Topical prostaglandin E analog restores defective dendritic cell-mediated Th17 host defense against methicillin-resistant *Staphylococcus aureus* in the skin of diabetic mice

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**Short title:** Topical PGE restores skin host defense in diabetic mice

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Abstract

Diabetics are more prone to *Staphylococcus aureus* skin infection than healthy individuals. Control of *S. aureus* infection is dependent on dendritic cell (DC)-induced Th17-mediated neutrophil recruitment and bacterial clearance. DC ingestion of infected apoptotic cells (IAC) drive prostaglandin E$_2$ (PGE$_2$) secretion to generate Th17 cells. We speculated that hyperglycemia inhibits skin DC migration to the lymph nodes (LN) and impairs Th17 differentiation that accounts for poor skin host defense in diabetic mice. Diabetic mice show increased skin lesion size, bacterial load, decreased PGE$_2$ and Th17 cells compared to nondiabetic mice after Methicillin-resistant *S. aureus* (MRSA) infection. Bone marrow-derived DCs (BMDCs) cultured in high glucose (25 mM) exhibits decreased *3Ptxes* mRNA expression, PGE$_2$ production, lower CCR7-dependent DC migration, and diminished maturation after recognition of MRSA-IAC than BMDCs cultured in low glucose (5 mM). Similar events were observed in DCs from diabetic mice infected with MRSA. Topical treatment of diabetic mice with the PGE analog Misoprostol improved host defense against MRSA skin infection by restoring dendritic cell migration to draining lymph nodes, Th17 differentiation, and increased antimicrobial peptide expression. These findings identify a novel mechanism involved in poor skin host defense in diabetes and propose a targeted strategy to restore skin host defense in diabetes.
Introduction

Diabetes mellitus is a clinical syndrome associated with deficiency in insulin secretion or action. As a consequence, diabetes causes hyperglycemia, which has been associated with undermined host defense and increased susceptibility to localized and systemic infections (1). Skin and soft tissues are sites of prevalent infections primarily caused by *Staphylococcus aureus* in diabetics (2; 3). Methicillin-resistant *Staphylococcus aureus* (MRSA) is a frequent causative pathogen of complicated skin and soft tissue infections in diabetics (2) that can be difficult to treat and often require hospitalization and surgery (4).

Innate immune recognition of *S. aureus* promotes pro-inflammatory signals that lead to neutrophil recruitment and abscess formation, a hallmark of *S. aureus* infection (5). Extensive neutrophil apoptosis is also a hallmark of *S. aureus* infections (5). An additional complication in diabetes is that neutrophils from diabetic patients have defective antimicrobial effector functions (6; 7).

Clearance of apoptotic cells (efferocytosis) has long been associated with resolution of inflammation (8). However, efferocytosis of infected apoptotic cells has been proposed to be an innate antimicrobial mechanism that results in pathogen destruction (9), and comprises a critical link to adaptive immune responses (10). Considering the increased neutrophil recruitment and apoptosis at sites of MRSA infection, we speculate that the uptake of infected apoptotic neutrophils by dendritic cells (DCs) dictate the type of host defense produced against *S. aureus* in skin infections.

In the skin, DCs are prompted to respond to intracellular and extracellular modifications that induce a rapid change in their function and phenotype, accompanied by
their migration into secondary lymphoid tissue (11). During this process, DCs require maturation, a process associated with up-regulation of class II MHC and costimulatory molecules (CD80, CD86, CD40) (12) and consequent migration to lymph nodes in a CC-chemokine receptor 7 (CCR7)-dependent response to its ligands CCL19 and CCL21 (13). In addition, it has been shown that the lipid mediator prostaglandin E$_2$ (PGE$_2$) is a key factor for DC maturation and migration via CCR7 (14). Moreover, PGE$_2$ is required to initiate skin immunity by increasing maturation and migration of Langerhans cells, a subtype of skin resident dendritic cells (14).

PGE$_2$ synthesis involves activation of phospholipase A2 family members that mobilize arachidonic acid (AA) from cellular membranes. Cyclooxygenases (COX)-1 and -2 then convert AA into PGH$_2$ that is further metabolized by specific synthases to generate specific prostanoids. Microsomal PGE synthases-1 (mPGEs) is induced during inflammatory response that acts mainly downstream to COX-2 to convert PGH$_2$ to PGE$_2$. The levels of PGE$_2$ are regulated by the local balance between the COX-2-driven synthesis and 15-hydroxyprostaglandin dehydrogenase (15-PGDH)-mediated degradation of PGE$_2$ or the actions of PG transporters (33). PGE$_2$ acts through four G protein-coupled receptors, designated EP1-4, which display different tissue distribution and deliver distinct intracellular signals (15). Moreover, efferocytosis has been shown to trigger PGE$_2$ production (16), although the role of PGE$_2$ on DC functions during efferocytosis remains to be determined.

While efferocytosis leads to secretion of anti-inflammatory mediators that promote expansion of regulatory Foxp3 T cells, efferocytosis of *E. coli*-infected neutrophils by DCs induces IL-6, IL-23, and TGF-β production, which triggers Th17 differentiation (10; 17).
Th17 cells are fundamental players in host defense against *S. aureus* infection (18) by activating keratinocytes, and resulting in the induction of neutrophil chemoattractants and antimicrobial peptides (5). Patients with defective Th17 responses exhibit increased susceptibility to *S. aureus* skin infections (5), however whether diabetics also exhibit defective Th17 during *S. aureus* skin infection is unknown. Reduced frequencies of IL-17-producing memory CD4\(^+\) T cells associated with elevated glucose and increased glycated hemoglobin A1c have been observed in response to *Streptococcus pneumoniae* in diabetic patients (19).

Here, we found that localized deficient PGE\(_2\) production impairs DC migration and maturation, which impairs generation of Th17 T cells and drives poor skin host defense against infection. Our data show that topical PGE\(_2\) treatment restores immune responsiveness and increases microbial clearance in diabetic mice.
Methods

Animals

WT C57BL/6 mice (8-12 weeks old) were obtained from The Jackson Laboratory. Mice were maintained according to NIH guidelines for the use of experimental animals in the Laboratory Animal Resource Center (LARC) at Indiana University School of Medicine in Indianapolis. Experiments were performed under a protocol approved by Indiana University School of Medicine Animal Care and Use Committee.

Induction of diabetes

Diabetes was induced by 5 daily intraperitoneal injections of low dose (40 mg/kg) Streptozotocin (STZ) as described (20). Mice with blood glucose >300 mg/dl were considered diabetics. Experiments were performed using mice at 30 days after diabetes onset.

MRSA-skin infection and misoprostol topical treatment

Control and diabetic mice were anesthetized (Ketamine/xylazine), and the backs were shaved and the MRSA USA300 LAC strain (5×10^6 CFU) was injected subcutaneously in 50 µL PBS. Lesion and abscess size were monitored daily and determined by affected area calculated using a standard formula for area (A = [π/2] × l × w) (21). Mice were topically treated at the site of infection twice a day for 7 days with 0.03% misoprostol (prepared by emulsifying 150 µg misoprostol in 0.5 g petroleum gel or vehicle control).
In vivo DC migration

Control and diabetic mice were injected subcutaneously with $5 \times 10^6$ CFU of MRSA plus 50 µg of CellTrace FarRed DDAO-SE as suggested by the vendor (Invitrogen). Brachial lymph nodes were harvested 36 h or 48 h post-infection. Migrating dendritic cells were identified as FarRed$^+$CD11c$^+$ cells detected by flow cytometry.

Skin biopsies and bacterial load

Punch biopsies (8 mm) from noninfected or infected skin were harvested at different time points and used for determining bacterial counts, cytokine production, RNA extraction, and cell isolation (22). For bacterial counts, skin biopsies were collected at day 7 post-infection, processed and homogenized in TSB media and serial dilutions were plated on TSB agar. Colonies were counted after incubation overnight at 37°C.

MRSA-Infected Apoptotic Cells (MRSA-IAC)

DMSO-differentiated neutrophil like-cells were incubated with GFP-MRSA (MOI 50) for 2 h. We observed by FACS that 90% of neutrophil-like cells were infected after 2 h of MRSA infection (not shown). After phagocytosis, cells were washed and apoptosis of infected and noninfected cells was induced using an ultraviolet irradiation crosslinker (5 mJ) and confirmed by Annexin-V and 7-AAD staining (Figure S2).

Co-culture of bone marrow-derived dendritic cells (BMDCs) with infected apoptotic cells
Bone marrow cells from control or diabetic mice were differentiated into DCs as previously described (23) in either 5 mM or 25 mM glucose. After differentiation, BMDCs were cocultured with infected apoptotic cells (ratio 1:3) for 18 hours in DMEM containing 5 or 25 mM glucose. Supernatants were then collected and inflammatory mediators were detected by ELISA or EIA; and maturation of BMDCs were analysed by FACS.

**In vitro BMDC migration**

After 18 h of co-culture with MRSA-infected apoptotic cells, BMDCs were isolated using CD11c microbeads (Miltenyi, Biotec) and cells (1×10^6) were suspended in serum-free DMEM with 5 mM or 25 mM glucose and plated into the upper chamber of a 24-well Corning Costar Transwell plate – 5 µM (Corning Inc., USA). To study CCR7-dependent directional migration, CCL19 (100 ng/mL) and CCL21 (100 ng/mL) were placed into the lower chamber in free-serum medium with 5 mM or 25 mM glucose. Cells that had migrated into the lower chamber 8 h later were photographed, harvested, and counted by FACS.

**Skin cell isolation and staining for flow cytometry**

Skin biopsies were digested with collagenase and processed to obtain a single-cell suspension. For lymphocyte staining, skin and lymph node cells were stimulated for 5 h with 0.1 µg/ml PMA (Sigma), 0.5 µg/ml Ionomycin calcium salt, and 10 µg/ml Brefeldin A. In all circumstances, prior to staining with antibodies, cells were treated with anti-FcγR antibodies to prevent nonspecific antibody binding. For lymphocyte staining, cells were fixed and permeabilized using Cytofix/CytoPerm (BD) and were stained with the
antibodies indicated in the figure legends. For dendritic cell staining, cells were fixed in 1% paraformaldehyde and stained with fluorescent labeled antibodies for 20 minutes.

Detection of cytokines and PGE₂
Cytokines including IL-6, IL-1β, IL-17A, IL-10, TGF-β (Ebioscience or R&D), production of PGE₂, PGE metabolites, 6-keto prostaglandin F1α and cytokines (Cayman Chemicals) were detected by ELISA or EIA in skin biopsy homogenates or supernatants from cultured BMDCs. In all circumstances, the sensitivity limit of the assays were ~ 4-5 pg/mL.

RNA isolation, reverse transcription, quantitative real-time PCR
Total RNA was isolated from skin biopsies using lysis buffer (RLT, Qiagen). cDNA and real time PCR were performed as previously published (20) using primers indicated in the legends on a CFX96 Real-Time PCR Detection System (Bio-Rad). Primers were purchased from Integrated DNA technologies. Relative expression was calculated as described (20).

Statistics Analysis
Results are shown as mean ± SEM and were analyzed using the software Prism 5.0 (GraphPad Software, San Diego, CA). For comparisons between two experimental groups Student's t-test was used, and for comparisons among three or more experimental groups, we used One-way Anova followed by Bonferroni multiple comparison test. Values of p<0.05 were considered significant.

Results
Decreased Th17 response during MRSA-skin infection in diabetic mice

Initially, we aimed to characterize the events involved in enhanced susceptibility to MRSA skin infection during diabetes. Mice that were diabetic for at least 30 days were more susceptible to MRSA skin infection than non-diabetic mice as seen by increased lesion size and bacterial load measured at day 7 after infection (Figure 1 A-C and data not shown). We observed that MRSA skin infection in diabetic mice led to increased number of neutrophil in the infected skin by 18 h post infection, an event that lasted for at least 7 days (Supplementary information, Figure S1). After ingestion and killing of bacteria, neutrophils become apoptotic and are eliminated by macrophages and dendritic cells (24). When clearance is not effective, infected apoptotic neutrophils become necrotic, releasing danger-associated molecular patterns that elicit inflammatory response. Initially, we determined the numbers of dead cells in the infected skin of diabetic and nondiabetic mice. We observed similar increased numbers of apoptotic cells among skin cells isolated 18 h or 7 days after MRSA-skin infection in both control and diabetic mice (Supplementary information, Figure S2 A-C) and increased apoptosis in a human neutrophil-like cell line (HL-60) cultured with MRSA for 18 h (Supplementary information, Figure S2 C-E).

Bacterial infections that are associated with significant induction of apoptosis in host tissues preferentially induce Th17 immunity (5). Thus, we first determined the profile of the T cell subsets in the skin of diabetic mice 7 days after infection. Diabetic mice showed decreased percentages of Th17, but no significant differences were observed in the percentage of regulatory T cells (Treg) or Th1 cells when compared with infected nondiabetic mice (Figure 1 D-G).
Next, we assessed the production of PGE₂, an inflammatory mediator involved in Th17 differentiation, as well as IL-17A and IL-10 in the skin of control and diabetic mice at different time points after infection. Diabetic mice showed significantly reduced PGE₂ levels at day 2 and decreased levels of IL-17A and IL-10 in the skin at day 7 post-infection compared with infected nondiabetic controls (Figure 1 H-J). Next, we determined whether decreased PGE₂ levels in DCs cultured in low or high glucose or infected skin of diabetic mice correlated with decreased mRNA expression of enzymes involved in PGE₂ synthesis, such as Cox-1 and -2 and Ptges (that encodes mPGEs-1). Our data clearly show that Ptges but not Cox-1 and -2 expression is decreased in in vivo and in vitro Indeed, another important regulatory step involved in decreased PGE₂ levels in diabetic mice could be increased PGE₂ metabolism by 15-PGDH or deficiency in PGE₂ transporter (PGT). We then studied the presence of PGE metabolites in the infected skin of diabetic and nondiabetic mice. While we did not detect the expression of in the skin of all groups tested, and MRSA skin infection increased the production of PGE metabolites in a similar manner in both diabetic and nondiabetic mice 48 h after infection (Supplementary information, Figure S5 A-C and data not shown).

Together, these findings show that decreased PGE₂, foreshadows the production of IL-17A and IL-10 suggesting a temporal relationship between PGE₂ and the generation of Th17 in the infected skin of diabetic mice.

**Dendritic cell migration to skin-draining lymph nodes is impaired in diabetic mice**

We next studied whether impaired DC migration to skin-draining lymph nodes is associated with the decrease in Th17 subsets during MRSA skin infection in diabetic mice.
We tracked DC migration \textit{in vivo} during MRSA-skin infection and determined the numbers and percentages of migrating DCs (FarRed+CD11c+) and Th17 cells in the skin draining lymph nodes. Diabetic mice exhibited decreased percentages and numbers of migrating DCs and Th17 cells in the lymph nodes compared to infected nondiabetic mice (Figure 2 A-F).

Langerhans cells have been described as the main cutaneous DC subset capable of inducing a Th17 response (25). Whereas migrating DCs were decreased in the lymph nodes, we observed an increased percentage of Langerin+ DCs in the infected skin of diabetic mice (Figure 3 A-C), suggesting that these cells are retained in the skin and cannot properly migrate to the LNs. While no differences were observed in numbers or expression levels of maturation markers in Langerin+ DCs (data not shown) in the naïve skin of control and diabetic mice, MRSA infection induced MHC II expression in about the same number of Langerin+ DCs in diabetic and control skin (Figure 3D), but Langerin+ DCs in diabetic skin had lower levels (median fluorescence intensity - MFI) than in control skin (Figure 3E). MRSA infection also induced a similar frequency of Langerin+ DCs expressing CD86 (Figure 3F) and at similar MFI in diabetic and control (Figure 3G). Interestingly, following MRSA infection, CCR7 was expressed by fewer Langerin+ DCs with reduced MFI in diabetic skin than in control skin (Figure 3 H-K), indicating that Langerhans cells from infected diabetic mice fail to express the machinery necessary to efficiently activate T cells in the draining lymph node.

**High glucose impairs BMDC migration and maturation after recognition of MRSA-infected apoptotic cells**
Next, we investigated whether a high glucose environment influenced maturation, migration, and generation of cytokines by DCs involved in Th17 commitment after engulfment of MRSA-infected apoptotic neutrophils.

Initially, BMDCs from control (nondiabetic) and diabetic mice were differentiated in the presence of 5 mM or 25 mM of glucose. We did not detect differences in spontaneous changes in the phenotype or production of cytokines by immature BMDCs from diabetic and nondiabetic mice under resting conditions (Supplementary information, Figure S3 A-E). However, after recognition of MRSA-infected apoptotic cells, under high glucose conditions BMDCs showed decreased expression of the maturation markers MHC II, CD86, and CCR7 expression (Figure 4 A, C, D) and decreased ability to migrate via CCR7 towards a gradient of CCL19/CCL21, compared to cells under normal glucose conditions (Figure 5 B-C).

In 5 mM or 25 mM glucose, BMDCs showed similar capacities to recognize MRSA-IAC, and no changes in efferocytosis index (Supplementary information, Figure S3 F-G) or cytokine production (Supplementary information, Figure S3 H-J) were observed. Moreover, BMDCs from control or diabetic mice cultured in either 5 mM or 25 mM glucose after recognition of MRSA-IAC produced increased amounts of Th17-driving cytokines IL-6, TGF-β, and IL-1β when compared to the control BMDCs before efferocytosis (Supplementary information, Figure S3 K-N). On the other hand, we observed decreased PGE₂ levels in BMDCs cultured in high glucose and challenged with MRSA-IAC (Figure 5 A). Efferocytosis of MRSA-IAC in BMDCs enhanced Cox-2, but not Cox-1 mRNA expression in cells cultured in 5 mM or 25 mM glucose (Supplementary information, Figure S4 A-B). However, we observed that efferocytosis of MRSA-IAC in
25 mM glucose led to decreased *Ptges* and *Ptger4* expression and no changes were seen in *Ptger 1,2, and 3* expression by BMDCs than efferocytosis in 5 mM glucose (Supplementary information, Figure S4 C, E-F), suggesting that high glucose milieu decreases PGE$_2$ production and signaling in DCs.

Next, we sought to determine whether decreased PGE$_2$ levels in high glucose might be responsible for impaired maturation and migration of BMDCs. To test this hypothesis, we added PGE$_2$ in BMDC co-cultures in high glucose, and observed that exogenous PGE$_2$ restored BMDC migration (Figure 5 B-C) and CD86 and CCR7 (Figure 4 C, D, G, H) expression. Together these data show that deficient PGE$_2$ production accounts for inadequate DC maturation and chemotaxis during efferocytosis of infected apoptotic cells, which influences later events involved in MRSA skin infection in diabetics.

**Topical treatment with PGE analog (misoprostol) improves DC migration to skin-draining lymph nodes in diabetic mice**

Considering that exogenous PGE$_2$ *in vitro* improved BMDC migration in diabetic mice, we investigated whether topical treatment with PGE$_2$ *in vivo* improved DC migration to skin-draining lymph nodes, as well as Th17 differentiation and host defense after MRSA skin infection in diabetic mice. For these studies, we used misoprostol, which is a PGE$_1$ analog with agonist activity for EP2, EP3, and EP4 receptors (26) used therapeutically in humans (27).

At two days post-infection we detected increased percentages of migrating DCs (FarRed$^+$CD11c$^+$) in lymph nodes from misoprostol treated-diabetic mice compared to untreated diabetics (Figure 6 A, C). We also observed that misoprostol treatment in
diabetics increased the Th17 population in skin-draining lymph nodes (Figure 6 B, D) but did not significantly alter Treg and Th1 cells (Figure 6 E, F).

**Misoprostol attenuates infection and improves host defense against MRSA-skin infection in diabetic mice**

Given that misoprostol improved DC migration and increased differentiation of Th17 in the lymph nodes, we speculated that misoprostol topical treatment could restore skin host defense in diabetic mice. Diabetic mice treated with misoprostol had smaller lesion and abscess size (Figure 7 A) and lower bacterial load (Figure 7 B) than vehicle-treated diabetic mice. Indeed, the treatment of diabetic mice with misoprostol promoted increased percentage (Figure 7 C, D) and number (Figure 7 E) of Th17 cells in the skin compared with vehicle-treated diabetic mice, but not Treg and Th1 percentages (Figure 7 F, H) or numbers (Figure 7 G, I) compared with vehicle-treated diabetic and control mice.

Next we determined whether increased skin host defense in misoprostol-treated diabetic mice was accompanied by changes in production of antimicrobial peptides, which are key mediators involved in controlling MRSA skin infection (28). We found that misoprostol-treated diabetic mice expressed significantly increased *mbd2* and *mbd3* in the skin compared to vehicle-treated diabetic and nondiabetic mice (Figure 7 K-L), and expressed increased, albeit not significantly, *mbd1, 4, 5, and Cramp* (Figure 7 J, M-O). Together, these data suggest that misoprostol treatment improves skin host defense in diabetic mice by increasing Th17 generation and expression of antimicrobial peptides.
Discussion

Skin and soft tissue infections are the most important chronic complications of diabetes, and may lead to hospitalization, amputation, osteomyelitis, and death (29). The higher incidence of infections in diabetic patients is related to hyperglycemia, which is known to cause immune dysfunction (30). In the current study, we provide evidence that reduced PGE₂ in the skin of diabetic mice compromises DC maturation and migration to lymph nodes, which inhibits the development of Th17 cells and host defense. These effects correlate with inefficient bacterial clearance and progressive skin infection in diabetics (Figure 7 P). These findings suggest new therapeutic opportunities for treating skin infections in diabetics.

After being activated by infectious or inflammatory stimuli, DCs upregulate the expression of maturation marks as they migrate to lymph nodes in a CCR7-dependent manner (12). Migrating mature DCs initiate adaptive immune responses through presentation of peripherally acquired antigens to T cells. PGE₂ signaling in Langerhans cells promotes skin immune responses by supporting maturation and migration to skin-draining lymph nodes (14). Notably, initiation of skin immune responses and DC homing is severely impaired in mice lacking the PGE₂ receptor EP4 (14), highlighting the importance of PGE₂ in DC-mediated skin immunity.

The influence of hyperglycemia on inhibition of PGE₂ by endothelial cells and defective maturation and function of DCs in diabetes has been reported (31; 32). It has been shown, as well, that during wound healing, diabetic mouse skin exhibits a decreased capacity to produce PGE₂ (33). Moreover, diminished production of cyclooxygenase products of arachidonic acid metabolism have been described as an abnormality of
polymorphonuclear leukocytes (PMNL) in diabetes (34). In the current study, we sought to
determine if such effects were responsible for decreased skin DC actions and inefficient
host defense in diabetes. Consistent with this hypothesis, at a late time point diabetic mice
maintained an open lesion, higher bacterial load, and diminished Th17 population in the
infected skin compared to nondiabetic mice.

The finding of increased apoptosis during MRSA skin infection directed us to
investigate whether efferocytosis by DCs was involved in PGE₂ production and Th17
differentiation during infection. Efferocytosis induces PGE₂ production (16) and is a
condition for restricting M. tuberculosis replication (9). In addition, it has been shown that,
in the absence of microbial signals, efferocytosis induces the differentiation of regulatory T
cells; whereas, the phagocytosis of E. coli-infected apoptotic neutrophils by DCs induces
Th17 cell differentiation (10). Thus, apoptosis during bacterial infection has been shown to
create a proper cytokine microenvironment promoted by efferocytosis that triggers Th17
(35). Here, we extend these findings to show that efferocytosis of MRSA-infected skin
cells in a hyperglycemic environment impairs PGE₂ production, which affects maturation
and migration of DCs, and consequently prevents Th17 development.

However, after efferocytosis of MRSA-IAC, we observed a specific reduction of
PGE₂ levels in the supernatant of BMDCs cultured in high glucose, which correlated with
decreased Ptges expression, but not COX enzymes. We did not detect any differences in
the production of PGI₂ (another prostanoid that exerts immunoregulatory properties) or
PGE₂ metabolites in the skin of diabetic mice infected with MRSA. These data contrast
with previous reports showing that hyperglycemia enhances prostaglandin degradation by
increasing the expression and activity of PGT, leading to diminished PGE₂ levels and
angiogenic signaling in diabetic skin (33). The reasons for such discrepancies are unknown, but it could be due to differences in animal models, cells involved in the inflammatory response during angiogenesis and infection. In addition to reduced PGE\(_2\) production, decreased expression of EP4 by BMDCs after efferocytosis under hyperglycemic conditions has been observed. This effect may be related to the capacity of PGE\(_2\) to enhance its own receptor expression, as it has been previously shown that PGE\(_2\) increases the levels of both \(Pgter4\) mRNA and protein expression in monocytoid cell lines (36) and bone marrow stromal cells, pointing to a mechanism of autoamplification of PGE\(_2\) action (37).

The fact that our results show that a high glucose environment causes similar maturation and migration defects in BMDC from control and diabetic mice suggests that high glucose is the sole driver of the PGE\(_2\)/CCR7/Th17 axis during MRSA infection in diabetes. The role of PGE\(_2\) in CCR7 expression has been controversial. Recently, it has been shown that PGE\(_2\) induces CCR7 receptor oligomerization, rather than expression, resulting in an efficient signaling pathway that enhances migration (13). However, together with other soluble mediators such as TNF-\(\alpha\), IL-1\(\beta\), and IL-6, it has been shown that PGE\(_2\) increases CCR7 expression (38). Our data showed that exogenous addition of PGE\(_2\) to medium containing high glucose restored BMDC migration to CCL19/CCL21 chemoattractants and induced CCR7 and CD86 expression, which prompted us to investigate the \textit{in vivo} effects of PGE\(_2\) treatment on host defense against MRSA skin infection in diabetes.

Because of their roles in angiogenesis and wound healing, PGE\(_2\) analogs have been used to treat patients with diabetic neuropathy (39). Here, we found that diabetic mice
receiving topical misoprostol treatment had decreased lesions and bacterial counts as a consequence of improved dendritic cell migration to skin-draining lymph nodes and enhanced Th17 immunity.

Th17-derived cytokines, such as IL-17A and IL-22, induce secretion of antimicrobial peptides by neutrophils and keratinocytes (40). We found that topical misoprostol treatment in diabetic mice increased expression of mbd2 and mbd3 antimicrobial peptides, which correlates with increased skin Th17 cells after infection. Although we did not explore the mechanism by which misoprostol promotes higher antimicrobial peptide expression or determine the cell source, it has been shown that keratinocytes have a stronger dependence on IL-17 for expression of antimicrobial peptides than do other cell types (40). On the other hand, it might be possible that misoprostol directly upregulate antimicrobial peptides expression on keratinocytes, without a particular dependence of IL-17. Therefore, further investigations are needed to fully elucidate the role of PGE₂ in the antimicrobial peptides machinery. Furthermore, it has been shown that COX-2 activity is important for optimal production of human β-defensin 1 and 2 by human keratinocytes in vitro (41). Therefore, we suggest that improving Th17 responses in diabetic mice promotes skin host defense against MRSA via induction of antimicrobial peptide expression. Indeed, considering the known effects of hyperglycemia in impairing macrophage and neutrophil phagocytosis and killing, which is associated with resistance to antibiotics, it is becoming attractive to consider endogenous antimicrobial peptides as sources of more sustainable antimicrobial agents.

Our data show that PGE₂ equilibrium is a key player in skin host defense. Reduced PGE₂ in diabetic skin impairs DC activation and migration to skin draining lymph nodes.
compromising the development of adaptive immunity. Delayed priming of Th1 responses against *M. tuberculosis* in pulmonary infections has been previously demonstrated in diabetic mice, although no association with decreased PGE$_2$ levels or dendritic cell migration were observed (42). In addition, PGE$_2$ has been shown to direct IL-23 secretion by DCs and be crucial for Th17 development (43).

PGE$_2$ may have both positive and negative immune regulatory roles, showing opposite effects depending on its concentration, microenvironment, cell type, and receptor subtype (15; 44; 45). It has been previously described that PGE$_2$ affects mainly macrophages phagocytosis and killing of bacteria and fungus (46). On the other side, the effects of PGE$_2$ on macrophage functions in the skin microenvironment are still unclear. Indeed, neutrophils and macrophages in diabetics have already poor phagocytosis and killing capacities, which lead to defective bacterial clearance compared to cells of controls (47; 48). Thus, enhancing endogenous anti-microbial machinery could be an attractive strategy to control bacterial growth when effector functions of innate cells are impaired. Thus, the use of PGE analog may have some limitations as well as delineating levels and mechanisms by which PGE$_2$ improves skin host defense in diabetics may provide an avenue toward development of an attractive therapeutic target.

In summary, our study provides evidence that PGE$_2$ production was compromised during recognition of MRSA-IAC by DCs in a hyperglycemic environment. Moreover, topical treatment with misoprostol improved host defense against MRSA by enhancing dendritic cell-mediated Th17 responses, and expression of antimicrobial peptides. Based on these results, targeting pathways to restore PGE$_2$ balance in the diabetic skin may be a strategy to improve innate and adaptive immune responses in diabetics skin infections.
Identifying and delineating levels and mechanisms by which PGE$_2$ improves skin host defense in diabetics may provide an avenue toward development of an attractive therapeutic target.
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Guarantor’s statement

Dr. C. Henrique Serezani is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Author Contributions

Conceived and designed the experiments: Dejani, NN; Serezani, CH, Brandt, S Glosson-Byers, N, Medeiros, AI. Performed the experiments: Dejani, NN, Brandt, S, Wang, S, Piñeros, A., Young min Son Analyzed the data: Dejani, NN, Piñeros, A, Serezani CH, Medeiros AI, Brandt, S, Glosson-Byers, N, Piñeros, A. Wrote the paper: Dejani, N.N., Serezani, C.H.

Disclosures

The authors have no conflicts of interest.
Fig. 1. Uncontrolled MRSA skin infection in diabetics correlates with decreased skin PGE2 and Th17 cells in the infected skin. (A) Control and diabetic mice were infected s.c. with MRSA (5×10^6 CFU). Lesion development was monitored every other day for 7 days. (B) Representative images of the skin of diabetic and control mice at day 7 post-infection. (C-J) Skin biopsies were collected at day 7 post-infection and isolated cells were examined for bacterial counts (C), expression of CD4 and IL-17a (D and E), and expression of T-bet (F) and Foxp3 (G) by CD4^+ lymphocytes. The lymphocyte population was gated on TCRβ^+CD4^+ cells. Levels of PGE2 (H), IL-17A (I), and IL-10 (J) were determined by ELISA in skin biopsy homogenates. Data show mean ± SEM of samples from 5-10 mice. *p<0.05 vs control.

Fig. 2. Impaired DC migration and Th17 generation in skin-draining lymph nodes of diabetic mice. (A) Representative dot plots of the DC (CD11c^+MHCII^+) population and histogram of the percentage of FarRed^+ DCs in the brachial lymph nodes of control and diabetic mice 36 h post-infection. (B, C) Percentage and number of migrating DCs (CD11c^+MHCII^+FarRed^+) in the lymph nodes. (D) Representative dot plots of the Th17 (CD4^+IL-17A^+) population in the brachial lymph nodes 36 h post-infection in control and diabetic mice. (E, F) Percentage and numbers of IL-17A-producing CD4^+ lymphocytes. Data show mean ± SEM of samples from 5-10 mice. *p<0.05 vs control.
Fig. 3. Decreased CCR7 and MHCII expression by Langerin$^+$ DCs in diabetic skin during MRSA infection. (A, B) Representative dot plots of CD11c$^+$CD11b$^+$ and Langerin$^+$ DCs in the skin of control and diabetic mice 18 h post-infection. (C) Percentage of Langerin$^+$ DCs in skin of control and diabetic mice 18 h post-infection. Percentage of Langerin$^+$ DCs expressing MHC II (D), CD86 (F), or CCR7 (H) 18 h post-infection. Intensity of staining (median fluorescence intensity, MFI) of MHC II (E), CD86 (G), and CCR7 (I) in Langerin$^+$ DCs from diabetics and controls 18 h post-infection. (J, K) Percentage of Langerin$^+$CCR7$^+$ DCs and CCR7 MFI on Langerin$^+$DCs in the skin of naïve control and diabetic mice or 18 h post-infection. Data show mean ± SEM of samples from 5-10 mice. *p<0.05 vs control.

Fig. 4. Exogenous PGE$_2$ restores CD86 and CCR7 expression in BMDCs cultured in high glucose and incubated with MRSA-IAC. Representative histograms of expression (median fluorescence intensity, MFI) of MHC II (A), CD80 (B), CD86 (C), and CCR7 (D). Fold change in expression (MFI) of MHC II (E), CD80 (F), CD86 (G), and CCR7 (H) by BMDCs from control or diabetic mice after efferocytosis of MRSA-IAC compared with inactivated BMDCs from control or diabetic mice. The analyzed populations were gated on CD11c$^+$CD11b$^+$. Data represent mean ± SEM of at least 3 independent experiments performed in triplicate. *p<0.05 vs control 5 mM glucose, &p<0.05 vs control 25 mM glucose, #p<0.05 vs diabetic 5 mM glucose, %p<0.05 vs diabetic 25 mM glucose.

Figure 5. Exogenous PGE$_2$ enhances BMDC migration in a CCR7-dependent manner after recognition of MRSA-IAC. (A) PGE$_2$ levels in the supernatants of BMDCs +
MRSA-IAC co-cultures after 18 h determined by EIA. (B) Number of migratory BMDCs using flow cytometry acquisition software. (C) Representative images of migrating BMDCs. Data represent mean ± SEM of at least 3 independent experiments performed in triplicate. *p<0.05 control vs 5 mM glucose, $p<0.05$ control vs 25 mM glucose, #p<0.05 diabetic control vs diabetic 5 mM glucose, %p<0.05 diabetic control vs diabetic 25 mM glucose.

**Fig. 6. Misoprostol improves DC migration and Th17 development in skin-draining lymph nodes in diabetes.** (A) Representative histograms of the percentage of migrating DCs in the brachial lymph nodes of MRSA infected control or diabetic mice treated or not with misoprostol. (B) Representative dot plots of the percentage of Foxp3 and IL-17A expressing T lymphocytes in the brachial lymph nodes of control and diabetic mice treated or not with misoprostol. (C) Percentage of migrating DCs (CD11c+FarRed+) in the brachial lymph nodes. The percentage of intracellular IL-17A (D), Foxp3 (E) and IFN-γ (F) producing TCRβ⁺CD4⁺ T lymphocytes determined by FACS analysis. Data show mean ± SEM of samples from 5-10 mice. *p<0.05 vs control and #p<0.05 vs untreated diabetic.
Fig. 7. Topical misoprostol decreases MRSA-skin infection in diabetics. (A) Lesion and abscess size are represented by affected area in mm³ as described in Materials and Methods. (B) Bacterial load determination in the skin of diabetic mice treated or not with misoprostol at day 7 after infection. (C) Representative dot plots of the percentage of Foxp3 and intracellular IL-17A expressing T lymphocytes determined by FACS analysis. Percentage and number of IL-17A (D, E), Foxp3 (F, G), and IFN-γ (H, I) expressing TCRβ⁺CD4⁺ lymphocytes determined by FACS analysis. mRNA expression of β-defensin1 (J), β-defensin2 (K), β-defensin3 (L), β-defensin4 (M), β-defensin5 (N) and Cramp (O). Results represent mean ± SEM of samples from 5-10 mice. *p<0.05 vs control, #p<0.05 vs untreated diabetic.

Figure 8. Proposed model of PGE₂ regulation of DC migration and Th17 generation during MRSA skin infection in diabetic mice. (Right) During MRSA skin infection, PGE₂ is produced in the skin and further enhances CCR7-dependent DC migration to lymph nodes and Th17 generation. Increased Th17 enhances defensin generation and bacterial clearance in infected nondiabetic mice. (Left) In diabetic mice, hyperglycemia decreases the Ptges mRNA expression that culminates in low PGE₂ production and deficient CCR7-dependent DC migration, and Th17 generation in the lymph nodes, which impairs skin host defense. Adding back topical PGE analog restores DC/Th17 axis and improves host defense in diabetic mice.
Fig. 1
Fig. 2

256x194mm (300 x 300 DPI)
fig. 3
fig. 4

249x200mm (300 x 300 DPI)
Fig. 5

201x199mm (300 x 300 DPI)
fig. 6
Fig. 7

255x200mm (300 x 300 DPI)
Fig. 8

Normoglycemia

MRSA-skin infection

\[ \text{AA} \quad \underbrace{\text{Cox-1}}_{\text{Cox-2}} \]

PGH$_2$

\[ \text{Ptges} \quad \uparrow \]

PGE$_2$

\[ \text{DC migration} \]

\[ \text{Th17 differentiation} \]

\[ \text{Bacterial clearance} \]

\[ \text{Decreased lesion size} \]

Hyperglycemia

MRSA-skin infection

\[ \text{AA} \quad \underbrace{\text{Cox-1}}_{\text{Cox-2}} \]

PGH$_2$

\[ \text{Ptges} \quad \downarrow \]

PGE$_2$

\[ \text{DC migration} \]

\[ \text{Th17 differentiation} \]

\[ \text{Topical misoprostol} \]

\[ \text{Bacterial clearance} \]

\[ \text{Decreased lesion size} \]
Figure S1. Diabetic mice exhibit a persistent neutrophil recruitment to the affected skin during MRSA infection. (A) Percentage of neutrophils in the naïve skin of control and diabetic mice determined by FACS analysis. (B) Percentage of neutrophils in the infected skin of control and diabetic mice 18 h post MRSA infection determined by FACS analysis. (C) Percentage of neutrophils in the infected skin of control and diabetic mice 7 d post MRSA infection determined by FACS analysis. Data show mean ± SEM from 5-10 mice. *p<0.05 vs control.
Sup. Fig. 2

Figure S2. MRSA infection induces apoptosis in the infected skin of control and diabetic mice and in a human neutrophil-like cell line in vitro. (A, B, C) Representative dot plots of apoptotic cells stained with 7-AAD and Annexin V, and (D) percentage of early (Annexin-V\(^+\)) and late apoptosis (Annexin-V\(^+\) 7-AAD\(^+\)) and necrosis (7-AAD\(^+\)) in naïve and infected skin of control and diabetic mice after 18 h and 7 d of MRSA infection determined by FACS analysis. (E) Representative dot
plots showing basal apoptosis in neutrophil-like HL-60 cells. (F) Number of apoptotic cells after 18 h of MRSA infection and (G) number of apoptotic cells after 4 h MRSA infection and UV irradiation. Representative dot plots of 3 independent experiments. *$p<0.05$ vs naïve control and diabetic. #$p<0.05$ vs 7d control (late apoptosis).
Figure S3. High Glucose does not influence BMDC phenotype after recognition of MRSA-IAC. (A) Percentage of CD11b<sup>+</sup>CD11c<sup>+</sup> BMDCs after 7 days of differentiation in 5 mM or 25 mM of glucose. Expression of CD86 (B), CD80 (C), MHC II (D), and CCR7 (E) analyzed by FACS on BMDCs at day 7 of differentiation. (F) CD11b<sup>+</sup>CD11c<sup>+</sup>-mediated efferocytosis of CFSE labeled MRSA-IAC (1:3 ratio) for 18 h in 5 mM or 25 mM glucose. (G) Representative histograms
showing percentage of CFSE\textsuperscript{+}CD11b\textsuperscript{+}CD11c\textsuperscript{+} BMDCs. Quantification of (H) IL-6, (I) TGF-\(\beta\), and (J) IL-1\(\beta\) levels in supernatants of BMDCs incubated or not with MRSA-IAC for 18 h determined by ELISA. (K-N) Representative dot plots of the expression of IL-17A by T lymphocytes differentiated with supernatants from co-cultures of BMDCs with MRSA-IAC. Data are mean \(\pm\) SEM (A-F; H-J), representative histograms (G) or representative dot plots (K-N) of 3 independent experiments.
Figure S4. High glucose inhibits PGE2 synthase (Ptges) expression. (A) Cox-1, (B) Cox-2, (C) Ptges, (D) Ptger1, (E) Ptger3, (F) Ptger2, and (G) Ptger4 mRNA expression by BMDCs from control or diabetic mice co-incubated or not with MRSA-IAC (1:3 ratio) for 18 h in 5 mM or 25 mM glucose determined by real time PCR. Data represent mean ± SEM from 3-5 individual experiments, each performed in triplicate. $p < 0.05$ vs BMDC control 5mM. $\%p < 0.05$ vs BMDC
diabetic 5mM. *p < 0.05 vs BMDC+IAC control 5mM. #p < 0.05 vs BMDC+IAC diabetic 5mM.

Figure S5. Ptges expression is decreased in diabetic skin after MRSA infection. (A) At different time points post-infection the expression of Ptges mRNA was analyzed in the skin of control and diabetic mice by qPCR. (B, C) Levels of PGE metabolite and 6-keto prostaglandin F1α were quantified in the naïve skin of control and diabetic mice as well as at 48 h and 7 d post-infection. Data show mean ± SEM from 2-4 mice.
Figure S6. Hyperglycemia does not modulate TGF-β and IL-6 levels in the skin. (A, B) Levels of IL-6 and TGF-β were determined in the skin of control and diabetic mice at 18 h post-infection. Data show mean ± SEM from 4-5 mice.
Figure S7. Misoprostol treatment enhances β-defensin 3 production. Levels of β-defensin 3 were quantified by Elisa in the naïve skin of control and diabetic mice, and in the skin of control, diabetic and diabetic treated with topical misoprostol mice at 7 d post-infection. Data show mean ± SEM from 4-5 mice. #p<0.05 vs control naïve skin. %p<0.05 vs diabetic naïve skin.
