

PTEN Limits Alveolar Macrophage Function against *Pseudomonas aeruginosa* after Bone Marrow Transplantation

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Hematopoietic stem cell transplant patients are susceptible to infection despite cellular reconstitution. In a murine model of syngeneic bone marrow transplantation (BMT), we previously reported that BMT mice have impaired host defense against *Pseudomonas aeruginosa* pneumonia due to overproduction of (PGE)₂ in lung. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is an effector in the PGE₂ signaling pathway that negatively regulates alveolar macrophage (AM) phagocytosis and bacterial killing. Therefore, we examined whether overproduction of PGE₂ after BMT inhibits AM host defense by up-regulating PTEN phosphatase activity. We found that PTEN activity is elevated in BMT AMs in response to increased PGE₂ signaling and that pharmacological inhibition of PTEN activity in BMT AMs fully restores phagocytosis of serum-opsonized *P. aeruginosa* but only partially restores phagocytosis of nonopsonized *P. aeruginosa*. In wild-type mice transplanted with myeloid-specific conditional PTEN knockout (PTEN CKO) bone marrow, bacterial clearance is improved after challenge with *P. aeruginosa* pneumonia. Furthermore, PTEN CKO BMT AMs display improved TNF- α production and enhanced phagocytosis and killing of serum-opsonized *P. aeruginosa* despite overproduction of PGE₂. However, AM phagocytosis of nonopsonized *P. aeruginosa* is only partially restored in the absence of PTEN after BMT. This may be related to elevated AM expression of IL-1 receptor-associated kinase (IRAK)-M, a molecule previously identified in the PGE₂ signaling pathway to inhibit AM phagocytosis of nonopsonized bacteria. These data suggest that PGE₂ signaling up-regulates IRAK-M independently of PTEN and that these molecules differentially inhibit opsonized and nonopsonized phagocytosis of *P. aeruginosa*.

Keywords: pneumonia; lung; neutrophil; eicosanoids; bacteria

Hematopoietic stem cell transplantation (HSCT) is commonly used for the treatment of various malignant and genetic disorders of the immune system. However, delays in immune reconstitution and reduced functionality of recovered immune cell compartments render patients susceptible to infectious complications, including bacterial pneumonia (1–3). This impairment in immune recovery and function extends for several months after transplant and occurs in autologous and allogeneic transplant recipients (4, 5).

Alveolar macrophages (AMs) are the primary immune cell type in the alveolar space and are critical for initiating immune responses against inhaled pathogens (6–8). However, AMs are

CLINICAL RELEVANCE

Our results demonstrate that transplant-induced prostaglandin E₂ may differentially inhibit opsonized and non-opsonized phagocytosis in alveolar macrophages via the up-regulation of phosphatase and tensin homolog deleted on chromosome 10 activity and IL-1 receptor-associated kinase M expression, respectively. Our studies provide a rationale for prostaglandin blockade as a therapy for pulmonary bacterial infections after stem cell transplant.

reported to have decreased host defense capability after allogeneic HSCT (9). Furthermore, neutrophils recruited to sites of infection display impaired chemotaxis and killing ability (10). These studies using allogeneic transplant patients, however, involve various confounding factors, such as immunosuppressive drug therapy and graft-versus-host disease, that are known to impair immune function (3, 4). Thus, the specific impact of conditioning and reconstitution alone on pulmonary innate immune function remains unclear.

To determine the impact of HSCT on pulmonary host defense, we previously developed a mouse model of syngeneic bone marrow transplantation (BMT). Compared with nontransplant control mice, BMT mice were more susceptible to *P. aeruginosa* pneumonia after intratracheal infection despite full hematopoietic reconstitution in the lung and periphery (11). Furthermore, donor-derived BMT AMs and recruited lung neutrophils displayed impaired host defense functions (12). We found that this reduction in innate immune function was induced by an elevated production of the immunosuppressive lipid mediator prostaglandin (PGE)₂ in the lung after BMT (2, 12, 13).

PGE₂ is known to inhibit bacterial killing, phagocytosis (14, 15), chemotaxis (16), and the production of proinflammatory mediators in leukocytes (17–19). At least one consequence of increased PGE₂ production after transplant is the up-regulation of IL-1 receptor-associated kinase (IRAK)-M, which limits AM function (including inhibition of phagocytosis of nonopsonized *P. aeruginosa*) (20). However, it is not known what downstream signaling pathways PGE₂ activates to up-regulate IRAK-M expression. One potential candidate in the PGE₂ signaling pathway that may regulate IRAK-M is the phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which has also been reported to negatively regulate Fc γ receptor (Fc γ R)-mediated phagocytosis and bacterial killing in AMs (21). It remains unknown whether PTEN regulates AM function in the BMT setting.

PTEN is a dual-specificity phosphatase that can dephosphorylate protein and lipid targets; however, PTEN is best characterized by its ability to dephosphorylate the lipid second messenger phosphatidylinositol (3–5)-trisphosphate (PIP₃), a key mediator of the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway (22). This dephosphorylation event results in the inhibition

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of Fc γ R-mediated phagocytosis of IgG-opsonized particles as mediated by PI3K/AKT signaling (23, 24). PTEN phosphatase activity is negatively regulated via a number of posttranslational mechanisms, including tyrosine phosphorylation (25). PGE₂ activation of the cAMP signaling cascade has been shown to induce PTEN activity via tyrosine dephosphorylation in a Src homology region 2 domain-containing phosphatase-1 dependent manner (21, 26). Furthermore, these studies showed that PGE₂-mediated increases in PTEN activity diminish AM phagocytosis and killing of IgG-opsonized particles (21). Additional roles for PTEN may exist in the inhibition of non-Fc γ R-mediated phagocytosis, given that PI3K activity has been reported to mediate phagocytosis of nonopsonized particles (27–29). Therefore, we hypothesized that, in our model of syngeneic BMT, increased PGE₂ signaling in the lung inhibits host defense against acute *P. aeruginosa* infection by elevating PTEN activity in AMs. Additionally, we wished to determine the influence of PTEN in opsonized and nonopsonized phagocytosis pathways and whether PTEN signaling is related to IRAK-M elevation after BMT.

To address our hypothesis, we measured PTEN activity and AKT phosphorylation levels in BMT and in nontransplant control AMs in the presence or absence of an inhibitor of endogenous PGE₂ production. In addition, we transplanted lethally irradiated wild-type (WT) mice with bone marrow from myeloid-specific PTEN conditional knockout (CKO) mice to determine whether PTEN plays a role in impaired pulmonary host defense after BMT. We demonstrate that increased PGE₂ signaling augments PTEN activity in BMT AMs and diminishes pAKT levels. Furthermore, we show that myeloid-specific ablation of PTEN in the bone marrow of transplant mice can restore AM phagocytosis of serum-opsonized bacteria and improve bacterial clearance after *P. aeruginosa* infection. In contrast, PTEN CKO BMT AMs do not have fully restored nonopsonized phagocytosis of *P. aeruginosa*, likely due to the up-regulation of IRAK-M in these phagocytes. Taken together, our results demonstrate that, after BMT, PGE₂ signals through IRAK-M independently of PTEN and that up-regulation of PTEN activity primarily inhibits serum-opsonized phagocytosis, whereas up-regulation of IRAK-M may specifically inhibit nonopsonized phagocytosis. Additionally, results from our PTEN CKO BMT studies demonstrate the importance of AM function for *in vivo* clearance of *P. aeruginosa* independent of neutrophil function.

MATERIALS AND METHODS

Additional details regarding all methods can be found in the online supplement.

Animals

WT C57BL/6 (B6), *PTEN*^{loxP/loxP}, and myeloid-specific LysM Cre mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Myeloid-specific PTEN KO mice (*PTEN*^{loxP/loxP}; *Cre*^{+/-}) were generated by breeding as previously described (30). For all experiments involving myeloid-specific PTEN KO mice, *PTEN*^{wi/wi}; *Cre*^{+/-} mice were used as WT bone marrow donors and *PTEN*^{loxP/loxP}; *Cre*^{+/-} mice were used as PTEN CKO bone marrow donors. Mice were housed under specific pathogen-free conditions and monitored daily by veterinary staff. All mice were killed by CO₂ asphyxiation. The University of Michigan Committee on Use and Care of Animals approved these experiments.

BMT

Total body irradiation and BMT were performed as previously described (20). All experiments with BMT mice were performed 5 to 6 weeks after BMT when mice were fully donor-cell reconstituted (13, 31).

P. aeruginosa PAO1 Preparation and Intratracheal Infection

As previously described, *P. aeruginosa* PAO1 inoculum was prepared, and mice were injected intratracheally with a sublethal dose of 5×10^5 CFU (12, 31).

Immune Serum Preparation and Opsonization

P. aeruginosa-specific immune serum was prepared (without heat inactivation) and used to opsonize *P. aeruginosa* as previously described (32).

Quantification of Bacterial Burden in Lung and Blood

Bacterial burden in whole lung and blood samples was assessed by CFU assay as previously described (12).

AM and Neutrophil Isolation

AMs and elicited lung neutrophils were harvested by bronchoalveolar lavage (BAL), counted, and adherence purified as previously described (31).

IgG-Sheep Red Blood Cell Fc γ R Stimulation Assay

AMs were pretreated with the drugs of interest, stimulated at a 1:10 ratio with IgG-opsonized or nonopsonized sheep red blood cells (MP Bio-medicals, Solon, OH), and prepared for Western blot analysis as described previously (21, 33).

Differential Cell Analysis of Total Lung Leukocytes and BAL Cells

Differential analysis was performed using BAL cells or total lung leukocytes isolated from collagenase-digested whole lung samples as previously described (20).

In Vitro Phagocytosis and Bacterial Killing Assays

AM phagocytosis of FITC-labeled *P. aeruginosa* was measured *in vitro* as previously described (20). Bacterial killing was quantified in AMs and lung neutrophils *in vitro* using a tetrazolium dye reduction assay as described previously (34).

Western Blot Analysis

Western blot analysis was performed as previously described (20, 21) using 15 μ g of whole-cell lysate or protein isolated by immunoprecipitation.

PTEN Phosphatase Activity Assay

PTEN was immunoprecipitated from AM whole-cell lysates to assess *in vitro* lipid phosphatase activity as previously described (21, 35).

ELISA/Enzyme-Linked Immunoassay

Enzyme-linked immunoassay samples were prepared as previously described (20). TNF- α production was measured using a DuoSet ELISA kit (R&D Systems, Minneapolis, MN), and PGE₂ production was measured using a PGE₂ enzyme-linked immunoassay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions.

Statistical Analysis

Statistical significance was analyzed using the Prism 5.01 (GraphPad Software, San Diego, CA). ANOVA with a *post hoc* Bonferroni test was used to compare groups as previously described (20). A value of $P < 0.05$ was considered statistically significant.

RESULTS

Increased PTEN Activity and Diminished pAKT Levels in BMT AMs

After syngeneic BMT, AMs display elevated production of PGE₂, which directly impairs AM phagocytosis and killing of

IgG-opsonized and nonopsonized particles (12). Given that PGE₂ is known to inhibit FcγR-mediated phagocytosis and killing in AMs by up-regulating PTEN activity (21), we determined whether overproduction of PGE₂ in BMT AMs results in elevated PTEN phosphatase activity. PTEN was immunoprecipitated from BMT and nontransplant control AMs, and the lipid phosphatase activity of the purified PTEN protein was assessed after incubation with PIP₃. Relative to control AMs, BMT AMs had an approximately 50% increase in PTEN activity (Figure 1A). This increase was blocked in BMT AMs after overnight treatment with indomethacin, an inhibitor of endogenous prostanoic production (Figure 1A). Because PGE₂ is the major prostanoid produced in BMT AMs (12, 13), the indomethacin treatment likely exerts its effect by blocking PGE₂.

PTEN activity is negatively regulated via a number of post-translational mechanisms, including tyrosine phosphorylation (25). Our preliminary data suggest that the increase we observed in PTEN activity in BMT AMs is related, at least in part, to diminished PTEN tyrosine phosphorylation (see Fig. E1, lane 2, in the online supplement). Furthermore, these data suggest that the reduction in PTEN tyrosine phosphorylation is due to BMT AM overproduction of PGE₂, given that PTEN tyrosine phosphorylation is improved in BMT AMs after treatment with indomethacin (Fig. E1, lane 3).

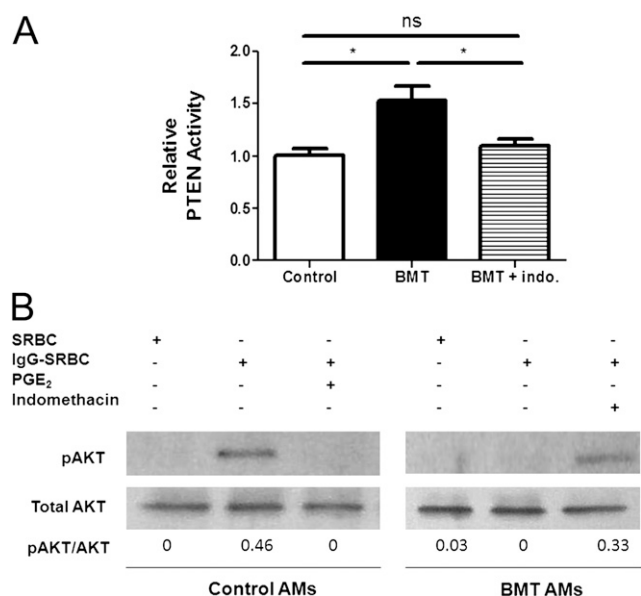


Figure 1. Overproduction of prostaglandin (PG)_{E2} elevates phosphatase and tensin homolog deleted on chromosome 10 (PTEN) activity and diminishes pAKT levels in bone marrow transplantation (BMT) alveolar macrophages (AMs). Mice were given syngeneic BMT and harvested 5 to 6 weeks after transplant. AMs were isolated by bronchoalveolar lavage as described in MATERIALS AND METHODS from BMT and nontransplanted control mice. (A) PTEN protein was immunoprecipitated from whole-cell lysates of AMs cultured at 5×10^5 per well in the presence of 5 μ M indomethacin or 0.05% vehicle control overnight. PTEN phosphatase activity was determined as described in MATERIALS AND METHODS. Results are represented as PTEN phosphatase activity relative to the untreated control group ($*P < 0.05$; $n = 3-4$ per group combined from two experiments). (B) AMs were pretreated with 5 μ M indomethacin for 2 hours or 100 nM PGE₂ for 15 minutes at 37°C. AMs were then stimulated with IgG-sheep red blood cells (SRBCs) (1:10 ratio) for 15 minutes at 37°C. After IgG-SRBC stimulation, AMs were prepared for Western blot analysis as described in MATERIALS AND METHODS. Relative band densitometry data are indicated under each lane. Data shown are representative of two experiments.

Increased PTEN activity inhibits the activation of a number of downstream targets in the FcγR-signaling pathway, such as AKT (22, 25). To determine whether increased PTEN activity in BMT AMs translates into functional Akt suppression, AMs from control and BMT mice were stimulated with IgG-opsonized sheep red blood cells to induce phosphorylation of AKT. FcγR stimulation of control AMs induced phosphorylation of AKT (Figure 1B, lane 2); however, as previously reported (21), pretreatment with 100 nM PGE₂ blocked FcγR-induced AKT phosphorylation (Figure 1B, lane 3). As expected, BMT AMs had no detectable levels of pAKT after FcγR stimulation (Figure 1B, lane 5). pAKT levels were restored in FcγR-stimulated BMT AMs after pretreatment with indomethacin (Figure 1B, lane 6). Taken together, these data suggest that elevated PTEN activity and diminished pAKT levels in BMT AMs are related to increased PGE₂ signaling.

Inhibition of PTEN Activity Restores Phagocytosis in BMT AMs

Because PTEN activity is known to inhibit FcγR-mediated phagocytosis in macrophages (21, 36), we determined whether pharmacologic inhibition of PTEN activity could restore phagocytosis in BMT AMs. The phosphatase inhibitor bpV(pic) specifically inhibits PTEN phosphatase activity at concentrations within the nanomolar range (37). We found that pretreating BMT AMs with 100 nM bpV(pic) restored phagocytosis of serum-opsonized *P. aeruginosa* to that of control AM levels (Figure 2A). In experiments using nonopsonized *P. aeruginosa*, bpV(pic) treatment was also able to improve the phagocytic function of BMT AMs, although not to control AM levels (Figure 2B). These data suggest that PTEN negatively regulates

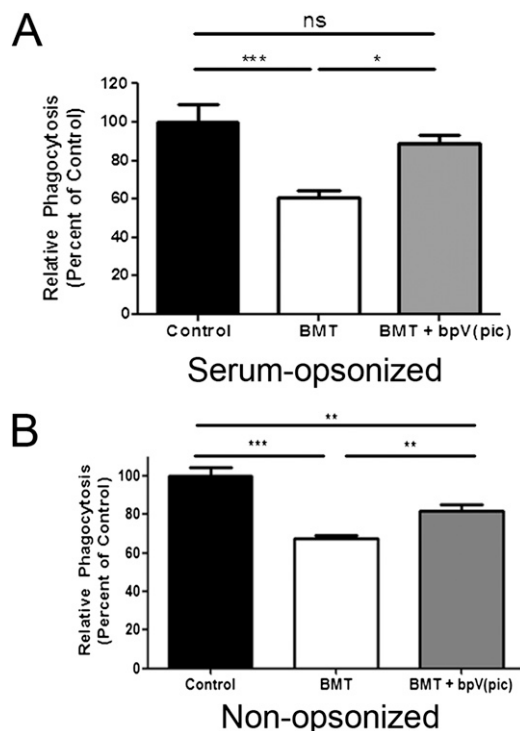


Figure 2. Inhibiting PTEN activity improves BMT AM phagocytic ability. AMs were harvested from control and BMT mice and cultured at 2×10^5 cells per well overnight. Phagocytosis of serum-opsonized (A) and non-serum-opsonized (B) FITC-*P. aeruginosa* was assessed as described in MATERIALS AND METHODS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

phagocytosis but may play a more important role in the inhibition of serum-opsonized phagocytosis as opposed to nonopsonized phagocytosis.

Myeloid-Specific Ablation of PTEN Expression in BMT Mice

Myeloid-specific ablation of PTEN enhances lung phagocyte function and pulmonary host defense against gram-negative bacterial infections (38); however, it is not known whether similar mechanisms are relevant in the setting of BMT. Because our data demonstrate that pharmacologic inhibition of PTEN activity improves BMT AM phagocytosis *in vitro* (Figure 2), we generated myeloid-specific PTEN KO mice to assess whether reconstituting irradiated WT mice with PTEN-deficient bone marrow would restore host defense function in AMs. To generate myeloid-specific PTEN KO mice, we obtained mice homozygous for a “floxed” PTEN mutant allele containing *loxP* sites on either side of exon 5 encoding the phosphatase domain. The PTEN floxed mice were bred to myeloid-specific Cre mice, which express the Cre recombinase gene under the control of the lysozyme M (*LysM*) promoter. As previously described, mice bred to express two copies of the floxed PTEN allele and at least one copy of *LysM*-Cre recombinase are deficient in PTEN expression in cells of the myeloid lineage (30). Furthermore, these mice do not display an abnormal phenotype and are viable, fertile, and of normal size (30).

To verify PTEN ablation in our BMT model, AMs were harvested from nontransplanted WT control mice and lethally total body irradiated WT mice transplanted with WT BMT or myeloid-specific (conditional) PTEN CKO BMT. Figure E2 demonstrates that these cells isolated by BAL from each group of mice are predominantly AMs. PTEN protein expression was comparable in control and WT BMT AMs (Figure 3A). As expected, PTEN protein expression was ablated in AMs from PTEN CKO BMT mice (Figure 3A), and preliminary data suggest that this is accompanied by a restoration in basal pAKT expression levels (Fig. E3). Ablation of PTEN was also observed in recruited lung neutrophils from PTEN CKO BMT mice (data not shown).

AMs from PTEN CKO BMT Mice Display Enhanced Phagocytosis and Killing of Serum-Opsonized *P. aeruginosa* Despite Overproduction of PGE₂

We performed experiments to determine whether myeloid-specific disruption of PTEN could improve opsonized phagocytosis and killing of serum-opsonized *P. aeruginosa* in BMT AMs. We found that AMs from PTEN CKO BMT mice displayed restored phagocytosis of serum-opsonized FITC-*P. aeruginosa* *in vitro* relative to WT BMT AMs (Figure 3B). Furthermore, WT BMT AMs had increased survival of ingested *P. aeruginosa* relative to control AMs, indicating defective bacterial killing ability (Figure 3C). However, the amount of surviving bacteria was significantly lower in PTEN CKO BMT AMs compared with control AMs, suggesting that bacterial killing is not only restored in AMs from PTEN CKO BMT mice but is also enhanced (Figure 3C). AMs from PTEN CKO BMT mice produce significantly more PGE₂ than control and WT BMT AMs, as measured from overnight culture supernatants (Figure 3D). These data suggest that PTEN ablation in AMs restores phagocytosis of serum-opsonized bacteria after BMT despite AM overproduction of PGE₂.

PTEN Ablation Improves but Does Not Fully Restore Nonopsonized Phagocytosis in AMs after BMT

Our results with the PTEN inhibitor bpV(pic) suggest that PTEN function may be less important for nonopsonized phagocytosis of

bacteria. To verify this with the genetic ablation, AMs were harvested from control, WT BMT, and PTEN CKO BMT mice, and phagocytosis of nonopsonized bacteria was tested *in vitro*. Similar to our findings shown in Figure 2, PTEN CKO BMT AMs showed improved phagocytosis compared with WT BMT AMs (Figure 3E). However, this improvement in phagocytic ability was not restored to control AM levels (Figure 3E). Thus, BMT-mediated increases in PTEN may play a lesser role in nonopsonized phagocytosis as compared with opsonized phagocytosis.

Restored AM and Lung TNF- α Levels in PTEN CKO BMT Mice

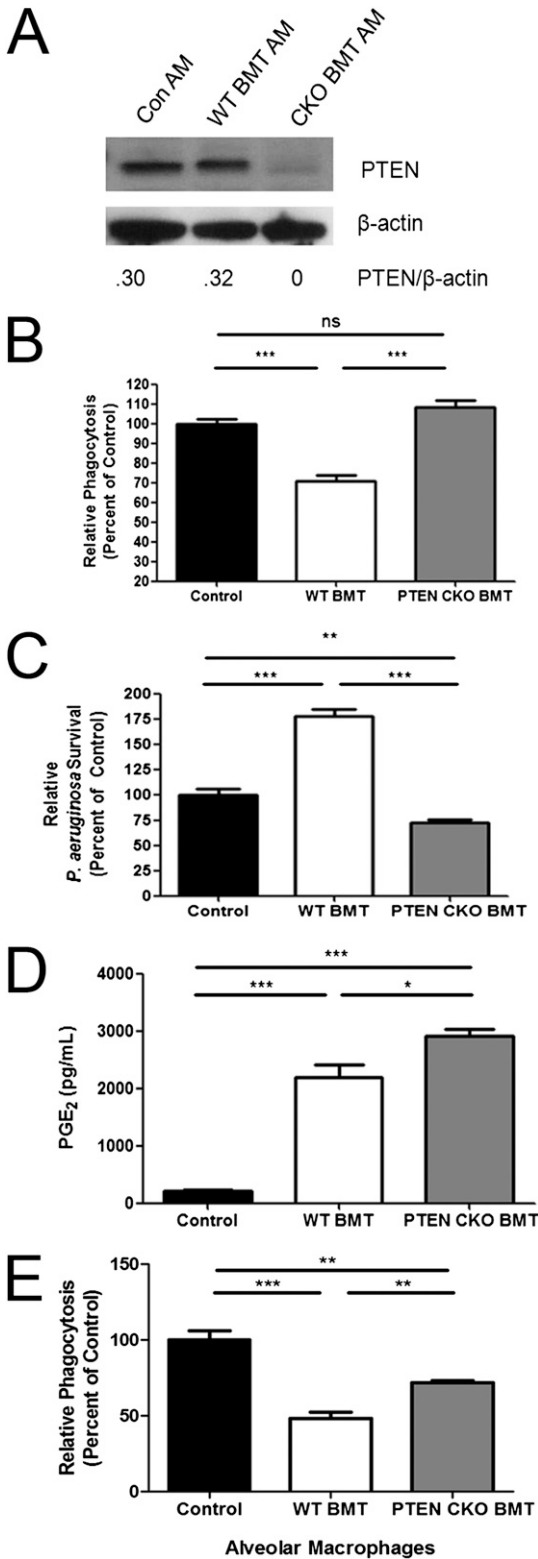
AMs from neutropenic myeloid-specific PTEN KO mice display enhanced proinflammatory cytokine profiles, including increased production of TNF- α , relative to neutropenic WT mice (38). Because TNF- α is a key proinflammatory mediator in pulmonary host defense against *P. aeruginosa* (8) and can enhance bacterial killing mechanisms in macrophages (39–41), we measured TNF- α levels in AM overnight culture supernatants and lung homogenates from mice challenged with an acute *P. aeruginosa* lung infection. As previously reported (11, 31), defective TNF- α production was observed in WT BMT AMs and lung homogenates relative to control samples (Figures 4A and 4B). However, PTEN CKO BMT mice displayed improved AM TNF- α production (Figure 4A), and lung homogenates from infected mice had fully restored TNF- α levels (Figure 4B).

Improved Bacterial Clearance in PTEN CKO BMT Mice after *P. aeruginosa* Infection

To determine whether PTEN CKO BMT mice have improved bacterial clearance after BMT, we challenged control, WT BMT, and PTEN CKO BMT mice with a sublethal *P. aeruginosa* lung infection. At 24 hours after infection, bacterial burden was assessed in lung and blood samples. In WT BMT mice, bacterial burden was significantly higher in lung and blood samples relative to control (Figures 5A and 5B). In PTEN CKO BMT mice, bacterial burden was significantly reduced in lung and blood samples relative to WT BMT mice, although it was not fully restored to control levels (Figures 5A and 5B). We speculate that the reason PTEN CKO BMT mice have an intermediate phenotype is that the acute *in vivo* infection model relies heavily on AM clearance of nonopsonized bacteria—a pathway in which PTEN may be less important.

IRAK-M Expression Is Elevated in WT BMT and PTEN CKO BMT AMs

We previously demonstrated that, in response to increased PGE₂ signaling, BMT AMs display elevated expression of IRAK-M, a well described negative regulator of TLR/IL-1R signaling (20). Furthermore, we found that, despite overproduction of PGE₂, ablation of IRAK-M in the hematopoietic compartment of BMT mice fully restores BMT AM phagocytosis of nonopsonized bacteria, killing, and production of proinflammatory mediators (20). Therefore, we wondered whether the partial restoration of nonopsonized phagocytosis in PTEN CKO BMT AMs was the result of elevated IRAK-M expression. IRAK-M protein expression was assessed in AMs from control, WT BMT, and PTEN CKO BMT mice. IRAK-M protein expression was significantly elevated in WT BMT and PTEN CKO BMT AMs compared with control AMs (Figure 6). These data suggest that, after BMT, IRAK-M may play a critical role in the negative regulation of nonopsonized phagocytosis, whereas PTEN primarily inhibits pathways of opsonized phagocytosis.



Lung Leukocyte Composition in PTEN CKO BMT Mice

We next assessed the total number of leukocytes and the frequency of leukocyte populations in the lungs of control, WT BMT, and PTEN CKO BMT mice at baseline and 24 hours after *P. aeruginosa* pneumonia. Total lung leukocyte numbers were increased in uninfected PTEN CKO BMT mice relative to uninfected WT BMT mice (Figure 7A). However, 24 hours after infection, total leukocyte numbers were comparable between

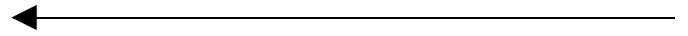


Figure 3. Restored AM phagocytosis and killing of serum-opsonized *P. aeruginosa* in PTEN conditional knockout (CKO) BMT mice despite AM overproduction of PGE₂. (A) Whole-cell lysates were prepared from control, wild-type (WT) BMT, and PTEN CKO BMT AMs after 1 hour of serum-free media adherence. PTEN and β -actin protein expression were analyzed by Western blot. Ablation of PTEN was verified in this manner for all experiments using PTEN CKO BMT mice. (B and C) Bone marrow from WT or myeloid-specific PTEN CKO mice was transplanted into lethally irradiated WT recipients. WT control, WT BMT (WT > WT), and PTEN CKO BMT (PTEN CKO > WT) AMs were harvested and cultured at 2×10^5 cells per well overnight. Phagocytosis of serum-opsonized FITC-*P. aeruginosa* (B) and killing of serum-opsonized *P. aeruginosa* (C) was assessed as described in MATERIALS AND METHODS ($n = 7-10$ per group). (D) PGE₂ levels were measured in overnight culture supernatants by Enzyme-linked immunoassay as described in MATERIALS AND METHODS ($n = 4-5$ per group). (E) AMs were harvested from control, WT BMT, and PTEN CKO BMT mice and cultured at 2×10^5 cells/well overnight. Phagocytosis of non-serum-opsonized FITC *P. aeruginosa* was assessed (E) as described in MATERIALS AND METHODS ($n = 7-10$ per group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

groups (Figure 7B). A shift was observed in whole lung macrophage and neutrophil counts in uninfected PTEN CKO BMT mice (Figure 7C). We found that the percentage of macrophages was significantly reduced and neutrophils increased relative to control and WT BMT lungs (Figure 7C). After infection, however, the percentages of neutrophils, macrophages, and lymphocytes were comparable between groups (Figure 7D).

Neutrophil Function Is Improved in PTEN CKO BMT Mice

Previous studies have suggested that PTEN-deficient neutrophils have improved host defense functions (38, 42). Given that PTEN CKO BMT mice have increased neutrophil numbers in the lung at baseline, we wanted to assess the functional status of these phagocytes with regard to bacterial killing. Relative to control neutrophils, WT BMT neutrophils displayed impaired bacterial killing of serum-opsonized *P. aeruginosa*, and this defect was

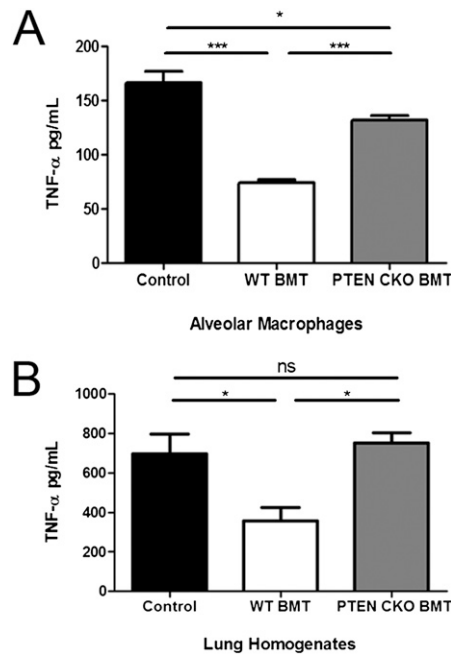


Figure 4. PTEN CKO BMT mice display improved TNF- α production. TNF- α production was measured by ELISA as described in MATERIALS AND METHODS using AM overnight culture supernatants (A) and lung homogenates from mice 24 hours after intratracheal *P. aeruginosa* infection (B) (* $P < 0.05$, *** $P < 0.001$; $n = 3-5$ per group).

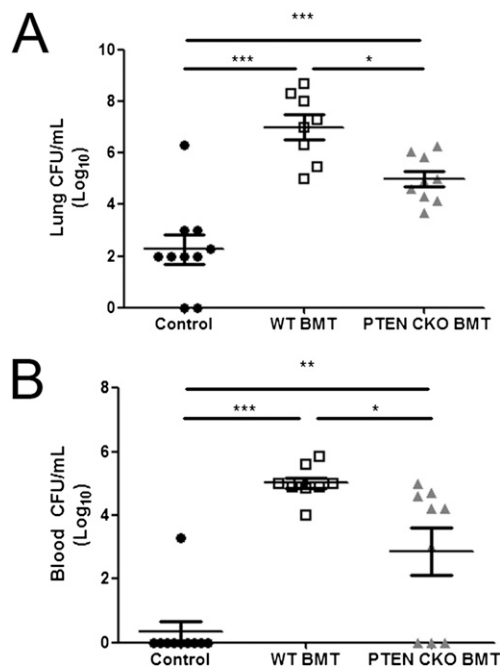


Figure 5. Improved bacterial clearance in PTEN CKO BMT mice after *P. aeruginosa* pneumonia. Mice were injected intratracheally with 50 μ L of 5×10^5 CFU *P. aeruginosa*. At 24 hours after infection, bacterial burden of whole lung (A) and blood (B) samples from each mouse was assessed by CFU assay (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; $n = 8$ –10 mice per group).

restored in the absence of PTEN after BMT (Figure 7E). Thus, despite the fact that PTEN CKO BMT mice have increased numbers of neutrophils in the lung at baseline and display normal killing of serum-opsonized *P. aeruginosa*, the *in vivo* clearance of bacteria in these mice is still not completely restored. In total, these data suggest that persistent impairment of non-opsonized phagocytosis by AMs likely contributes to the partial restoration of host defense against acute *P. aeruginosa* lung infection in PTEN CKO BMT mice, despite an increased presence of functional neutrophils.

DISCUSSION

AMs are the primary immune cell type in the alveolar space and serve as the first line of cellular defense against inhaled pathogens (6–8). Defects in AM phagocytosis/killing and production of inflammatory signals hinder the ability of the host to clear pathogens. In the case of HSCT, donor-derived AMs reconstituting the lung airspaces have a significantly impaired ability to ingest and kill bacterial pathogens (9, 11), leaving the host susceptible to organisms such as *P. aeruginosa*.

Our previous work has shown that overproduction of PGE₂ BMT directly impairs AM host defense (12). One effector in the PGE₂ signaling cascade, PTEN, has recently been shown to inhibit AM phagocytosis and killing of IgG-opsonized particles by negatively regulating the Fc γ R signaling pathway in control mice (21). In this work, we show a similar mechanism after BMT, where PGE₂ is overproduced by AMs and increases the lipid phosphatase activity of PTEN (see Figure 1A). PTEN activity inhibits BMT AM phagocytosis of serum- and non-serum-opsonized *P. aeruginosa* (see Figure 2), although it appears that PTEN may play a more critical role in the negative regulation of opsonized phagocytosis. In addition, we show that myeloid-specific ablation of PTEN restores bacterial host defense

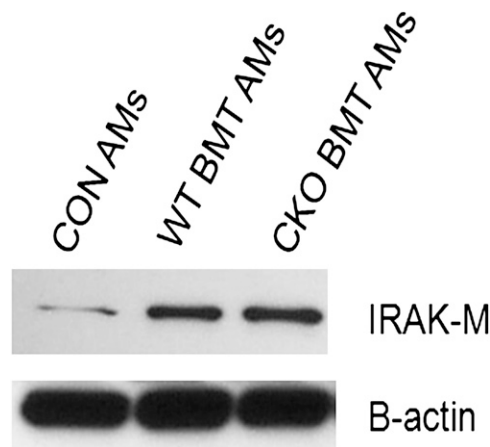


Figure 6. Elevated IL-1 receptor-associated kinase (IRAK)-M expression in WT BMT and PTEN CKO BMT AMs. Whole-cell lysates were prepared from pooled control, WT BMT, and PTEN CKO BMT AMs after 1 hour of serum-free media adherence. IRAK-M and β -actin protein expression were analyzed by Western blot as described in MATERIALS AND METHODS. Blot is representative of data from two experiments.

functions in AMs (see Figure 3) and neutrophils (Figure 7E) after BMT.

Our data demonstrate that BMT AMs have increased PTEN activity relative to nontransplant control AMs (see Figure 1A). Because PGE₂ has been shown to increase PTEN activity via activation of the cAMP signaling cascade, we examined whether BMT AM overproduction of PGE₂ mediated this increase in PTEN activity. Indomethacin treatment, which inhibits COX-2 synthesis of prostaglandins, effectively abrogated the increase we observed in PTEN activity (see Figure 1A). Enhanced PTEN activity translated into the functional consequence of suppressed AKT activity after Fc γ R stimulation in BMT AMs, but we observed that AKT function could be restored with indomethacin pretreatment (see Figure 1B). Thus, these data suggest that PGE₂ may increase BMT AM PTEN activity and diminish AKT activation after Fc γ R activation.

During acute bacterial lung infections, AM phagocytosis likely involves uptake of bacteria that are nonopsonized or opsonized by serum-derived complement (6, 43). It has previously been shown that PI3K activity, which is tightly regulated by PTEN, is involved in complement-mediated and nonopsonized phagocytosis (27–29) in nontransplant settings. Therefore, we examined whether inhibiting PTEN activity could restore BMT AM phagocytosis of serum and non-serum-opsonized FITC-*P. aeruginosa*. Pharmacologic PTEN inhibition increased phagocytosis of nonopsonized *P. aeruginosa* but did not restore levels to those seen in control AMs. In contrast, pharmacologic inhibition of PTEN fully restored serum-opsonized phagocytosis to control levels (see Figure 2). It is likely that the presence of IgG (in addition to complement) in our immune serum contributed to the greater magnitude of improvement we observed in phagocytosis of serum-opsonized bacteria in bpV(pic)-treated BMT AMs compared with nonopsonized bacteria. As discussed below, these pharmacologic results were corroborated by studies using genetic deletion of PTEN.

It is interesting to speculate on the role that PTEN activation may play during acute infection in control mice. Our data demonstrate that PTEN activity is low in control AMs, allowing AKT to be phosphorylated in response to Fc γ R signaling (see Figure 1). It is likely, however, that during the course of infection, inflammatory mediators (including PGE₂) may up-regulate

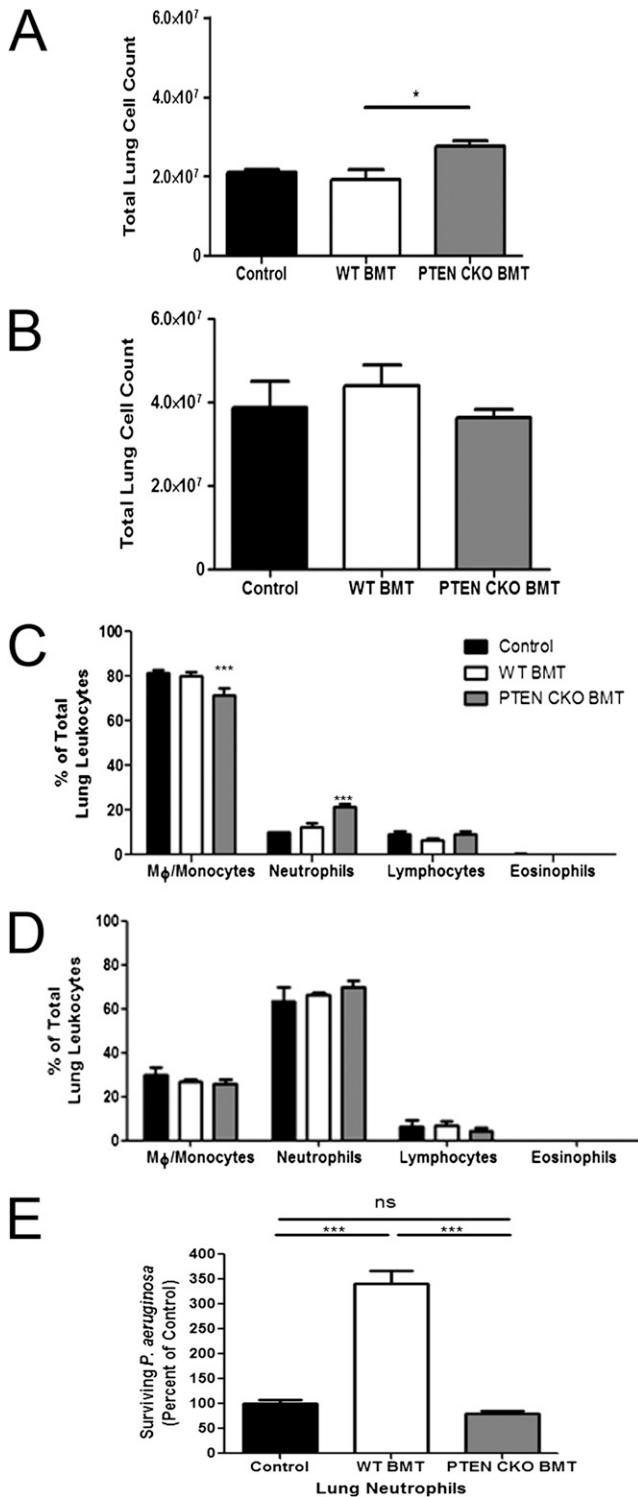


Figure 7. Lung leukocyte composition before and after *P. aeruginosa* infection and neutrophil function. Control, WT BMT, and PTEN CKO BMT mice were injected intratracheally with 50 μ L of 5 \times 10⁵ CFU *P. aeruginosa*. Lung samples were harvested from uninfected and infected mice 24 hours later, and lung leukocytes were isolated, counted, and stained for differential cell analysis as described in MATERIALS AND METHODS. (A and B) Total lung leukocyte count in naive (A) and infected (B) mice. (C and D) Percentage of macrophages/monocytes, neutrophils, lymphocytes, and eosinophils in the lungs of naive (C) and infected (D) mice (*n* = 4 per group). Neutrophils were elicited to the lung and harvested by BAL from control, WT BMT, and PTEN CKO BMT mice as described in MATERIALS AND METHODS. Killing of serum-opsonized *P. aeruginosa* was assessed in neutrophils (E) as described in MATERIALS AND METHODS (*n* = 7–10 per group; * *P* < 0.05, *** *P* < 0.001).

defense after transplantation of myeloid-specific PTEN KO bone marrow into WT recipients. Despite overproduction of PGE₂ (see Figure 3D), we found that AM phagocytosis and killing of serum-opsonized *P. aeruginosa* is restored (see Figures 3B and 3C) and that AM TNF- α production is improved in the absence of PTEN expression after BMT (see Figure 4A). Phagocytosis of nonopsonized bacteria is not fully restored in PTEN CKO BMT AMs (see Figure 3E). Furthermore, bacterial clearance in PTEN CKO BMT mice challenged with *P. aeruginosa* lung infection is only partially recovered (see Figure 5), despite the fact that lung TNF- α levels are restored (see Figure 4B). Taken together, these data suggest that PTEN may be necessary for PGE₂ to mediate its suppressive effects on BMT AM function against opsonized targets; however, BMT AMs retain PTEN-independent defects in nonopsonized phagocytosis.

After infection, total lung leukocyte numbers and neutrophil recruitment are similar in WT BMT and PTEN CKO BMT mice (Figures 7B and 7D). However, uninfected PTEN CKO BMT mice display an increase in total leukocyte number relative to WT BMT mice (Figure 7A). We found significantly more neutrophils and fewer macrophages in the lungs of uninfected PTEN CKO BMT mice compared with uninfected control and WT BMT mice (Figure 7C). In Figure 7E, we demonstrate that neutrophil killing of serum-opsonized *P. aeruginosa* is restored to control levels in PTEN CKO BMT mice. Taken together, our results indicate that PTEN CKO BMT mice have fully restored AM phagocytosis of serum-opsonized bacteria, more neutrophils at baseline, and normal neutrophil killing ability.

Despite these improvements in host defense, *in vivo* clearance of *P. aeruginosa* in PTEN CKO BMT mice is not fully restored to control levels. We believe this is best explained by the observation that nonopsonized bacterial phagocytosis is still impaired in PTEN CKO BMT AMs relative to control AMs (see Figure 3E). This is likely because IRAK-M is elevated in the AMs from PTEN CKO BMT mice (see Figure 6). This elevation in IRAK-M in the PTEN CKO BMT AMs may also explain why TNF- α production is not fully restored to control levels (see Figure 4A), assuming the signal to up-regulate TNF- α is mediated via TLR signaling. We have previously shown that increased PGE₂ production after BMT elevates IRAK-M expression in AMs (20). Transplantation of IRAK-M^{-/-} bone marrow into WT mice fully restores AM phagocytosis of nonopsonized *P. aeruginosa* and *in vivo* clearance of *P. aeruginosa* after an acute (24-h) infection. Putting our two studies together, we conclude that nonopsonized phagocytosis of *P. aeruginosa* by AMs plays a critical role in clearance of acute infection and is negatively regulated by PGE₂-induced IRAK-M. Although we believe the partial restoration of bacterial clearance in our PTEN CKO BMT mice is best explained by our observation that nonopsonized phagocytosis by AMs is not fully recovered,

PTEN activity at later time points after infection. Up-regulation of PTEN activity at later time points may serve as a negative-feedback control mechanism to limit further macrophage activation via IgG-opsonized targets. This may prevent potentially harmful and prolonged activation of the innate immune response.

Previous studies have shown that in murine models of neutropenia, myeloid-specific ablation of PTEN can enhance opsonized bacterial clearance and proinflammatory cytokine production in AMs (38). Our data demonstrate a similar enhancement of host

another possibility is that neutrophils in the PTEN CKO BMT may display prolonged survival (30, 38), resulting in increased neutrophil damage to the lung, delayed immune resolution, and diminished tissue repair. Because PTEN is a critical regulator of cellular apoptosis, this may well be occurring.

IRAK-M protein expression is elevated in WT BMT and PTEN CKO BMT AMs relative to control AMs (see Figure 6). These data suggest that PGE₂-mediated up-regulation of IRAK-M may occur independently of PTEN to regulate AM host defense functions. Thus, our data support a model where PGE₂ signaling induces two unique pathways that regulate host defense. The up-regulation of PTEN by PGE₂ primarily causes inhibition of opsonized AM phagocytosis and neutrophil killing after BMT. Simultaneously, PGE₂-mediated up-regulation of IRAK-M inhibits nonopsonized phagocytosis in AMs after BMT.

Susceptibility to *P. aeruginosa* is widely considered to be a consequence of neutropenia (44). However, our data demonstrate that isolated defects in AM phagocytosis of nonopsonized bacteria can render the host susceptible to this infection. Our findings are supported by previous observations demonstrating that clodronate liposome depletion of AMs negatively affects lung host defense against *P. aeruginosa* *in vivo* (44).

Overall, our data suggest that PGE₂ may inhibit multiple mechanisms of phagocytosis in BMT AMs via the up-regulation of PTEN activity and the induction of IRAK-M. Blocking PTEN activity mitigates the suppressive effects of PGE₂ on opsonized bacterial phagocytosis in AMs and restores neutrophil killing after BMT. However, ablation of PTEN may not be sufficient to fully restore host defense against acute *P. aeruginosa* infection, given that nonopsonized phagocytosis by PTEN CKO BMT AMs appears to be inhibited by elevated IRAK-M expression. As such, our results suggest that a better strategy to restore host defense after BMT may be to target the production of PGE₂ or EP2 receptor signaling. In fact, pharmacologic blockade of PGE₂ production by indomethacin after BMT can fully restore host defense (12). An important future goal will be to determine whether these same PGE₂-induced alterations are present in human AMs after HSCT.

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