of control mice. PMA effectively induced NET release from bone marrow neutrophils, a process significantly inhibited following inhibition of autophagy (wortmannin treatment) or inhibition of reactive oxygen species (diphenyleneiodonium (DPI) treatment) (Fig.4-9E).Interestingly, when examining rapamycin-induced NETosis, PGE_2 was more effective in limiting this pathway than either wortmannin or DPI treatment (Fig.4-9F).

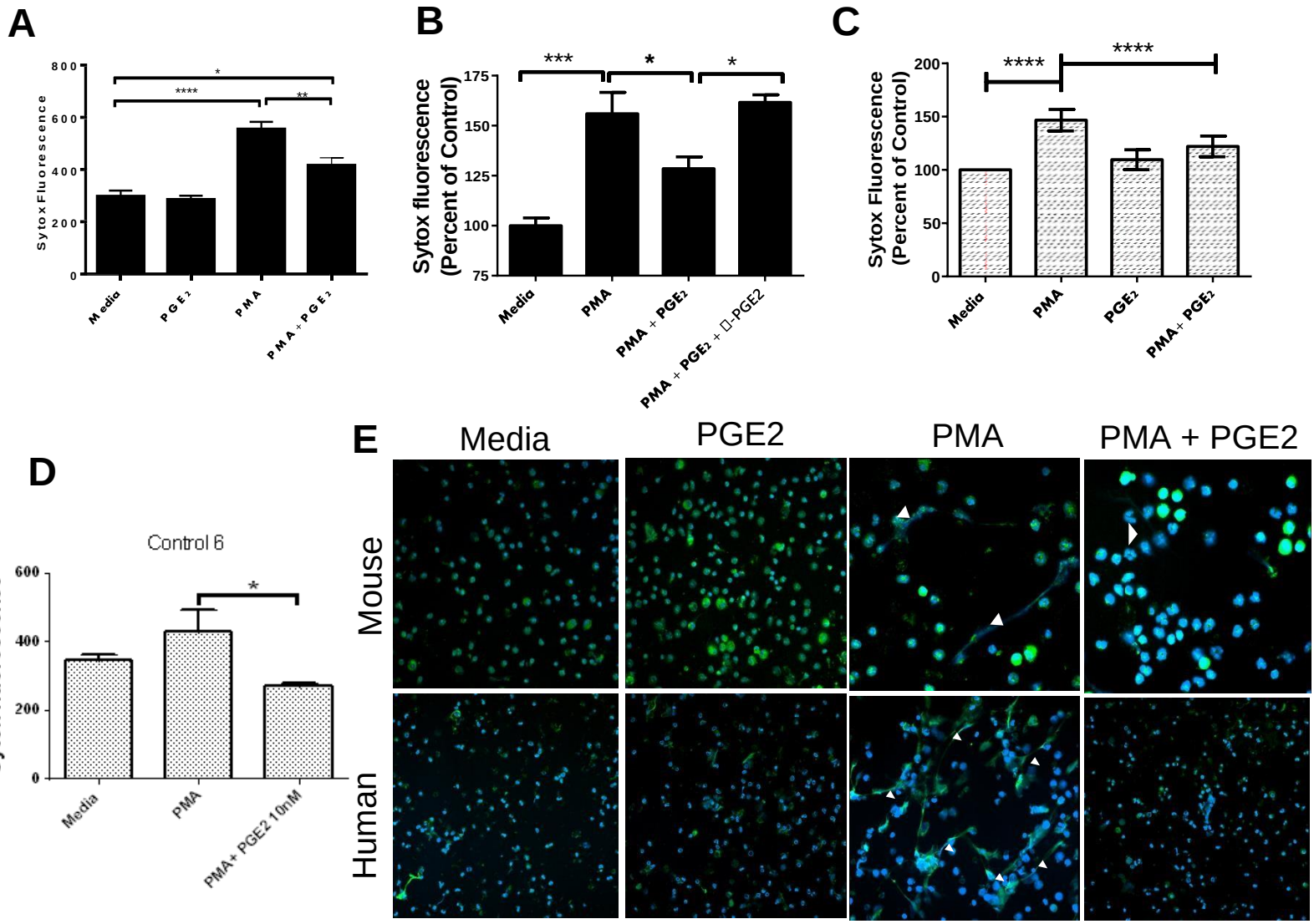

Figure 4-5: PGE_2 inhibits PMA-induced murine and human NETs. (A) Murine neutrophils were stimulated with PGE_2, PMA, combination or with (B) anti-PGE_2 for 5 h and measured for NETosis via Sytox fluorescence (SF). (C) Human neutrophils were stimulated with PMA, PGE_2, PMA + PGE_2 or (D) 10nM PGE_2 or were left untreated for 3h before NETosis was measured by SF or (E) immunofluorescence. (Hoechst, DNA, blue; neutrophil elastase (green); 60x magnification; arrowheads denote NETs); *P <0.05, **P <0.01, ***P<0.001, ****P <0.0001.

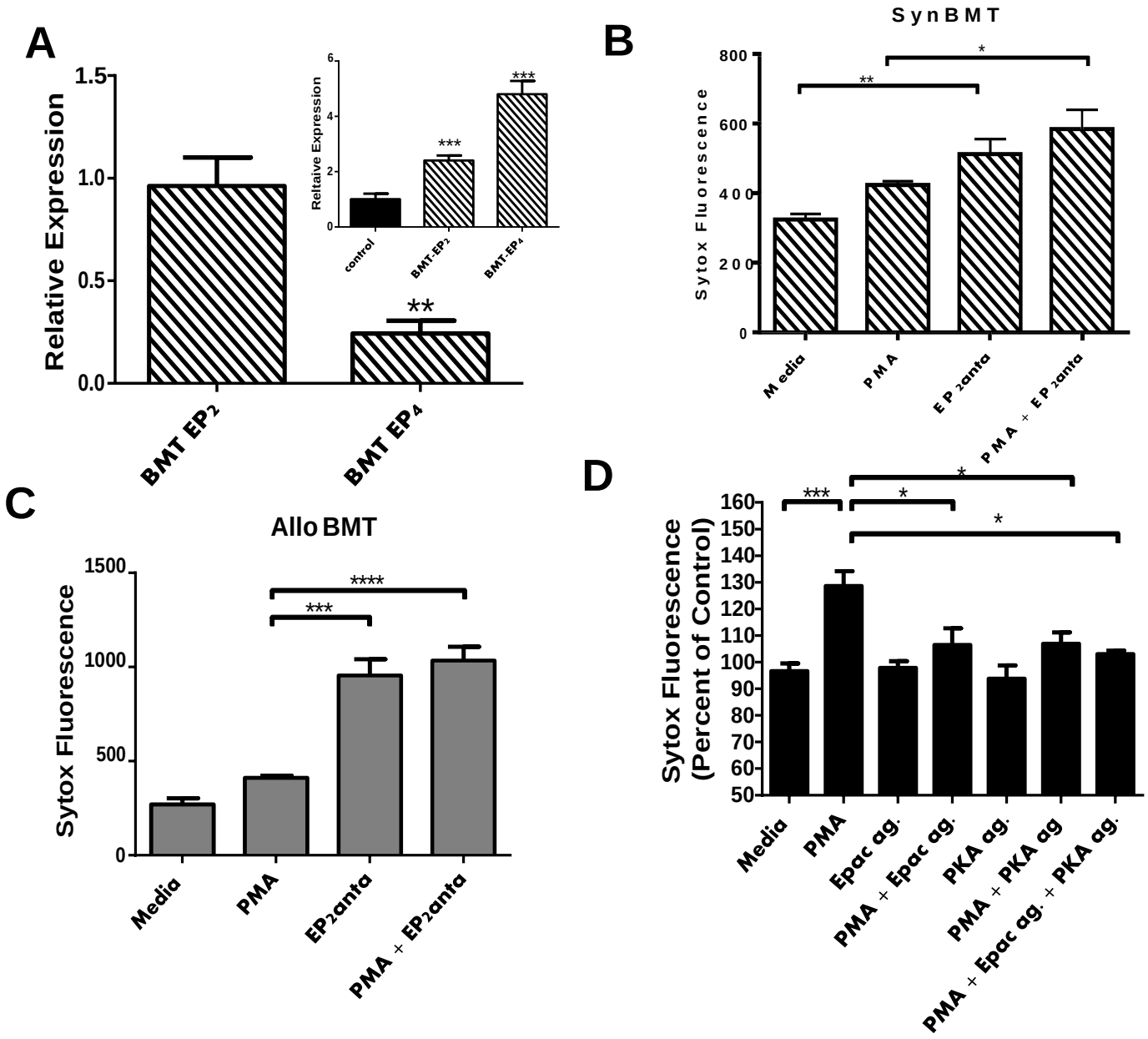

Figure 4-6: Inhibition of PGE_2 signaling rescues murine NET production post-BMT. (A) Total RNA was isolated from neutrophils collected from control and syn BMT mice (n=6 per group) and real-time RT-PCR was performed to analyze expression of EP2 and EP4 receptors. Inset shows levels of EP2 and EP4 in BMT samples compared to their respective levels in cells from control untransplanted mice (normalized to 1). The large panel shows the levels of EP4 when compared to EP2 (normalized to 1) in the syn BMT cells alone. LPS recruited neutrophils were collected from (B) syn (n=5-6/group) and (C) allo (n=4-5/group) BMT mice were treated with PMA (100 nM), an EP2 antagonist (EP2 anta; 10 nM), or PMA and EP2 anta for 5 h and NETs were measured by Sytox fluorescence. (D) Effects of downstream PGE_2 signaling on NETosis were determined with 5 h PKA agonist (PKA ag=6-Bnz-cAMP; 500 µM) and Epac agonist (EPAC ag=8-pCPT-2'-O-Me-cAMP; 500 µM) treatment plus or minus PMA on untransplanted control neutrophils. NET production was determined by Sytox fluorescence (n=4-6 pooled from 2 separate experiments; shown as "percent of control"); *P <0.05, **P <0.01, ***P<0.001.

When neutrophils from an age-matched control and HSCT patient were collected and analyzed on the same day, the cells from the HSCT patient were defective in NETosis

induced both by PMA and rapamycin relative to the control subject (Fig.4-10A). In human control neutrophils, rapamycin effectively induced NETosis (Fig.4-10B). This rapamycin-stimulated NETosis was reduced by PGE_2, PKA agonists or Epac agonists (Fig.4-10B).

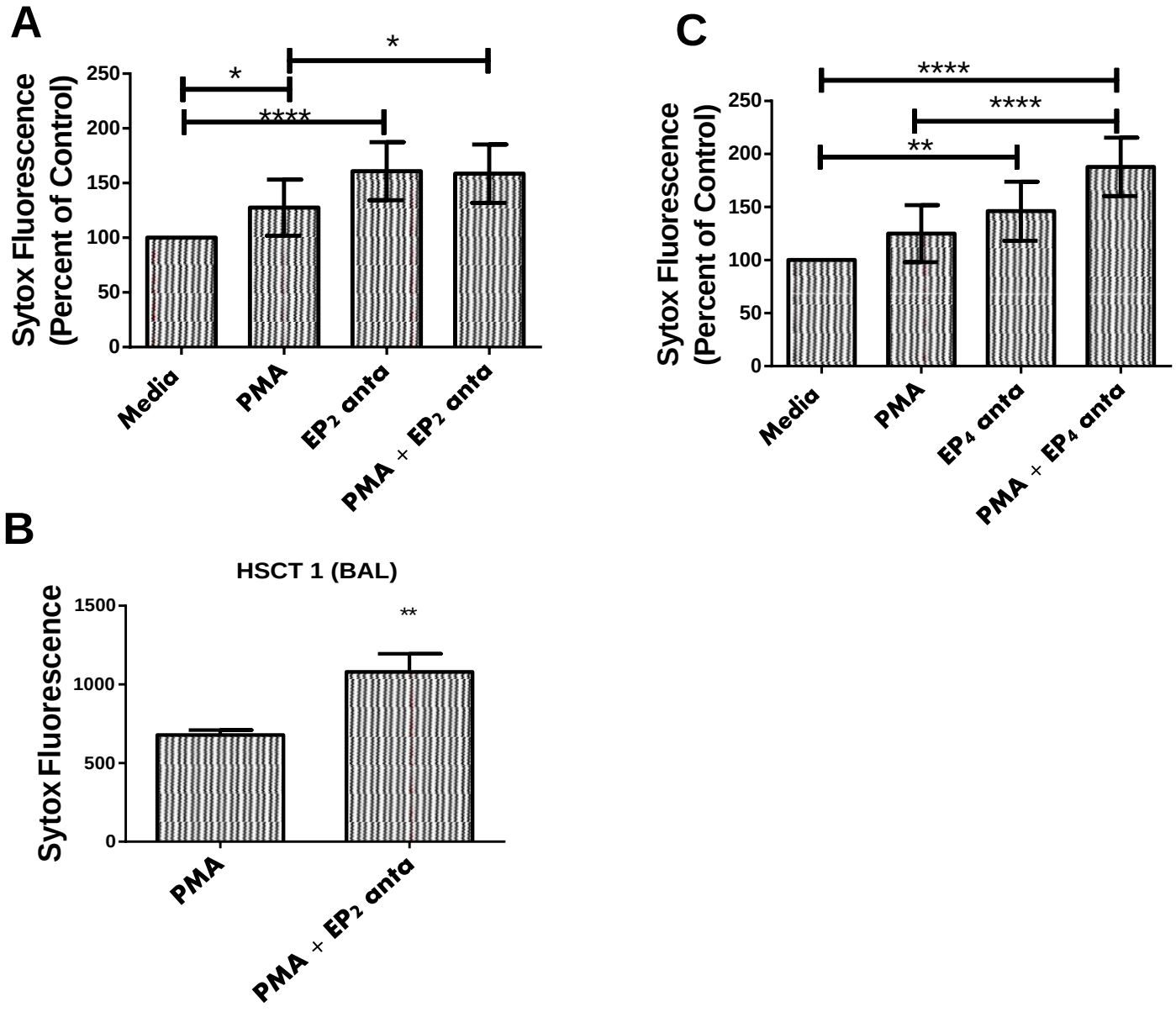

Figure 4-7: Blocking EP receptor signaling rescues NETs in allogeneic HSCT patients. Neutrophils collected from peripheral blood (A and C) or BAL (B) from allo HSCT patients were incubated with media, PMA (100 nM), EP2 antagonist (10 nM), or EP4 antagonist (AE3-208, 1µM) as indicated and NETosis was measured by Sytox fluorescence. Panel A reports the mean ± 95% CI as estimated via mixed effect linear models for n=5 HSCT patients and panel C reports data from n=6 HSCT patients (see supplemental table S1C). *P <0.05, **P <0.01, ****P <0.0001.

Overall, these data suggest that the inhibition of NETs by PGE_2 signaling is a universal feature of neutrophils obtained from both lung and bone marrow compartments in mice and from lung and peripheral blood of humans. In addition, PGE_2 can inhibit NETosis induced by a variety of stimuli.

4.3 Discussion

Neutrophil responses have been shown to be particularly important for host response to pulmonary infection. We have previously shown that neutrophils are unable to clear engulfed pathogens post-BMT [25]. Here we show that neutrophils recruited to the lung also exhibit impaired NETosis following either syngeneic or allogeneic BMT implying that extracellular killing mechanisms may also be defective post-BMT. This impairment is caused by overproduction of PGE_2 and signaling via the EP2 or EP4 receptor. We verified the ability of physiologic doses of PGE_2 to limit NETosis and utilized anti-PGE_2 antibodies to confirm specificity. We previously measured EP4 receptor expression to be 4.8-fold higher in BMT PMNs while EP2 was 2.4-fold higher compared to untransplanted controls [inset Fig. 4-6A [25]]. EP4 has higher affinity for PGE_2 than does EP2 (0.79 nM vs 4.9 nM) [37], but given the fact that EP2 levels are over 4-fold higher than EP4 levels (Fig.4-6A large panel) when compared to each other on BMT neutrophils and both of these receptors signal via a cAMP intermediate, it is not surprising that both EP2 and EP4 antagonists are effective in restoring NETosis post-transplant. We were only able to obtain one BALF sample with sufficient neutrophils for analysis, but BALF-derived cells behaved similarly to peripheral-blood derived cells from other HSCT patients in responses to EP2 antagonists (Fig.4-7A-B). While it is possible that other changes in the lung post-BMT (e.g. elevated IL-6 and GM-CSF; decreased

TNFα, IFN-γ, and leukotrienes) [24, 30, 38, 39] or other COX-2 effectors (e.g. thromboxane and prostacyclin) may also serve roles in regulating NETosis, our studies prove that PGE_2 signaling limits NETosis.

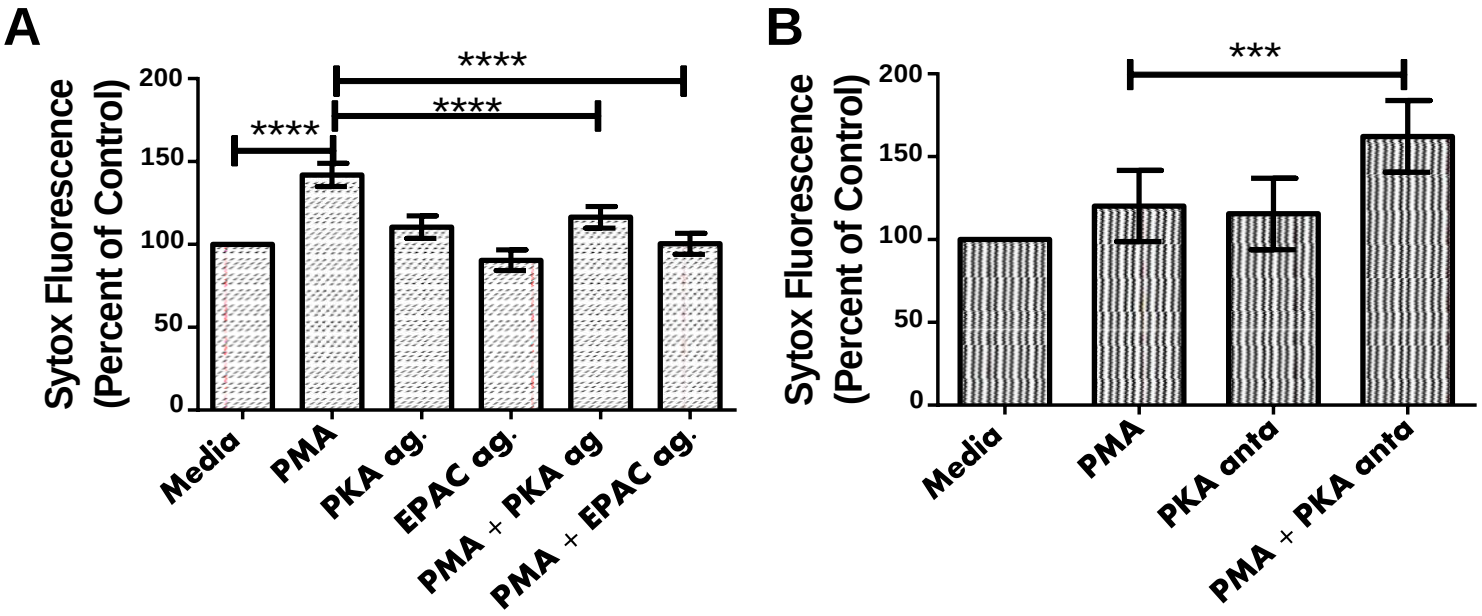

Figure 4-8: Alterations in PGE_2 signaling can influence NETosis in Control and HSCT patients. Neutrophils were collected from peripheral blood of 4 normal subjects (A) or from 8 HSCT patients (B). Cells were cultured in the presence of media, PMA (100 nM), Epac agonist (EPAC ag, 8-pCPT-2'-O-Me-cAMP; 500 µM), PKA agonist (PKA ag, 6-Bnz-cAMP; 500 µM) or PKA antagonist (H89, 20 µM; Sigma) as indicated for 3 hours and NETosis was measured by Sytox fluorescence; ***P<0.001, ****P <0.0001. Graphs represent the mean ± 95% CI as estimated via mixed effect linear models (see supplemental tables S1D and S1E).

As PGE_2-EP2 or PGE_2-EP4 intracellular signaling relies on PKA and/or Epac [40], we explored the contribution of both pathways in NET regulation. Interestingly, the use of either PKA or Epac agonists was sufficient for the inhibition of NETosis in murine and human cells and the use of both agonists did not result in an additive effect. Similar inhibitory effects of PGE_2 on NETosis were observed regardless of source of neutrophils (BAL or peripheral blood) or species (humans or mice) or stimulus (PMA, rapamycin, bacteria), suggesting that this is a well-conserved mechanism. It is interesting that the neutrophils collected from allogeneic HSCT patients showed

spontaneous NETosis following treatment with EP2 or EP4 antagonists alone (Fig.4-7) which may indicate that cells from these patients were stimulated *in vivo*, yet unable to respond.

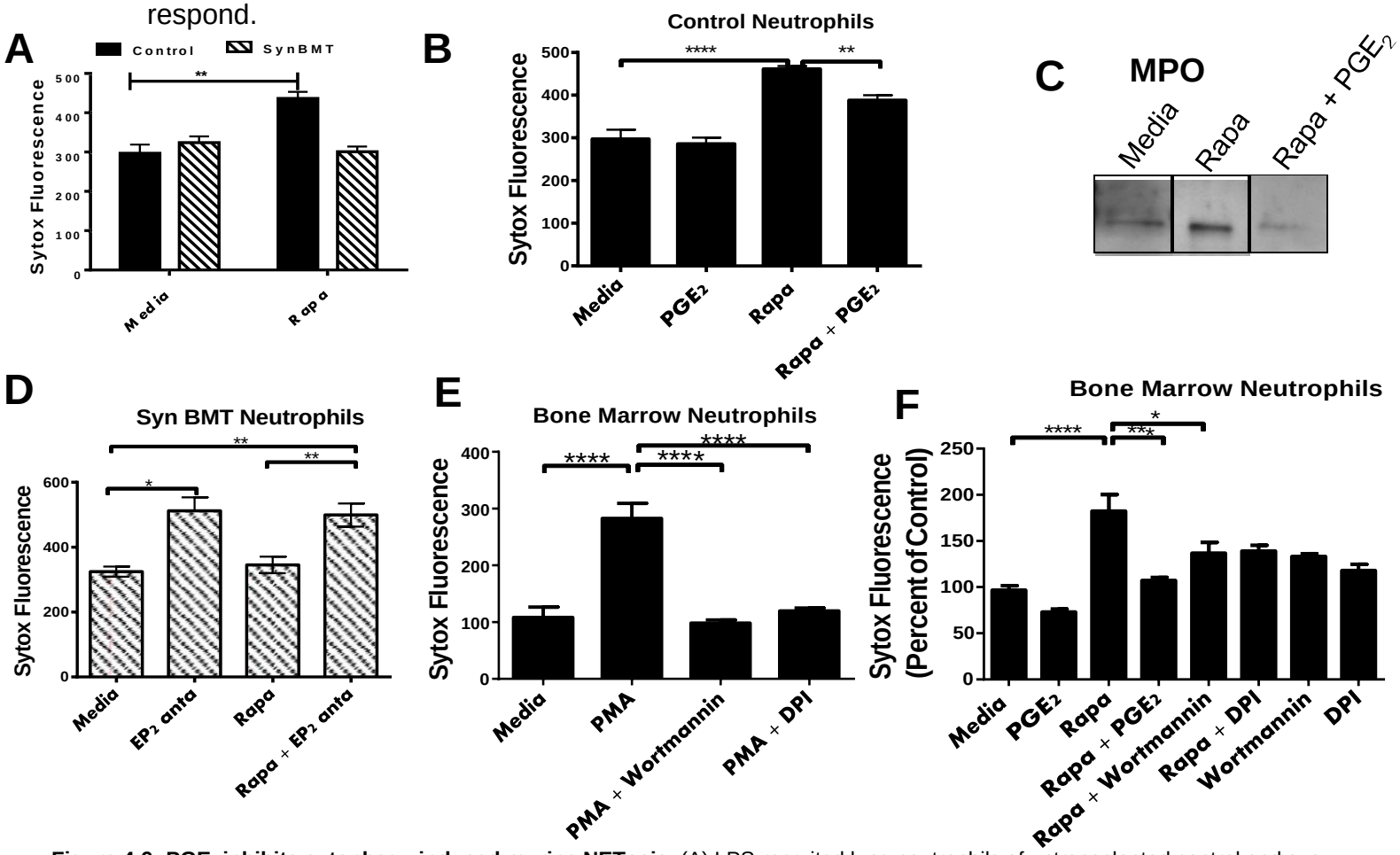

Figure 4-9: PGE_2 inhibits autophagy-induced murine NETosis. (A) LPS-recruited lung neutrophils of untransplanted control and syn BMT mice were treated with 200 nM rapamycin or were left untreated for 5 h prior to Sytox fluorescence detection; n=6/group. (B) LPS-recruited lung neutrophils from untransplanted control mice were treated with 10 µM PGE_2, rapamycin, or rapamycin + PGE_2 for 5 h prior to Sytox fluorescence detection; n=6/group. (C) LPS-recruited lung neutrophils of untransplanted control mice were stimulated with rapamycin, rapamycin + PGE_2, or were unstimulated for 5 h prior to removal of supernatants and western blot analysis of this supernatant for myeloperoxidase. The western blot depicts dividing lines to denote altered lane order from the original blot. (D) LPS-recruited lung neutrophils from syn BMT mice were treated with EP2 antagonist (10 nM), rapamycin, rapamycin +EP2 antagonist, or were untreated for 5 h prior to detection of Sytox fluorescence, n=6/group. (E) Bone marrow neutrophils from control mice were treated with PMA, PMA + wortmannin (100 nM), or PMA + diphenyleneiodonium (DPI; 10 µM) for 5 h prior to Sytox measurement, n=5/group. (F) Bone marrow neutrophils from control mice were treated as above with media, PGE_2, wortmannin, DPI or combinations of these drugs with rapamycin for 5 h prior to detection of Sytox fluorescence, n=5-8/condition. *P <0.05, **P <0.01, ***P <0.001, ****P <0.0001.

Remijsen et al. previously showed that autophagy was required for the productive release of NETs [29]. Our data demonstrate that recruited lung neutrophils from untransplanted control mice as well as neutrophils isolated from murine bone marrow or normal volunteers induce NET formation following stimulation of autophagy via

rapamycin (Fig.4-9B,F and 4-10B); however, NETosis was defective in syn BMT lung neutrophils despite exposure to rapamycin (Fig.4-9D). When cells from a control and HSCT patient were analyzed simultaneously, the response to rapamycin was diminished in the HSCT sample relative to the control (Fig.4-10A). There were differences in the collection of the cells as murine neutrophils recruited to the lung were exposed to LPS prior to treatment with rapamycin whereas murine bone marrow and human peripheral blood neutrophils are unstimulated. LPS alone can induce NET formation [14, 18], however it is likely that not all neutrophils produce NETs following the initial recruitment with LPS as our studies with murine lung neutrophils show clear changes in NET release following treatments. Furthermore, the fact that rapamycin was sufficient to induce NETs from human neutrophils in the absence of any priming factors, like fMLP, suggests that autophagy induction is sufficient to induce NET release, challenging findings from a previous study [29].

Hydrogen peroxide, a reactive oxygen species (ROS), was decreased in syn BMT neutrophils. As ROS production is required for NETosis, it is possible that PGE_2-dependent inhibition of NETosis is mediated through the Epac-mediated inhibition of ROS. Recently, a renal ischemia-reperfusion study found that activation of Epac reduced mitochondrial ROS production via interaction with Rap1 in tubular epithelial cells [41]. Thus, it is possible that Epac signaling upstream of NAPDPH oxidase or mitochondrial ROS production mediates inhibition of NETs. Alternatively, the effect of PGE_2 and Epac on autophagy is unknown. However, previous studies have shown a possible role for PKA in autophagy inhibition via phosphorylation of LC3 [42] or activation of TORC1[43]. Thus, it is possible that BMT neutrophils exhibit defective NETosis as a

consequence of reduced autophagy and/or ROS production secondary to PGE_2 stimulation. These pathways are likely linked, and this may also explain why inhibition of either PKA or Epac restored NETosis post-transplant.

Previous studies from our lab demonstrated that blocking EP2 signaling improves lung alveolar macrophage function post-HSCT by restoring bactericidal killing [25]. Our current study extends this work to suggest that EP2 or EP4 antagonists may also restore both intracellular and extracellular killing mechanisms in neutrophils post-transplant. It is worth noting that the EP2 and/or EP4 antagonists or PKA antagonists restored function to neutrophils of HSCT patients despite the fact that these patients were often on immunosuppressive medications (Table 1). Thus, this strategy may be a way to improve innate immune function while maintaining immunosuppressive control of graft vs. host disease. A limitation of our study is that the sample size for the human studies is small; however, findings were highly reproducible between mice and humans, and all findings are consistent with our conclusion that PGE_2 signaling inhibits NETosis.

Another implication of our work is that there may be a therapeutic role for EP2 or EP4 agonists for the inhibition of pathological NETosis in diseases where uncontrolled NET formation induces exacerbation of disease [44-46]. Currently, therapies under development or in use for targeting NETs in autoimmune diseases like Lupus include DNase [47, 48], anti-histone antibodies [49, 50], and anti-proteases [51]. In the case of anti-histone antibodies, the possibility of promoting autoimmunity may outweigh the benefits of this therapy. Prior to our studies, physiological factors aside from DNase that could potentially suppress NETosis were unknown. Here, we reveal PGE_2 signaling via EP2 or EP4 as a negative regulator of NETosis.

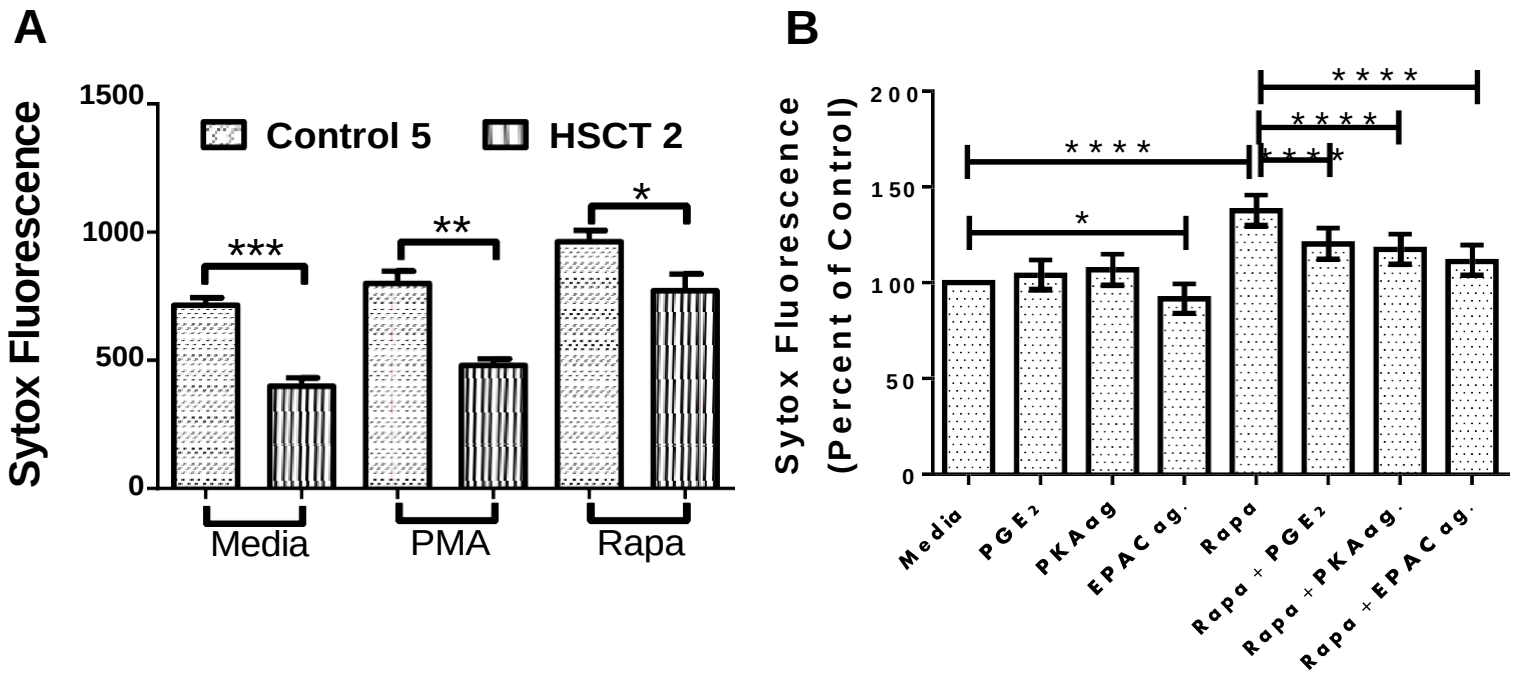

Figure 4-10: PGE_2 regulates NETosis induced by autophagy in human neutrophils. (A) Human peripheral blood neutrophils from control subject 5 (age 50) and HSCT 2 (age 47) were collected on the same day and stimulated for 3 h in the presence of media, PMA (100nM) or rapamycin (200 nM) before being analyzed by Sytox fluorescence, n=10 replicates per patient per group. (B) Peripheral blood neutrophils collected from 4 normal volunteers were cultured for 3 h in the presence of media, PGE_2 (10 µM), the Epac agonist (EPAC ag, 8-pCPT-2'-O-Me-cAMP; 500 µM), PKA agonist (PKA ag, 6-Bnz-cAMP; 500 µM), or combinations of these agents with rapamycin (200 nM) for 3 hours prior to Sytox detection; *P <0.05, ****P <0.0001.

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Table 4-1: Human Subject Characteristics

Subject ID	Age	Gender	HSCT Type	Days post-HSCT	Indication for HSCT	Conditioning Regimen	Chronic GVHD?	Current Immunosuppressive Regimen
HSCT 1 (BALF)	65	M	Allo	806	Acute Myelomonocytic Leukemia	Fludarabine and Busulfan	Present	Dexamethasone Prednisone
HSCT 2	47	F	Allo	158	Acute Myelomonocytic Leukemia	Fludarabine and Busulfan	Absent	Tacrolimus
HSCT 3	64	F	Allo	616	Acute Myeloid Leukemia	Fludarabine and Busulfan	Present	Predinosone
HSCT 4	70	F	Allo	58	Myelodysplastic Syndrome	Fludarabine and Busulfan	Absent	Tacrolimus Methotrexate (low dose)
HSCT 5	63	F	Allo	91	Myelofibrosis	Fludarabine and Busulfan	Absent	Tacrolimus Prednisone
HSCT 6	67	M	Allo	20	Acute Myeloid Leukemia	Clofaradine Bulsufan	Absent	Tacrolimus Cellecept
HSCT 7	30	M	Allo	176	Acute Myeloid Leukemia	Fludarabine and Busulfan	Absent	Tacrolimus Restasis

HSCT 8	27	M	Allo	26	T cell Acute Lymphoblastic Leukemia	Fludarabine and Busulfan	Absent	Tacrolimus
HSCT 9	55	M	Allo	55	Myelodysplastic Syndrome	Fludarabine and Busulfan	Absent	Tacrolimus Carfilozomib
HSCT 10	71	M	Allo	35	Myelodysplastic Syndrome-Refractory Anemia with Excess Blasts-2	Fludarabine and Busulfan	Absent	Tacrolimus Restasis
HSCT 11	46	M	Allo	83	Philadelphia chromosome+ Acute Lymphoblastic Leukemia	Fludarabine and total body irradiation	Present	Prednisone Sirolimus
HSCT 12	55	F	Allo	51	Angio-immunoblastic T-cell Lymphoma post auto transplant	Fludarabine and Busulfan	Present	Prednisone Tacrolimus
Control 1	50	F	none					
Control 2	23	M	none					

Control 3	23	F	none	
Control 4	26	F	none	
Control 5	51	F	none	
Control 6	53	M	none	

Chapter 5. Discussion

5.1 Elevated PGE_2 post-BMT mediates IL-1β-related acute lung injury (ALI)

HSCT transplant patients are susceptible to opportunistic bacterial infections particularly with *Pseudomonas aeruginosa*. This Gram-negative bacterium has been shown to cause much of its lung pathology by inducing IL-1β-dependent acute lung injury (ALI)[1]. PGE_2 is elevated in HSCT patients and reducing its levels has shown improvement in clearance of *P. aeruginosa*[2, 3]. Yet, the role of PGE_2 in regulating IL-1β was not well understood. Thus**,** in chapter 3 of this dissertation, I aimed to study the role of PGE_2 in IL-1β related lung injury caused by *P. aeruginosa*. Using a BMT mouse model (described in chapter 1), I detected a more severe ALI, and more bacterial burden in BMT mice compared to control mice[4]. Interestingly, using an LPS-induced ALI model, I determined that the severe ALI in BMT mice was not dependent on the increased bacterial burden, but rather, it was dependent on excessive secretion of IL-1β. Moreover, I found that IL-1β release did not just correlate with elevated levels of PGE_2, but also, PGE_2 dependent stimulation of the EP2/EP4 receptors in alveolar macrophages (AMs) was responsible for the exacerbated IL-1β pathology. Furthermore, I performed treatments with a non-selective COX-inhibitor, indomethacin, in BMT mice and observed reduced lung injury, less IL-1β and improved bacterial clearance. These findings suggest ideas for the development of new therapeutic strategies to improve outcomes of HSCT patients with pulmonary infections with *P. aeruginosa*. However, the translatability of our findings is limited for multiple reasons

including use of our mouse model and choice of experimental reagents as discussed below. Such limitations should be addressed before fully considering this pathway as a therapeutic target.

One of the limitations in the work performed in chapter 3 is that all murine experiments were in syngeneic BMT models, where total body irradiation (TBI) was used for conditioning, $5x10^6$ total bone marrow cells were infused to reconstitute the immune system, and all experiments were performed at 5 weeks post-transplant (post-engraftment phase). To a large extent, these BMT mice replicate the immunosuppression to bacterial infections and the elevated levels of PGE_2 found in humans post-HSCT[5] making it a good system to study post-transplant infections; however, around 47% of transplants performed each year are allogeneic[6]. Thus, the findings in this dissertation should be reproduced or tested using an allogeneic BMT murine model. Additionally, in the clinics, TBI is not the standard conditioning given to all HSCT patients. The conditioning regimen will depend on the disease. Combinations of chemotherapy and TBI are mostly given to the patients' pre-transplant[7]. Research in the laboratory of my advisor has shown differences in lung and spleen immune reconstitution post-TBI or chemotherapy. It was reported that mice (C57BL/6J) exposed to 13Gy of TBI prior to BMT have an immune reconstitution of 95% donor cells in the spleen and around 81% donor cells in the alveolar compartment[8]; whereas chemotherapy performed by busulfan and cyclophosphamide treatment lead to an immune reconstitution of 72% spleenocytes from donor cells and 57% of alveolar cells from donor sources[8]. Both conditioning regimens lead to elevated levels of PGE_2 secreted by AMs, and AMs were defective in bacterial phagocytosis[8]. Thus, we can

hypothesize that elevated levels of PGE_2 in the lung, independent of the conditioning regimen, would exacerbate IL-1β related ALI. However, findings discussed in chapter 3 should be replicated in a BMT mouse model where different conditioning regimens including chemotherapy are performed.

As mentioned before, our findings show that improved bacterial clearance in BMT mice can be achieved by decreasing the levels of PGE_2. These experiments were all performed with *P. aeruginosa* infected mice, and no other bacteria were used *in vivo*. My focus was on *P. aeruginosa* because it is one of the main pathogens affecting HSCT individuals, and this bacterium is now considered to be a critical public threat. However, multiple bacteria can present lung complications in HSCT individuals including methicillin-resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pneumoniae*[5, 9-11]. In chapter 3, using *in vitro* assays, I was able to demonstrate that PGE_2 can exacerbate IL-1β release when cells are challenged with MRSA and *S. pneumoniae* suggesting that exacerbated IL-1β is not pathogen specific, and it can also occur without stimulation of the Gram-negative sensor, TLR4, as both MRSA and *S. pneumoniae* are gram-positive bacterium. Yet, to make inhibition of PGE_2 a global treatment to combat bacterial infections, we should perform MRSA and *S. pneumoniae* infections, along with other bacteria, in BMT mice and test the ability of PGE_2 signaling inhibitors to decrease bacterial burden. Apart from bacterial infections, PGE_2 has been reported to play both positive and negative roles in the outcome of multiple viral and fungal pathogens[12]. So implications of blocking PGE_2 in HSCT subjects may go beyond bacterial pathogens, and extend to all pathogens whose pathology is known to be regulated by PGE_2. However, we would not expect that inhibition of PGE_2 in HSCT

would confer protection to all pathogens. For example, additional work in the laboratory of my advisor has shown that BMT mice have impaired immunity to gamma herpesvirus, but this was independent of high levels of PGE_2[13, 14]. However, HSCT patients are highly susceptible to infections with fungal pathogens including *Candida albicans* and the pathology caused by this pathogen is known to be partially regulated by PGE_2[15]. Thus, we can hypothesize that inhibition of PGE_2 may also play a positive role in BMT subjects post-*C. albicans* infection. Thus, it would be of high importance to test inhibition of PGE_2 in BMT mice infected with not just bacterial pathogens, buy also fungal and viral pathogens.

An additional limitation of our findings is the sole use of the non-selective COX inhibitor, indomethacin, in *P. aeruginosa* infected mice to reduce PGE_2. Indomethacin will not just reduce the levels of PGE_2 but also other COX metabolites, which might also be playing a role in *P. aeruginosa* pathogenesis*. P. aeruginosa* lung infection in mice has been shown to dysregulate levels of eicosanoids including elevating levels of PGE_2 and prostacyclin (PGI_2). This is due to the expression of a type three secretion system effector molecule, ExoU, a homologue of phospholipase A_2 [16]. ExoU is a marker of high virulence in *P. aeruginosa*[17]. Previously, the laboratory of my advisor showed that PGI_2 is also found elevated in BMT mice[8]. As our approach to reduce PGE_2 is also affecting all COX metabolites including PGI_2, future experiments should focus on using specific PGE_2 signaling inhibitors. Additionally, experiments should also address the effect of PGI_2, *in vivo*, post-*P. aeruginosa* infection in BMT mice.

5.2 PGE_2 mediated IL-1β increase is dependent on EP2/EP4 signaling and activation of CREB

In chapter 3, we demonstrated that PGE_2 stimulation in murine macrophages and human monocyte-derived macrophages lead to increased IL-1β transcript levels. This serves as "signal 1" of inflammasome-mediated IL-1β secretion which is normally restricted to pathogen recognition receptors (PRRs) including toll-like receptors (TLRs)[18]. Signal 2 of IL-1β secretion is mediated by a wide array of signals leading to activation of caspase-1 and one or multiple members of the inflammasome family (NLP3, NLRC4, etc.). Some stimulations such as LPS can lead to both signal 1 and 2. For example, we can achieve increased IL-1β secretion in macrophages*, in vitro*, with prolonged LPS stimulation. Prolonged stimulation with LPS doesn't just lead to a TLR4-dependent increase in pro-IL-1β, but also to a moderate activation of caspase-1[19]; thus, activating both signal 1 and signal 2 for IL-1β secretion. This is an effect unique to murine macrophages as only signal 1 is necessary for IL-1β secretion in human monocytes, probably because of the constant expression of caspase-1[20, 21]. Single stimulation with PGE_2 did not affect caspase-1. To understand how PGE_2 was mediating the increase in pro-IL-1β, we decided to focus on the PGE_2 signaling pathway. PGE_2 can signal through 4 different G-protein-coupled receptors termed EP1-EP4[12]. The mechanism that we found to contribute to this increase was activation of cAMP-PKA by stimulation of EP2 and EP4 receptors, and not the EP3 receptor. However, we did not test the EP1 receptor as we were lacking a specific agonist for EP1 that would not activate other EP receptors. Hence, the role of EP1 in PGE_2 dependent increased IL-1β should be addressed. Nevertheless, we do not anticipate an

increase in IL-1β expression post-EP1 stimulation. This is because our data suggest that IL-1β increase is mediated by the activation of PKA and not by the EP1 intracellular signaling pathway mediators, calcium and protein kinase C (PKC). PKA stimulation leads to the activation of two downstream transcription factors, NFκB and CREB[22]. We demonstrated that inhibition of the transcription factor CREB, with the use of a selective antagonist, was able to abolish the increase in IL-1β. Thus, we concluded that CREB was the main transcription factor necessary for PGE_2-dependent IL-1β increase. However, we never tested the role of the master transcription factor, NFκB. Interestingly, shortly before we published our findings, another research laboratory reported that PGE_2 increases IL-1β production[23]. They attributed this increase to activation of NFκB dependent on EP2/EP4 stimulation. Thus, CREB might not be the only transcription factor playing a role in increasing levels of IL-1β post-EP2/EP4. If our results are taken together with the other laboratories, both NFkB and CREB are likely involved.

5.3 Improving bacterial clearance post-IL1β inhibition

As mentioned before, in the lung of BMT mice, *P. aeruginosa* induces exacerbated levels of IL-β secreted mainly by AMs leading to ALI and high mortality. As we demonstrated that increased levels of PGE_2, in BMT mice, regulated the exacerbated levels of IL-1β post- *P. aeruginosa* infection, we hypothesized that reducing PGE_2 would further reduce the levels of IL-1β in the lung which would in turn reduce ALI. In accordance with our hypothesis, we detected less ALI and less IL-1β after indomethacin (non-selective COX inhibitor) treatments in *P. aeruginosa* infected BMT mice. Additionally, not to our surprise, we detected improved bacterial clearance. While PGE_2

can regulate IL-1β secretion, it also participates is multiple pathways of inflammation and immunity. Thus, the improved bacterial clearance post-indomethacin treatment can be due to the many immunosuppressing effects that PGE_2 has on immune cells including, but not limited, to inhibition of autophagy, NETs, phagocytosis and ROS production rather than inhibition of IL-1β. However, others have shown that inhibition of IL-1β specifically, independent of PGE_2 inhibitors, can lead to improved *P. aeruginosa* clearance. For example, $Il1R1^{-/-}$ mice and administration of neutralizing antibodies to IL-1β can improve bacterial clearance against *P. aeruginosa* in the lung [1, 24]. The reasons behind improved bacterial clearance post-IL-1β neutralization are not well understood. It is worth noting that specific inhibition of other cytokines including IL-18, TNF-α and IL-36 cytokines [1, 25, 26] have also been shown to improve *P. aeruginosa* clearance in the lung. These findings suggest that reducing inflammation seems to be an efficient method to improve *P. aeruginosa* clearance in the lung and might not be specific to one cytokine including IL-1β. Thus, the improved *P. aeruginosa* clearance post-IL1β inhibition might be due to reduced inflammation rather than an IL-1β specific effect on immune cells.

5.4 Control of IL-1β by PGE_2

PGE_2 is involved in many physiological processes and plays a role in both pro and anti-inflammatory responses by regulating different inflammatory mediators including cytokines[27, 28]. IL-1β is a pro-inflammatory cytokine that plays an essential role in controlling immune responses to pathogens. However, if not regulated, elevated levels of IL-1β can be pathologic as it has been reported in the lung post-*P. aeruginosa* infection[29]. Hence, our findings showing that decreasing PGE_2 can lead to decreased

IL-1β-mediated pathology post-*P. aeruginosa* infection are of great clinical interest. However, our *in vivo* therapeutic approach to reduce PGE_2 was using a COX-inhibitor, which will not just decrease the levels of PGE_2, but also all COX metabolites including other prostaglandins (PGD_2, PGI_2, and $PGF_{2\alpha}$) and thromboxane A2. Thus, we cannot completely attribute decreased IL-1β to decreased PGE_2. To overcome this limitation, future experiments should focus on specifically inhibiting PGE_2 and measuring the effect on IL-1β. We could perform this experiment with the use of a selective EP2/EP4 antagonist *in vivo*[23]. Additionally, decreasing inflammation by a COX inhibitor might affect multiple cytokines, not just IL-1β. Thus, future experiments should focus on using IL-1β specific inhibitors such as Anakinra and or neutralizing IL-1β monoclonal and polyclonal antibodies in BMT mice post *P. aeruginosa* infection to determine the specific role of IL-1β.

5.5 AMs post-BMT mediate IL-1β related pathology post-*P. aeruginosa* infection

Secretion of IL-1β by AMs post-*P. aeruginosa* infection has been shown to create lung pathology[1]. In our BMT mice, we were able to detect that AMs, but not interstitial macrophages, were responsible for elevated levels of IL-1β and we attributed this to increased COX-1 and COX-2 expression in this cell type. However, neutrophils have been reported to secrete high levels of IL-1β post lung bacterial infection[30]. We did not address the role of neutrophils in secreting IL-1β post-BMT. To address this issue, future experiments should focus on depleting AMs before bacterial challenge in BMT mice. We can achieve this by treating BMT mice with clodronate liposomes which will deplete AMs[1]. If we can still detect exacerbated levels of IL-1β post-bacterial infection in BMT mice it will mean that AMs are not the main source of IL-1β but rather other

immune cells such as neutrophils can also contribute. Additionally, we can measure IL-1β in the lung of BMT mice post-bacterial challenge with or without neutrophil depletion with anti-Ly6G antibodies. If we reduce the levels of lung infiltrating neutrophils, and still detect the elevated levels of IL-1β, we can clearly attribute exacerbated levels of IL-1β to AMs.

5.6 PGE_2 inhibits autophagy-mediated clearance of *Pseudomonas aeruginosa*

Autophagy is an intracellular homeostatic process for energy conservation in which the cell engulfs cytoplasmic proteins and organelles for degradation[31]. It is characterized by formation of a double membrane vesicle termed an “autophagosome” which fuses with the lysosome to create the “autolysosome”, where engulfed material will be destroyed by lysosomal hydrolases[32]. Interestingly, autophagy has been shown to play a pivotal role in the destruction of invading microorganisms, control of inflammation, as well as an essential role in antigen presentation[31]. Autophagy can be induced in different ways including amino acid starvation, mammalian target of rapamycin (mTOR) inhibition, inflammatory cytokine stimulation, as well as PRR (e.g. TLR4) activation[33-37]. AMs, particularly, have been shown to primarily clear *P. aeruginosa* using autophagy machinery[38]. As explained in chapter 3, in our efforts to explain why PGE_2 inhibits clearance of *P. aeruginosa*, we decided to study autophagy. We demonstrated that PGE_2 stimulation in AMs was able to interrupt the autophagy-mediated killing of *P. aeruginosa*. The direct pathway of how PGE_2 inhibits autophagy-mediated bacterial clearance was not determined, but the EP4-mediated activation of ERK1/2 signaling might explain autophagy inhibition as ERK1/2 is a well-known activator of mTOR, a negative regulator of autophagy[39]. However, we did not test this, rather, we showed

that PGE_2 stimulation in macrophages downregulates the lipidation of microtubule-associated protein 1A/1B-light chain 3 (LC3), an autophagy-related protein, and these results were considered a direct measurement of autophagy inhibition. However, recently, a role for LC3 in others pathways has been reported including a role in the newly identify pathway, LC3-mediated phagocytosis[40]. Thus, with the set of experiments that we performed in chapter 3, we cannot completely conclude that PGE_2 specifically inhibits autophagy. As a future direction, we should perform autophagy related canonical stimulations including rapamycin treatment with and without the presence of PGE_2, and measure autophagy by visualizing the double membrane vesicle using electron microscopy. Using this method, we could conclude whether PGE_2 inhibits autophagy, specifically.

5.7 Link between PGE_2, autophagy, and the inflammasome.

The inflammasome controls the release of IL-1β, which if dysregulated can lead to a decrease in pathogen clearance, or to the development of different IL-1β-driven auto-inflammatory syndromes. Here, we show that PGE_2 plays a role in exacerbating the secretion of IL-1β in macrophages. We have found that this exacerbation is due to priming the cells for IL-1β secretion, rather than activating the inflammasome. We determined that this was due to elevation of intracellular cAMP, and activation of PKA mediated by the receptors EP2 and EP4. However, others have reported that PGE_2 inhibits release of IL-1β when pathogenic signals are activators of the specific NLRP3 inflammasome[41, 42]. PGE_2-mediated inhibition of the NLRP3 inflammasome was found to be mediated by PKA-mediated phosphorylation of the Ser295 residue which inhibits its function[41]. We found that these differences were due to the time of exposure to

PGE_2. A short exposure (5 minutes in culture) to high levels of PGE_2 inhibits the NLRP3-mediated secretion of IL-1β by a post-translational modification[41]. However, longer exposures (4 hours in culture) to PGE_2 boost the levels of intracellular IL-1β by stimulating NFkB and CREB-dependent transcription of the IL-1β gene; thus, amplifying amounts of pro-IL-1β for processing after exposure to microbial compounds [4, 23]. Additionally, although we did not test, another mechanism for PGE_2-mediated increase of IL-1β can be by the inhibition of autophagy. It has been reported that autophagy vesicles can target inflammasome components for degradation to reduce IL-1β secretion [43, 44]. Thus, PGE_2 mediated inhibition of autophagy could lead to a non-regulated activation of the inflammasome. To asses this, future experiments should focus on the role of PGE_2-mediated inhibition of autophagy on IL-1β release.

5.8 PGE_2 mediated inhibition of neutrophil extracellular traps (NETs)

Neutrophils are the most abundant innate immune effector cell in humans, and their antimicrobial responses are essential for combating bacterial infections[45]. One of the major strategies to combat pathogens is the release of NETs, which are decondensed chromatin web-like structures loaded with antimicrobial peptides including myeloperoxidase (MPO) and neutrophil elastase. NETs can neutralize and kill bacteria, fungi, viruses, and parasites[45-47]. The mechanism of NETs release involves the translocation of NE protein into the nucleus by reactive oxygen species (ROS)-dependent MPO activation leading to NE-dependent chromatin disruption and histone citrullination by protein-arginine deiminase type 4 (PAD4)[45]. In chapter 4, we tested the ability of neutrophils from HSCT patients to secrete NETs by PMA stimulation. We detected an impaired ability of neutrophils from HSCT patients to induce NETs.

Neutrophils from BMT mice also had an impaired ability to release NETs. We confirmed increased levels of PGE_2 in our human HSCT subjects (n=12) compared to healthy donor volunteers. Increased PGE_2 in our patients was independent of age, gender, conditioning regimen, and days post-transplant (dpt) (20-806, dpt). Interestingly, indomethacin treatment was able to restore normal function for NET release in neutrophils coming from HSCT patients. We reported that autocrine stimulation of EP2/EP4 in HSCT/BMT neutrophils was responsible for the impairment in NET release. Shortly after our publication, another laboratory published similar findings supporting our work in a non HSCT context[48]. The mechanism is still unknown, but we provided evidence that might involve the inhibition of autophagy which is a known NET regulator[49]. However, our experimental design was to induce autophagy-mediated release of NETs in neutrophils by rapamycin treatments, and measure the impact of PGE_2 on NET release. We detected decreased release of NETs. However, we did not fully measure autophagy in these cells. Thus, as mentioned before, future experiments should focus on understanding the possible mechanism of autophagy inhibition by PGE_2 with a focus on both AMs and neutrophils. Apart from inhibiting autophagy, PGE_2 could be inhibiting NETs by other mechanisms including inhibition of the NADPH oxidase. In chapter 4 we reported inhibition of NETs release in neutrophils post-PMA stimulation. PMA induction of NETs release has been shown to be dependent on free superoxide production with a downstream phosphorylation of the enzymes p38, MAPK and ERK. As we, and others, have shown that inhibiting the NADPH oxidase completely abolished NETs release, we believe this is a pathway that PGE_2 might be interfering with as PGE_2 has been shown to inhibit NADPH oxidase[50]. Of note however, PGE_2

inhibition of NAPH oxidase was shown in AMs and not in neutrophils. To overcome this limitation, future experiments should target the PGE_2-mediated regulation of NADPH oxidase in neutrophils stimulated for NETs release.

One of the limitations in chapter 4 is that all experiments performed to prove the role of PGE_2 regulation of NETs were done post-PMA stimulation and not with other stimulants including bacterial infections. We can speculate that PGE_2 will also inhibit NETs release upon bacterial infections as NADPH oxidase plays an essential role in microbial clearance. However, NETs release post-bacterial and fungal infections has been shown to be dependent on scavenger receptors, in particular Dectin-1. As PGE_2 has been shown to alter expression of many scavenger receptors, the effects of PGE_2 in regulation of NETs release upon microbial infection might be different and should be addressed. Furthermore, all experiments performed in chapter 4 were performed by measuring NETs in an *in vitro* model; future experiment should focus in measuring NET release impairment *in vivo* using BMT mice. We can do that this by flow cytometry[51] post-*S. pneumoniae* infection, as we provided evidence that PGE_2 decreases *S. pnuemoniae*-mediated NETs release. We can additionally develop BMT chimeras with the use of bone marrow from mice lacking essential proteins for NETs release such as $MPO^{-/-}$ (MPO deficiency does not block, but delays NETs release[52]), $NE^{-/-}$ and/or $PAD4^{-/-}$ mice. In these mice we can inhibit PGE_2 post-*S. pneumoniae* infection and determine whether PGE_2 inhibition is unable to improve bacterial clearance. We could also consider experiments to measure NETs in histology sections or to treat WT BMT mice with DNAse to degrade NETs *in vivo* and determine outcomes.

Even though the mechanism of PGE_2 inhibition of NETs release is unknown, its implications can provide benefit for combating multiple infections. For example, the bacterial pathogens *S. aureus*[46, 53] and *P. aeruginosa*[54], the viral pathogens respiratory syncytial virus (RSV) [55] and influenza virus[56] as well as the fungal pathogen *C. albicans*[57] are all known inducers of NETs and they are all known to cause post-transplant infectious complications[10, 58-62]. So our future experiments should focus on understanding the therapeutic benefit that PGE_2 inhibition can provide in NETs regulation against these microbes in an *in vivo* model.

5.9 Polarization of PGE_2 in macrophages, and the role of metabolism in this process.

Macrophages are tissue resident immune cells capable of inducing pathogen clearance and stimulating adaptive immunity upon encountering foreign molecules. However, apart from its many immunological functions, macrophages oversee physiologic homeostatic functions including removal of apoptotic cells and iron recycling from erythrocytes. Thus, upon pathogen exposure the macrophages adopt a pro-inflammatory environment that needs to be tightly regulated. This is known as the M1 stage. One of the characteristics of the M1 macrophage stage is overexpression of inducible nitric oxide synthase (iNOS) as well as pro-inflammatory cytokines including IL-1β and TNF-α[63]. In culture, we can achieve this by stimulating macrophages with a pathogen associated molecular patter such as LPS in combination with macrophage activating signals such as IFN-γ. In the absence of a pathogenic stimulation, macrophages are in an anti-inflammatory stage termed M2. In culture we can achieve an M2 stage by stimulating macrophages with IL-4 and IL-13 cytokines. As we noticed

that PGE_2 stimulation induced high levels of IL-1β, we decided to investigate if it was also inducing a M1 stage. To our surprise, when we stimulated macrophages with PGE_2 we couldn't detect any difference in the M1 marker, iNOS (Figure 5-1 top left). However, PGE_2 stimulation did have an effect in inducing a higher M2 stage as seen by elevation of the M2 marker, Arginase-1, post-M2 stimulation. This phenotype has been reported and was determined to be mediated via the cyclic AMP-responsive element binding (CREB)-mediated induction of Krupple-like factor 4 (KLF4)[64]. Even though, PGE_2 does not induce high levels of iNOS it does induce high levels of IL-1β which is a phenotype of M1 polarization. Interestingly when comparing the increased elevation of IL-1β transcript levels we find that a single stimulation of PGE_2 can raise IL-1β transcript levels as strong as dual stimulation with LPS and IFN-y (Figure 5-1, bottom). Moreover, together with M1 stimulants, PGE_2 can further increase levels of IL-1β. We detected this phenotype just with IL-1β and not with IL-6 or TNF-α (data not shown). Our data show that PGE_2 is not polarizing macrophages into a M1 nor M2 stage, rather it has a direct effect in increasing IL-1β transcript levels. One finding that is still not explained is why polarization to M2 can limit the ability of PGE_2 to induce IL-1β transcript levels (Figure 5-1, bottom). The most likely explanation is that M2 polarization may induce an epigenetic change that limits access of transcription factors to the IL-1β promoter. This is another area for future investigation.

Others have reported macrophage polarization stages with characteristic of both M1 and M2. These macrophages are termed regulatory macrophages and it can be achieved by prostaglandin stimulation as well as by stimulation with apoptotic bodies and IL-10[65]. However, the direct effect on IL-1β but no other pro-inflammatory cytokines,

makes us believe that PGE_2 might not be inducing a regulatory stage. To clearly study the effects of PGE_2 in macrophage polarization we might want to study its effects in macrophage metabolism rather than measuring canonical markers of macrophage polarization. This is because macrophage polarization and metabolism are tightly

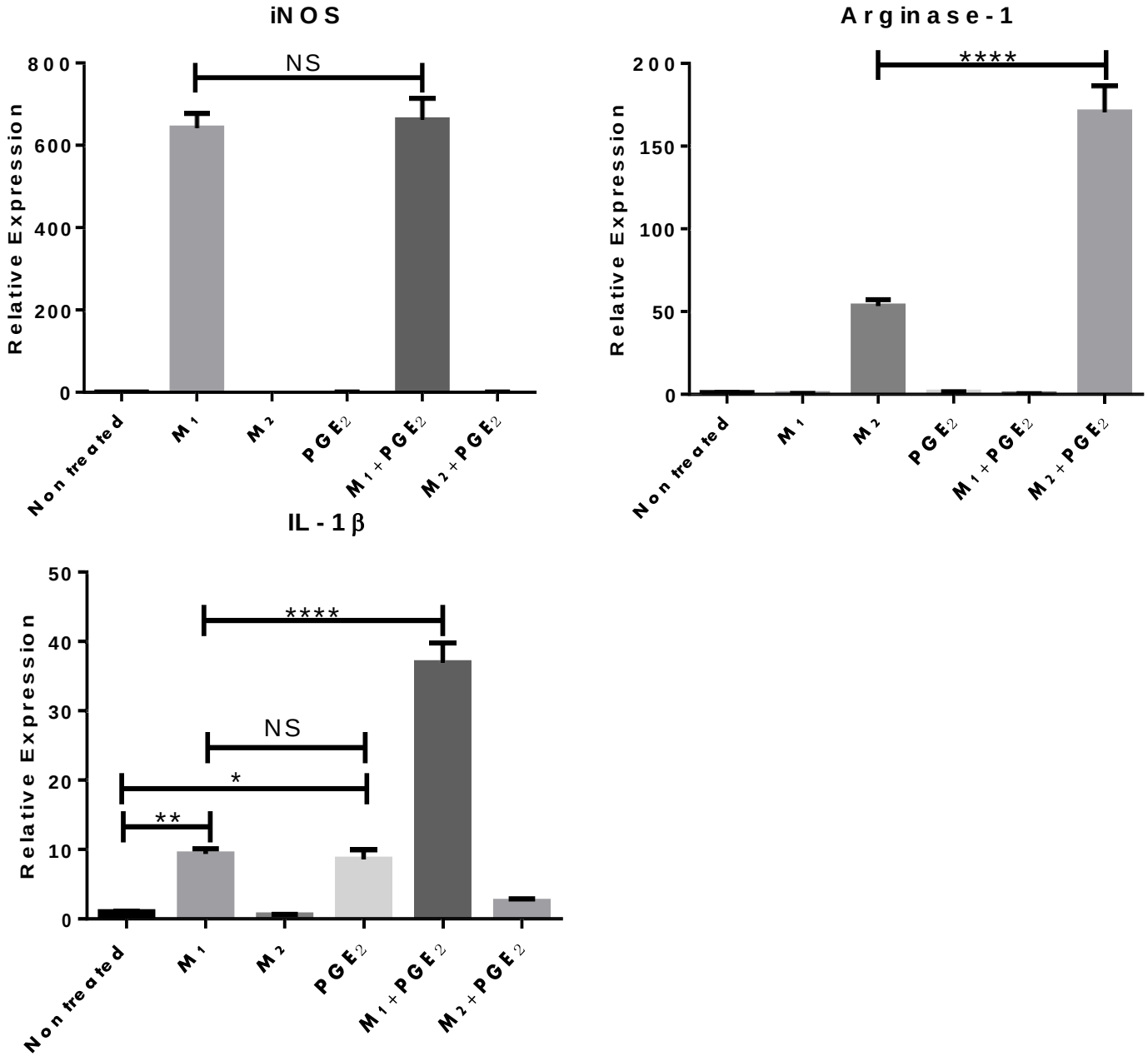

Figure 5-1 PGE_2 increases IL-1β expression without inducing a canonical M1 polarization stage. BMDMs were treated or not with LPS (100ng/ml) and IFN-γ (50ng/ml) to induce M1 stage or treated with IL-14 (10ng/ml) and IL-13 (10ng/ml) to induce M2 stage together or not with PGE2 (1μM). All stimulations were performed for 6 hours and iNOS (top left), Arginase-1 (top right), and IL-1β (bottom) gene expression was assesses by RTq-PCR. One-way ANOVA with Bonferroni's post-test. *P<0.05 ,**P<0.01, ***P<0.001, ****P<0.0001.

associated. M1 macrophages increase glucose consumption and lactate release and decrease oxygen consumption rate whereas M2 macrophages mainly employ oxidative glucose metabolism pathways[66]. Thus, to understand the role of PGE_2 in polarization, future experiments should focus on the effect of PGE_2 in macrophage metabolism.

5.10 Cyclooxygenase (COX) inhibitors as a therapeutic strategy

In this dissertation, I successfully used a non-selective COX inhibitor, indomethacin, to restore immunity to bacterial pathogens in BMT mice and HSCT neutrophils. However, indomethacin, together with other non-steroidal inflammatory drugs (NSAIDs), especially selective COX-2 inhibitors, have been shown to be highly toxic leading to serious gastrointestinal and cardiovascular injury[67, 68]. Thus, therapeutic strategies should focus not just on decreasing PGE_2 levels but also on reducing toxicity. To overcome this, we should use more selective inhibitors of the PGE_2 signaling pathway. In mice, researchers have shown improved outcome in disease models with selective antagonists for EP2 and EP4[23]. Additionally, we could consider specific inhibitors of microsomal PGE synthase-1 (mPGES1), the enzyme downstream of COX and responsible for PGE_2 synthesis, which has been shown not to induce cardiovascular injury. Thus, future experiments should focus on replicating our findings with the use of selective antagonists for EP2 and EP4 as well as inhibitors of mPGES1. Additionally, we can opt for treating our BMT mice with indomethacin loaded nanoparticles[69], which will be ingested by phagocytic cells leading to decreased toxicity, prior to bacterial challenge. This therapeutic strategy has been tried as an inhaled therapy, with other drugs, to treat pulmonary bacterial infections[70].

HSCT patients are routinely prescribed immunosuppressive drugs to treat or prevent GVHD (allogeneic transplant) and or other drugs including antibiotics, antifungal or antiviral drugs to prevent infections[9, 71-73]. Our findings showing a role for PGE_2 inhibition in improving antimicrobial response is not intended to be used as a single treatment post-HSCT, but rather, to be used in combination with other drugs that HSCT patients may also be prescribed. Thus, our findings need to first be tested in Phase 1 clinical trials to help understand the right dose and frequency with which the drug needs to be given in order to reduce PGE_2 levels systemically or locally in the lung and to detect whether there is a significant decrease in frequency of infections compared to placebo control.

In this dissertation we focus particularly on HSCT patients, who are known to have exacerbated levels of PGE_2[74]. However, our findings can be relevant also in a non-HSCT context as naturally secreted PGE_2 can be inhibited to improve bacterial clearance even in cells from control subjects[50, 75-80]. Additionally, a research article, shortly published after our findings in chapter 3, showed how endogenous PGE_2 is necessary for IL-1β induction[81]. In that article, inhibition of PGE_2 by indomethacin led to decreased LPS-induced IL-1β in macrophages. Thus, our findings related to inhibition of PGE_2 to reduce IL-1β-ALI should be tested in a non-HSCT setting as well and may be able to improve outcomes for ALI caused by many stimuli.

5.11 Summary

My doctoral dissertation research work aims to identify cell signaling pathways, mediators, and/or strategies to combat respiratory bacterial infections in HSCT patients. These patients have high levels of PGE_2 in the serum and BALF. PGE_2 is a known

immune suppressor against multiple pathogens. Here, I decided to focus on the implication of high levels of PGE_2 in regulating immunity to bacterial infections. Two of my major research projects are well described in chapter 3 and 4 of this dissertation. In chapter 3, I identified that high levels of PGE_2 induces exacerbated levels of IL-1β in BMT mice leading to severe ALI post-*P. aeruginosa* infection. This is dependent on adenyl cyclase (AC) activation by EP2 and/or EP4 receptor stimulation which leads to activation of the transcription factor CREB. Furthermore, reducing levels of PGE_2 with indomethacin treatments was able to decrease IL-1β levels, improve bacterial killing, and reducing ALI in BMT mice post-*P. aeruginosa* infection. In chapter 4, together with a fellow graduate student at the time, we showed how PGE_2 production impaired NET release in murine and human neutrophils. Furthermore, indomethacin treatments were able to restore NET release. Our findings suggest new therapeutic strategies aimed at blocking PGE_2 production or signaling may have positive impacts against bacterial infections in HSCT subjects.

5.12 References

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Chapter 6.

Influenza-induced immune suppression to Staphylococcus aureus is mediated by TLR9

6.1 Abstract

Bacterial infections, particularly with methicillin-resistant *Staphylococcus aureus* (MRSA), increase mortality following influenza infection, but the mechanisms remain unclear. Expression of TLR9, a microbial DNA sensor, is increased in murine lung immune cells including macrophages, dendritic cells, and $CD8^+$ T cells, post-influenza infection. $TLR9^{-/-}$ mice did not show differences in handling single viral nor bacterial infection. Surprisingly however, $TLR9^{-/-}$ mice have improved survival and bacterial clearance in the lung post-influenza and MRSA coinfection, with no difference in viral load. Improved bacterial clearance post-coinfection was specific for MRSA, as there was no difference in the clearance of *Streptococcus pneumoniae*. Additionally, inhibition of TLR9 *in vivo* reduces lung bacterial burden post-MRSA coinfection. IFN-γ does not play a role in MRSA clearance. Rather, $TLR9^{-/-}$ macrophages exhibit improved bacterial clearance, phagocytosis, and inducible nitric oxide synthase expression (iNOS) post-influenza infection. Thus, TLR9 inhibition improves the clearance of MRSA coinfection potentially by increasing macrophage antimicrobial function.

6.2 Background

Influenza viruses are single-stranded RNA viruses with a segmented genome (8 RNA molecules, encoding 10 proteins) capable of mutating their antigens to evade host immunity and causing seasonal outbreaks, leading to over a half million deaths per year, worldwide (World Health Organization, 2016)[1]. Their nature allows them to overcome traditional vaccine strategies such as inoculation with attenuated viruses, as this will not confer protection to antigenic alterations [2]. There are three types of influenza viruses that can infect humans (A, B, and C). Influenza A virus (IAV) is the most severe and the main cause of seasonal outbreaks. IAV infections can be complicated by bacterial pathogens including *Staphylococcus aureus*, and *Streptococcus pneumoniae* leading to increased morbidity and mortality [3]. Retrospective studies have shown that 95% of the deaths caused by the 1918 influenza pandemic (Spanish Flu) were complicated by bacterial superinfections [3, 4]. Furthermore, whole-blood transcriptome analysis of over 225 influenza-infected patients showed a shift and enrichment in gene signatures from viral response to bacterial response in critically ill patients [5]. Thus, to decrease morbidity and mortality of IAV infections we need a better understanding of how to treat secondary bacterial infections. Past studies have found that IAV infections can lead to secondary bacterial infections in different ways including increasing the attachment sites for bacteria, reducing responsiveness of immune cells, and reducing efficiency of antibiotics [6-8]. Yet, the mechanism underlying influenza-induced mortality is poorly understood and we are still lacking better therapeutic strategies to improve outcomes for influenza-infected individuals.

Toll-like receptors (TLRs) are germline encoded pathogen recognition receptors capable of initiating innate immune responses, and regulating adaptive immunity to both viral and bacterial pathogens[9]. They are primarily expressed in immune cells, and are membrane bound and distributed in the extracellular membrane and endosomes making them able to recognize extracellular and intracellular microbial components [10]. Manipulation of TLRs has shown great potential in combating bacterial infections. For example, agonistic stimulation of TLR4, a lipopolysaccharide (LPS) receptor, has been shown to improve bacterial clearance in *Pseudomonas aeruginosa* infected mice [11]. IAV has been shown to alter the expression of TLRs including downregulation of TLR2, a bacterial lipopeptide sensor, in human monocytes and dendritic cells [12]. TLR2 agonist stimulation was shown to have therapeutic potential when reported to improve survival as well as bacterial and viral clearance in a mouse model of viral-bacterial coinfection [13]. Little is known about the role of other TLRs that are altered in IAV infections and their implications in secondary bacterial infections.

IAV infection was shown to increase the expression of TLR9 in human monocytes and dendritic cells [12]. TLR9 is an endosomal receptor that recognizes unmethylated cytosine and guanine (CpG) motifs which are rich in viral and bacterial DNA and mitochondrial DNA (mDNA)[14, 15]. Here, we aim to study the role of TLR9 in IAV associated bacterial secondary infections, particularly with the bacterial pathogen methicillin-resistant *Staphylococcus aureus* (MRSA). Studying MRSA secondary infections is of high importance as in recent pandemics it has been the main cause of secondary pneumonia in IAV infected individuals [16]. Additionally, MRSA is the leading cause of bacterial

infections in humans worldwide, and infections are difficult to treat as MRSA is resistant to all known β-lactam antibiotics [17].

With the use of a mouse-adapted IAV strain, A/Puerto Rico/8/1934 (PR8), we found that TLR9 expression is elevated in lung macrophages, dendritic cells, and CD8 T cells from PR8 infected mice. TLR9$^{-/-}$ mice infected with PR8 or MRSA did not differ from wildtype (WT) mice, but they experience improved survival post PR8-MRSA coinfection. Even though TLR9$^{-/-}$ mice didn't have a difference in viral titers or acute lung injury in the measured time points, they did experience improved bacterial clearance post coinfection. The resistance to coinfection found in TLR9$^{-/-}$ mice was specific to the synergism between PR8 and MRSA, as TLR9$^{-/-}$ mice did differ in bacterial clearance when challenged with PR8 and *Streptococcus pneumoniae*. Interestingly, TLR9$^{-/-}$ macrophages post-PR8 infection experience increased bacterial phagocytosis and intracellular killing. Additionally, TLR9$^{-/-}$ macrophages have increased inducible nitric oxide synthase (iNOS) expression post-PR8 compared to WT. Our findings show a previously unrecognized role of TLR9 in influenza infection, in which TLR9 inhibition can serve to combat secondary MRSA superinfections.

6.3 Results

IAV increases expression of TLR9 in macrophages. Changes in expression of different toll-like receptors (TLRs) (TLR2, TLR3, TLR4, TLR7, TLR8, and TLR9) have been reported before in human monocytes and dendritic cells from seasonal influenza infected patients [12]. In our murine experiments, we noticed that just TLR9 gene expression is increased in lung immune cells 5 days post-PR8 infection (Fig.6-1A).

Protein expression was also increased in these cells post-PR8 infection as measured by TLR9 immunoblotting (Fig.6-1B). To understand which cells were upregulating TLR9, we used flow cytometry to characterize the major immune cells in the lung compartment. We noticed that CD8 T cells, macrophages (interstitial and alveolar), and dendritic cells were the main cells expressing higher TLR9 expression post-PR8 (Fig.6-1C). We did not notice changes in CD4 T cells, NK cells, B cells, and neutrophils (Fig.6-1C). Adherence selection of lung macrophages, after lung collagenase digestion, allowed us to detect gene expression changes in multiple TLRs in lung monocytes/macrophages after influenza infection. We noticed that apart from TLR9 being increased, TLR3 and TLR2 were also altered with increased and decreased expression, respectively (Fig.6-1D). These later observations were also seen in human monocytes of influenza-infected individuals [12]. Interestingly, direct infection of isolated alveolar macrophages with PR8 shows an increase in TLR9 and TLR3, with no changes in TLR2, 4, and 7 (Fig.6-1E). We also detected TLR9 increased 24 hours post-PR8 infection in cultured bone marrow derived macrophages (BMDMs) (Fig.6-2A). To our knowledge, we are the first to report that direct infection of macrophages with IAV can increase TLR9 expression. This increase was likely independent of IAV recognition by TLR7, or by IAV-induced release of CpG rich mDNA as TLR7, and TLR9 agonist stimulation lead to downregulation of TLR7 and TLR9 gene expression (Fig.6-2B).

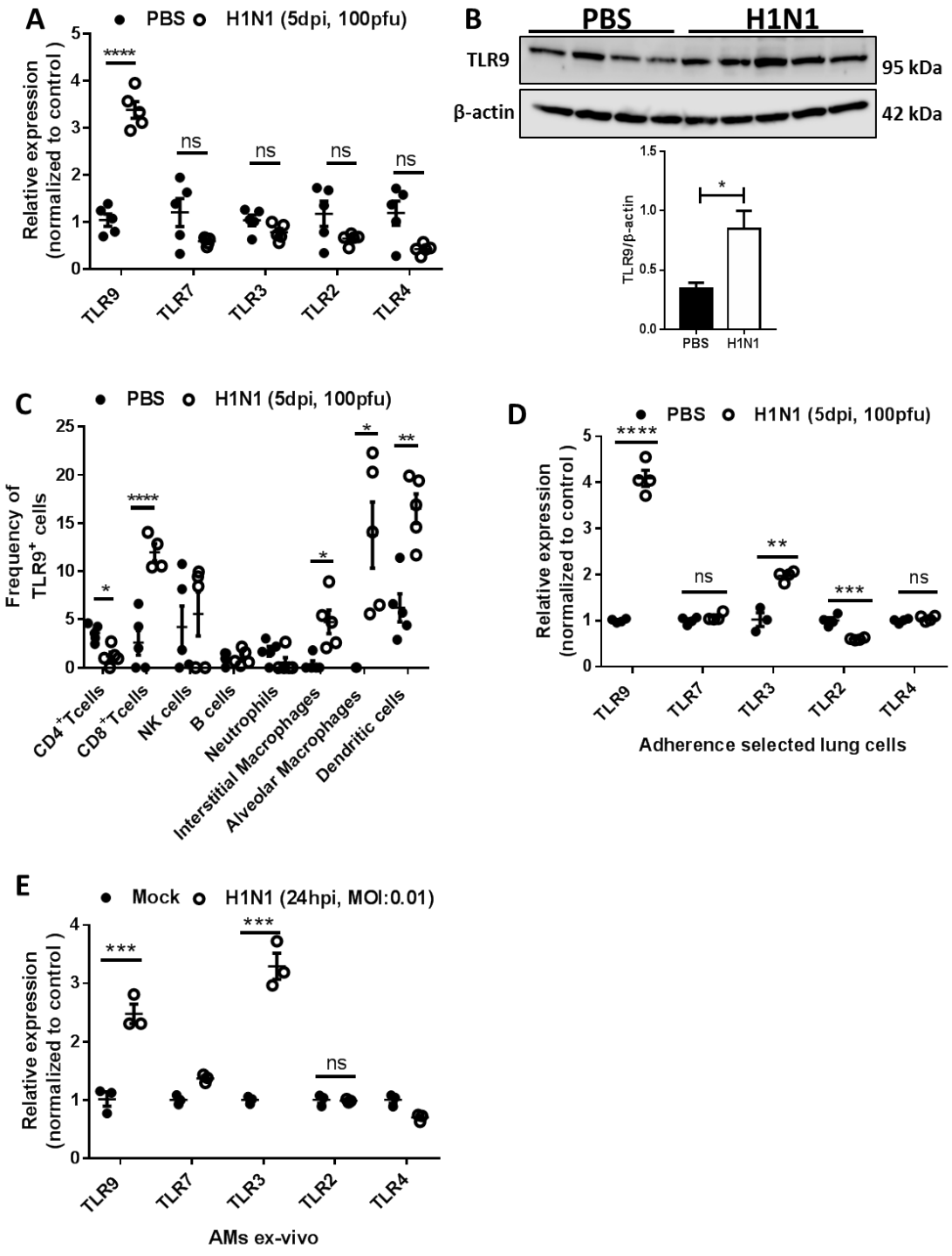

Figure 6-1: TLR9 overexpression in lung immune cells post-IAV infection. (A) Relative gene expression of TLRs by RTqPCR and (B) western blotting of TLR9 and β-actin. RNA and protein were isolated from lung immune cells post-collagenase digestion in mice infected with 100 PFUs of H1N1 (PR8) for 5 days or placebo (PBS). β-actin was used to normalize RNA in samples. (C) Frequency of TLR9$^+$ cells measured by flow cytometry in lung immune cells post-collagenase digestion. Gating was as follows: CD4$^+$ T cells (CD45$^+$,CD90.2$^+$, CD3$^+$, CD4$^+$), CD8$^+$ T cells (CD45$^+$,CD90.2$^+$, CD3$^+$, CD4$^-$, CD8$^+$), natural killer cells (CD45$^+$,CD90.2$^+$, NKp46$^+$), B cells (CD45$^+$,CD90.2$^-$, CD19$^+$), neutrophils (CD45$^+$, CD11b$^+$, LY6G$^+$), interstitial macrophages (CD45$^+$, CD64$^+$, CD11b$^+$, F4/80$^+$), alveolar

macrophages (CD45^{+}, CD64^{+}, CD11C^{+}, Siglec F^{+}) and dendritic cells (CD45^{+}, CD64^{-}, CD11c^{+}, MCHII^{high}). (D) Relative expression of TLRs after lung macrophage isolation by adherence selection from PBS or IAV-infected mice. (E) Relative expression of TLRs in alveolar macrophages infected or not ex-vivo with H1N1 (MOI:0.01) for 24 hours. Statistics are student T test between comparative groups. *P<0.05 ,**P<0.01, ***P<0.001, ****P<0.0001.

TLR9$^{-/-}$ mice are resistant to IAV and MRSA coinfection. Secondary bacterial infections in influenza infected individuals are a main cause of mortality and morbidity [18]. To study this synergistic effect between the virus and the bacteria, we used a well-known IAV-MRSA coinfection model consisting of an initial viral infection followed by a secondary bacterial infection (Fig.6-3A); where we can detect susceptibility to MRSA infection as early as 5 days post-PR8 infection (Fig.6-4). We observed a dramatic survival difference between TLR9$^{-/-}$ and WT mice, where 70% of TLR9$^{-/-}$ mice survived the secondary bacterial infection compared to 10% of WT mice (Fig.6-3B). There were no significant differences in weight changes between TLR9$^{-/-}$ and WT mice noted (Fig.6-3C).

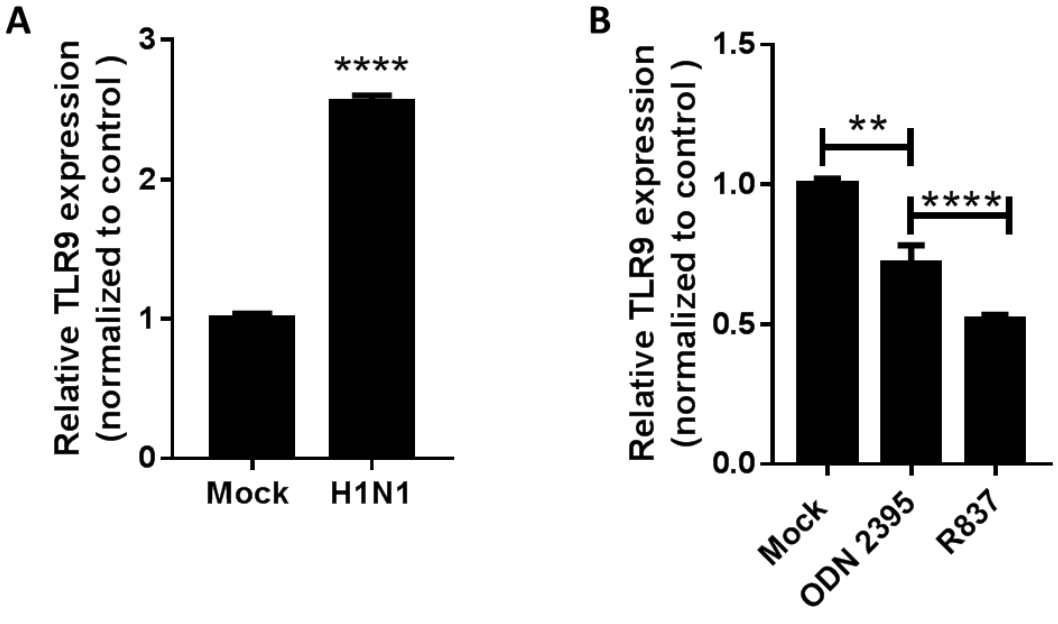

Figure 6-2: TLR7 or TLR9 agonist stimulation does not increase expression of TLR9. (A) BMDMs infected or not with IAV at a MOI of 0.01 for 48 hours. (B) BMDMs were treated with the TLR9 agonist, ODN 2395, or with the TLR7 agonist, imiquimod (R837), for 24 hours at a concentration of 1μM or 1μg/ml, respectively. RNA was isolated

and TLR9 transcript expression was measured by RTqPCR. Statistics are student T test between comparative groups;***P<0.001.

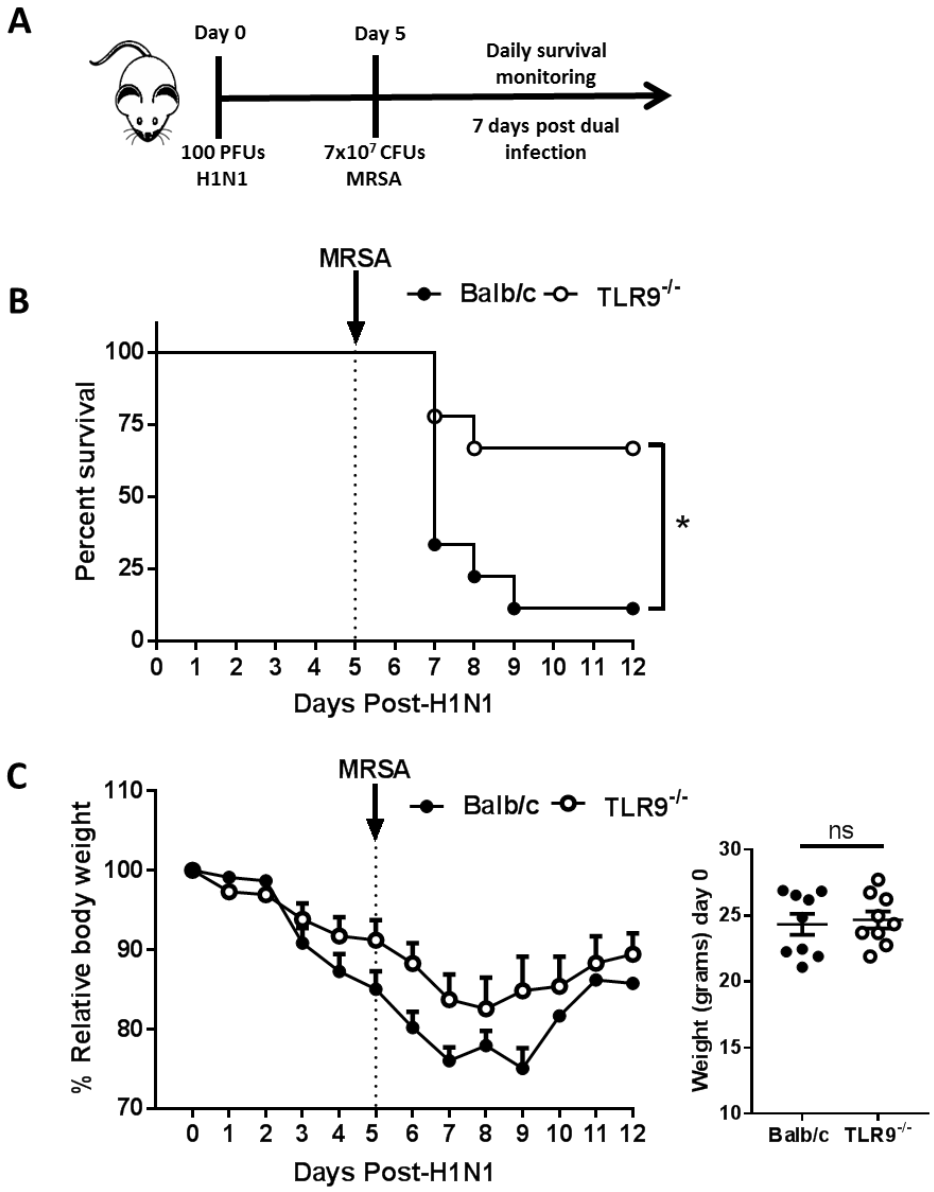

Figure 6-3: TLR9$^{-/-}$ mice are resistant to secondary bacterial infection. (A) Sketch of infection model where Balb/c and TLR9$^{-/-}$ mice are infected with 100 PFUs of H1N1 5 days prior to infection with 7x10^7 CFUs of MRSA (US300). Mice are monitored daily to check for survival. (B) Survival assay of Balb/c (n=9) and TLR9-/- (n=9) mice following coinfection. (C) Weight changes (left) and initial weight (right) of all mice used in survival assay. Statistics for weight changes and initial weight were student T test, non-significant (ns). Statistics in survival assay were done with the Log-rank (Mantel-Cox), *P<0.05.

TLR9$^{-/-}$ and WT mice show similar susceptibility to MRSA infection alone.

Currently, there are conflicting results regarding the role of TLR9 in single MRSA infection [19, 20]. To understand if TLR9$^{-/-}$ mice are susceptible to a single MRSA infection

independent of the initial IAV infection, we performed a survival assay post-MRSA infection. We did not detect a difference in survival between TLR9$^{-/-}$ and WT mice (Fig.6-5A). Interestingly, weight recovery during the infection was slower in TLR9$^{-/-}$ mice, but mice were able to recover the lost weight during the survival monitoring (Fig.6-5B). We detected reduced immune cell infiltration in the alveolar compartment post-MRSA infection in TLR9$^{-/-}$ mice (Fig.6-5C). However, this did not affect bacterial clearance or lung injury which was reduced significantly 48 hours post-infection (Fig.6-5D-E). Previous work has shown that TLR9$^{-/-}$ mice have reduced TNF-α in the BALF post-MRSA [20]. We detected lower amounts of TNF-α, IL-6, and IL-10 in the BALF of MRSA-infected TLR9$^{-/-}$ mice (Fig.6-5F). Yet, lower amounts of these cytokines did not have a negative effect on bacterial clearance, lung injury, or survival. Reduced immune cell infiltration and cytokine profile might be explained by lower NF-κB activation post-MRSA infection as TLR9 is a sensor of bacterial DNA [21].

TLR9$^{-/-}$ mice experience improved MRSA clearance post-coinfection. We were able to detect better bacterial clearance in the IAV-infected TLR9$^{-/-}$ mice 24 hours post-MRSA infection, but no difference in tissue injury or viral load (Fig.6-6A-C). Synthetic oligonucleotides (ODNs) are single-stranded synthetic DNA molecules containing unmethylated CpG which can be modified to work as either agonists or antagonists of TLR9. To test whether TLR9 signaling inhibition during viral-bacterial coinfection can lead to improved bacterial clearance, we treated WT mice with 3 equal doses of a TLR9 antagonist, ODN 2088 (Fig.6-6D). As expected, mice that were treated with TLR9 antagonist experience improved bacterial clearance compared to mice treated with

control ODN (Fig.6-6D). These findings suggest that TLR9 blockade during IAV infection can improve immunity to a secondary MRSA infection.

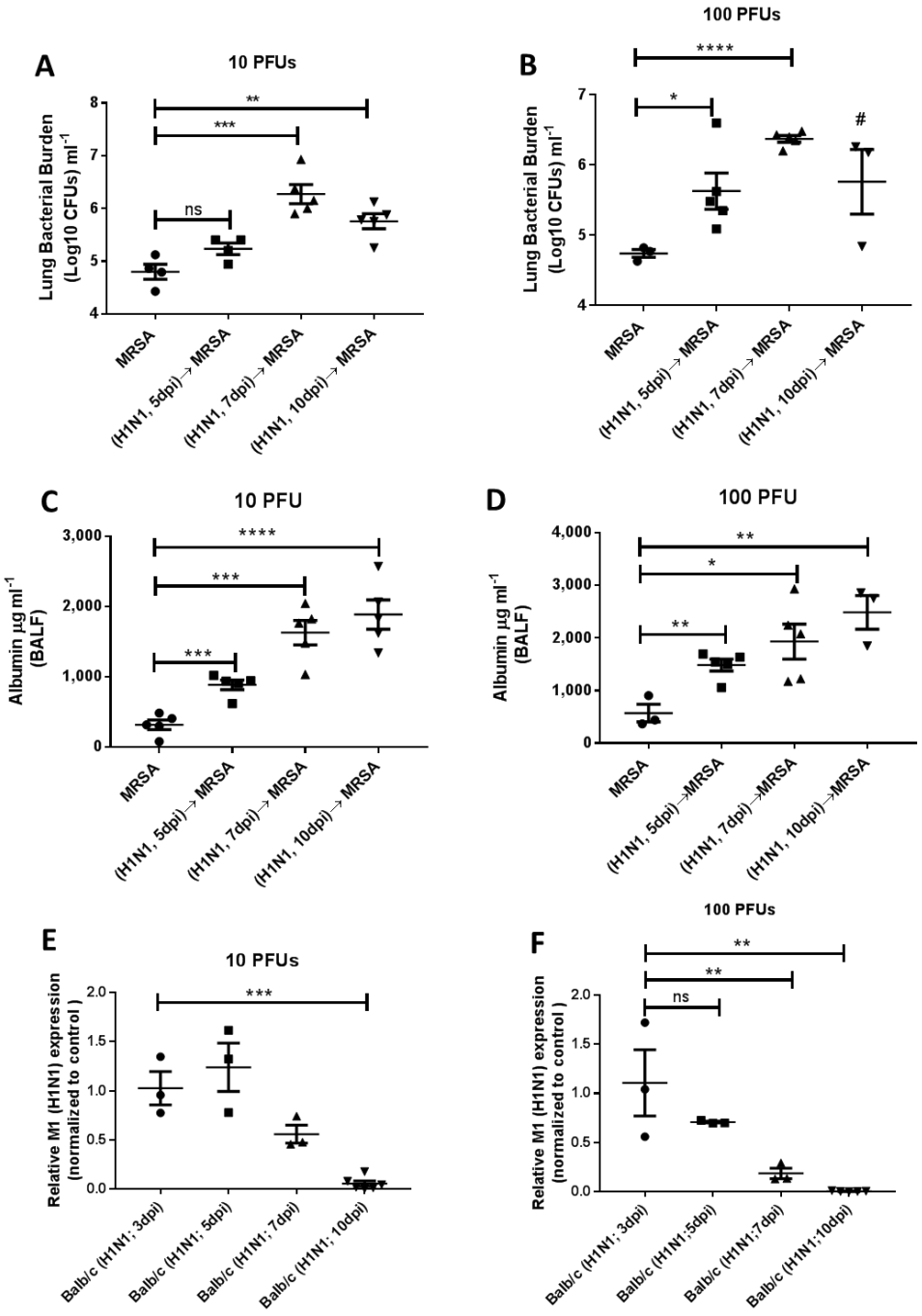

Figure 6-4: Reduced MRSA clearance, and exacerbated lung tissue injury in the lung post-IAV infection. Bacterial load measurement in the whole lung of mice infected, or not, with (a) 10 PFUs or (b) 100 PFUs and co-infected with MRSA 5, 7 or 10 days post-H1N1 infection. Albumin measurements from the BALF of (c) 10 PFUs or (d) 100PFUs IAV-infected or not mice for 5, 7 and 10 days and co-infected with MRSA for 24 hours. Relative expression

of M1 viral gene in lungs of mice infected with 10 PFUs (E) or 100 PFUs (F) of IAV, samples were taken on days 3, 5, 7, and 10 post-infection. Statistics are student T test between comparative groups. *P<0.05 ,**P<0.01, ***P<0.001, ****P<0.0001; # two mice died in this group before bacterial load measurement. 0 dpi mice were infected with placebo, PBS, 5 days before MRSA coinfection.

Higher bacterial clearance in TLR9$^{-/-}$ mice post-coinfection is independent of exacerbated levels of IFN-γ. To understand whether TLR9$^{-/-}$ mice are clearing the bacteria better post coinfection due to differences in their cytokine profile, we measured the levels of different pro and anti-inflammatory cytokines. We detected reduced amounts of TNF-α, IL-1β, IL-6, and IL-17, but increased levels of IFN-γ (Fig.6-7A). High levels of IFN-γ have been shown to improve MRSA clearance due to full activation of macrophages [22]. Together with this, we detected higher numbers of TH1 (CD45^{+},CD90.2^{+},CD4^{+},IFN-γ^{+}), CD8 T (CD45^{+},CD90.2^{+},CD4^{-},CD8^{+},IFN-γ^{+}), and NK (CD45^{+},CD90.2^{+},NKP46^{+},IFN-γ^{+}) cells, but no difference in other immune cells including B cells (CD45^{+},CD90.2^{-},CD19^{+}), and CD4 T (CD45^{+},CD90.2^{+},CD4^{+}) cells (Fig.6-7C). To test whether high levels of IFN-γ in the bronchoalveolar lavage fluid (BALF) were responsible for the improved bacterial clearance in TLR9$^{-/-}$ mice, we neutralized IFN-γ during IAV-MRSA coinfection. To our surprise, there was no difference between isotype-treated and IFN-γ-neutralized mice (Fig.6-7C-D). Thus, improved bacterial clearance in TLR9$^{-/-}$ mice is independent of high levels of IFN-γ.

IFN-γ inhibits *Streptococcus pnuemoniae* (SPS3) clearance but has no effect on MRSA. IFN-γ has been shown to play a negative role in SPS3 clearance post-IAV infection by decreasing the expression of macrophage receptor with collagenous structure (MARCO) [23]. Just like MRSA, SPS3 is a Gram-positive bacterial pathogen that

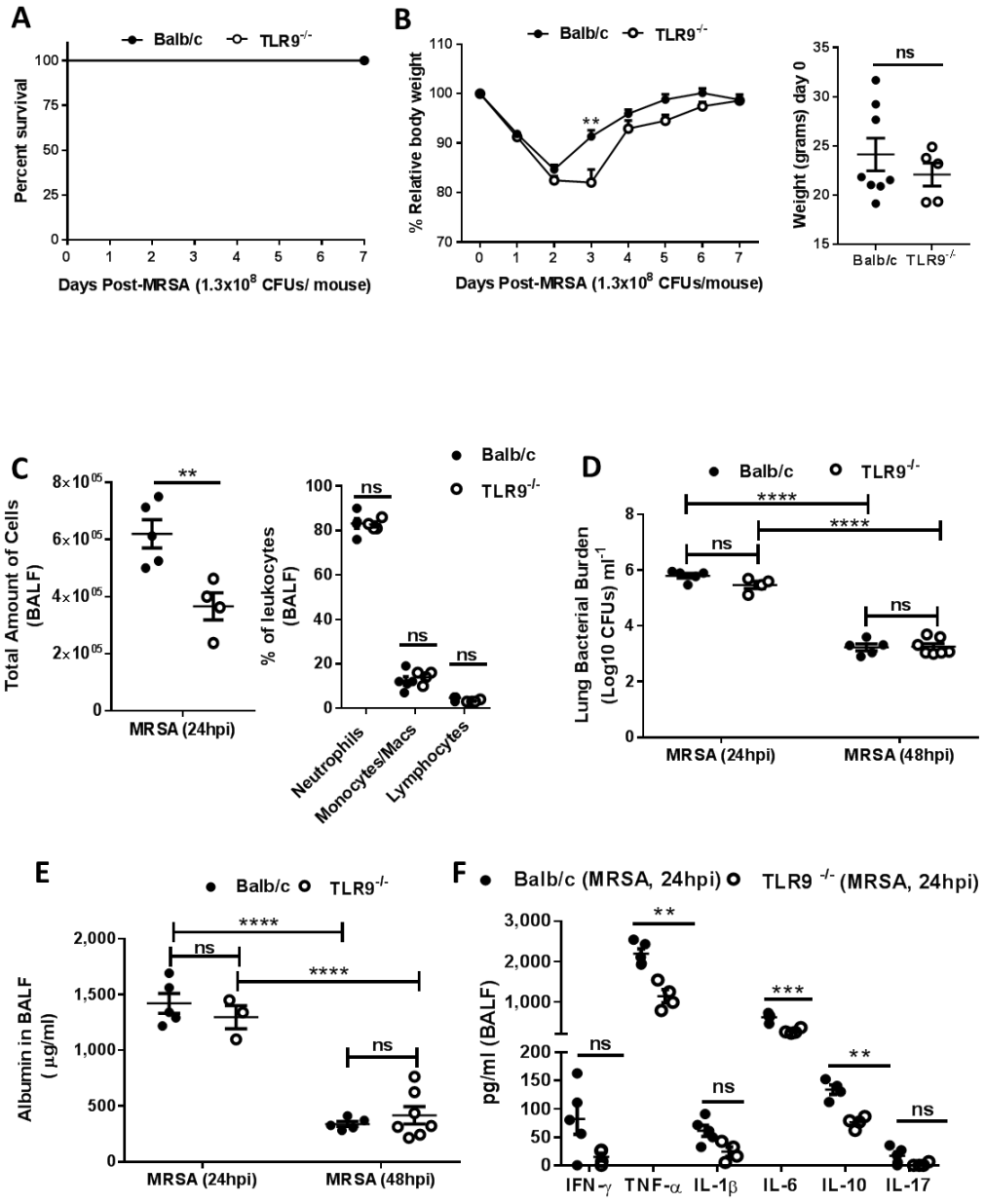

Figure 6-5: TLR9$^{-/-}$ mice experience no difference in MRSA clearance. (A) Survival assay of BALB/c (n=8) and TLR9$^{-/-}$ (n=5) mice infected with 1.3x10^8 CFUs of MRSA (US300). (B) Weight changes (left) and initial body weight (right) of mice used in survival assay. (C) Total amount of cells (left) and percentage of leukocytes (right) in the alveolar compartment of BALB/c and TLR9$^{-/-}$ mice quantified using a hemocytometer and differential staining; samples were taken by BAL 24 hours post-MRSA (7x10^7 CFUs) infection. (D) Lung bacterial burden & (E) Albumin measurements in the BALF 24 and 48 hours post-MRSA (7x10^7 CFUs) infection in BALB/c and TLR9$^{-/-}$ mice. (F) Cytokine levels in the BALF of BALB/c and TLR9$^{-/-}$ mice infected with MRSA (7x10^7 CFUs) for 24 hours. Cytokines were measured by ELISA. (B), (C) & (F) Statistics are student T test between comparative groups; ns=non-significant, **P<0.01, ***P<0.001. (D) and (E) statistics were obtained by one-way analysis of variance with Bonferroni's posttest. ****P<0.0001.

is a high threat to influenza-infected individuals [24, 25]. When examining outcomes with IAV and SPS3 more fully, we found that TLR9$^{-/-}$ mice have no difference in SPS3 clearance with or without an initial influenza infection (Fig.6-8A). Thus, improved bacterial clearance in TLR9$^{-/-}$ mice post-IAV seems to be specific to MRSA. INF-γ levels in the BALF of TLR9$^{-/-}$ mice after IAV-SPS3 infection were not significantly higher than in WT mice (Fig.6-8B). To test whether high levels of IFN-γ have a different effect on MRSA and SPS3 clearance, we gave recombinant IFN-γ intratracheally before the bacterial infection and measured lung bacterial burden 24 hours after infection. As expected, we were able to detect immunosuppression to SPS3, but IFN-γ had no effect on the clearance of MRSA. The negative role of IFN-γ in the pathogenesis of SPS3 post-IAV was attributed to decreased expression of MARCO. We detected lower expression of MARCO with higher expression of SRAI/II in macrophages from mice infected with IAV (Fig.6-9). Previous work reported that decreased expression of MARCO but increased expression of SRAI/II can improve phagocytosis of MRSA [26]. To test if IFN-γ has different effects on phagocytosis of MRSA and SPS3, we treated BMDMs with IFN-γ and assessed their phagocytosis capability. We found that IFN-γ improves MRSA uptake, but decreases SPS3 uptake (Fig.6-10B). This effect was not seen in opsonized bacteria, suggesting the effect is specific to alteration in scavenger receptors (Fig.6-10C). Thus, even though IFN-γ improves MRSA uptake, it doesn't improves its clearance (Fig.6-10D).

TLR9$^{-/-}$ lung macrophages have increased phagocytosis, bacterial killing, and iNOS expression post-IAV infection. To determine if IAV was inducing changes to

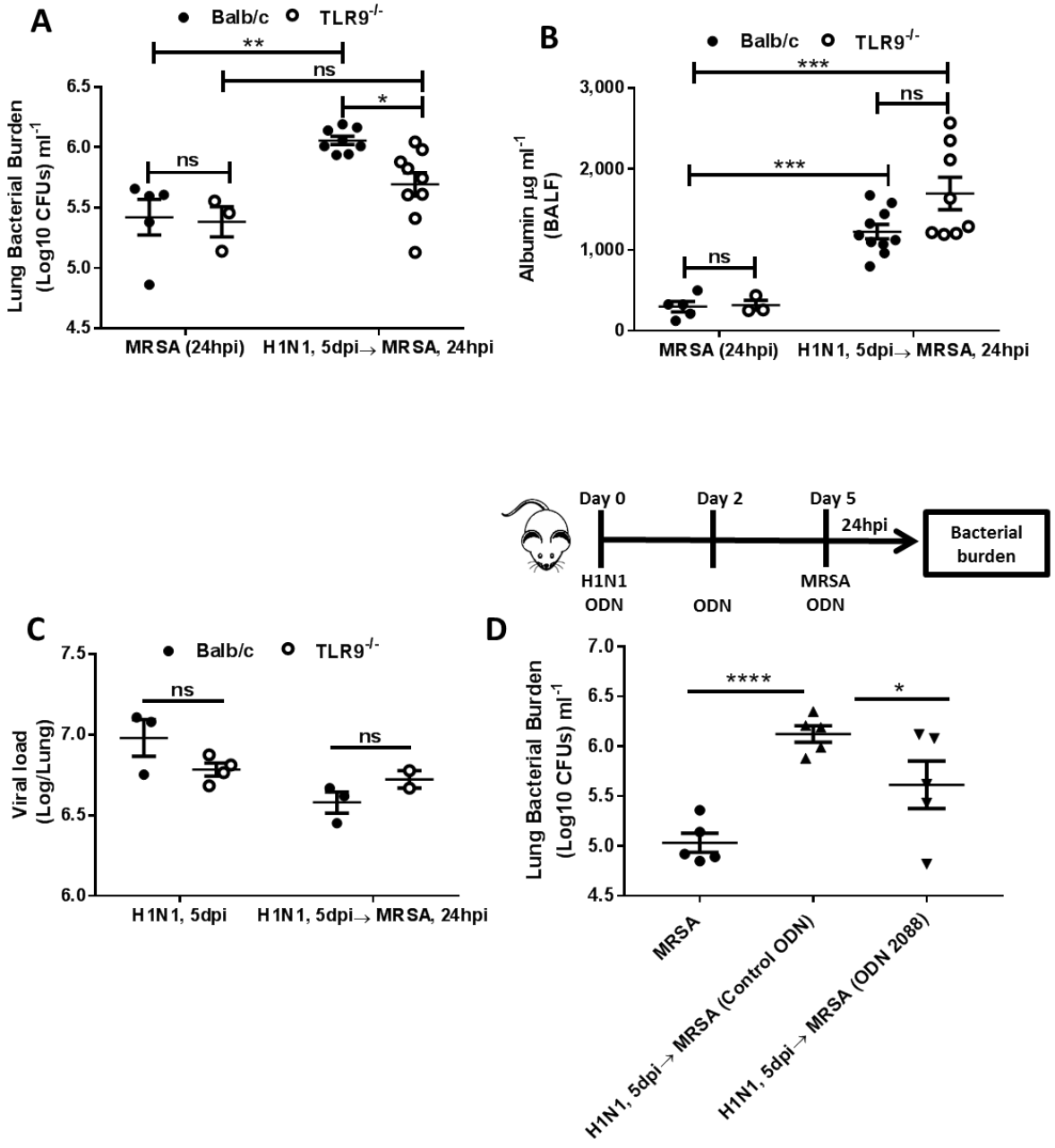

Figure 6-6: TLR9$^{-/-}$ mice experience improved bacterial clearance post-IAV and MRSA coinfection. (A) Bacterial burden in lungs & (B) Albumin levels in BALF from BALB/c and TLR9$^{-/-}$ mice that were infected with IAV (100 PFUs, H1N1) or treated with placebo, PBS, 5 days prior to MRSA ($7x10^7$ CFUs) infection; samples were harvested 24 hours post-MRSA infection. (C) Quantification of influenza titers in whole lung of BALB/c and TLR9$^{-/-}$ mice infected with influenza (100 PFUs, H1N1) 5 days prior to MRSA ($7x10^7$ CFUs) infection or PBS treatment. (D) Lung bacterial burden from BALB/c mice treated with three doses (50µg) of control ODN or TLR9 inhibitor (ODN 2088) and infected or not with IAV (100 PFUs, H1N1) 5 days prior to MRSA ($7x10^7$ CFUs) infection. (A-C) Statistics were obtained by one-way analysis of variance with Bonferroni's posttest; ns=non-significant, *P<0.05, **P<0.01, ***P<0.001. (D) Statistics are student T test between comparative groups; ns=non-significant, **P<0.01, ***P<0.001.

TLR9$^{-/-}$ mice that were leading to resistance to a MRSA infection, we measured the cytokine profile in the BALF, and lung immune cell profile in TLR9$^{-/-}$ mice post-single IAV infection. There was no difference in cytokines or lung immune cells 5 days post-IAV infection (Fig.6-11A and Fig.6-12). Interestingly, macrophages isolated from IAV-infected TLR9$^{-/-}$ mice have increased intracellular MRSA clearance and phagocytosis (Fig.6-11B-C). Additionally, TLR9$^{-/-}$ lung macrophages have higher levels of iNOS post-IAV compared to WT (Fig.6-11D). Nitric oxide production has been shown to be crucial in clearing MRSA infection [27]. To test whether TLR9 expression might suppress iNOS increase, we infected BMDMs from WT and TLR9$^{-/-}$ mice and measured iNOS expression. TLR9$^{-/-}$ macrophages have higher expression of iNOS post-IAV infection suggesting that TLR9 is a negative regulator of iNOS expression post-IAV infection.

6.4 Discussion

Lower respiratory infections are the fourth leading cause of death with 3 million deaths each year worldwide (WHO, 2018). The influenza virus infects the upper and lower respiratory tract and is successful at infecting 3-5 million individuals each year, taking the life of nearly a half million of these individuals [1]. Severe illness and death in influenza infections are seen mostly in high risk subjects, the very young and elderly. However, recent influenza outbreaks have taken the life of young healthy citizens creating public health concerns. Secondary bacterial superinfections are responsible for the high morbidity and mortality in influenza-infected patients [18]. Even with proper care including influenza vaccines, hygiene, and antibiotics, influenza-associated secondary bacterial infections are a burden to public health [28]. Additionally, the over-use of

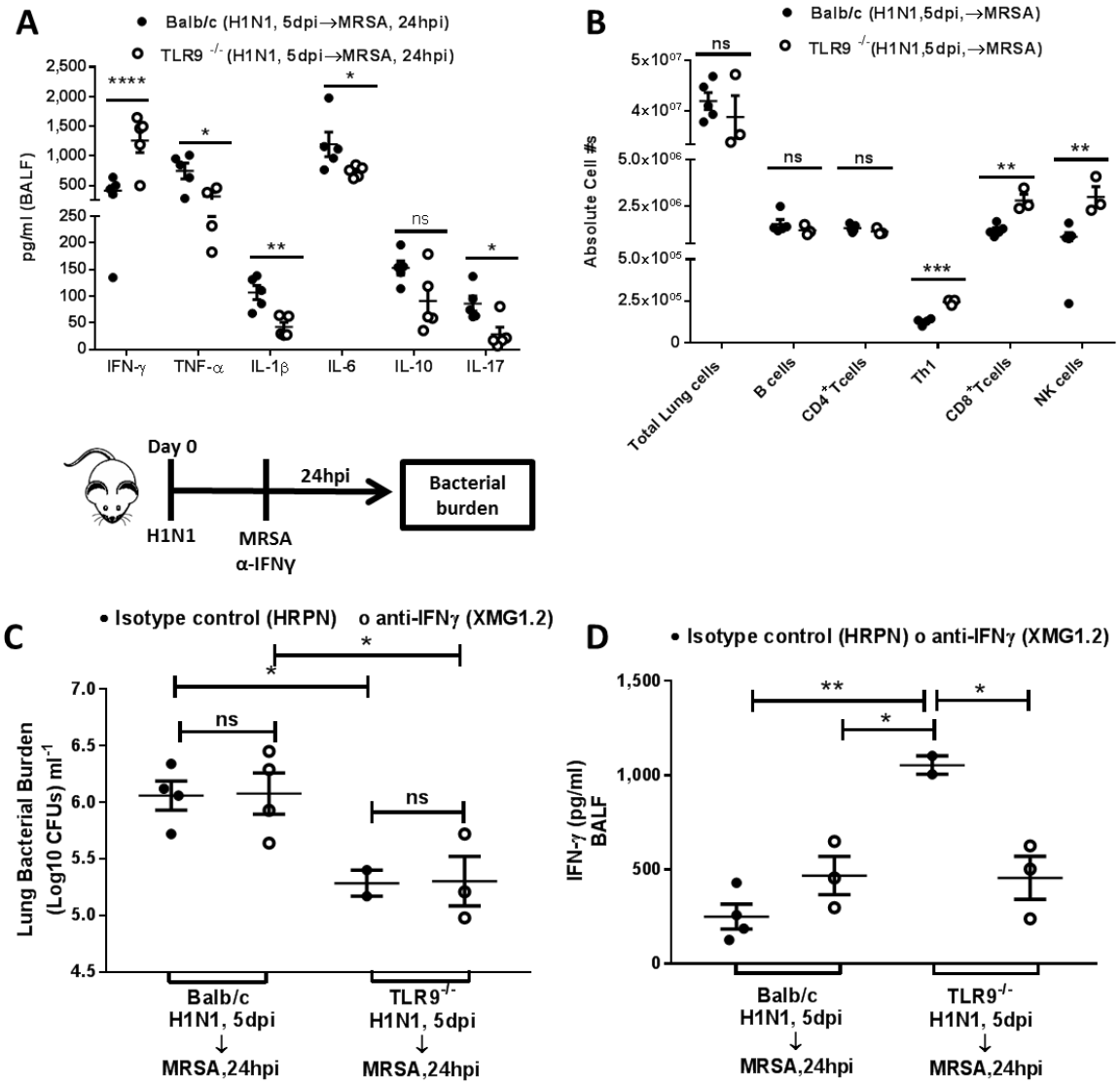

Figure 6-7: Higher bacterial clearance in TLR9$^{-/-}$ mice post-IAV and MRSA coinfection is independent of exacerbated levels of IFN-γ. (A) Cytokine measurement in BALF from BALB/c and TLR9$^{-/-}$ mice that were infected with IAV (100 PFUs, H1N1) 5 days prior to MRSA (7x10^7 CFUs) infection. (B) Absolute number of lung immune cells post-lung collagenase digestion in BALB/c and TLR9$^{-/-}$ mice that were infected with IAV (100 PFUs, H1N1) 5 days prior to MRSA (7x10^7 CFUs) infection. Cells were quantified by flow cytometry; gating was as follow: B cells (CD45$^+$CD90.2$^-$CD19$^+$); CD4$^+$ T cells (CD45$^+$CD90.2$^+$CD4$^+$); TH1 (CD45$^+$CD90.2$^+$CD4$^+$,IFN-γ$^+$); CD8$^+$ T cells (CD45$^+$CD90.2$^+$CD4$^-$,CD8$^+$,IFN-γ$^+$); NK cells (CD45$^+$CD90.2$^+$NKP46$^+$,IFN-γ$^+$). (C) Lung bacterial burden and (D) IFN-γ levels in BALB/c and TLR9$^{-/-}$ mice that were treated with 200μg of IFN-γ neutralizing antibody or isotype control and infected with IAV (100 PFUs, H1N1) 5 days prior to MRSA (7x10^7 CFUs) infection. (A-B) Statistics are student T test between comparative groups; ns=non-significant, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. (C-D) Statistics were obtained by one-way analysis of variance with Bonferroni's posttest; ns=non-significant, *P<0.05, **P<0.01.

antibiotics has led to the selection of multidrug resistant bacterial pathogens making it harder to reduce the severity of bacterial infections [17]. Thus, we are currently in need of better therapeutic strategies against viral-bacterial co-infections that can improve public health. Influenza infections have been shown to alter the expression of TLRs in immune cells [12]. Manipulation of TLRs, in particular TLR2, has been shown to improve survival, and microbial clearance in mice coinfected with influenza and bacterial pathogens [13]. However, little is known about the potential roles that other TLRs can have in controlling viral-bacterial coinfections.

TLR9 is an intracellular receptor that recognizes unmethylated CpG motifs which are rich in microbial DNA [14]. TLR9 expression was reported to be elevated in monocytes and dendritic cells from influenza-infected patients compared to healthy individuals [12]. Here, we noted that IAV infection similarly increases TLR9 expression in murine immune cells from mice infected with a mouse-adapted IAV strain (PR8) (Fig.6-1A-D). This increase can also be achieved in cultured alveolar macrophages (Fig.6-1E) and BMDMs (Supplemental Fig.6-1A) infected *in vitro*. We tested whether stimulation of TLR7, the innate influenza sensor, could lead to increased expression of TLR9 as it is known that NF-κB activation by TLRs can induce TLR expression [29]. However, we did not detect upregulation of TLR9 after TLR7 stimulation (Fig.6-2A). Influenza infections can lead to mitochondrial membrane permeabilization and release of mitochondrial components[30]. Release of mDNA can also lead to activation of TLR9 due to mDNA's high concentration of unmethylated CpG [15, 21]. However, we found that CpG oligonucleotide stimulation of TLR9 did not increase TLR9 expression in our

experiments (Fig.6-2B). Thus, there's still no clear evidence for how the IAV virus leads to the increased TLR9 expression noted in mice and humans.

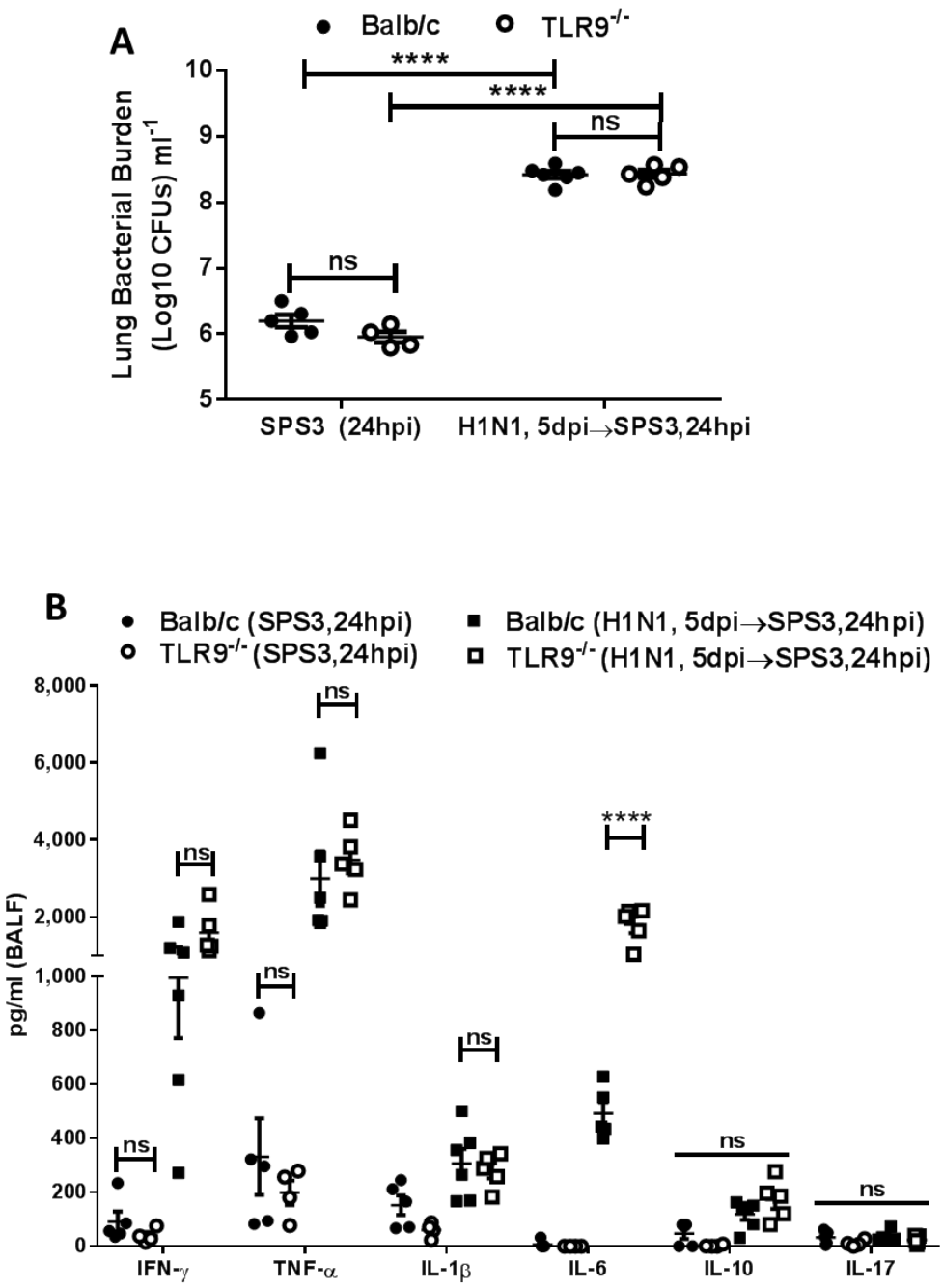

Figure 6-8: TLR9$^{-/-}$ mice have no difference in clearance of *Streptococcus pnuemoniae* and have no difference in IFN-γ. (A) Lung bacterial burden and (B) cytokine levels in BALB/c and TLR9$^{-/-}$ mice infected with IAV (100 PFUs, H1N1), or treated with PBS, 5 days prior to *Streptococcus pneumoniae* (SPS3) (3x10^5 CFUs) infection; samples were taken 24 hours post SPS3 infection. Statistics are student T test between comparative groups. Non-significant (ns), *P<0.05 ,**P<0.01, ***P<0.001, ****P<0.0001.

Mice lacking TLR9 (TLR9$^{-/-}$ mice) did not differ in viral response against IAV compared to WT as there was no difference in measured viral titers, cytokine profiles, tissue injury, or immune cell infiltration (Fig.6-6C, Fig.6-8A, and Fig.6-12). However, TLR9$^{-/-}$ mice were resistant to an IAV-MRSA coinfection with improved bacterial clearance (Fig.6-3 and Fig.6-6A). Interestingly, TLR9$^{-/-}$ mice did not clear the virus differently and there was no difference in acute tissue injury (ATI) post coinfection (Fig.6-6B-C). The improved clearance of MRSA in TLR9$^{-/-}$ mice was not due to a preexistent resistance to the bacteria either as there was no difference in single MRSA infection (Fig.6-5) between WT and TLR9$^{-/-}$ mice. Previous reports focused on the role of TLR9 in single MRSA infection have shown conflicting results. MRSA was shown to induce a type I interferon response dependent on TLR9, and TLR9$^{-/-}$ mice were reported to have lower TNF-α and improved bacterial clearance[19]. In contrast, TLR9$^{-/-}$ mice were reported to have decreased MRSA clearance despite the lower amount of TNF-α [20]. Similar to the previous findings, we noticed a decrease in cytokine secretion in TLR9$^{-/-}$ mice, specifically TNF-α was lower (Fig.6-5F); however, TLR9$^{-/-}$ mice did not differ from WT in bacterial clearance, survival and ATI despite lower lung immune cell infiltration and cytokine release (Fig.6-5). Thus, TLR9 seems to play a unique role in resistance to MRSA only in the context of secondary bacterial infection post-IAV.

TLR9$^{-/-}$ mice have increased IFN-γ in the BALF together with higher numbers of IFN-γ producing cells (TH1, CD8 T cells, and NK cells) in the lung post coinfection (Fig.6-7A-

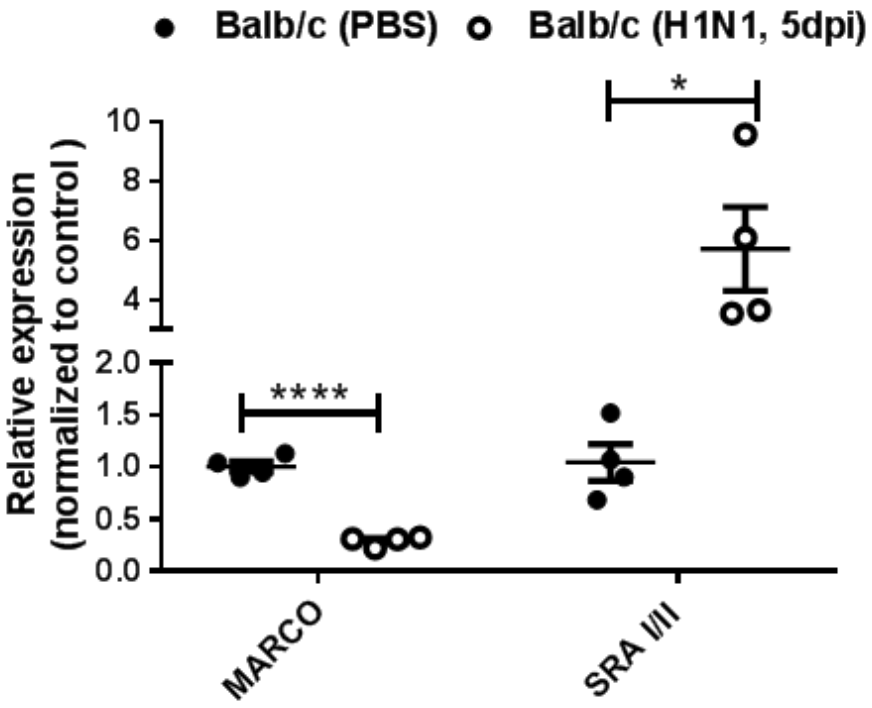

Figure 6-9: IAV infection alters scavenger receptor levels. MARCO and SRA I/II relative expression in macrophages from BALB/c mice 5 days post-IAV infection at a 100 PFUs. Statistics are student T test between comparative groups; *P<0.05, ****P<0.0001

B). This increase in IFN-γ provided a potential explanation for the improved bacterial clearance in TLR9$^{-/-}$ mice as IFN-γ has been shown to increase clearance of MRSA [22]. However, in our studies, INF-γ neutralization (confirmed by ELISA (Fig 6-7D) and bioassay (data not shown) was not able to decrease clearance of MRSA in TLR9$^{-/-}$ mice (Fig.6-7C). Therefore, the enhanced clearance on MRSA is independent of IFN-γ. As this cytokine was shown before to decrease clearance of SPS3 post-IAV [23], we investigated whether IFN-γ can have a different effect between MRSA and SPS3. Treating mice with IFN-γ and then challenging with MRSA or SPS3 showed that IFN-γ can decrease clearance of SPS3 but has no effect on MRSA clearance (Fig.6-10A). In culture, IFN-γ can decrease phagocytosis of SPS3 whereas this cytokine actually improves the phagocytosis of MRSA (Fig.6-10B). However, despite improved

phagocytosis, macrophages can't effectively kill intracellular MRSA (Fig.6-10D). Previous findings have shown that *S. aureus* is able to evade immunity and survive inside cells including phagocytic cells [17]. This is consistent with our data showing that IFN-γ can improve phagocytosis of MRSA without improving its clearance.

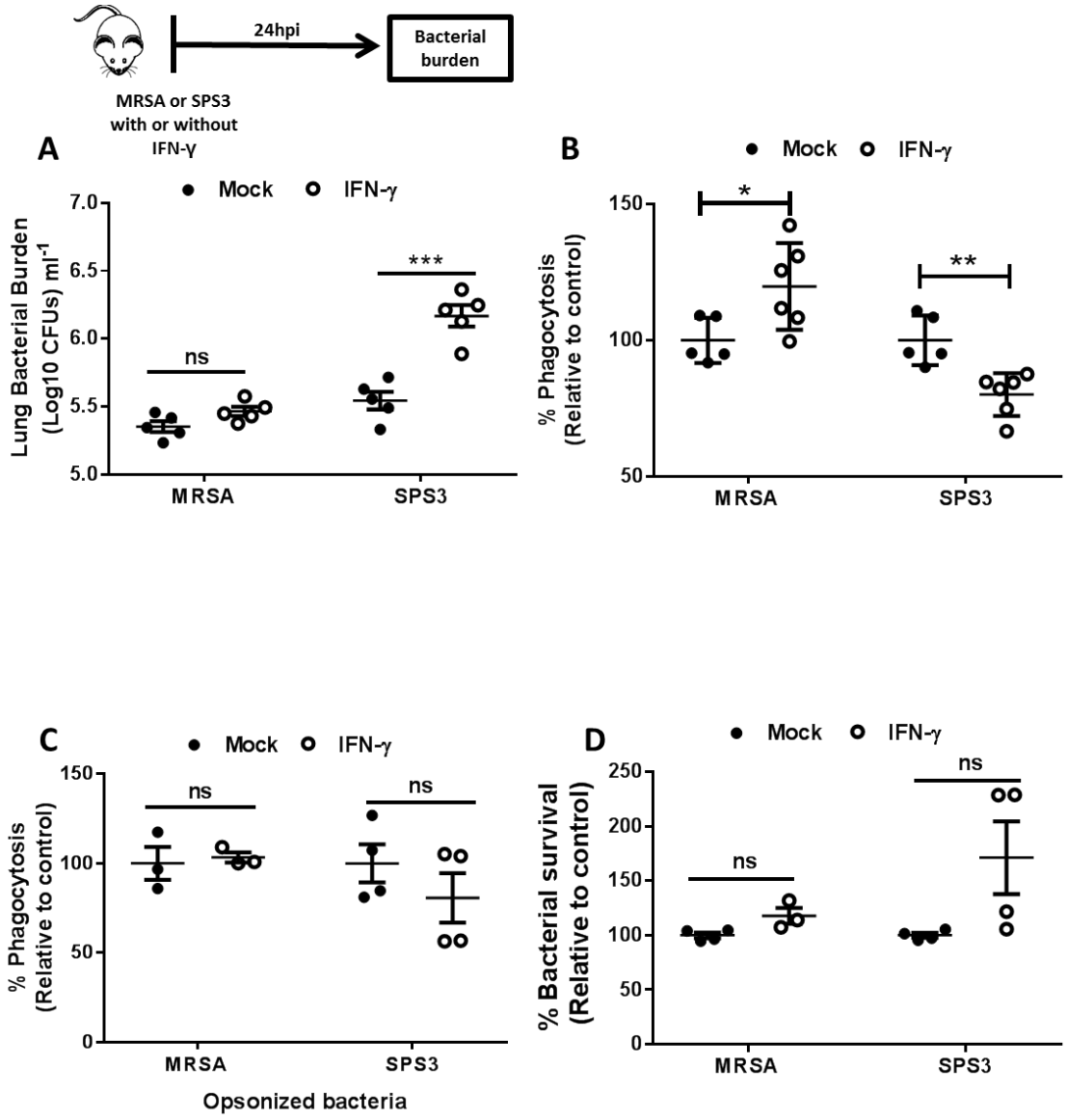

Figure 6-10: Administration of IFN-γ inhibits *S. pnuemoniae* clearance but has no effect in MRSA clearance, *in vivo*. (A) Lung bacterial burden of BALB/c mice that were treated or not with recombinant IFN-γ (2μg) prior to infection with MRSA ($7x10^7$ CFUs) or SPS3 ($3x10^5$ CFUs) for 24 hours. (B) In vitro phagocytosis assay where BMDMs were treated with IFN-γ (10ng/ml), or mock, for 24 hours prior to treatment with heat inactivated and FITC-labeled MRSA and/or SPS3. (C) In vitro phagocytosis assay and (D) in vitro bacterial survival assay where BMDMs were treated with IFN-γ (20ng/ml), or mock, for 24 hours prior to challenge with opsonized MRSA and/or SPS3. Statistics are student T test between comparative groups. Non-significant (ns), *P<0.05 ,**P<0.01, ***P<0.001.

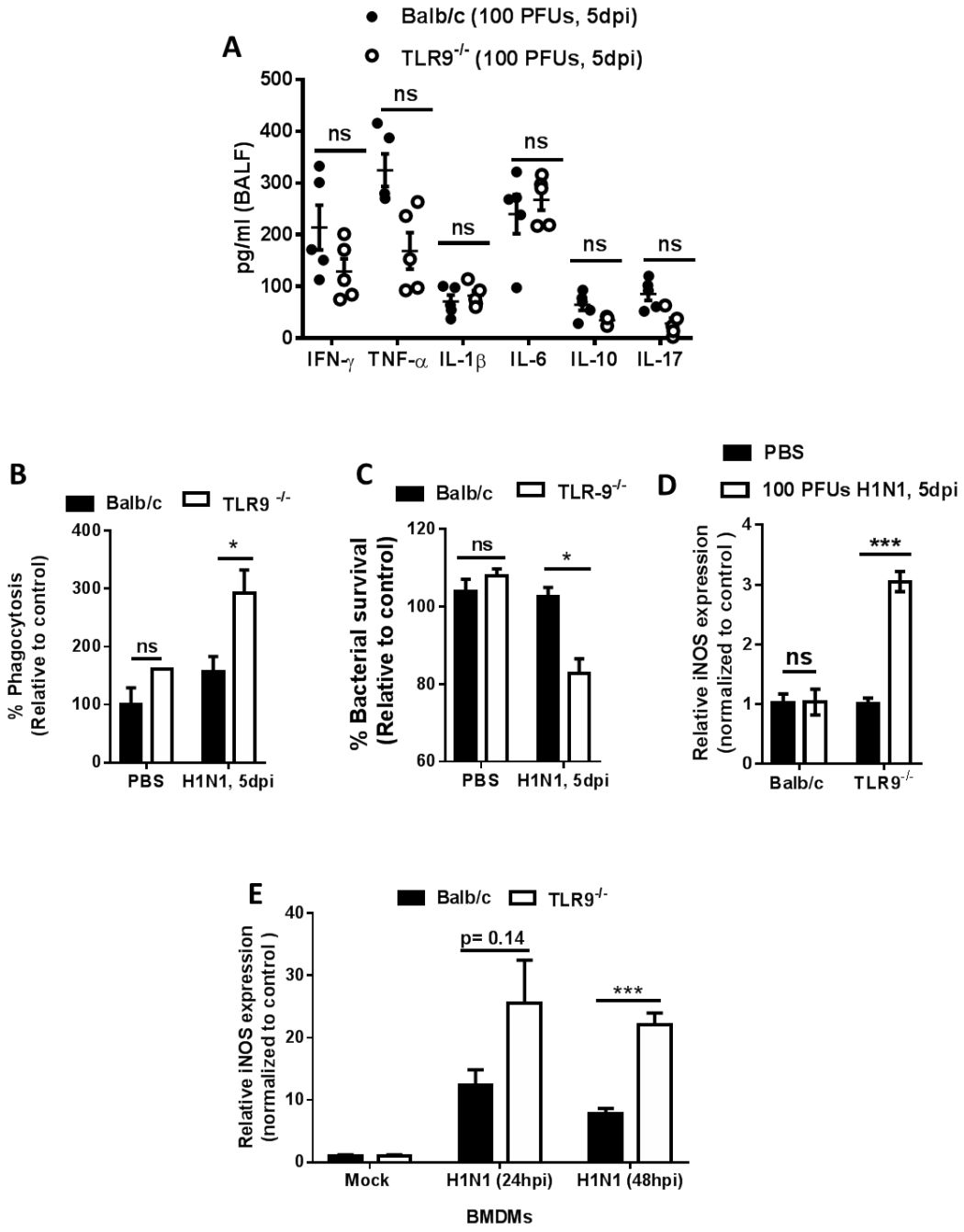

Figure 6-11: TLR9$^{-/-}$ lung macrophages have increased phagocytosis, bacterial killing, and iNOS expression post-IAV infection. (A) Cytokine measurement in BALF from BALB/c and TLR9$^{-/-}$ mice that were infected with IAV (100 PFUs, H1N1) for 5 days. (B) Ex vivo MRSA phagocytosis and (C) Intracellular survival assay using adherence selected lung macrophages from BALB/c and TLR9$^{-/-}$ mice that were infected with IAV (100 PFUs H1N1) for 5 days or treated with PBS. (D) Quantitative reverse transcriptase-PCR measurement of relative gene expression of iNOS from adherence selected lung macrophages from BALB/c and TLR9$^{-/-}$ mice that were infected with IAV for 5 days or treated with PBS; RNA samples were normalized to their β-actin levels. (B-C) Statistics were obtained by one-way analysis of variance with Bonferroni's posttest; ns=non-significant, *P<0.05. (A and D) Statistics are student T test between comparative groups. Non-significant (ns), ***P<0.001

Shortly after infection, MRSA is engulfed by phagocytes [31]. Macrophages, especially M1 (antimicrobial) polarized macrophages, play an essential role in the clearance of MRSA [32]. So, we tested the ability of macrophages from TLR9$^{-/-}$ mice to clear MRSA in culture. TLR9$^{-/-}$ macrophages isolated from uninfected mice had no difference in phagocytosis or intracellular bacterial clearance compared to WT. However, macrophages from PR8-infected TLR9$^{-/-}$ mice are capable of improving bacterial clearance and killing (Fig.6-11B-C). Interestingly, TLR9$^{-/-}$ macrophages from infected mice have higher expression of iNOS (Fig.6-11D). Mice lacking iNOS expression are deficient in clearance of MRSA and more than 50% of mice will not survive a MRSA infection past 24 hours[27]. Thus, the induction of iNOS is the most likely explanation for why TLR9$^{-/-}$ macrophages are more effective at clearing MRSA post-IAV infection.

In conclusion, our findings provide evidence that TLR9 plays a negative role in IAV-associated secondary MRSA infections. Blocking of TLR9 post-IAV infection can improve MRSA clearance by increasing macrophage bacterial phagocytosis, intracellular killing, and increasing production of nitric oxide. This also suggests that TLR9 antagonism may be an effective therapeutic for MRSA complicated influenza infections. However, care should be taken to know the nature of the secondary infection as TLR9 regulates MRSA, but not SPS3 coinfection. Future work will be focused on elucidating the mechanism(s) of influenza-induced upregulation of TLR9.

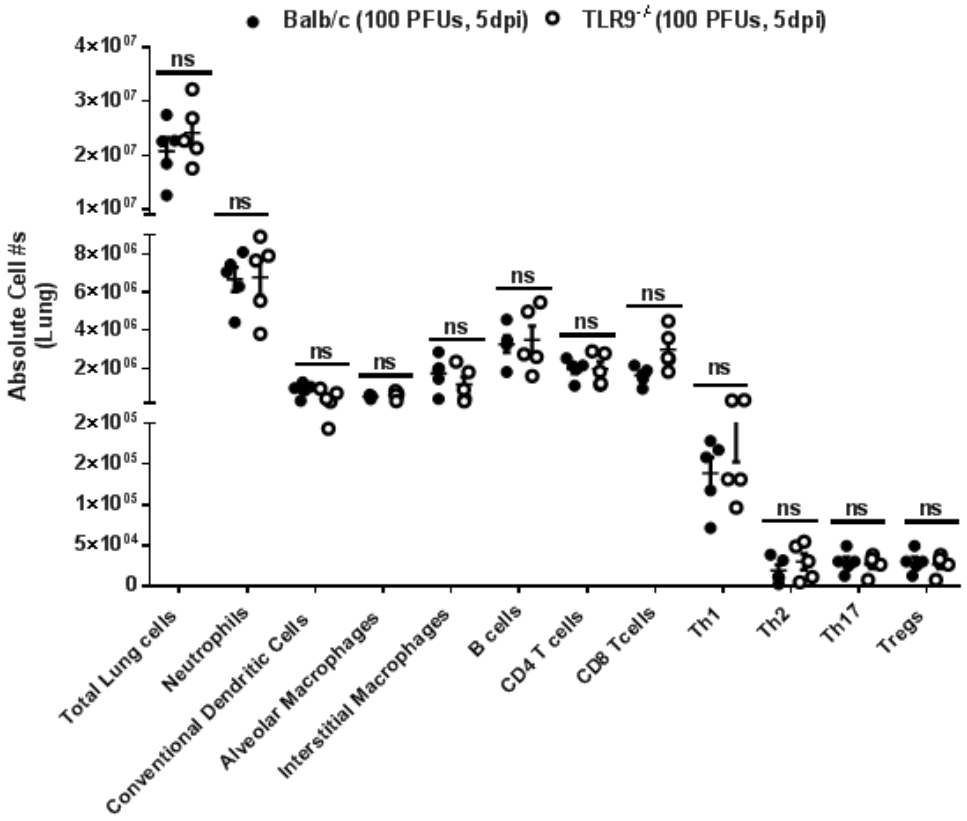

Figure 6-12: Immune cell profiles in TLR9$^{-/-}$ mice post-IAV infection. Absolute number of lung immune cells post-lung collagenase digestion in BALB/c and TLR9$^{-/-}$ mice that were infected with IAV (100 PFUs, H1N1) for 5 days. Total lung cells counted by hemocytometer and immune cell quantification was done by flow cytometry; gating was as follows: neutrophils (CD45^{+},CD11b^{+},MHCII^{-},Ly6G^{+}); conventional dendritic cells (CD45^{+},CD11c^{+},MHCII^{+},CD64^{-}); AMs (CD45^{+},CD11c^{+},Siglec F^{+},CD64^{+}); interstitial Macs (CD45^{+},CD11b^{+},MHCII^{+},Siglec F^{-}, CD64^{+}); B cells (CD45^{+}CD90.2^{-} CD19^{+}); CD4^{+} T cells (CD45^{+}CD90.2^{+}CD4^{+}); CD8^{+} T cells (CD45^{+}CD90.2^{+}CD4^{-}); Th1 (CD45^{+}CD90.2^{+}CD4^{+},IFN-γ^{+}); Th2 (CD45^{+}CD90.2^{+}CD4^{+}IL-4^{+}); Th17 (CD45^{+}CD90.2^{+}CD4^{+}IL-17a^{+}); Tregs (CD45^{+}CD90.2^{+}CD4^{+}Foxp3^{+}). Statistics are student T test between comparative groups; ns= non-significant.

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