Intracellular *Staphylococcus aureus* triggers pyroptosis and contributes to inhibition of healing due to Perforin-2 suppression

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**Intracellular *Staphylococcus aureus* Triggers Pyroptosis and Contributes to Inhibition of Healing Due to Perforin-2 Suppression**

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**Conflict of interest statement**

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Abstract

Impaired wound healing associated with recurrent *Staphylococcus aureus* infection and unresolved inflammation are hallmarks of non-healing diabetic foot ulcers (DFU). Perforin-2, an innate immunity molecule against intracellular bacteria, limits cutaneous infection and dissemination of *S. aureus* in mice. Here we report the intracellular accumulation of *S. aureus* in the epidermis of DFU with no clinical signs of infection due to marked suppression of Perforin-2. *S. aureus* residing within the epidermis of DFU triggers AIM2-inflammasome activation and pyroptosis. These findings were corroborated in mice lacking Perforin-2. The effects of pyroptosis on DFU clinical outcomes were further elucidated in a 4-week longitudinal clinical study in DFU patients undergoing standard of care. Increased AIM2-inflammasome and ASC-pyroptosome coupled with induction of IL-1β were found in non-healing when compared to healing DFU. Our findings reveal the mechanism that includes Perforin-2 suppression, intracellular *S. aureus* accumulation and associated induction of pyroptosis that contribute to healing inhibition and prolonged inflammation in patients with DFU.
Introduction

Diabetic foot ulcers (DFU) are a serious complication of diabetes mellitus and a frequent cause of morbidity, including infection and lower leg amputation (1-4). More than a half of DFU are clinically infected and are especially associated with poor outcomes (5, 6). *Staphylococcus aureus* has been identified as the most prevalent pathogen in DFU (7-12). DFU are also characterized with unresolved inflammation preventing progression to healing (8, 13-17). Despite the high rate of recurrent *S. aureus* colonization and infection, the mechanisms by which this pathogen contributes to prolonged inflammation and inhibition of healing in DFU are not yet fully understood.

Perforin-2 (P-2) is a membrane-attack-complex-perforin (MACPF) domain containing proteins that are associated with broad spectrum bactericidal activity against intracellular bacteria (18-23). P-2 is encoded by the Mpeg1 gene and was initially identified based on homology to the MACPF domain of perforin 1, the cytolytic protein expressed by NK cells and CTL (24). We have shown previously that constitutive expression of P-2 in the epidermis and primary human keratinocytes plays important role in protection from infection and invasion by *S. aureus* (20, 23). *Mpeg1*−/− mice, are unable to limit the proliferation and dissemination of bacteria, and succumb to bacteremia after epicutaneous exposure to *S. aureus* at a dose nonlethal to wild type (WT) littermates (20). Recent studies show that *S. aureus* wound infection results in striking suppression of P-2 and inhibition of healing in *ex vivo* human acute wound healing (23). However, despite the importance of P-2 in cutaneous bacterial defense, the role of P-2 in the pathological chronicity of DFU remains unknown.

Pyroptosis, is a caspase-1-dependent, highly inflammatory form of cell death that results in cell lysis and release of its pro-inflammatory contents (25-29). Execution of pyroptosis is an inflammasome-mediated process induced by the presence of intracellular pathogens (27, 30-32). Absent in melanoma 2 (AIM2) was identified as an inflammasome-forming protein in response to cytosolic dsDNA originating from intracellular pathogens (32-35). Activation of AIM2 leads to the oligomerization of the inflammasome adaptor protein apoptosis-associated speck like protein containing a CARD domain (ASC) into a large supramolecular complex termed the pyroptosome (28, 32, 34-36). Pyroptosome assembly induces cleavage and activation of procaspase-1, which in turn activates caspase-1, resulting in proteolytic processing of the pore-
forming protein, gasdermin D. This cascade triggers formation of pores in the plasma membrane resulting in cell lysis and release of intracellular contents and inducing an inflammatory response (27, 37, 38). Recent studies have shown that inflammasome components are expressed in several skin cell types including keratinocytes (25, 33, 36, 39, 40), however the role of pyroptosis and its relevance to healing inhibition in DFU have not been reported.

We investigated the role of P-2 in the pathophysiology of chronic DFU in the absence of clinical signs of infection, which are characterized by unresolved inflammation (17). We found striking suppression of P-2 in DFU keratinocytes, that allows intracellular *S. aureus* accumulation in the epidermis. We show induction of ASC pyroptosome assembly and AIM2 activation by intracellular *S. aureus* in primary human keratinocytes and DFU tissue, which is corroborated in vivo in the P-2KO (*Mpeg −/−*) mice. Furthermore, induction of pyroptosis in DFU was associated with activation of gasdermin D and increased levels of IL-1β, which contribute to prolonged inflammation. AIM2 inflammasome activation and increased levels of pyroptosome components demonstrated correlation with the non-healing outcome of DFU. We conclude that *S. aureus*, by suppressing P-2, persists within DFU keratinocytes and contributes to prolonged inflammation and inhibition of healing by induction of pyroptosis.
Results

Perforin-2 is suppressed in the epidermis of DFU allowing intracellular accumulation of S. aureus

Systemic dissemination of S. aureus due to epicutaneous infection in the P-2KO mice coupled with suppression of P-2 in human ex vivo wound model infected with S. aureus (22, 23) leads to premise that human cutaneous conditions with P-2 suppression or insufficiency may be susceptible to S. aureus intracellular infection (41). To test this, we evaluated the expression of P-2 in chronic DFU by qPCR and found suppression of P-2 in the ulcer tissue compared to location matched controls (Fig 1A). Next, we analyzed P-2 expression in DFU on a single cell level by FISH-Flow (23) and found marked suppression of P-2 expression in CD104+ basal DFU keratinocytes (Fig 1B). Previously, we showed that S. aureus suppresses P-2 in keratinocytes during wound infection of human skin (23). Thus, we postulate that P-2 suppression in DFU results in accumulation of intracellular S. aureus. To evaluate presence of the intracellular pathogen in DFU we isolated epidermis and dermis from the ulcer tissue in the presence of gentamicin, lysozyme and lysostaphin in order to remove all extracellular bacteria, and subjected cell suspension to hypotonic lysis, releasing bacteria from skin cells. Higher intracellular load of S. aureus was found in the DFU epidermis compared to dermis (Fig 1C), whereas it was absent in location matched controls. Presence of intracellular S. aureus in the epidermis of DFU was confirmed by immunohistochemistry, co-staining with epidermal marker keratin 17 (K17) and confocal microscopy (Fig D & F, Suppl Movie 1).

Intracellular S. aureus induces pyroptosis in DFU tissue and in human keratinocytes

To determine potential presence of pyroptosis in DFU due to intracellular S. aureus, we analyzed the histology of tissue samples obtained from the ulcer edge, where pathologically stalled keratinocytes accumulate. Location matched non-ulcerated plantar diabetic foot skin (FS) served as control. Morphological analyses revealed a thick cornified layer, characteristic of plantar skin in all samples (Fig 2A). However, presence of hole-like structures indicative of pyroptosis were found only in DFU tissue and were absent in control samples (Fig 2A).
marker for pyoptosis is the assembly of the pyroptosome, a supramolecular assembly of ASC oligomers. In order to confirm the presence of pyoptosis in tissue samples, chemical cross-linking of the ASC pyroptosome was performed followed by assessment of oligomers by Western blot. In line with histology assessments, FS controls showed absence, whereas DFU tissue samples showed induction of pyroptosome assembly as indicated by the dimer and higher-ordered oligomer structures of ASC (Fig 2B). To rule out the possibility of cell death by apoptosis, we assessed an apoptotic marker, caspase-3 activation, in DFU by immunoblotting. In both control and DFU samples, only the pro-form of caspase-3 (39 kDa) was found, whereas its cleaved activated form (19 kDa) was not detected (Suppl Fig 1), confirming absence of apoptotic cells in DFU. In line with this finding, we did not detect apoptotic DNA fragmentation in DFU tissue (Suppl Fig 2).

Next, we assessed if presence of intracellular bacteria induces pyoptosis in primary human epidermal keratinocytes (HEK). To test this, we used a S. aureus strain isolated from a DFU (8). Infected and uninfected HEK were either treated by lysostaphin to eliminate extracellular bacteria or left untreated (20, 23), collected at multiple time points upon infection and pyoptosis was assessed by chemical crosslinking of ASC (Fig 2C). The presence of intracellular S. aureus was confirmed by immunostaining and confocal microscopy corresponded to induction of ASC pyroptosome, while bacteria attached to the cell wall did not (Fig 2D). We confirmed induction of pyroptosome assembly in HEKs infected with intracellular S. aureus as indicated by the oligomer structures of ASC in human epidermal keratinocytes (Fig 2 C&D).

**Induction of pyoptosis is associated with increased IL-1β**

Activation of AIM2 inflammasome has been shown to induce the oligomerization of ASC into the pyroptosome, which in turn activates procaspase-1 leading to cleavage and activation of the pore-forming protein, gasdermin D and pro-IL-1β, resulting in inflammatory cell lysis. We evaluated AIM2 levels in DFU by immunoperoxidase staining (Fig 3A). DFU samples showed induction of AIM2, in contrast to control FS that showed absence of AIM2 (Fig 3A). Further, we assessed AIM2 inflammasome activation by western blot. We confirmed AIM2 induction specifically in DFU, whereas it was absent in control FS (Fig 3B). To corroborate
findings from human tissue we performed transcutaneous infection in P-2KO and WT mice. Infection with methicillin resistant S. aureus (MRSA) induced AIM2 in the P-2KO skin compared to WT skin in vivo and in vitro (Fig 3C&D; Suppl Fig 3). Accumulation of intracellular MRSA in the keratinocytes of P-2KO was evident, whereas it was absent in the WT skin, as confirmed by confocal imaging (Fig 3E).

Next, we evaluated the downstream effector of AIM2, Caspase 1 in DFU samples. The Caspase-1 cleaved activated form (10 kDa) was selectively found in DFU samples, and not in control location matched foot skin (FS) (Fig 3F). To further confirm the induction of pyroptosis in DFU samples, we assessed the main effector of pyroptosis, gasdermin D (Fig 3G). We found cleavage and activation of gasdermin D to be present only in DFU, whereas it was absent in control FS (Fig 3G). To assess expression levels of genes regulated by induction of pyroptosis we utilized DFU gene expression dataset (8). We found deregulated set of genes known to be involved in pyroptosis, including IL-1β, gasdermin D and AIM2 (Fig 3H). To confirm the microarray data and test if induction of pyroptosis in DFU is associated with increased inflammation, IL-1β levels were assessed by ELISA in DFU tissue and FS controls. We found an increased levels of IL-1β in DFU (Fig 3I) suggesting that induction of pyroptosis induces IL-1β, a pro-inflammatory cytokine, thus contributing to the persistent inflammation seen in DFU. Furthermore, increased levels of IL-1β were confirmed in the skin of P-2KO mice post-infection with MRSA when compared to control WT skin (Fig 3J).

**Pyroptosis correlates with the healing outcomes of DFU**

Next, we performed a 4 week longitudinal clinical study to investigate whether intracellular accumulation of S. aureus followed by induction of the AIM2 inflammasome and pyroptosis contributes to a non-healing phenotype. We obtained tissue samples from a prospective set of patients at the initial visit (Week 0, W0), and after 4 weeks of standard wound care (Week 4, W4). The healing outcome was determined by a surrogate endpoint, as a percent reduction in wound size at W4 compared to W0 (42). Patients were grouped in two categories: healers, in which wound reduction was greater than or equal to 50%; and non-healers, in which wound reduction was less than 50% (Suppl Fig 3). Pyroptosis was assessed by chemical cross-
linking of the ASC pyroptosome and ASC oligomers were evaluated and quantified by Western blot. We found that pyroptosome assembly was induced in all DFU samples at W0. However, healers showed a decrease in pyroptosome assembly at W4, whereas pyroptosome assembly persisted or increased in non-healing DFU at W4 (Fig 4A&B). In addition, we tested the correlation of the AIM2 inflammasome levels with the healing outcome of DFU and found a similar pattern. All DFU tissue samples showed presence of AIM2 at W0 (Fig 4C). Decrease of AIM2 at W4 compared to W0 was found in healing DFU, whereas AIM2 was found increased at W4 in non-healing DFU (Fig 4C&D). We then assessed if gasdermin D activation correlates with the healing in the prospectively collected tissue and found cleaved and activated gasdermin D decreased at W4 compared to W0 in healing DFU, whereas gasdermin D was increased in non-healing DFU (Fig 4E&F). Next, we assessed the levels of S. aureus in the formalin fixed paraffin embedded (FFPE) tissue from the healing and non-healing DFU by qPCR. DNA from the DFU FFPE samples was extracted and the presence of the S. aureus-specific thermonuclease gene (nuc) was quantified and normalized to total bacterial load by qPCR (8, 43). All DFU tissue samples were positive for the nuc, confirming accumulation of S. aureus in the ulcer even without clinical signs of infection (Fig 1G), however increased levels of S. aureus were detected in non-healing DFU tissue (Fig 1G). Taken together, our results demonstrate that pyroptosome assembly and AIM2 inflammasome are induced in DFU due to the accumulation of intracellular S. aureus likely as a result of the suppression of innate immune molecule P-2 in the epidermis. Importantly, AIM2 inflammasome and ASC pyroptosome activation persist in patients with non-healing DFU and correlate with higher levels of S. aureus.

Discussion

Here we show that S. aureus by suppressing P-2 can survive and persist in keratinocytes of the DFU epidermis. Intracellular S. aureus triggered AIM2 inflammasome activation, ASC pyroptosome assembly and resulted in increased IL-1β levels in tissue samples obtained from chronic ulcers of diabetic patients that exhibit no clinical signs of infection (Fig 5). S. aureus internalization within epidermal keratinocytes was confirmed in the P-2KO animals, resulting in subsequent activation of AIM2 and induction of IL-1β. Furthermore, when correlated with ulcer
healing, we found persistence or increase of pyroptosis in non-healing DFU, whereas a decrease in pyroptosis was associated with healing. To our knowledge, these findings are the first to demonstrate the clinical relevance of P-2 suppression in DFU in the context of intracellular *S. aureus* and unresolved inflammation triggered by pyroptosis. Suppression of indispensable antimicrobial effector P-2 in DFU tissue and accumulation of intracellular *S. aureus* in non-healing ulcers even without clinical signs of infection may identify patients at the higher risk of infection and further complications.

P-2 and its bactericidal role against *S. aureus* has only recently been described (19, 20), and this is the first report showing correlation between P-2 expression levels and the chronicity of DFU. Downregulation of P-2 is unique characteristic of DFU, our previously published data on gene expression profiles from human diabetic foot skin have shown lack of P-2 regulation in the diabetic vs. healthy non-diabetic foot skin (44). Intracellular *S. aureus* is of major clinical importance as it may provide antimicrobial resistance residing within this niche and contribute to recurrence of DFU. While certain virulence factors of *S. aureus* have been recently associated with healing inhibition of DFU (7), their potential role in P-2 suppression and underlying mechanisms remain to be elucidated. The potent bactericidal activity of P-2 (18, 20, 45) mandates suppression of the P-2 expression in order for bacteria to establish even a temporary residence in the host’s cells. Indeed we have shown recently the ability of *S. aureus* to inhibit P-2 in human skin resulting in the inhibition of healing, whereas over-expression of P-2 results in reduction of intracellular *S. aureus* in human keratinocytes (23). This leads to a conundrum: decrease of P-2 expression leads to invasion of intracellular *S. aureus* that further suppresses P-2 expression and, in turn, facilitates accumulation of intracellular *S. aureus*. In contrast to pathogenic *S. aureus*, commensal *S. epidermidis* is capable of inducing P-2 in keratinocytes and resident GDT cells, opening the possibilities for therapeutic intervention (46).

Chronic unresolved inflammation is a major contributor to delayed healing in DFU (8, 13, 14, 17, 47, 48). We have recently identified major transcriptional networks deregulated in DFUs that result in decreased neutrophils and macrophages recruitment and overall, poorly controlled inflammatory response (17). Here we demonstrate the role of inflammatory cell death, pyroptosis, triggered by suppression of P-2 and intracellular *S. aureus* to inhibition of healing in DFU. Cytosolic dsDNA originating from intracellular pathogens has been shown to trigger
AIM2 inflammasome activation (32-35). We confirmed AIM2 inflammasome activation in DFU and primary human keratinocytes due to intracellular S. aureus. Consequently, release of the host factors from the microbial-induced pyroptotic cells may perpetuate AIM2 inflammasome activation and pyroptosis, promoting deregulation of the inflammatory response in DFU, whereas the lack of functional neutrophils and macrophages may further affect bacterial clearance in DFU (17). Release of intracellular S. aureus from pyroptotic cells may also promote ulcer colonization and perpetuate infection and its recurrence (Fig 5). Our data also demonstrate AIM2 correlation with the healing outcomes, suggesting that AIM2 has an important role in modulating the inflammatory response in DFU. We confirmed P-2 role in AIM2 activation in the epidermis of P-2KO animals. In the P-2KO skin AIM2 inflammasome levels were higher compared to WT and correlated with accumulation of intracellular S. aureus and IL-1β post infection. This finding underscores the importance of the P-2 in skin barrier protection and maintenance.

Induction of ASC pyroptosome, and subsequent activation of Casp-1 are in agreement with previous findings of increased levels of activated IL-1β in DFU (48-51), suggesting that high levels of this pro-inflammatory cytokine in DFU may be the result of increased pyroptosis. High levels of pro-inflammatory cytokines can have detrimental effects on wound healing, due to subsequent induced expression of metalloproteases that excessively degrade the extracellular matrix and impair cell migration (50, 51). In addition, our laboratory has previously shown that IL-1β induces synthesis of epidermal cortisol, a potent wound healing inhibitor, resulting in impaired cell migration and wound closure (52-54). Therefore, it is possible that induction of pyroptosis in DFU may promote excessive cortisol production further contributing to unresolved inflammation and inhibition of healing.

We also found cleavage and activation of the casp-1 substrate, gasdermin D, to be uniquely present in DFU. Gasdermin D has been shown to be the main effector of pyroptosis by acting as the pore-forming protein that results in cell lysis (37). While previous studies have implicated aberrant apoptosis to play a role in impaired wound healing in mice (55-57), our data show absence of apoptosis in DFU and prevalence of pyroptosis. Caspase-3, a unique molecule found specifically in apoptosis, but not in pyroptosis was absent from DFU, suggesting pyroptosis to be the predominant form of cell death in DFU. While the absence of apoptosis in
DFU was confirmed, the possibility of additional forms of cell death such as necroptosis remains to be validated. Furthermore, induction of Gasdermin D in DFU coupled with Casp-1 activation supports recent data documenting Gasdermin D requirement for Casp-1 mediated pyroptosis (58).

Taken together, induction of pyroptosis due to intracellular *S. aureus* and its relationship with the healing outcome of DFU identifies a pathway for persistent and recurrent bacterial colonization and prolonged inflammation in DFU (Fig 5). Correlation of pyroptosis and the healing outcome of DFU suggest that targeting intracellular bacterial niche with the goal of inhibition of the pyroptotic pathway may promote healing. The identification of therapeutics that restore and/or increase P-2 levels may prevent *S. aureus* intracellular accumulation and allow the skin to successfully combat bacterial pathogens, prevent wound infection and promote healing. In addition, evaluation of the P-2 levels in DFU tissue in the future may serve as a biomarker that can predict infection.
Methods

Skin Specimens

Eligible participants were adults aged 21 or older with type 2 diabetes mellitus and at least 1 clinically non-infected ulcer on the plantar aspect of their foot larger than 0.5 cm². Inclusion and exclusion criteria are listed in Suppl. data. Full thickness skin specimens were obtained from wound edges of DFU \((n = 24, \text{mean age } \pm \text{standard deviation } = 57 \pm 11.9, 21 \text{ males, 3 females})\) and location matched diabetic (DFS) and non-diabetic foot skin (NFS) \((n = 12, \text{mean age } \pm \text{standard deviation } = 58 \pm 14.9, 7 \text{ males, 5 females})\). Patient demographics and sample characteristics are included in Suppl. Table S1. DFU specimens were collected from discarded tissue after surgical debridement at initial visit (W0) and 4 weeks post standard wound care (W4). Wounds were measured by planimetry and patients were grouped as healing if there was >50% reduction or as non-healing if there was <50% reduction at W4 compared to W0 (59). Specimens were fixed in formalin and processed for paraffin embedding and stained with hemotoxylin and eosin to assess tissue morphology, as previously described (60).

FISH-Flow P-2 RNA Assay and Flow cytometric analysis

Single cell suspensions were obtained from full thickness DFU tissue and control skin as described before (23). Cells were first labeled with live/dead detection kit followed by labeling with fluorescently labeled antibodies CD45-Alexa Fluor 700 (catalog, 368514, Clone 2D1, Biolegend), CD31-PacBlue (catalog 303114, Clone WM59, Biolegend) CD104-FITC (catalog 327806, Clone 58XB4, Biolegend). P-2 mRNA was detected using an amplified signal FISH technique (PrimeFlow; Affymetrix/eBioscience- Thermo Fisher Scientific). For mRNA detection, target probe hybridization was performed using type 1 (AlexaFluor647) probes for P-2 as previously described (23). Approximately 20,000 cell events were acquired from each sample on flow cytometer equipped with 405 nm, 488 nm, 642 nm, and 785 nm (SSC) lasers (Fortessa, BD, San Jose, CA). Spectral compensation was completed using single color control samples. Live, CD45 negative, CD31 negative and CD104+ cells were analyzed for expression of P-2 mRNA using FlowJo© software (Tree Star, Ashland, OR, USA, Version V10).
**RNA isolation, qPCR and genomic analyses**

Total RNA was extracted using the miRNeasy kit (QIAGEN, Valencia, CA) per manufacturer’s instructions. cDNA was made with qScript™ Synthesis kit (Quanta BioSciences Inc., Gaithersburg, MD). P-2 real-time PCR (qPCR) reactions were performed in triplicate using PerfeCTa® SYBR® Green SuperMix (Quanta BioSciences), P-2 specific primers (forward 5’-AAGGACCAAGCTATAACTACCG-3’; reverse 5’-CGTCAGAGATGTCAAGGAGTTTC-3’), and ARPC2 (forward 5’-TCCGGGACTACCTGCACTAC-3’; and reverse 5’-GGTTACGACCTTGAGGAAG-3’) was used as a reference gene for normalization. Microarray data set (Gene Expression Omnibus accession number GSE80178) was previously described (8). Raw data (.CEL) files were GCRMA normalized and filtered using Genespring 13.0, and a list of differentially expressed probes/corresponding genes were determined using moderated t test, \( p < 0.05 \), fold change >2.

**Isolation of intracellular bacteria**

Full thickness skin samples were minced and incubated at 37°C for 3 hours with 2 mg/ml Collagenase D (Roche) at 37°C under constant agitation in the presence of 100 \( \mu \)g/ml gentamycin, 25 \( \mu \)g/mL lysostaphin and 100 \( \mu \)g/ml lysozyme (23). Obtained cell suspensions were washed with DMEM (Gibco-Thermo Fisher Scientific) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 0.15% sodium hydrogencarbonate, 1 mM sodium pyruvate, nonessential amino acids, 50 \( \mu \)g/ml gentamycin, 25 \( \mu \)g/mL lysostaphin and 100 \( \mu \)g/ml lysozyme. To separate epidermis and dermis skin samples were cut in 1-2 mm strips and incubated overnight with Dispase II (2.4 U/ml) at 4°C in the presence of 100 \( \mu \)g/ml gentamycin, 25 \( \mu \)g/mL lysostaphin and 100 \( \mu \)g/ml lysozyme. Epidermal cells were obtained by physical dissociation of epidermal sheets and vigorous pipetting. Dermis was additionally incubated for 1 h with 2 mg/ml collagenase D (Roche) at 37°C. The cells were pelleted, washed with PBS and subsequently lysed with 100 \( \mu \)l of 0.2% saponin in 1× PBS on ice for 20 minutes. Serial dilutions were plated on mannitol salt agar for bacterial quantification. Standard identification methods and PCR detection of the *S. aureus*-specific DNA gyrase A (*gyrA*) and staphylococcal nuclease (*nuc*) gene were used to identify *S. aureus* DFU isolates as described (8, 61).
**Bacterial strains and growth conditions**

Methicillin-resistant *S. aureus* (MRSA) USA300-0114 (62), USA300 JE2 (63), and *S. aureus* intracellular isolate from the DFU were used. MRSA USA300 AH1726 [MRSA LAC (AH1263) + pCM29 (CmR)] (46, 64) was used for murine in vivo skin infection. Todd-Hewitt broth supplemented with 0.2% yeast extract (THY) served as growth medium; oxacillin resistance screening agar base (OXOID, Carlsbad, CA) was used as selective media for MRSA colony forming units (CFU) quantification.

**Intracellular infection assay**

Primary human keratinocytes were grown to approximately 50% confluency and infected with MRSA USA300-0114 or *S. aureus* isolate from the DFU at an MOI of 100 (20). Cells were infected with MRSA at MOI of 100 for 15, 30 and 60 minutes, washed 3 times with PBS and collected at 15-minute time increments to allow for bacterial adhesion to keratinocytes. In addition, primary human keratinocytes were infected for 60, 120 and 180 minutes, washed 3x with sterile PBS to further remove any remaining extracellular bacteria then switched to 25 µg/mL lysostaphin to kill remaining extracellular bacteria, and collected at 30, 60 and 120 minutes to allow for intracellular infection (20). Cells were harvested at indicated time increments for ASC pyroptosome assay, immunofluorescence staining and confocal imaging.

**Mouse skin infection assay**

Wild type mice, C57Bl/6 (n=7) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). P-2KO mice (n=9) were generated and bred at University of Miami (20, 65). Mice were shaved and tape-striped (7 applications) with Transpore tape (3M, Minneapolis, MN, United States) to disrupt the epidermal barrier (20). An inoculum of 10⁹ MRSA USA300 AH1726 in 0.02 ml of phosphate-buffered saline (PBS) or PBS control was added to ~1 cm² of skin and the area bandaged with plastic sheet and overwrapped with dressings of Transpore tape and Nexcare waterproof tape (3M) for 24 hr. Upon 24 hours of infection mice were sacrificed and skin processed for protein isolation, CFU enumeration and cryopreserved for imaging.

**ASC Pyroptosome Assay**

Tissue or cells were homogenized using 20mM HEPES, pH 7.5; 150mM KCl; 1% NP-40; and 1X protease and phosphatase inhibitors (Cell Signaling). Lysates were centrifuged at 1800 rpm
for 5 minutes and supernatant was collected. Equal amount of protein (50 -100 µg) was used to crosslink the ASC pyroptosome with 2 mM DSS (disuccinimidyl suberate) for 30 minutes. Pyroptosome was pelleted by centrifugation at 6000 rpm for 10 minutes. Pellet was re-suspended with 2x SDS sample buffer and subjected to electrophoresis and immunoblot analysis with anti-ASC described below.

**Western Blot**

Protein from each sample was resolved on 4-20% Criterion TGX pre-cast gels (Bio-Rad) and transferred onto PVDF membranes (Bio-Rad). Membranes were blocked with I-Block (Applied Biosystems) in Phosphate-buffered saline containing 0.1% Tween-20, and then probed with anti-ASC (catalog NB1-78977, 1:1000, Novus Biologicals and catalog 13833S, 1:500, Cell Signaling), human anti-AIM2 (catalog D5X7K, 1:1000, Cell Signaling), mouse anti-AIM2 (catalog AB93015, 1:1000, Abcam), anti-caspase-1 (catalog 2225S, 1:1000, Cell Signaling), anti-Gasdermin D (catalog NBP2-33422, 1:1000, Novus Biologicals) or anti-caspase-3 (catalog 9662S, 1:1000, Cell Signaling). After washing, membranes were incubated with their appropriate horseradish peroxidase-conjugated secondary antibodies (catalog 7074S, 1:10000; catalog 7076S, 1:10000, Cell Signaling) and developed using an ECL Prime chemiluminescence detection system according to the instructions of the manufacturer (GE Healthcare). Immunoblots were stripped with Restore Plus Western Blot Stripping Buffer (Pierce). Anti-β-actin antibody (catalog, AS411. 1:50000, Sigma Aldrich) was used for loading control.

**Immunofluorescence staining**

Paraffin embedded tissue sections of DFU were used for staining with anti-S. aureus (catalog ab20920, 1:100; Abcam) as previously described (62). S. aureus was visualized with Alexa Fluor 488-conjugated goat anti-rabbit antibody (catalog #A11008, 1:1000, Invitrogen), and mounted with Prolong DAPI Gold antifade reagent (Invitrogen) to visualize cell nuclei. Keratin 17 (K17) (Gift from Dr. P. Coulombe, Johns Hopkins University, Baltimore) was used for staining of keratinocytes in DFU (66). For primary human keratinocytes staining, cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature after infection with S. aureus as described above followed by 3x washes with PBS. Cells were then permeabilized with 0.5% Triton X-100
for 5 minutes and washed 3x with PBS. Cells were then stained with anti-
\textit{S. aureus} (catalog ab37644, 1:500; Abcam) for 1 hour. \textit{S. aureus} staining was visualized with Alexa Fluor 488-
conjugated goat anti-mouse antibody (catalog A11029, 1:500; Invitrogen) and mounted with
Prolong DAPI Gold antifade reagent (Invitrogen) to visualize cell nuclei. Cells were then incu-
bated with 200 nM Acti-stain 555 phalloidin (catalog PHDR1, Cytoskeletal) for 30 minutes
followed by 3x PBS washes. Cryosectioned infected and control mouse skin embedded in OCT
was fixed in 4\% paraformaldehyde for 15 minutes at room temperature, rinsed in PBS, and
permeabilized in 0.5\% Triton X-100-PBS for 20 minutes. Slides were rinsed twice in PBS,
blocked in 5\% BSA in PBS with 0.1\% Tween20 (PBST) for 1 hour and labeled with rabbit anti-
\textit{S. aureus} (catalog ab20920, 1:500, Abcam) for 1 hour at room temperature. After 3 rinses in
PBST, slides were fluorescently labeled with Alexa Fluor 488-conjugated goat anti-rabbit
antibody (catalog A11008, 1:1000, Invitrogen) for 1 hour. The samples were then incubated
overnight at 4 °C with Rhodamine conjugated Phalloidin (200nM, catalog PHDR1,
Cytoskeleton) and mounted with Prolong Diamond Antifade Reagent with DAPI (Invitrogen) to
visualize the cellular nuclei. Images were obtained with a Zeiss LSM 800 confocal microscope
(Carl Zeiss GmbH, Oberkochen, Germany) in epifluorescence mode for lower resolution images
and in laser scanning mode for higher resolution images. Image analysis was performed using
Zeiss Zen 2.5 (blue edition) Version 2.5.75.00006.

\textbf{DNA extraction from formalin fixed paraffin embedded DFU tissue (FFPE) and
quantification of \textit{S. aureus} by PCR}

Total DNA from DFU FFPE blocks, was extracted from 3 sections (10μm thick) using the
GeneRead DNA FFPE kit (QIAGEN, Valencia, CA) and qPCR quantification of \textit{S. aureus}
performed as described (8). The primers used were: Nuc-F-162
GTTGTAAGTTTCAAGTCTAAGTC, Nuc-R-162 AACCCTATCACCTCAATC, Nuc-
probe-162 ATCCACAGTTATATAGTGCAAC (43). To measure microbial load, qPCR assays
of the 16S rRNA gene were performed as previously described (9).

\textbf{ELISA}

Active IL-1β from DFU was assessed by ELISA using the human IL-1β/IL-1F2 Quantikine
ELISA Kit (R&D Systems) while IL-1β from murine skin using mouse IL-1β /IL-1F2
Quantikine ELISA Kit (R&D Systems) according to the manufacturer’s instructions. Samples were measured in duplicates and mean optical density values were used to normalize to total protein.

**Statistical analyses**

The numbers of healthy foot skin and DFU samples, normal or P-2 knockout mice, ELISA replicates, or replicates of cell were indicated in each figure. All data were presented as mean ± SD. Student’s t test and either 1-way or 2-way ANOVA were performed. In all descriptions, *p < 0.05, **p < 0.01, and ***p < 0.001. Cultured cell experiments were repeated at least three times and similar results were obtained.

**Study approval**

Ulcer tissue specimens were obtained after Institutional Review Board approval (UM protocol #20150222), written informed consent was obtained prior to participation. All animal experiments were performed in accordance with University of Miami Animal Care and Use Committee guidelines under IACUC approved protocol (#16-006).

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References


Figure 1. Suppression of P-2 in the epidermis of DFU allows intracellular accumulation of S. aureus. A. Relative expression P-2 was assessed by qPCR normalized to Arpc2 in control foot skin (FS; n=5 biological replicates, 15 technical replicates) and diabetic foot ulcers (DFU; n=6 biological replicates, 18 technical replicates). B. P-2 mRNA levels in control and DFU keratinocytes quantified using FISH-Flow. Live, CD104+ CD45- CD31- cells were analyzed for the P-2 RNA expression within epidermal keratinocytes (n=5 biological replicates). C. Quantification of intracellular bacterial load in the epidermis and dermis per mg of tissue (n=5 biological replicates, 15 technical replicates) D. Immunofluorescence staining of FS and DFU with S. aureus specific antibody (green). Arrows indicate presence of intracellular S. aureus. DAPI=nuclei; dashed line demarcates epidermal-dermal boundary; Epi=epidermis, Der=dermis. scale bar = 50 µm. E. Confocal imaging of DFU tissue stained with keratin 17 (K17; red) and S. aureus specific antibody (green). DAPI (blue)=nuclei; images taken at 63x magnification, scale bar = 10 µm.
Figure 2. Intracellular presence of *S. aureus* induces ASC pyroptosome assembly in diabetic foot ulcers. **A.** Hematoxylin and eosin staining of healthy foot skin (FS) and diabetic foot ulcers (DFU) revealing presence of hole-like structures indicative of cell lysis, suggesting presence of pyroptosis. **B.** Western blot of ASC pyroptosome showing ASC oligomerization in DFU (n=3 per group), demonstrating induction of pyroptosis in DFU; Ctrl= human keratinocytes treated with poly dA:dT to induce pyroptosis. **C.** Western blot of ASC pyroptosome demonstrates lack of ASC oligomerization during shorter infection allowing for bacterial adhesion (left blot) and induction of pyroptosis during longer infection allowing for MRSA internalization (right blot). Primary human keratinocytes were infected with MRSA at MOI of 100 for 15, 30 and 60
minutes, washed with PBS and collected at 15-minute time increments (white triangles), or infected for 60, 120 and 180 minutes, washed and collected at 30, 60 and 120 minutes (gray triangles) to allow bacteria to be internalized (20); Ctrl=uninfected cells. D. Representative confocal imaging, corresponding to western blots, of primary human keratinocytes infected with MRSA USA300 (green); DAPI (blue) = nuclei, rhodamine conjugated phalloidin (orange) used to visualize actin; Ctrl=uninfected cells. Upper panel shows MRSA attached to keratinocytes during shorter infection time; bottom panel confirms intracellular localization of MRSA (white arrows); scale bar = 10 μm. Results depict representative experiment from three biological replicates.
Figure 3. AIM2 inflammasome activation induces pyroptosis and increases IL-1β activation in DFU. A. Immunoperoxidase staining of AIM2 in FS and DFU. AIM2 is induced in DFU compared to FS. Epi=epidermis; Der=Dermis, dashed line demarcates epidermal/dermal boundary; Scale bar = 50 µm. B. Western blot of AIM2 in FS and DFU (n=3 per group). C. Western blot of Caspase-1 in FS and DFU (n=3 per group).
Representative Western blot and quantification (D) of AIM2 levels in WT (n=4) and P-2KO (n=5) from murine skin in response to infection with MRSA USA3000 AH1726, validating that MRSA infection induces AIM2 with amplified induction in the skin from P2KO mice; *p<0.05  E. Confocal imaging confirming intracellular MRSA in the epidermis of P-2KO mice but not in the WT; Epi=epidermis; Der=Dermis, dashed line demarcates epidermal/dermal boundary, arrows indicate intracellular MRSA, scale bar = 10 µm; inset scale bar = 2 µm. F. Western blot Caspase-1 in FS and DFU (n=3 per group). Caspase-1 is uniquely activated in DFU compared to FS. G. Western blot of gasdermin D (GsdmD; n=3 per group). Activation of GsdmD was found to be present in prospectively collected DFU. H. Gene signature of DFU compared to control foot skin of a subset of genes involved in pyroptosis indicated strong induction of IL-1β. I. ELISA of IL-1β in FS and DFU confirmed increased levels of IL-1β in DFU (n=6 biological replicates for FS, n=3 biological replicates for DFU). Data are represented as mean ± SEM and analyzed by unpaired 2-tailed t-test, **p<0.01. J. ELISA of IL-1β confirmed increased levels of IL-1β in the murine skin lacking P-2 post-infection (n=4 biological, 8 technical replicates for WT, n=4 biological, 7 technical replicates for P2KO). Data are represented as mean ± SEM and analyzed by unpaired 2-tailed t-test, **p<0.01.
Figure 4. Pyroptosis correlates with the healing outcome of DFU. A. Western blot and corresponding quantification plot (B, the ratio of Week 4/Week 0 (W4/W0) tissue levels for ASC) of ASC pyroptosome in healing (n=3) and non-healing DFU (n=4); *p<0.05. C. AIM2 Western blot and corresponding quantification plot (D; the ratio of W4/W0 AIM2 tissue levels) in healing (n=3) and non-healing DFU (n=4). ASC pyroptosome and AIM2 levels decreased in healing DFU over time, whereas it persisted or increased in non-healing DFU after 4 weeks (W4) of standard of care; **p<0.01 (n=4 per group). E. Western blot of GsdmD in prospective healing and non-healing DFU. F. Quantification of the ratio W4/W0 levels for GsdmD in DFU-H and DFU-NH. Activation of GsdmD was found to persist or increase in non-healing DFU at W4. **p<0.01 (n=3 per group). G. Quantification of S. aureus levels by qPCR in tissue from DFU-H and DFU-NH **p<0.01 (n=5 per group).
Figure 5. A current model of P-2/intracellular *S. aureus* - mediated induction of pyroptosis in DFU. Accumulation of intracellular *S. aureus* in the DFU epidermis due to suppression of Perforin-2 triggers AIM2 inflammasome activation, leading to caspase-1 mediated IL-1β activation and proteolytic processing of the pore-forming protein, gasdermin D. This cascade triggers formation of pores in the plasma membrane resulting in pyroptosis and release of intracellular contents including inflammatory mediators and accumulated intracellular *S. aureus*, contributing to chronic inflammation and inhibition of wound healing.