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PGE₂-induced changes in alveolar macrophage scavenger receptor profiles differentially alter phagocytosis of *P. aeruginosa* and *S. aureus* post-bone marrow transplant

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Abstract

The effectiveness of hematopoietic stem cell transplantation as a therapy for malignant and nonmalignant conditions is complicated by pulmonary infections. Using our syngeneic bone marrow transplant (BMT) mouse model, BMT mice with a reconstituted hematopoietic system displayed increased susceptibility to *Pseudomonas aeruginosa* and *Staphylococcus aureus*. BMT alveolar macrophages (AMs) exhibited a defect in *P. aeruginosa* phagocytosis while *S. aureus* uptake was surprisingly enhanced. We hypothesized that the difference in phagocytosis was due to an altered scavenger receptor (SR) profile. Interestingly, MARCO expression was decreased while SR-AI/II was increased. To understand how these dysregulated SR profiles might affect macrophage function, CHO cells were transfected with SR-AI/II and phagocytosis assays revealed that SR-AI/II was important for *S. aureus* uptake but not *P. aeruginosa*. Conversely, AMs treated *in vitro* with soluble MARCO exhibited similar defects in *P. aeruginosa* internalization as BMT AMs. The 3'UTR of SR-AI contains a putative target region for miR-155 and miR-155 expression is decreased post-BMT. Anti-miR-155-transfected AMs exhibited an increase in SR-AI/II expression and *S. aureus* phagocytosis. Elevated PGE₂ has been implicated in driving an impaired innate immune response post-BMT. *In vitro* treatment of AMs with PGE₂ increased SR-AI/II, and decreased MARCO and miR-155. Despite a difference in phagocytic ability, BMT AMs harbor a killing defect to both *P. aeruginosa* and *S. aureus*. Thus, our data suggest that PGE₂-driven alterations in scavenger receptor and miR-155 expression account for the differential phagocytosis of *P. aeruginosa* and *S. aureus* but impaired killing ultimately confers increased susceptibility to pulmonary infection.

Keywords

alveolar macrophage; transplantation; *Staphylococcus aureus*, *Pseudomonas aeruginosa*; scavenger receptors; lung

Background

Hematopoietic stem cell transplant (HSCT) is a widely used treatment to address a variety of inherited and genetic disorders in patients. Though effective, HSCT patients show increased susceptibility to numerous complications, many involving the lung, in the months to years following treatment (1–3). Infectious and non-infectious complications arise in HSCT recipients despite differences in stem cell source (2, 4). Autopsy reports found 80–89% pulmonary complication rates in both allogeneic and autologous HSCT patients (4, 5) and a recent study found that pulmonary complications manifested in more than 25% of autologous HSCT recipients, with the majority of the complications being infectious (3).

Bacterial pulmonary complications in HSCT recipients manifest at different times post-transplant and are varied in pathogen species (Gram-positive vs. Gram-negative). Although allogeneic transplant recipients develop pulmonary infectious complications more frequently (potentially due to barrier disruptions secondary to graft vs. host disease), autologous HSCT patients also experience infectious complications and there is no difference in the types of infections that manifest in autologous and allogeneic HSCT recipients in the first 30 days post-transplant (pre-engraftment phase) (6). While it is not surprising that infections are common in the pre-engraftment phase (7), or in allogeneic HSCT patients on immunosuppressive therapy, it is less understood why infections have also been reported to occur in autologous HSCT recipients in the late post-transplant phase (8). Both Gram-negative and Gram-positive infections can be problematic in HSCT patients. Prophylactic antibiotics have lowered the incidence of *Pseudomonas aeruginosa* infection, but invasive *Pseudomonas* remains a concern in this population (9, 10). Additionally, infections caused by Gram-positive bacteria, particularly the *Streptococcus* and *Staphylococcus* species, have risen as predominant infections in HSCT recipients (6).

In order to better understand the immunological alterations that may characterize innate immune cells in the lung and predispose the host to bacterial infection post-engraftment, our lab developed a syngeneic (syn) BMT animal model. This model allows for an investigation of transplant-related immune alterations that are not caused by immunosuppressive drugs or impaired barrier function secondary to graft vs. host disease. We previously demonstrated that C57BL/6 mice that have received a syn BMT are more susceptible to infection by Gram-negative *P. aeruginosa* (11). Increased susceptibility to *P. aeruginosa* was related to overproduction of prostaglandin E₂ (PGE₂) post-BMT and this increase in PGE₂ was regulated by hypomethylation of the cyclooxygenase (COX)-2 gene (12, 13). Elevations of PGE₂ post-BMT impaired alveolar macrophage (AM) phagocytosis and killing of ingested bacteria, and also limited neutrophil-mediated killing (12). Some of the effects of PGE₂ signaling on AMs post-BMT were to upregulate interleukin-1 receptor associated kinase (IRAK)-M and activation of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (14, 15). These changes limited inflammatory cytokine production and non-opsonized and opsonized phagocytosis of *P. aeruginosa* as well as bacterial killing.

The current studies were undertaken to determine whether syn BMT mice also displayed increased susceptibility to a Gram-positive, *S. aureus* infection and if so, whether the mechanisms of susceptibility were related to PGE₂ production and impaired phagocytosis mediated by scavenger receptors (SRs). Similar to our previous studies with *P. aeruginosa*,

we find syn BMT mice are more susceptible to *S. aureus* and this is related to overproduction of PGE₂. Surprisingly however, phagocytosis of *S. aureus* is enhanced, not diminished post-BMT. Our mechanistic studies identify PGE₂-induced changes, including alterations in miRNAs which alter the functional profile of scavenger receptors to differentially regulate phagocytosis. Despite the fact that phagocytosis of *S. aureus* is improved post-BMT, bacterial killing is impaired, explaining the overall defect in host defense.

Materials and Methods

Animals

Wild type C57BL/6 (B6) and SRAI/II^{-/-} mice (C57BL/6 background) were obtained from The Jackson Laboratory (Bar Harbor, Maine). Mice were housed under specific pathogen-free conditions and monitored daily by veterinary staff. All mice were euthanized by CO₂ asphyxiation. All animal experiments were approved by the University of Michigan Committee on Use and Care of Animals.

Bone marrow transplantation

Bone marrow was harvested from C57BL/6 donor mice and infused by tail vein injection into lethally irradiated C57BL/6 recipients. Ablation of recipient-derived HSCs was achieved by the administration of a fractionated 13 Gy dose of total body irradiation (TBI) from an X-ray orthovoltage source. Complete immune reconstitution is achieved five weeks following infusion of 5×10^6 whole bone marrow cells into TBI recipients (16, 17). The percentage of donor-derived cells was approximately 95% \pm 1% in the spleen and 82% \pm 2% in the lung at this time point, as assessed by transplanting CD45.1⁺ bone marrow into C57BL/6 CD45.2⁺ mice (16).

P. aeruginosa PAO1 and *S. aureus* (USA300/NRS384) preparation

P. aeruginosa PAO1 and *S. aureus* USA300 stock were grown in tryptic soy broth and nutrient broth (Difco; BD, Sparks, MD), respectively. The culture concentration was determined via absorbance measurements as previously described (11). For fluorescein isothiocyanate (FITC)-labeling, a culture was centrifuged and washed two times by resuspending the pellet in 1 mL sterile PBS and transferring into a sterile tube. The bacteria were heat-killed by autoclaving for 20 minutes and resuspended at $10^9 - 10^{10}$ CFU/mL in 0.1 M NaHCO₃ (pH 9.2). 0.2 mg/mL FITC (Sigma, St. Louis, MO) in DMSO was added to heat-killed *P. aeruginosa* or *S. aureus* and allowed to incubate in the dark for 1 hour on a rocker at room temperature. Following FITC-labeling, heat-killed *P. aeruginosa* or *S. aureus* were washed three times and resuspended in sterile PBS at 6×10^9 CFU/ml.

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

A culture of *P. aeruginosa* or *S. aureus* was grown as described above, and an inoculum was prepared. Mice were anesthetized and i.t. injected with 50 μ L of inoculum to provide either a sublethal dose of 5×10^5 PAO1 CFU as previously described (11, 12) or a sublethal dose of 7×10^7 USA300 CFU.

Quantification of bacterial burden in lung

Mice were euthanized 24 hours following i.t. infection with *P. aeruginosa* or *S. aureus*. As previously described (12), lungs were collected from each mouse, homogenized and the bacterial burden of each specimen was assessed by performing a CFU assay. Data are expressed as total CFU per lung.

Harvesting AMs

Resident AMs from mice were obtained via ex vivo lung lavage, using a previously described protocol (12). Briefly, these cells were collected in lavage fluid consisting of complete medium (DMEM, 1% penicillin-streptomycin, 1% L-glutamine, 10% FCS, 0.1% Fungizone) and 5 mM EDTA. The cells were enumerated by counting on a hemocytometer before plating.

In vitro phagocytosis assays

AMs were harvested as described above and the ability of the AMs from control and BMT mice to phagocytose via opsonin-independent pathways was examined using a 300:1 ratio of FITC-labeled *P. aeruginosa* or *S. aureus* to AMs. Briefly, 2×10^5 AMs were plated on a half-area black 96-well plate and were incubated overnight at 37°C. The next day, the media was changed to serum-free media and 10 μ l of FITC-labeled *P. aeruginosa* or *S. aureus* was added to each well. After 2 h at 37°C, trypan blue was added to quench extracellular fluorescence and phagocytosis was quantified as previously described (18).

Tetrazolium dye reduction assay of bacterial killing

The ability of AMs from control and BMT mice to kill *P. aeruginosa* and *S. aureus* was quantified using a tetrazolium dye reduction assay, as described elsewhere (19, 20). Briefly, AMs from WT and BMT mice were aliquoted into duplicate 96-well plates: one experimental (37°C) plate and one control (4°C). Cells from both plates were infected with IgG-opsonized *P. aeruginosa* or *S. aureus* (2×10^8 CFU/ml; multiplicity of infection (MOI), 50:1) for 30 min at 37°C. The cells on the experimental plate were washed then incubated at 37°C for 90 min, while the cells on the control plate were washed then lysed with TSB and 0.5% saponin (Sigma, St. Louis, MO) and placed at 4°C. After 90 min, the cells from the experimental plate were lysed with TSB and 0.5% saponin. Both plates were then incubated at 37°C for 2.5 h. Five mg/ml of MTT (Sigma, St. Louis, MO) 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. Results were expressed as percentage of survival of ingested bacteria normalized to the percent of control, where the A_{595} experimental values were divided by the average of the A_{595} control values. Survival of ingested bacteria = (A_{595} experimental plate/ A_{595} control plate) \times 100%.

Real-time RT-PCR

Gene-specific primers and probes were designed using Primer Express software (PE Biosystems, Foster City, CA) as published previously (12, 21). Sequences for primers and probes used can be found in Table 1. SnoR142 and miR-155 expression were measured using TaqMan® MicroRNA Assays (Applied Biosystems/Life Technologies, Foster City, CA). Each AM sample was pooled from two to three mice and was run in duplicate. MicroRNA expression was determined by first converting TRIzol-isolated RNA into cDNA using TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems/Life Technologies, Foster City, CA). Real-time RT-PCR was performed on an ABI Prism 7000 thermocycler (Applied Biosystems, Foster City, CA). Average cycle threshold (C_T) was determined for each sample and was normalized to β -actin or snoR142. Relative gene expression was calculated as described previously (22).

Flow Cytometry

5×10^5 primary AMs from untransplanted control and BMT mice were stained for flow cytometry using a primary antibody (2 μ g/mL) against the cell surface receptor MARCO (Hycult Biotech, Plymouth Meeting, PA) after incubation with anti-CD16/CD32 (FcBlock; BD Biosciences, San Jose, CA). Fluorochrome-conjugated secondary antibody, donkey anti-

rat PE, (Jackson Immunoresearch, West Grove, PA) was used to detect the primary antibody. Cells were resuspended in PBS and fixed with 4% paraformaldehyde. Data were analyzed using flow cytometry analysis software (FlowJo, version 7.5; Tree Star, Inc., Ashland, OR).

***In vitro* MicroRNA Transfection**

Primary AMs were harvested from untransplanted C57BL/6 mice as described above. 2×10^5 cells were transfected with the antagomir of miR-155 (Dharmacon/Thermo Fisher Scientific, Lafayette, CO) or anti-miR™ miRNA inhibitor negative control #1 (Ambion®/Life Technologies, Grand Island, NY) for 24 hours when measuring mRNA expression and 48 hours for phagocytosis assays. Transfections were performed using Lipofectamine® RNAiMAX Reagent (Invitrogen/Life Technologies, Grand Island, NY) as described by the manufacturer. Briefly, antagomir or control miR were diluted in Opti-MEM® I Reduced Serum Media (Life Technologies, Grand Island, NY) and mixed with Lipofectamine® RNAiMAX Reagent prior to transfecting the cells.

Statistical analysis

Statistical significance was analyzed using the GraphPad Prism 5.0 statistical program (La Jolla, CA). Comparisons between two experimental groups were performed using the Student t test and error bars denote the standard error of the mean. Comparisons between three or more values used an ANOVA analysis with a post hoc Bonferroni comparison. A p value <0.05 was considered statistically significant.

Results

Syn BMT mice are more susceptible to *S. aureus*

To determine the susceptibility of BMT mice to *S. aureus*, untransplanted (control) and syn BMT mice were i.t. injected with a methicillin-resistant strain of *S. aureus* or with the PAO1 strain of *P. aeruginosa* for comparison. Lungs were harvested 24 h post-infection for CFU analysis. BMT mice were more susceptible to both *P. aeruginosa* (Fig. 1A) and *S. aureus* (Fig 1B) pulmonary infections.

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

Because AMs are the predominant immune cell in the lung during an initial infection (23), we hypothesized that AM function was compromised in a BMT setting. Using AMs harvested from BAL of control and syn BMT mice, phagocytosis of *P. aeruginosa* by AMs was measured. As we have previously published (12), syn BMT AMs exhibited decreased phagocytosis of FITC-*P. aeruginosa* compared to controls (Fig. 2A). Surprisingly, syn BMT AMs incubated with FITC-*S. aureus* displayed augmented phagocytosis compared to controls (Fig. 2B).

SR profiles are altered post-BMT

Class A SRs are pattern recognition receptors reported to play important roles in the recognition of non-opsonized Gram-positive and -negative bacteria and endogenous ligands (24). Studies have shown that the macrophage receptor with collagenous structure (MARCO), a class A SR, is important for *in vivo* host response against *Streptococcus pneumoniae*, a Gram-positive bacterium (25), and the macrophage scavenger receptor A (MSR-A/SR-A) types I and II have been previously reported to recognize *S. aureus* (26). However, it remains unclear which SR is important for recognition of *P. aeruginosa* in the lung. As MARCO and SRAI/II have been implicated in the recognition and initiation of an

immune response within macrophages (24), we wanted to determine whether the difference in phagocytosis between *P. aeruginosa* and *S. aureus* could be explained by an alteration in SR expression on AMs. Using RNA isolated from control and BMT AMs, MARCO mRNA expression decreased post-BMT (Fig. 3A, left) while SR-AI/II mRNA increased (Fig. 3B) compared to control. A decrease in MARCO surface expression was confirmed by flow cytometry (Fig. 3A, right), correlating with MARCO mRNA analysis. Commercially available Abs against SRAI/II do not recognize the allele in the C57BL/6 mice, precluding our ability to measure protein expression of this receptor by flow cytometry in our model.

MARCO is negatively regulated by PGE₂ and is important for *P. aeruginosa* phagocytosis

To further understand how an altered SR profile on AMs post-BMT contributes to AM function, control AMs were treated with class A SR inhibitors and AM phagocytic capacity against non-opsonized *P. aeruginosa* was determined. Interestingly, soluble MARCO (sMARCO) inhibited internalization of *P. aeruginosa* significantly compared to no treatment (no txt) and treatment with control compounds poly C and chondroitin sulfate (ChSO₄) (Fig. 4A). Furthermore, the decrease in AM phagocytosis induced by sMARCO was comparable to the inhibition noted with the pan class A SR inhibitors [fucoidan and polyinosinic acid (poly I)], suggesting that MARCO may be the primary SR for *P. aeruginosa* phagocytosis by AMs. Cytochalasin D (cyto D) is an inhibitor of actin polymerization and was included as a negative control for phagocytosis. Although scavenger receptor with C-type lectin (SRCL) mRNA expression decreased in syn BMT AMs (data not shown), blocking SRCL did not affect uptake of non-opsonized *P. aeruginosa* in control AMs (Fig 4A) suggesting that this receptor is not used for *P. aeruginosa* uptake. Soluble mannan did not inhibit *P. aeruginosa* engulfment either, suggesting the mannose receptor is not involved in recognition of *P. aeruginosa* (not shown). Finally, Figure 4B shows that control AMs treated with sMARCO exhibited a similar defect in engulfment of *P. aeruginosa* as syn BMT AMs when compared to untreated control AMs. This highlights MARCO as the dominant *P. aeruginosa*-recognizing SR in AMs.

Our laboratory has previously shown that the defect in AMs post-BMT is, in part, mediated by upregulated COX-2 expression and overproduction of PGE₂. Treatment with indomethacin, a COX inhibitor, rescues host defense in BMT mice *in vivo* and restores the ability of BMT AMs to phagocytose *P. aeruginosa* (12). However, whether decreased MARCO post-BMT is driven by PGE₂ remains unknown. Therefore, untransplanted AMs were treated with 1 μM PGE₂ or vehicle DMSO *in vitro*. Following 24 h, RNA was harvested and real-time RT-PCR analysis of MARCO mRNA expression indicated that decreased MARCO expression is PGE₂-driven (Fig 4C).

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

Syn BMT AMs exhibited increased *S. aureus* phagocytosis compared to control AMs (Fig 2B) and SR-AI/II mRNA expression was increased post-BMT (Fig. 3B). To determine whether the observed AM phagocytosis of *S. aureus* may be due to increased SR-AI/II on macrophages following transplant, CHO cells (which do not express class A SRs) were transfected with SR-AI. Transfected and untransfected CHO cells were measured for internalization of *S. aureus*. SR-AI-transfected CHO cells were more efficient at phagocytosing *S. aureus* than untransfected CHO cells (Fig. 5A left), while *P. aeruginosa* internalization was unaffected by SR-AI transfection in CHO cells (Fig. 5A right). These data verify that SR-AI is utilized for the recognition of *S. aureus* and explain why SR-A elevations do not influence *P. aeruginosa* uptake. To confirm this result post-BMT, AMs harvested from BMT mice that received HSCs from SR-AI/II^{-/-} mice (SR-AI/II^{-/-} BMT) were unable to phagocytose *S. aureus* compared to WT BMT AMs (Fig 5B).

SR-AI/II negatively regulates MARCO expression

The fact that the phagocytic ability of AMs from SR-AI/II^{-/-} BMT mice was not completely abolished (Fig 5B) suggested that there may be some compensatory upregulation of MARCO expression in these mice which allowed phagocytosis of *S. aureus*. MARCO has previously been shown to mediate *S. aureus* uptake (28, 29). To determine if this were true, we compared the percent of MARCO expressing AMs in untransplanted WT and SR-AI/II^{-/-} mice (Fig 6A). In fact, SR-AI/II exerts a negative influence on MARCO expression. To study the effect of this *in vivo*, we created all combinations of chimeric BMT mice. In Fig 6B, untransplanted control C57Bl/6 mice or SR-AI/II^{-/-} mice were compared to BMT mice created with the following donor→host combinations: WT→WT, SR-AI/II^{-/-}→SR-AI/II^{-/-} or SR-AI/II^{-/-}→WT. When looking at *in vivo* host defense against *S. aureus*, non-transplanted SR-AI/II^{-/-} mice had similar lung CFU as control C57Bl/6 mice, presumably reflecting adequate MARCO expression in each strain at baseline. All BMT combinations were more susceptible than untransplanted mice, but there was no difference between the BMT groups. This suggests that uptake of *S. aureus* is mediated by either SR-AI/II or MARCO post-BMT as long as either receptor is available. This uptake was sufficient to maintain the infection in the lung as no dissemination of the *S. aureus* to the blood was noted (not shown). In contrast, when looking at *in vivo* responses to *P. aeruginosa*, a bacterium that can be recognized by MARCO but not SR-A, the SR-AI/II^{-/-}→WT mice (which likely have a compensatory MARCO upregulation) showed decreased lung and blood CFU burden when compared to the WT→WT BMT mice. There was no difference between WT→WT and WT→SR-AI/II^{-/-} BMT mice.

SR-AI/II expression is regulated by PGE₂ and miR-155 post-BMT

Figure 5 suggests SR-A plays a prominent role in the recognition and phagocytosis of *S. aureus*. However, why SR-AI/II is upregulated post-BMT is unclear. To determine whether PGE₂ signaling could upregulate SR-AI/II, control AMs were treated with PGE₂ *in vitro* and SR-AI/II mRNA expression was analyzed by real-time RT-PCR. Figure 7A demonstrates that PGE₂ signaling increases SR-AI/II mRNA expression. Elevations in SR-AI/II mRNA in AMs post-BMT (Fig 3B)(which have a high basal PGE₂ production (12)) and in exogenous PGE₂-treated macrophages (Fig 7A), suggest that changes in the transcriptional machinery or actions of key microRNAs indeed play a role in PGE₂-enhanced SR-AI/II expression. To our knowledge, this receptor does not have a cyclic AMP responsive binding element (CREB) (the main transcription factor induced by cAMP) secondary to PGE₂ signaling within the promoter region. Rather, SR-A type I contains a predicted target sequence in its 3' UTR for miR-155 (TargetScan). Thus, we hypothesized that miRNAs are involved in SR regulation post-BMT. Since SR-AI/II mRNA expression increased post-BMT and PGE₂ treatment, the data suggested the loss of a regulatory miRNA.

Analysis of miRNA expression profiles with a focused miRNA array (SABioscience) revealed miR-155 as the only miRNA significantly downregulated (approximately 7-fold post-syn BMT; Supplemental Fig. 1). To confirm that miR-155 was in fact decreased after BMT, miR-155 expression was determined by real-time RTPCR using miR-155-specific primers. Our data show that there was a 6-fold decrease in miR-155 expression in syn BMT (Fig. 7B) correlating well with the PCR array data. To determine whether the decrease in miR-155 expression was related to overproduction of PGE₂ in the lung after BMT, control AMs were harvested and treated with 10 μM PGE₂ for 24 h. miR-155 mRNA expression decreased upon treatment with PGE₂ compared to AMs treated with DMSO (vehicle; Veh) control (Fig. 7C).

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

Because miR-155 is decreased post-BMT, regulation of SR-AI/II by miR-155 was further explored. An antagomir of miR-155 (anti-miR-155) was transfected into control AMs to inhibit miR-155 endogenous expression. SR-AI/II mRNA expression was increased upon transfection of anti-miR-155 compared to cells transfected with a control antisense oligomer (anti-miR) (Fig. 8A). As SR-AI/II was shown in Fig. 5 to be important for *S. aureus* internalization, we were interested in understanding whether a decrease in miR-155 would have a functional effect on *S. aureus* internalization. AMs transfected with anti-miR-155 at increasing concentrations were measured for their ability to phagocytose *S. aureus*. Figure 8B shows *S. aureus* internalization increased dose-dependently with anti-miR-155. Transfection with anti-miR-155 had no effect on MARCO expression within this 48 h time point (data not shown).

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

Bacterial clearance by AMs relies not only on phagocytosis of the pathogen, but also on intracellular killing of the organism. While a defect in phagocytosis, as shown in Fig. 2A, would increase susceptibility to *P. aeruginosa*, AMs collected from syn BMTs are also unable to kill *P. aeruginosa* (Fig. 9A) as we have previously published (12). Because the BMT mice are more susceptible to *S. aureus in vivo* (Fig 1B), yet display enhanced phagocytosis of this Gram-positive bacteria (Fig 2B), we examined the ability of BMT AMs to kill ingested *S. aureus* and found that killing of this pathogen was impaired in syn BMT AMs when compared to control AMs (Fig 9B). Thus, killing defects likely account for the *in vivo* susceptibility to infection despite enhanced phagocytosis. Taken together, it is interesting to speculate that because both phagocytosis and killing are defective for *P. aeruginosa*, this could explain the approximately 4-log increase in bacterial susceptibility to this pathogen in BMT mice *in vivo*, whereas the improved phagocytosis of *S. aureus* moderates the killing defect to account for an approximate 1 log difference in susceptibility to this pathogen in BMT mice *in vivo*.

Discussion

Syn BMT mice show increased *in vivo* susceptibility to both *S. aureus* and *P. aeruginosa*, with the latter observations confirming previously published data (11, 12). While this impaired host response can be explained by an inability of BMT AMs to engulf *P. aeruginosa*, the same is not true with *S. aureus*. In fact, BMT AMs exhibit enhanced phagocytosis of *S. aureus*. This difference suggested that receptors mediating phagocytosis may have been altered following transplantation. Although MARCO and SR-AI/II have both been reported to have the ability of recognizing Gram-positive and – negative bacteria (25–28) and SR-AI/II has previously been linked to recognition of *S. aureus* (26), it was unclear which receptor was important for *P. aeruginosa* in control cells, and nothing was known about the regulation of these SRs post-BMT. Here, we show that MARCO is significantly downregulated at both the mRNA and protein levels, while SR-AI/II mRNA expression is increased post-BMT. These data indicate that MARCO is important for *P. aeruginosa* internalization as elevated levels of SR-AI/II were unable to rescue phagocytosis of this Gram-negative pathogen in BMT AMs or CHO cells. This observation was further supported by the finding that control AMs pretreated with soluble Marco were unable to phagocytose *P. aeruginosa* as efficiently.

A surprising result was the enhanced phagocytosis of *S. aureus* given that BMT mice were more susceptible to infection *in vivo*. As SR-AI/II can regulate *S. aureus* phagocytosis (26), elevations in SR-AI/II may explain this result. Here we show that CHO cells expressing SR-AI only, were able to phagocytose *S. aureus* but not *P. aeruginosa*. Furthermore, recipient

mice reconstituted with SR-AI/II^{-/-} donor marrow showed a reduction in *S. aureus* phagocytosis when compared to mice transplanted with wild-type (C57Bl/6) marrow (Fig 5B). These data support the importance of SR-AI/II for *S. aureus* and MARCO for *P. aeruginosa* phagocytosis. Interestingly, the fact that phagocytosis of *S. aureus* in the SR-AI/II^{-/-} BMT mice was not completely abolished suggested there may be some compensatory upregulation of MARCO in these mice as MARCO has been shown to recognize *S. aureus* (28, 29). These data highlight an interesting inhibitory role of SR-AI/II on MARCO expression which we have verified in Fig 6A. SR-AI/II^{-/-} untransplanted mice have a higher percentage of MARCO positive cells in the absence of infection. Therefore, it is also possible that the overexpression of SR-AI/II observed post-BMT may contribute to the decreased expression of MARCO in addition to the PGE₂ signaling. We believe the inhibitory effects of SR-AI/II on MARCO may not be immediate as transfection of the anti-miR-155 which upregulated SR-AI/II expression within 48 h (Fig 8A) did not yet alter MARCO expression, whereas PGE₂ stimulation reduced MARCO in 24 h (Fig 4C). Ultimately, SR-AI/II^{-/-} BMT mice may upregulate MARCO to improve their host defense against both pathogens, which is consistent with the observations in Fig. 6. Obviously the co-regulation of these receptors is complex, but on the whole our *in vivo* results are consistent with the conclusion that MARCO is uniquely responsible for *P. aeruginosa* uptake while *S. aureus* can be recognized by either MARCO or SRAI/II. Our findings of SR-AI/II inhibition of MARCO expression are also consistent with previously published data that suggest SRAI/II may have an inhibitory role on inflammatory responses (30, 31).

MicroRNA analysis of BMT AMs indicated that altered miRNA expression may be responsible for the upregulation of SRAI/II post-BMT. Specifically, miR-155 expression was decreased 6-fold and SR-AI contains a putative target sequence for miR-155 in its 3'UTR. As miRNAs generally function to destabilize or inhibit protein translation of the targets they regulate, we hypothesized decreased expression of miR-155 would be responsible for the increase in SR-AI/II observed post-BMT. Anti-mir-155-transfected AMs showed a significant increase in SR-AI/II expression. To determine whether this correlated with the functional phenotype observed after transplant, anti-mir-155-transfected AMs measured for phagocytosis of *S. aureus* exhibited a dose-dependent increase in *S. aureus* internalization. This provided convincing evidence that after transplant, the elevation of SR-AI/II was regulated by decreased miR-155 expression. Our data also demonstrate that this altered miR expression profile is secondary to PGE₂ signaling. PGE₂ also decreases MARCO, but the mechanism does not appear to involve miR-155. This finding provides new insight into intracellular regulation of scavenger receptors by microRNA. A recent study suggested miR-155 as a novel therapeutic target for improving graft vs. host disease (32). This is likely to also cause an inhibition of SR-AI/II which may impair phagocytosis of some pathogens, and the long term effects of this treatment on MARCO expression would need to be evaluated.

COX-2 and PGE₂, are elevated following transplantation (12, 33) and inhibition of COX-2 by indomethacin treatment rescues host defense (12). PGE₂ production is not influenced by SR expression as PGE₂ levels were similarly elevated in all chimeric BMT mice in this study (not shown), however PGE₂ can dramatically alter SR profiles and also inhibit bacterial killing. Here, we show that *in vitro* treatment with PGE₂ decreases miR-155 and MARCO, while increasing SR-AI/II expression. Although increased SR-AI/II expression enhances *S. aureus* phagocytosis, syn BMT mice remain susceptible to bacteria *in vivo*. Further investigation showed that effective killing of both *P. aeruginosa* and *S. aureus* was impaired in syn BMT mice compared to their untransplanted controls. Therefore, a better understanding of how killing is impaired remains an area of future investigation. We have previously shown that BMT AMs express an immature phenotype (low CD11a and CD11c, high CD11b)(11) and that the BAL fluid of BMT mice contains aberrant mediator

expression (elevated IL-6, GM-CSF, and PGE₂; decreased TNF α , IFN- γ , H₂O₂ and leukotrienes)(11, 12, 22, 34). Overall, BMT AMs appear impaired in their ability to become activated, which is likely important for intracellular bacterial killing. Phagocytosis and killing function is rescued upon treatment with indomethacin (12) indicating that the impairment is due to overexpression of the cyclooxygenase pathway. Microarray analysis also revealed increased expression of miR-27b (8-fold) and -29b (17-fold) (Supplemental Fig 1). Overexpression of these miRNA on primary AMs did not, however, induce a change in expression of either SR-AI/II or MARCO indicating that these miRs are not involved in regulating SR profiles (data not shown). However, NF κ B suppresses miR-29b expression (35) and miR-27b was found to inhibit NF- κ B translocation into the nucleus (36) supporting the idea that these miRNA function in an anti-inflammatory and perhaps functionally suppressive manner and that their expression pattern is influenced by BMT. Interestingly, miR-29b has been shown to directly regulate DNA methyltransferases and affect COX-2 expression in lung epithelial cells(37). Thus, upregulation of miR-29b may be promoting COX-2 overexpression in BMT AMs as the COX-2 promoter is significantly hypomethylated following transplantation (13). Here we show that PGE₂ produced by the COX pathway can decrease miR-155 and affect SR-AI/II expression. Furthermore, miR-155 has been shown to regulate TNF α by mRNA stabilization. TNF α is important for macrophage activation and immune function, and it is decreased post-BMT (22). Our results suggest that miR-155 loss post-BMT may also play a role in destabilizing TNF α . These differences related to dysregulated microRNA expression, may, in part, be responsible for the defective killing post-BMT.

Taken together, our results show that AMs undergo significant functional alterations in the setting of syngeneic BMT. We have previously shown that DNA hypomethylation accounts for the increased production of PGE₂ in AMs post-BMT (13). Our current studies highlight the fact that elevated PGE₂ leads to alterations in the scavenger receptor profile (decreases MARCO and increases SR-AI/II via downregulation of miR-155). These epigenetic changes differentially affect phagocytosis of *P. aeruginosa* and *S. aureus*; however, bacterial killing of both pathogens is impaired post-BMT. It is important to note that these changes observed in AMs post-BMT are only seen in the setting of myeloablative conditioning. Tarling et al. suggested that AMs derive from a lung resident stem cell that is naturally radio-resistant (38). At lower doses of irradiation, AMs likely repopulate from these radio-resistant host stem cells and only upon myeloablative conditioning (8–13 Gy, or using high dose chemotherapy combinations) did AM reconstitution derive from the donor bone marrow resulting in the PGE₂-induced impaired function (16). Thus, interventions to limit PGE₂ production post-HSCT or reverse these epigenetic alterations may improve outcomes for patients developing bacterial infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this article

AM	alveolar macrophage
BAL	bronchoalveolar lavage
BMT	bone marrow transplantation
CHO	chinese hamster ovary
COX	cyclooxygenase
HSC	hematopoietic stem cell
HSCT	HSC transplant
LDH	lactate dehydrogenase
TBI	total body irradiation
SR	scavenger receptor
PGE₂	prostaglandin E ₂
MARCO	macrophage receptor with collagenous structure
SR-A	macrophage scavenger receptor A
syn	syngeneic
TBI	total body irradiation
UTR	untranslated region

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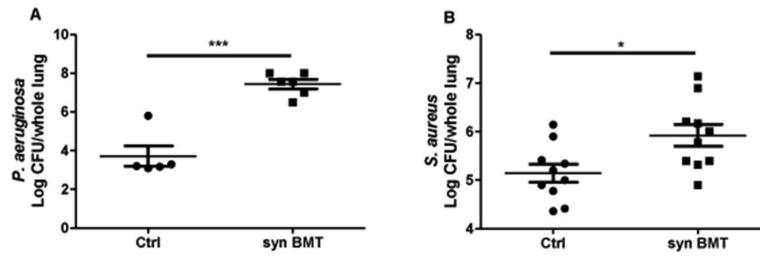


Fig 1. Syn BMT mice are more susceptible to *P. aeruginosa* and *S. aureus*
 Control (ctrl) or syn BMT mice received (A) *P. aeruginosa* (n=5–6/group) or (B) *S. aureus* (n=10/group) i.t. and lungs were collected for CFU analysis 24 h later; *p<.05, ***p<.001.

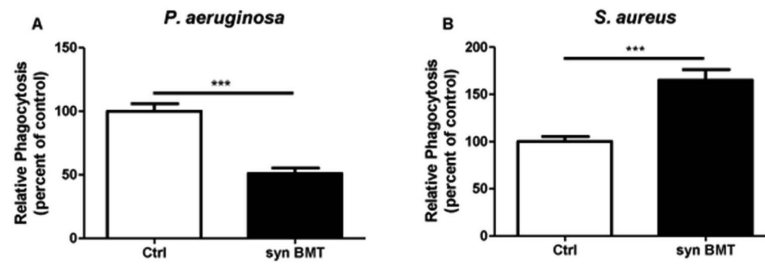


Fig 2. Syn BMT AMs exhibit defective phagocytosis of *P. aeruginosa* but not *S. aureus*
Primary AMs were harvested and phagocytosis of (A) FITC-*P. aeruginosa* or (B) FITC-*S. aureus* was determined (n=5–10 per group). Data are normalized to lactate dehydrogenase (LDH) levels per well to standardize for cell numbers. Results are presented relative to the average of untransplanted control values set to 100%; ***p<.001.

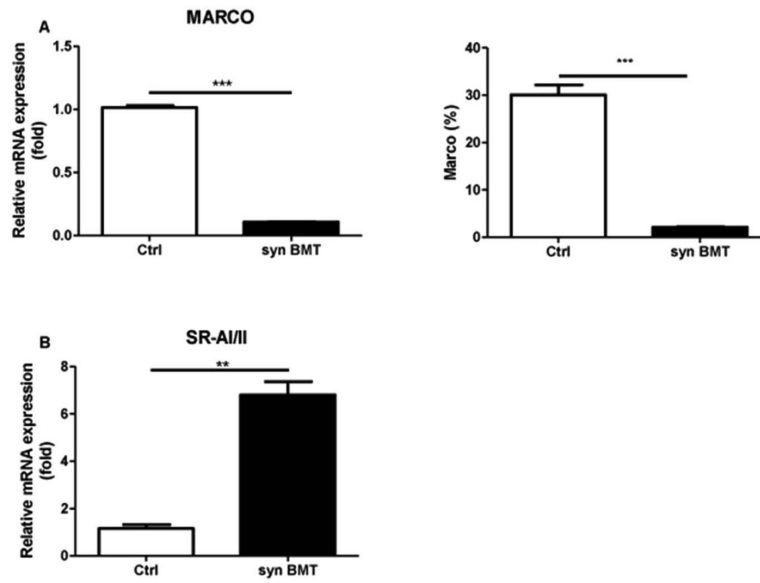


Fig 3. MARCO is decreased on syn BMT AMs while SR-AI/II is increased

AMs were harvested from untransplanted control B6 and syn BMT mice and MARCO (A, left) mRNA was detected by real-time RT-PCR. Expression of one control AM sample was set to n=1 for comparisons, n=3. (A, right) MARCO protein levels were detected by flow cytometry, n=3 individual mice. (B) SR-AI/II mRNA expression was measured by real-time RT-PCR. Analysis was performed as described with MARCO above, n=3. **p<.01, ***p<.001.

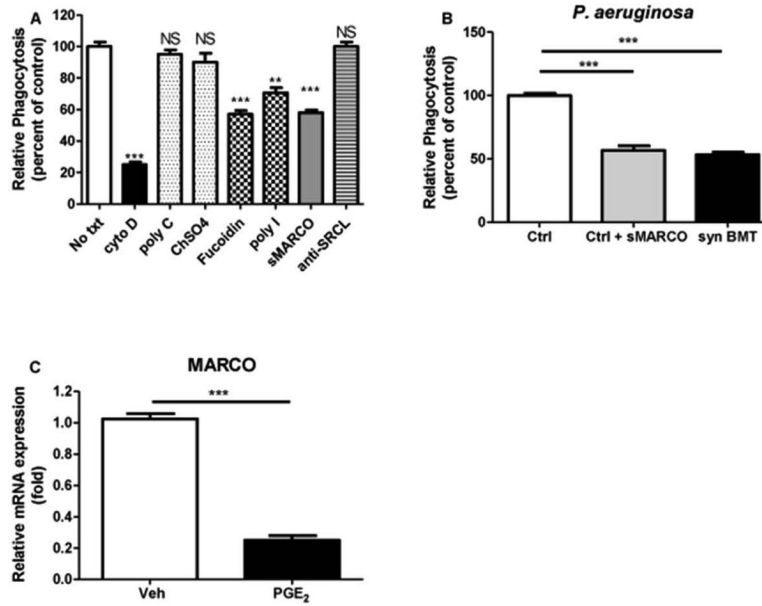


Fig 4. MARCO is necessary for *P. aeruginosa* phagocytosis and expression is regulated by PGE₂ post-BMT

(A) AMs were harvested and pretreated with pan class A SR inhibitors (fucoidin, poly I) or the control compounds poly C and ChSO₄, as well as with anti-SRCL or soluble MARCO and phagocytosis of *P. aeruginosa* was measured. Cytochalasin D (cyt D) inhibits phagocytosis by limiting actin polymerization. Values are presented relative to the no treatment (no txt) control set to 100% for one sample; n=3 per group. (B) Phagocytic ability of control, soluble MARCO-treated control, or syn BMT AMs was measured. Results are presented relative to the average of untransplanted control values set to 100%, n=3. (C) AMs from control mice were treated for 24 h with 1μM PGE₂ or control DMSO (veh). MARCO mRNA levels were determined by real-time RT-PCR. Expression of one control AM sample was set to n=1 for comparisons; n=3. **p<.01, ***p<.001.

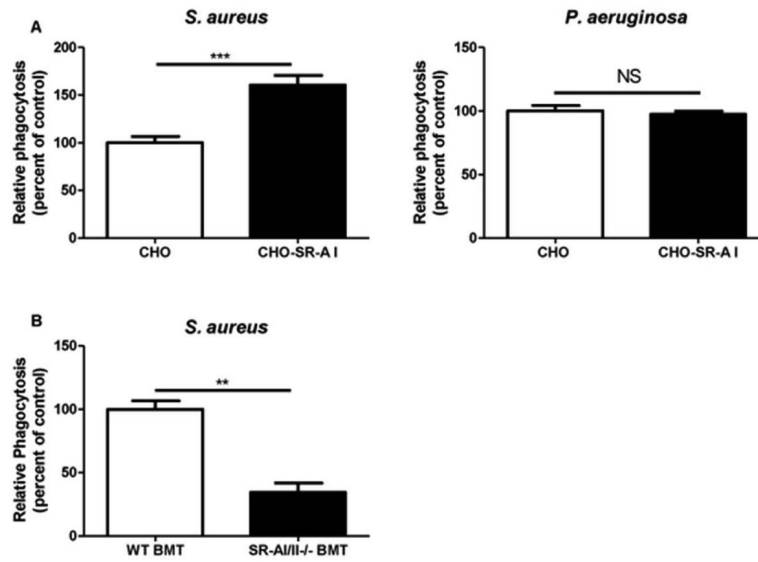


Fig 5. SR-AI/II is important for AM phagocytosis of *S. aureus*

(A) Phagocytosis of FITC-*S. aureus* (left) or FITC-*P. aeruginosa* (right) by control CHO or SR-A-I-transfected CHO cells was measured. Results are presented relative to the average of control CHO values set to 100%; n=12/group; (B) Phagocytosis of FITC-*S. aureus* by WT BMT (n=4) or SR-AI/II-/- BMT (n=3) AMs was measured. Data are normalized to LDH levels per well. Results are presented relative to the average of WT BMT values set to 100%; **p<.01, ***p<.001.

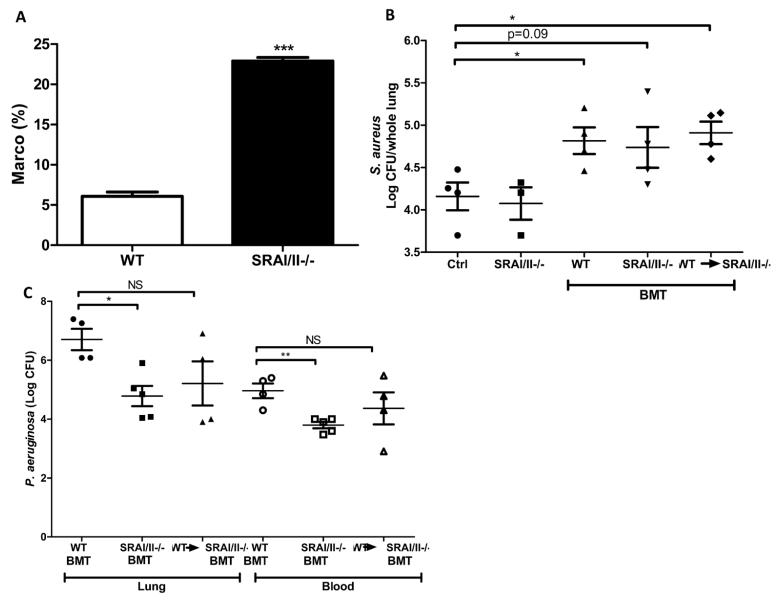


Fig 6. SR-AI/II negatively regulates MARCO expression

(A) MARCO protein expression was determined on AMs from untransplanted SR-AI/II^{-/-} mice by flow cytometry; n=3 individual mice, ***p<.001. Untransplanted B6 (Ctrl), untransplanted SR-AI/II^{-/-} or chimeric BMT mice were challenged *in vivo* with (B) *S. aureus* (n=3–4) or (C) *P. aeruginosa* (n=4–5) via i.t. injection for 24 hours prior to lung and blood harvest for CFU analysis; *p<.05, **p<.01.

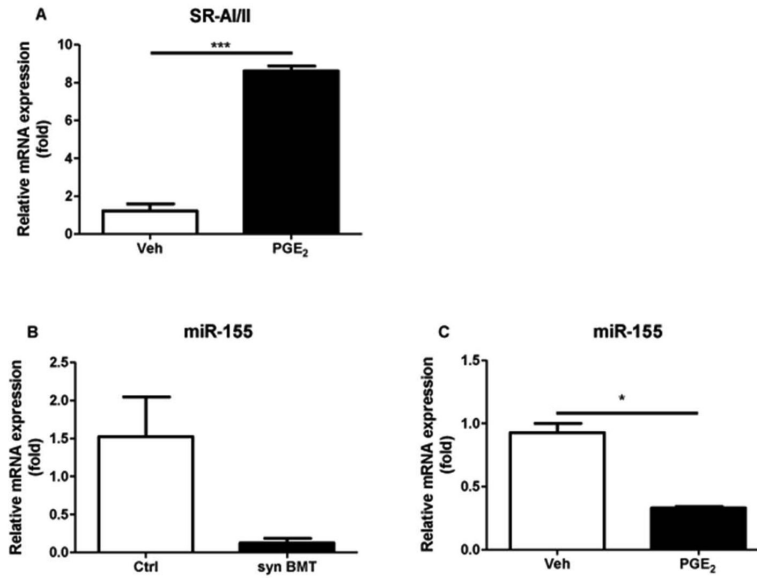


Fig 7. miR-155 mRNA expression is decreased in syn BMT AMs

(A) SR-AI/II expression was measured in AMs treated with 10 μ M of PGE₂ or DMSO (Veh) for 24 h; n=3. Expression of one control AM sample was set to n=1 for comparisons. Similar results were also noted with treatment with 1 μ M PGE₂ (not shown). (B) miR-155 expression was measured by real-time RT-PCR in syn BMT AMs (n=2 samples pooled from multiple mice). Expression was normalized to snoR142 and one control AM sample was set to n=1 for comparison. (C) Primary AMs were treated with 10 μ M PGE₂ or control DMSO (Veh) for 24 h prior to RNA isolation and cDNA conversion. miR-155 expression was compared to snoR142; n=2 samples pooled from multiple mice, *p<.05, ***p<.001.

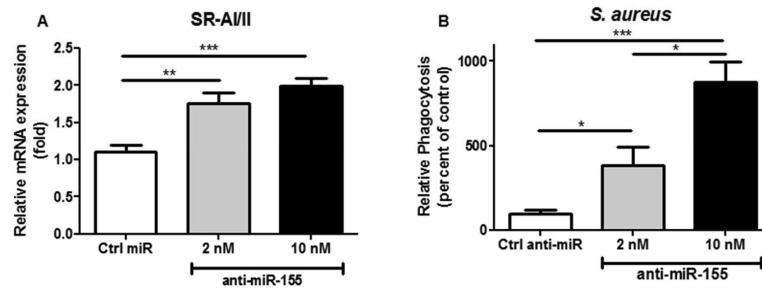


Fig 8. Anti-miR-155-transfected AMs exhibit increased expression of SR-AI/II and increased phagocytosis of FITC-SA

(A) Primary AMs were transfected with either control anti-miR (10 nM) or anti-miR-155 (2 or 10 nM) for 24 h prior to harvesting RNA. SR-AI/II mRNA expression was determined by real-time RT-PCR and the expression of one control AM sample was set to n=1 for comparison; n=3–4 from 3 combined experiments. (B) Primary AMs were transfected with either control anti-miR (10 nM) or anti-miR-155 (2 or 10 nM) for 48 h prior to the two hour incubation with FITC-SA for phagocytosis measurement. Values are normalized to LDH levels per well. Results are presented relative to the average of control values set to 100%; n=3–4; *p<.05, **p<.01, ***p<.001.

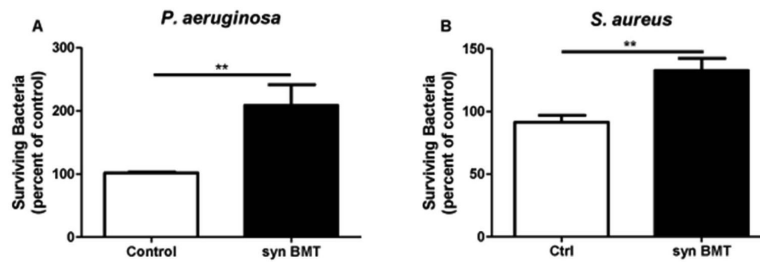


Fig 9. Syn BMT mice have defective killing of *P. aeruginosa* and *S. aureus*

Primary AMs from untransplanted control B6 and syn BMT mice were assessed for killing of serum-opsonized (A) *P. aeruginosa* (n=8–10/group) and (B) *S. aureus* (n=5–6/group). Results are shown relative to the average of control values set to 100%; **p<.01.

Table I

Primers and probes for semiquantitative real-time RT-PCR

RT-PCR primers and probes	Sequence (5'– 3')
β-Actin forward	CCGTGAAAAGATGACCCAGATC
β-Actin reverse	CACAGCCTGGAGGCTACGT
β-Actin probe	TTTGAGACCTTCAACACCCCAGCCA
MARCO forward	CCTGGACGAGTCGGTCAGAA
MARCO reverse	CTTCAGCTCGGCCTCTGTT
MARCO probe	CCAACGCGTCCGGATCATGGGT
SR-AI/II forward	TGAAGGACTGGGAACACTCACA
SR-AI/II reverse	CAGTAAGCCCTCTGTCTCCCTTT
SR-AI/II probe	TTCATTCAAGGGCCTCCTGGACCC