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A Role for IL-1 Receptor-Associated Kinase-M in Prostaglandin E2-Induced Immunosuppression Post-Bone Marrow Transplantation

Leah L. N. Hubbard,* Megan N. Ballinger,† Peedikayil E. Thomas,‡ Carol A. Wilke,§ Theodore J. Standiford,† Koichi S. Kobayashi,§ Richard A. Flavell,§ and Bethany B. Moore†

Following immune reconstitution, hematopoietic stem cell transplant patients often display reduced immune function and are especially susceptible to lung infections. In a mouse model of syngeneic bone marrow transplantation (BMT), we previously reported that PGE2 is overproduced in lungs of BMT mice, significantly impairing host defense against Pseudomonas aeruginosa. This impairment in host defense post-BMT is also marked by diminished alveolar macrophage (AM) phagocytosis, bacterial killing, and production of TNF-α and cysteinyl leukotrienes. However, a mechanism by which overproduction of PGE2 suppresses pulmonary host defense post-BMT is unknown. As IL-1R–associated kinase (IRAK)-M is a known inhibitor of MyD88-dependent IL-1R/TLR signaling and macrophage function, we sought to determine whether IRAK-M is involved in PGE2-induced immunosuppression post-BMT. We found that IRAK-M expression is elevated 3.5-fold in BMT AMs relative to control AMs, and this is related to AM overproduction of PGE2. Furthermore, genetic ablation of IRAK-M in the bone marrow of BMT mice restores host defense against P. aeruginosa. Despite AM overproduction of PGE2 and elevated E prostanoid 2 receptor expression, AM phagocytosis, killing, and production of cysteinyl leukotrienes and TNF-α are restored in the absence of IRAK-M post-BMT. Also, treatment with PGE2 does not inhibit AM phagocytosis in the absence of IRAK-M. These data suggest that the absence of IRAK-M in the hematopoietic compartment post-BMT enhances pulmonary host defense and mitigates AM sensitivity to the inhibitory effects of PGE2. Therefore, strategies to limit IRAK-M elevation post-BMT may be efficacious in reducing patient susceptibility to infection. The Journal of Immunology, 2010, 184: 000–000.

Hematopoietic stem cell transplantation (HSCT) is an effective treatment for certain forms of cancer and genetic diseases. However, delays in immune reconstitution render HSCT patients susceptible to infection (1, 2). Even after immune reconstitution, donor-derived cells are often functionally immature, and patients remain at risk for infectious complications months to years posttransplant. This susceptibility is noted in both allogeneic and autologous transplant recipients (3, 4).

Bacterial pneumonia is a common infectious complication post-HSCT and remains one of the leading causes of infection-related patient mortality (2, 5). This incidence is likely related to impaired pulmonary innate immunity. Alveolar macrophages (AMs) constitute up to 95% of the immune cells in the alveolar space and are essential in mediating innate immune responses in the lung (6–8). After allogeneic HSCT, AMs have a decreased ability to phagocytose and kill bacteria (9). Furthermore, neutrophils recruited to sites of infection display impaired chemotaxis and killing ability (10).

Allogeneic transplants, however, have multiple confounding factors, such as immunosuppressive drug therapy and graft–versus-host disease, which can impair immune function (1, 3). Therefore, autologous or syngeneic transplants, which are not confounded by such factors, are useful for investigating the effect of conditioning and reconstitution alone on pulmonary host defense.

We previously developed a mouse model of syngeneic bone marrow transplant (BMT) to determine how HSCT alone impacts host defense in the lung. Compared with nontransplant controls, BMT mice are more susceptible to pneumonia following intratracheal (i.t.) challenge with Pseudomonas aeruginosa (11). Furthermore, BMT AMs display impaired host defense mechanisms in ex vivo assays, including decreased phagocytosis, bacterial killing, and TNF-α production. Impaired bacterial killing is also observed in recruited lung neutrophils (12). These defects in host defense were directly related to overproduction of PGE2 in the lung post-BMT (5, 12, 13). PGE2 is a lipid mediator with a variety of immunosuppressive properties. Several studies have shown that PGE2 inhibits bacterial killing, phagocytosis (14, 15), chemotaxis (16), and production of proinflammatory mediators in leukocytes (17–19). However, a mechanism for this is not well described, and it remains...
unclear how overproduction of PGE₂ post-BMT impairs pulmonary host defense.

In this study, we suggest a role for IL-1R–associated kinase (IRAK)-M as a mediator of PGE₂-induced immunosuppression post-BMT. IRAK-M is a member of the IRAK family of serine/threonine kinases involved in MyD88-dependent IL-1R/TLR signaling. In mice, IRAK-M is expressed mainly in monocytic cells in response to repeated endotoxin exposure (20–22). To prevent excessive inflammatory responses to endotoxin, IRAK-M limits proinflammatory cytokine production by inhibiting MyD88-dependent IL-1R/TLR signaling (20, 22). This inhibitory response may also be induced during sepsis, where increased IRAK-M expression in macrophages downregulates excessive proinflammatory cytokine production (23, 24). Given the role for IRAK-M in immunosuppression, we hypothesize that overproduction of PGE₂ post-BMT upregulates IRAK-M expression in AMs, thus inhibiting host defense.

To address this hypothesis, expression of IRAK-M was measured in BMT and nontransplant control AMs. In addition, experiments were setup using IRAK-M–deficient mice to determine the role of IRAK-M in pulmonary host defense post-BMT. We show for the first time that PGE₂ signaling can upregulate IRAK-M expression in AMs, and hematopoietic expression of IRAK-M post-BMT impairs pulmonary host defense.

Materials and Methods

Animals

Wild-type (WT) C57BL/6 (B6) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). IRAK-M–deficient mice bred on a B6 background have previously been described (22) and were bred at the University of Michigan (Ann Arbor, MI). Mice were housed under specific pathogen-free conditions and monitored daily by veterinary staff. All mice were euthanized by CO₂ asphyxiation. The University of Michigan Committee on Use and Care of Animals approved these experiments.

Bone marrow transplantation

Recipient mice received 13.5 Gy of total body irradiation (TBI; orthovoltage x-ray source) split in two fractions, 3 h apart. Bone marrow cells were harvested from donor mice and resuspended in serum-free medium (SPF; DMEM, 0.1% BSA, 1% penicillin-streptomycin, 1% l-glutamine, and 0.1% amphotericin B). Bone marrow cells (5×10⁶) were administered by tail vein injection into TBI recipient mice. All experiments with BMT mice were performed 5–6 wk post-BMT when mice were fully donor-cell reconstituted (12, 13, 25). Spleen cells were >94% donor-derived, and AMs were >80% donor derived at this time point (13).

P. aeruginosa PA01 preparation and FITC labeling

P. aeruginosa PA01 stock was grown in tryptic soy broth (Difco; BD, Sparks, MD), and the culture concentration was determined via absorbance measurements as described previously (11). For FITC labeling, a P. aeruginosa culture was centrifuged and washed twice with resuspending cell pellet in 1 ml sterile PBS and transferring into a sterile tube. P. aeruginosa was heat-killed by autoclaving for 20 min and resuspended at 10⁵–10⁶ CFU/ml in 0.1 M NaHCO₃ (pH 9.2). A total of 0.2 mg/ml FITC (Sigma-Aldrich, St. Louis, MO) in DMEM was added to heat-killed P. aeruginosa and allowed to incubate in the dark for 1 h on a rocker at room temperature. Following FITC labeling, heat-killed P. aeruginosa were washed three times and resuspended in 1 ml sterile PBS. Aliquots were prepared and stored at −80°C until use.

i.t. injection with P. aeruginosa

A culture of P. aeruginosa was grown as described above, and an inoculum was prepared. Mice were anesthetized and i.t. injected with 50 µl inoculum to provide either a sublethal dose of 5×10⁵ CFU or a lethal dose of 1.5×10⁶ CFU as described previously (11, 12).

Quantification of bacterial burden in lung and blood

Mice were euthanized 24 h following i.t. infection with P. aeruginosa. As previously described (12), whole-lung and blood samples were collected from each mouse, and bacterial burden of each specimen was assessed by performing a CFU assay.

AM isolation and adherence purification

AMs were harvested by bronchoalveolar lavage (BAL), counted, and adherence purified as described previously (25). Where indicated, AMs were cultured overnight on 24-well tissue culture plates in the presence or absence of PGE₂ (Cayman Chemical, Ann Arbor, MI) or in the presence or absence of indomethacin (Sigma-Aldrich) or vehicle (0.25% ethanol) in complete medium (CM; DMEM, 10% FCS, 1% penicillin-streptomycin, 1% l-glutamine, and 0.1% amphotericin B). Following treatment, samples were prepared as described below for real-time RT-PCR or Western blot analysis.

Neutrophil recruitment to lung and isolation

As previously described, neutrophils were recruited to the lung via i.t. injection of a 25-µg dose of P. aeruginosa-derived LPS (Sigma-Aldrich) and isolated by BAL 24 h later (12). Neutrophils made up ~80% of all BAL cells at this time point in all groups.

In vivo inhibition of endogenous PGE₂ production

As reported previously (12, 26), BMT mice were injected i.p. with a 100-µl dose of either 1.2 mg/kg indomethacin (Sigma-Aldrich) or 1% DMSO (vehicle) in sterile PBS. Twenty-four hours following i.p. injection, AMs were harvested from mice by BAL and purified as described above.

Total lung leukocyte preparation

Whole-lung samples were harvested from mice and collagenase digested as previously described (23) to isolate lung leukocytes. For each sample, total viable cell number was counted on a hemacytometer by trypan blue exclusion. Where indicated, remaining cells were either stained for flow cytometry (as described below) to identify lymphocyte populations or stained with modified Wright-Giemsa stain to determine the percentage of neutrophils, monocytes/macrophages, lymphocytes, and eosinophils in each sample.

Flow cytometry

Following total lung leukocyte preparation described above, 1×10⁶ cells were stained using fluorochrome-conjugated Abs against the cell surface markers CD45, CD4, CD8, CD19, NK1.1, and TCRβ (BD Pharmingen, San Diego, CA) following incubation with anti-CD16/CD32 (Fc Block; BD Pharmingen). To enumerate lymphocyte subsets, gates were first set on CD45-expressing cells followed by gating on the lymphocyte-sized subset. AMs were identified as TCRβ⁺CD4⁺CD8⁻. CD8 T cells were identified as TCRβ⁺CD8⁺CD4⁻. NK cells were identified as TCRβ⁺NK1.1⁺. B cells were identified as CD19⁺. NK cells were identified as TCRβ⁺NK1.1⁻.

In vitro phagocytosis assay

AMs isolated by BAL were plated at 2×10⁵ cells/well and cultured overnight in CM on a 96-well, flat-bottomed, half-area tissue culture plate (Costar, Cornning, NY). The following day, wells were aspirated and replaced with 50 µl CM. Where indicated, AMs were pretreated with or without 10 nM PGE₂ for 15 min (Cayman Chemical). AMs were then incubated with either FITC-labeled Escherichia coli using the Vybrant Phagocytosis Assay kit, according to the manufacturers’ instructions (Invitrogen, Carlsbad, CA), or FITC-labeled heat-killed P. aeruginosa (prepared as described above) at 300:1 multiplicity of infection. Two hours following incubation at 37°C in dark, 50 µl trypan blue (250 µg/ml in 0.09 M citrate buffer solution; Sigma-Aldrich) was added to each well for 1 min to quench fluorescence of non-phagocytosed FITC-labeled bacteria. AM phagocytosis of FITC-labeled bacteria was measured using a microplate fluorimeter and expressed in arbitrary fluorescence intensity units as described previously (11, 14). For possible differences in AM adherence to tissue culture plate, data were normalized for cell number using a LDH Cytotoxicity Detection Kit (Roche Diagnostics, Indianapolis, IN) as described previously (27).

Bacterial killing tetrazolium dye reduction assay

AMs isolated by BAL were plated at 2×10⁵ cells/well in replicate on two 96-well tissue culture plates (one control and one experimental plate). AMs were cultured overnight in CM, and the following day, AM killing of P. aeruginosa was quantified using a tetrazolium dye reduction assay as described previously (28). Briefly, AMs on the control plate were allowed only to phagocytose bacteria, whereas AMs on the experimental plate were
permitted to both phagocytose and kill ingested bacteria. After 5 h of incubation, the amount of surviving ingested bacteria was quantified using an MTT assay according to the manufacturer’s instructions (Sigma-Aldrich). Results were expressed as percent surviving ingested bacteria = (A595 of control/A595 experimental) × 100%.

**Real-time RT-PCR**

Real-time RT-PCR was performed on an ABI Prism 7000 thermocycler (Applied Biosystems, Foster City, CA). Gene-specific primers and probes were designed using Primer Express software (PE Biosystems, Foster City, CA) as published previously (12, 23). Sequences for all primers and probes used can be found in Table I. Each AM sample was pooled from two to three mice and was run in duplicate. Average cycle threshold (Ct) was determined for each sample and was normalized to β-actin. Relative gene expression was calculated as described previously (25).

**Western blot analysis**

Whole-cell lysates of AMs or neutrophils were obtained by treating cells with radioimmunoprecipitation assay buffer as described previously (23). Protein concentration in lysates was determined by the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA). Protein samples (10 μg) were electrophoresed in a 4–20% Tris-Glycine Novex Pre-Cast Gel (Invitrogen) and transferred to a nitrocellulose membrane. Membranes were blocked for 1 h at room temperature with 5% skim milk and probed with one of the following primary Abs: rabbit anti–IRAK-M (StressGen Bioreagents, Ann Arbor, MI); mouse anti–β-actin (Pierce Chemical Co., Rockford, IL); rabbit anti–cPLA2, and rabbit anti–5-lipoxygenase–activating protein (Santa Cruz Biotechnology, Santa Cruz, CA); and rabbit anti–cyclooxygenase-2, rabbit anti-PGE synthase 1, rabbit anti–EP2, rabbit anti–EP4, and rabbit anti–5-LOX (Santa Cruz Biotechnology, Santa Cruz, CA). To assess protein expression, band intensity was quantified using ImageJ Software available for download at http://rsweb.nih.gov/ij/download.html.

**ELISA/Enzyme immunoassay**

AMs were cultured overnight at 2 × 10⁶ cells/ml in a 96-well tissue culture plate, and supernatants were collected the following day for enzyme immunoassay (EIA). For cytokine leukotriene (cys-LT) determination, AMs were cultured in SFM instead of GM and lung homogenates were prepared using the SepPak procedure to isolate lipids prior to EIA analysis as described previously (29). TNF-α production was measured using a DuoSet ELISA kit (R&D Systems, Minneapolis, MN). Production of PGE₂ and total cys-LTs (LTC₄, LTD₄, and LTE₄) was measured by EIA (Cayman Chemical), according to the manufacturer’s instructions.

**Statistical analysis**

Statistical significance was analyzed using the Prism 5.01 statistical program (GraphPad Software, San Diego, CA). The Kaplan-Meier and log-rank methods were used to analyze survival rates. Comparisons between two experimental groups were performed using the Student t test. Comparisons among three or more experimental groups were performed with ANOVA and a posthoc Bonferroni test. A value of p < 0.05 was considered statistically significant.

**Results**

**BMT AMs have elevated IRAK-M expression**

IRAK-M can limit proinflammatory cytokine production in macrophages (20, 22). Given that AMs have impaired TNF-α production post-BMT (11), we wanted to determine whether IRAK-M expression was elevated in BMT AMs. AMs from BMT and non-transplant control mice were harvested and analyzed for IRAK-M mRNA using primers found in Table I and protein expression. Relative to control AMs, BMT AMs had approximately a 3.5-fold increase in both IRAK-M mRNA (Fig. 1A) and protein (Fig. 1B, 1C) expression. IRAK-M protein expression was also measured in neutrophils recruited to the lungs of control and BMT mice following LPS i.t. injection. No appreciable differences were observed in IRAK-M protein expression between control and BMT neutrophils (Fig. 1D).

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### Table I. Primers and probes used in real-time RT-PCR analysis

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**Elevated IRAK-M expression in BMT AMs is related to overproduction of PGE₂**

Our previous work has demonstrated that BMT AMs display increased PGE₂ production relative to control AMs (12), and this elevation in PGE₂ production is accompanied by increased BMT AM expression of PG synthetic enzymes (Supplemental Fig. 1). To determine whether elevated IRAK-M expression in AMs is...
related to increased PGE$_2$ signaling, WT nontransplant AMs were cultured overnight with or without 10 nM PGE$_2$. PGE$_2$ treatment increased IRAK-M protein expression 2.5-fold over untreated AMs (Fig. 2A, 2B). Similar results were observed by treating AMs with 10 nM butaprost-free acid (data not shown), an agonist selective for the E prostanoid 2 (EP2) receptor, which is known to mediate the suppressive effect of PGE$_2$ on AM phagocytosis and killing (12, 14, 30).

We next sought to determine whether overproduction of PGE$_2$ was elevating IRAK-M expression in BMT AMs. AMs from BMT and control mice were cultured overnight in the presence or absence of 5 μM indomethacin (or vehicle control) to inhibit endogenous PGE$_2$ production. Relative to untreated control AMs, vehicle-treated BMT AMs expressed 3.1-fold more IRAK-M protein; however, treatment with indomethacin reduced elevation of IRAK-M protein in BMT AMs by 40% (Fig. 2C, 2D). Inhibition of endogenous PGE$_2$ production was verified by EIA using overnight culture supernatants (data not shown). We then wanted to determine whether in vivo inhibition of endogenous PGE$_2$ production could also reduce elevation of IRAK-M in AMs post-BMT. Twenty-four hours following i.p. injection of either indomethacin or vehicle control into BMT mice, we found that AMs from vehicle-treated BMT mice had a 3.2-fold increase in IRAK-M expression relative to AMs from untreated control mice. In vivo administration of indomethacin reduced IRAK-M elevation in BMT AMs by 51%, relative to BMT AMs from vehicle-treated mice (Fig. 2E, 2F). We previously demonstrated that in vivo treatment with indomethacin improved host defense of BMT mice when infected with *P. aeruginosa* (12). Collectively, these data indicate that AM overproduction of PGE$_2$ may contribute to elevated IRAK-M expression in AMs and decreased host defense post-BMT.

**IRAK-M$^{-/-}$ BMT mice are protected from pneumonia following *P. aeruginosa* infection**

Expression of IRAK-M is associated with reduced host defense following in vivo bacterial challenge (22, 23). Because IRAK-M is elevated in BMT AMs (Fig. 1), we wanted to determine whether the absence of IRAK-M in the hematopoietic cell compartment could improve pulmonary host defense post-BMT. Control, WT BMT (WT > WT), and IRAK-M$^{-/-}$ BMT (IRAK-M$^{-/-}$ > WT) chimeric mice were challenged with a 24 h, acute *P. aeruginosa* lung infection. As previously published, WT BMT mice had increased bacterial burden in the lung (Fig. 3A) and blood (Fig. 3B) relative to control mice. However, IRAK-M$^{-/-}$ BMT mice had enhanced clearance of *P. aeruginosa* from the lung and reduced dissemination to the blood relative to WT BMT mice. In fact, the host defense capabilities of IRAK-M$^{-/-}$ BMT mice were not significantly different from control mice. Overall, these results suggest that hematopoietic IRAK-M expression post-BMT impairs host defense against *P. aeruginosa* infection, and genetic ablation of IRAK-M in the bone marrow donor can be protective.

**FIGURE 2.** PGE$_2$ increases IRAK-M expression in BMT AMs. A, Protein lysates were prepared from WT nontransplant AMs cultured overnight at $5 \times 10^5$ cells/well in the presence or absence of 10 nM PGE$_2$ in CM. IRAK-M and β-actin protein expression was analyzed by Western blot. The blot shown is from a single experiment representative of two. B, Densitometry quantification of blots from two experiments as shown in A. IRAK-M expression was normalized to β-actin for each sample, and expression was graphed as fold change above control. C, Protein lysates were prepared from control and BMT AMs cultured overnight at $5 \times 10^5$ cells/well in the presence or absence of 5 μM indomethacin (to inhibit PGE$_2$) or vehicle (0.05% ethanol) in CM. IRAK-M and β-actin protein expression was analyzed by Western blot. The blot shown is from a single experiment representative of two. D, Densitometry quantification of blots from two experiments as shown in C. E, BMT mice were i.p. injected with a 100-μl dose of either 1.2 mg/kg indomethacin (Sigma-Aldrich) or 1% DMSO (vehicle) in sterile PBS. Twenty-four hours following i.p. injection, AMs were harvested from control and BMT mice, and protein lysates of AM samples were prepared. IRAK-M and β-actin protein expression was analyzed by Western blot. The blot shown is from one experiment representative of two. F, Densitometry quantification of blots from two experiments as shown in E. For D and F, *p < 0.05; **p < 0.01.

**FIGURE 3.** Restored host defense in IRAK-M$^{-/-}$ BMT mice following acute *P. aeruginosa* lung infection. Bone marrow from WT or IRAK-M$^{-/-}$ mice was transplanted into lethally TBI WT recipients. Control, WT BMT (WT > WT), and IRAK-M$^{-/-}$ BMT (IRAK-M$^{-/-}$ > WT) mice were i.t. injected with a 50-μl dose of $5 \times 10^5$ CFU *P. aeruginosa* 5–6 wk post-BMT. Twenty-four hours following infection, bacterial burden of whole-lung (A) and blood (B) samples from each mouse was assessed by CFU assay. *p < 0.05; **p < 0.01; combined data from two independent experiments; n = 6–10 mice/group.
IRAK-M\(^{-/-}\) BMT mice have a survival advantage following lethal P. aeruginosa infection

IRAK-M\(^{-/-}\) BMT mice show restored bacterial clearance relative to WT BMT mice following acute P. aeruginosa lung infection (Fig. 3). To determine whether enhanced bacterial clearance in IRAK-M\(^{-/-}\) BMT mice would provide a survival advantage following a lethal P. aeruginosa lung infection, we i.t. injected control, WT BMT, and IRAK-M\(^{-/-}\) BMT mice with \(1.5 \times 10^6\) CFU P. aeruginosa. As predicted, the rate of survival of IRAK-M\(^{-/-}\) BMT and control mice was not statistically different, but WT BMT mice had a significantly increased mortality rate compared with both control and IRAK-M\(^{-/-}\) BMT mice (\(p < 0.05\)) (Fig. 4).

Inflammatory response during early P. aeruginosa infection

We next wanted to determine whether improved host defense in IRAK-M\(^{-/-}\) BMT mice was a result of differential accumulation of leukocytes in the lung during early infection. Control, WT BMT, and IRAK-M\(^{-/-}\) BMT mice were injected with P. aeruginosa, and 4 h later, lungs were harvested to assess leukocyte populations. Total lung leukocyte number was similar among all three groups (Fig. 5A). The percentage of lung macrophages, lymphocytes, neutrophils, and eosinophils was not statistically different between WT BMT and IRAK-M\(^{-/-}\) BMT mice 4 h postinfection (Fig. 5B). Percent neutrophil accumulation ranged between 8 and 25% among control, WT BMT, and IRAK-M\(^{-/-}\) BMT mice at 4 h postinfection (Fig. 5B) but increased to 67–78% 24 h postinfection in all groups (data not shown). Frequency of lung lymphocyte populations was also determined among uninfected mice, and no significant differences were observed between WT BMT and IRAK-M\(^{-/-}\) BMT mice (Supplemental Fig. 2).

IRAK-M\(^{-/-}\) BMT AMs display enhanced phagocytosis and killing of P. aeruginosa

Restored pulmonary host defense in IRAK-M\(^{-/-}\) BMT mice may be explained by enhanced AM function in the absence of IRAK-M. To verify this hypothesis, ex vivo phagocytosis of FITC-P. aeruginosa was compared among AMs harvested from control, WT BMT, and IRAK-M\(^{-/-}\) BMT mice. Similar to previous reports, WT BMT AMs displayed a 35% decrease in phagocytosis relative to control AMs; however, IRAK-M\(^{-/-}\) BMT AMs had restored phagocytic ability (Fig. 6A). Bacterial killing was also assessed in control and BMT AMs ex vivo. Relative to WT BMT AMs, survival of ingested bacteria was significantly diminished in the IRAK-M\(^{-/-}\) BMT AMs (Fig. 6B). The amount of surviving bacteria ingested by IRAK-M\(^{-/-}\) BMT AMs was not statistically different from control AMs, indicating restoration of bacterial killing by BMT AMs in the absence of IRAK-M.
IRAK-M−/− BMT AMs have restored TNF-α and cys-LT production

Macrophage production of cys-LTs enhances phagocytosis, microbial killing, and production of proinflammatory cytokines such as TNF-α (28, 31–33). We have previously demonstrated that AMs from BMT mice are defective in production of both of these mediators (11, 12). We therefore wanted to determine whether improved host defense mechanisms in IRAK-M−/− BMT AMs were also marked by restored AM cys-LT and TNF-α production. AMs harvested from control, WT BMT, and IRAK-M−/− BMT mice were cultured unstirnated overnight, and supernatants were analyzed for cys-LT and TNF-α production. WT BMT AMs had significantly reduced levels of TNF-α (Fig. 7A) and cys-LTs (Fig. 7B) relative to control AMs. In contrast, levels of both TNF-α and cys-LTs were restored in IRAK-M−/− BMT AMs to levels seen in control AMs. Interestingly, this same trend was observed in whole-lung homogenates from control, WT BMT, and IRAK-M−/− BMT mice 4 h post-P. aeruginosa infection. TNF-α and cys-LT production was significantly reduced in WT BMT lung samples relative to control; however, IRAK-M−/− BMT mice had restored production of lung TNF-α and cys-LTs (Fig. 7C, 7D).

AM TLR, macrophage receptor with collagenous structure, and triggering receptor expressed on myeloid cells-1 expression

Enhanced macrophage production of proinflammatory cytokines and phagocytosis can be related to altered/elevated expression of cell surface receptors such as TLRs or scavenger receptors (34, 35). We wondered whether such alterations could explain the differences observed between WT BMT and IRAK-M−/− BMT AM host defense function. Expression of mRNA for TLR-2, -4, and -9, was measured (Fig. 8A–C). TLR2 mRNA expression was downregulated in both BMT AM groups relative to control AMs. TLR4 mRNA expression was similar between control, WT BMT, and IRAK-M−/− BMT AMs. TLR9 mRNA expression was upregulated ~1.7- and 1.9-fold in WT BMT and IRAK-M−/− BMT AMs, respectively. Additionally, we measured mRNA expression of the scavenger macrophage receptor with collagenous structure (MARCO) (Fig. 8D). Interestingly, we found that MARCO mRNA expression was downregulated not only in WT BMT AMs relative to control AMs, but MARCO was also downregulated in IRAK-M−/− BMT AMs. Finally, we looked at expression of the cell surface molecule, triggering receptor expressed on myeloid cells (TREM)-1. However, TREM-1 mRNA expression was reduced ~50% in both WT BMT and IRAK-M−/− BMT AMs relative to controls.

Both WT and IRAK-M−/− BMT AMs have elevated PGE2 production and EP2 receptor expression

Post-BMT, elevated PGE2 production in the lung significantly impairs AM phagocytosis and killing (5, 12, 13), and this is associated with elevated EP2 receptor expression in BMT AMs (12, 25). Therefore, we wanted to determine whether improved pulmonary host defense in IRAK-M−/− BMT AMs was a result of reduced PGE2 production and/or altered EP2 receptor expression in AMs. AMs were harvested from control, WT BMT, and IRAK-M−/− BMT mice, and AM EP2 mRNA expression was measured by real-time RT-PCR. It should be noted that reliable Abs to detect EP2 in the mouse are not available. Expression of EP2 in AMs was elevated in both transplant groups relative to control (Fig. 9A). As noted previously (12), analysis of overnight culture supernatants revealed an 8-fold increase in WT BMT AM PGE2 production over control AMs (Fig. 9B). Interestingly, we found that IRAK-M−/− BMT AMs overproduced PGE2 by 32-fold relative to control AMs. This phenotype of PGE2 overproduction was unique to the transplant environment, given that PGE2 production by AMs isolated from IRAK-M−/− nontransplant mice was comparable to WT control AMs (data not shown). Thus, AMs from IRAK-M−/− BMT mice display similar PGE2 and EP2 receptor profiles that characterize AMs from WT BMT mice.

IRAK-M is necessary for PGE2-mediated inhibition of AM phagocytosis

IRAK-M−/− BMT AMs have restored host defense (Figs. 6, 7), despite overproduction of PGE2 and elevated EP2 receptor expression (Fig. 9). Therefore, we wanted to determine whether IRAK-M was required for PGE2 to mediate inhibition of AM host defense. Ex vivo phagocytosis of FITC-labeled bacteria was assessed in nontransplant WT and IRAK-M−/− AMs pretreated in the presence or absence of 10 nM PGE2. PGE2 significantly inhibited WT AM phagocytosis but did not inhibit IRAK-M−/− AM

FIGURE 7. IRAK-M−/− BMT mice have restored TNF-α and cys-LT production. AM TNF-α (A) and cys-LT (B) production was measured by ELISA/EIA as described in Materials and Methods using overnight culture supernatants from unstimulated AMs plated at 2 × 106 cells/ml in CM and SFM, respectively. **p < 0.01; n = 4–7/group. TNF-α (C) and cys-LT (D) levels in lung homogenates 4 h post-i.t. infection with a 50-μl dose of 5 × 103 CFU P. aeruginosa. *p < 0.05; ***p < 0.001; n = 3–4/group.
phagocytosis (Fig. 10). Thus, in the absence of IRAK-M, AMs were not sensitive to the inhibitory effects of PGE₂.

**Discussion**

Overproduction of PGE₂ in the lung post-BMT significantly diminishes AM phagocytosis, killing, and TNF-α production (12, 13, 25). Although a mechanism has not been defined, our current study suggests a role for IRAK-M in PGE₂-induced immunosuppression post-BMT. We are the first to show that IRAK-M is elevated in BMT AMs, and this phenotype is related to increased PGE₂ signaling. Furthermore, we demonstrate a suppressive role for IRAK-M in host defense post-BMT, as genetic ablation of IRAK-M in the bone marrow restores AM function and host defense against *P. aeruginosa* lung infection.

Our results demonstrate that IRAK-M expression is elevated in AMs but not elicited lung neutrophils post-BMT (Fig. 1). IRAK-M expression has been associated mainly with monocytic cells; however, expression has also been detected in tissues as well as fibroblasts, B cells, and alveolar epithelial cells (20–22, 36). IRAK-M expression is rapidly induced by MyD88-dependent IL-1R/TLR signaling as part of a negative feedback mechanism. As a result, IRAK-M stably binds the MyD88 adaptor complex and prevents activation of pathways that initiate NF-κB–induced proinflammatory cytokine production (20, 22). This negative regulation of MyD88-dependent IL-1R/TLR signaling induces endotoxin tolerance as well as macrophage deactivation in sepsis and cancer (23, 24, 37). In our studies, we show that post-BMT, elevated IRAK-M expression in AMs is related to increased PGE₂ signaling (Fig. 2). It is interesting to speculate that this may also be a mechanism involved in macrophage deactivation in cancer, because certain tumors are known to overproduce PGE₂ (38).

PGE₂ is a part of the eicosanoid family of lipid mediators with potent immunomodulatory functions (31). PGE₂ in particular has...
a number of immunosuppressive properties. By elevating intracellular cAMP, PGE₂ signaling activates two key effectors: protein kinase A and Epac-1 (14, 15). In undefined pathways, activation of these two molecules collectively induces production of IL-10 and inhibits NFκB–induced proinflammatory cytokine production, FcR-mediated phagocytosis, and microbial killing pathways. Our results suggest that upregulation of IRAK-M may be one mechanistic pathway by which PGE₂ signaling limits AM host defense. PGE₂ can increase the transcription of IRAK-M within 3 h of stimulation (our unpublished observation), and protein levels are elevated by 24 h posttreatment (Fig. 2A). However, our results cannot determine whether IRAK-M has direct inhibitory effects on phagocytosis and killing or whether the improved antibacterial host defense seen in the absence of IRAK-M post-BMT is secondary to the restoration of proinflammatory cytokine and/or eicosanoid production.

Although exogenous and endogenous PGE₂ can elevate IRAK-M protein expression in AMs as much 3.5-fold above controls (Fig. 2), it is possible that factors aside from PGE₂ may also contribute to elevation of IRAK-M in AMs post-BMT. We previously reported that BMT AMs overproduce GM-CSF relative to control AMs (25). Recent studies have shown that GM-CSF regulates expression of IRAK-M in addition to other components of the TLR4 signaling pathway in primary mouse AMs (39). However, we did not detect increases in IRAK-M protein expression in nontransplant AMs following overnight treatment with 50 ng/ml GM-CSF (data not shown), whereas we could demonstrate augmentation of IRAK-M expression by exogenous PGE₂ treatment. In addition, it is possible that IRAK-M is upregulated because of increased endogenous endotoxin levels following BMT. Epithelial cell damage caused by TBI and/or chemotherapy conditioning regimens can result in loss of gut integrity (40). This can increase gut bacterial leakage and raise levels of circulating endotoxin. However, IRAK-M levels in BMT AMs remain elevated to a similar degree if the AMs are cultured overnight in CM or if they are assayed directly from mice. This suggests to us that the elevation in IRAK-M is related to autocrine production of PGE₂ in those cultures, rather than gut-derived LPS, which would presumably have washed out of the culture.

We assessed whether the alteration in AM function between the WT BMT and IRAK-M⁻/⁻ BMT mice could be related to increased TLRs and/or scavenger receptor expression post-BMT (Fig. 8). We found that expression of TLR2 was decreased, and TLR9 was increased in AMs from both WT BMT and IRAK-M⁻/⁻ BMT mice relative to control. Additionally, expression of TLR4 was not different among the three groups. MARCO is the major receptor on AMs for binding of unopsonized particles (41), and MARCO-deficient mice are more susceptible to pneumococcal infection (42). Expression of the scavenger receptor MARCO was decreased in both BMT groups relative to control AMs. The same trend was observed in regard to BMT AM mRNA expression of TREM-1, which has been shown to improve bacterial clearance from the lung following *Streptococcus pneumoniae* lung infection, possibly via alterations in IRAK-M expression (36). Thus, although there are some reductions in these surface molecules between the control and BMT groups, the differences we observe in WT BMT and IRAK-M⁻/⁻ BMT AM host defense functions are likely not related to altered expression of TLRs, MARCO or TREM-1. Furthermore, we conclude that IRAK-M elevation is not secondary to TREM-1 expression and that IRAK-M⁻/⁻ BMT AMs are capable of appropriate phagocytosis and killing despite reductions in MARCO and TREM-1.

Our data demonstrate that IRAK-M⁻/⁻ BMT mice display enhanced clearance of *P. aeruginosa* from the lung and diminished dissemination of *P. aeruginosa* to the blood (Fig. 3). Furthermore, IRAK-M⁻/⁻ BMT mice have a significantly reduced mortality rate relative to WT BMT mice following lethal *P. aeruginosa* lung infection (Fig. 4). These data suggest that hematopoietic expression of IRAK-M increases susceptibility to *P. aeruginosa* lung infection post-BMT, and ablation of hematopoietic IRAK-M expression provides more effective clearance of *P. aeruginosa* to improve survival rates in BMT mice following infection. Other studies have also demonstrated a protective role for IRAK-M deficiency in improved host defense. In both septic patients and mice, IRAK-M expression is elevated in monocytes and is associated with increased susceptibility to secondary infections, particularly bacterial pneumonia (23, 24). Interestingly, impaired pulmonary host defense following sepsis is improved in IRAK-M⁻/⁻ mice (23). In addition, IRAK-M⁻/⁻ mice display an enhanced ability to clear infection, following challenge with *Salmonella typhimurium*. This was associated with enhanced macrophage TLR signaling and proinflammatory cytokine production in response to TLR ligand and pathogen stimulus, respectively (22). Therefore, targeting IRAK-M may serve a number of therapeutic roles in reducing bacterial infections in immunosuppressed patients.

We also found that the absence of IRAK-M in BMT AMs enhances phagocytosis and killing (Fig. 6). Whether or not TLR signaling regulates phagocytosis is unclear. It has been argued that phagocytosis is a mechanical process independent of the TLR signals produced from endocytosed cargo (43–45). However, other studies indicate the possibility for TLR signaling to enhance phagocytosis via increases in phagosome maturation or upregulation of gene transcription programs associated with phagocytosis (46–48). Interestingly, cys-LTs are known to enhance a number of host defense mechanisms in AMs, such as phagocytosis, killing, and proinflammatory cytokine production (28, 31–33). Therefore, restored cys-LT production in IRAK-M⁻/⁻ BMT AMs (Fig. 7) likely contributes to enhanced phagocytosis, killing, and TNF-α production post-BMT. It is important to note, however, that AMs from nontransplanted IRAK-M⁻/⁻ mice display similar phagocytic ability as AMs from WT nontransplant mice (Fig. 10), and TNF-α production is comparable between groups (data not shown). Thus, these differences may not be apparent under basal conditions where expression of IRAK-M is low.

Given that IRAK-M has been identified in other cell types, we questioned whether IRAK-M could also be detected in neutrophils and, if so, whether expression of IRAK-M in these cells was altered in the setting of BMT. After eliciting neutrophils to the lungs of control or BMT mice via i.t. injection of LPS, we found no difference in total numbers of recruited neutrophils (data not shown). In these recruited lung neutrophils, we could detect IRAK-M by Western blot analysis, but there was no increase in BMT neutrophil IRAK-M expression relative to control neutrophils (Fig. 1D). This may reflect the fact that IRAK-M is maximally elevated in these cells in response to the LPS recruitment signal. However, there was no clear evidence that neutrophils upregulated IRAK-M in response to BMT. This is consistent with our earlier findings, which demonstrated no defect in the ability of neutrophils from BMT mice to phagocytose bacteria, despite elevations in neutrophil PGE₂ production post-BMT (11, 12). Additionally, we wondered whether there was a defect in neutrophil recruitment to the lung in response to *P. aeruginosa* challenge between WT BMT and IRAK-M⁻/⁻ BMT mice. Both WT BMT and IRAK-M⁻/⁻ BMT mice were effectively able to recruit neutrophils in response to bacterial challenge, and no differences were noted between the groups (Fig. 5B). Taken together, our results suggest that the suppressive actions of IRAK-M post-BMT may be restricted to...
AMs. These results are also consistent with a previous report that demonstrated impaired host defense against P. aeruginosa following clodronate liposome depletion of AMs (49).

Our earlier studies demonstrated that impaired host defense in AMs post-BMT requires elevation in PGE2 production and concomitant increases in expression of the inhibitory EP2 receptor. It is not known for certain why expression of PGE2 is elevated post-BMT, but it is likely to be the result of a general increase in the transcription or stability of all key PG synthetic enzymes (Supplemental Fig. 1). Interestingly, improved host defense in IRAK-M−/− BMT mice is observed despite elevated PGE2 production and EP2 receptor expression in IRAK-M−/− BMT AMs (Fig. 9). Furthermore, IRAK-M−/− AMs were insensitive to PGE2-mediated inhibition of non–Fc-mediated phagocytosis (Fig. 10). This suggests that PGE2 may require IRAK-M to inhibit AM function, and therefore, IRAK-M−/− BMT AMs may not be responsive to PGE2-induced immunosuppression despite the upregulation of the inhibitory receptor. PGE2 is also known to signal via another inhibitory receptor, EP4 (50). However, EP4 mRNA expression is of suppression necessary to improve AM function.

It will be to find ways to target IRAK-M and to determine the level of response to infection. An important aspect for our future studies targeting of IRAK-M post-BMT to improve patient outcomes in defense post-BMT. This presents the possibility for therapeutic ablation of IRAK-M in bone marrow improves pulmonary host response following clodronate liposome depletion of AMs (49).

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DISCLOSURES

The authors have no financial conflicts of interest.

REFERENCES


SUPPLEMENTAL FIGURE 1. Elevated BMT AM expression of PGE₂ synthetic enzymes and reduced expression of leukotriene synthetic enzymes. AMs were harvested by BAL as described in Materials and Methods from BMT and control mice. Protein lysates of control and BMT AMs were prepared, and expression of cPLA₂ (A); PGES-1 and -2 (B); COX-2, 5-LO, and FLAP (C); and β-actin (βAC) was measured by Western blot analysis. cPLA₂ releases arachidonic acid from membrane phospholipids. COX-2 and the PGES enzymes are necessary for PGE₂ synthesis. 5-LO and FLAP are necessary for leukotriene synthesis. Blots are representative of an n=3 per group.
SUPPLEMENTAL FIGURE 2. Lung lymphocyte populations in control and BMT mice. Lungs were harvested from uninfected control, WT BMT, and IRAK-M -/- BMT mice 5-6 weeks post-BMT. Lung leukocytes were isolated and analyzed by flow cytometry for specific cell surface markers as described in Materials and Methods to determine the total number of CD4 T cells (A), CD8 T cells (B), NK-T cells (C), B cells (D), and NK cells (E) in each sample (*, p < 0.05 compared to the control group; n=4-5 per group).