Sustained Inflammasome Activity in Macrophages

Impairs Wound Healing in Type 2 Diabetic Humans and Mice

Rita E. Mirza\textsuperscript{1}, Milie M. Fang\textsuperscript{1}, Eileen M. Weinheimer-Haus\textsuperscript{1,3}, William J. Ennis\textsuperscript{2,3}, Timothy J. Koh\textsuperscript{1,3}

\textsuperscript{1}Department of Kinesiology and Nutrition

\textsuperscript{2}Department of Surgery

\textsuperscript{3}Center for Tissue Repair and Regeneration

University of Illinois at Chicago

To whom correspondence should be addressed:
Timothy J. Koh, PhD
Department of Kinesiology and Nutrition
University of Illinois at Chicago
1919 W. Taylor St. (m/c 994, Rm 650), Chicago, II 60612, USA
Tel: 312-413-9771, Fax: 312-413-0319
Email: tjkoh@uic.edu

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ABSTRACT

The hypothesis of this study was that sustained activity of the Nod-like receptor protein (NLRP)-3 inflammasome in wounds of diabetic humans and mice contributes to the persistent inflammatory response and impaired healing characteristic of these wounds. Macrophages (Mp) isolated from wounds on diabetic humans and db/db mice exhibited sustained inflammasome activity associated with low level of expression of endogenous inflammasome inhibitors. Soluble factors in the biochemical milieu of these wounds are sufficient to activate the inflammasome, as wound conditioned medium activates caspase-1 and induces release of IL-1β and IL-18 in cultured Mp via a reactive oxygen species-mediated pathway. Importantly, inhibiting inflammasome activity in wounds of db/db mice using topical application of pharmacological inhibitors improved healing of these wounds, induced a switch from pro-inflammatory to healing-associated Mp phenotypes and increased levels of pro-healing growth factors. Furthermore, data generated from bone marrow transfer experiments from NLRP-3 or caspase-1 knockout to db/db mice indicated that blocking inflammasome activity in bone marrow cells is sufficient to improve healing. Our findings indicate that sustained inflammasome activity in wound macrophages contributes to impaired early healing responses of diabetic wounds and that the inflammasome may represent a new therapeutic target for improving healing in diabetic individuals.
INTRODUCTION

Tissue repair involves overlapping phases of hemostasis, inflammation, proliferation and remodeling (1). Diabetes can disrupt the timely progression through each phase of healing through its effects on many different cell types and molecular effectors (2). Macrophages (Mp) are involved in each phase of healing and are thought to play an important role in the repair of a variety of tissues (3-7). Thus, diabetes-induced disruptions in macrophage function might be expected to impair healing.

Mp promote tissue repair by killing pathogens, clearing damaged tissue and producing growth factors that induce angiogenesis, collagen deposition and wound closure (4; 8-11). During impaired healing associated with diabetes, wounds exhibit prolonged accumulation of Mp associated with elevated levels of pro-inflammatory cytokines and proteases and reduced levels of various growth factors (2; 12; 13). We recently demonstrated that Mp exhibit a sustained pro-inflammatory phenotype in wounds of diabetic mice (14) and that the persistence of the pro-inflammatory Mp phenotype appears to be mediated at least in part by the pro-inflammatory cytokine IL-1β (15). However, much remains to be learned about the dysregulation of Mp in diabetic wounds.

Pro-inflammatory danger signals induce activity of a multi-protein complex called the Nod-like receptor protein (NLRP)-3 inflammasome (16-18). During inflammasome activation, the pro-form of caspase-1 is recruited to the NLRP-3 complex and cleaved to produce active caspase-1, which then cleaves and activates the potent pro-inflammatory cytokines IL-1β and IL-18. Elevated levels of IL-1β have been found in wounds of diabetic humans and mice (12; 15; 19; 20), consistent with increased inflammasome activity. Since IL-1β, in turn, is known to induce a pro-inflammatory Mp phenotype (15; 21), the NLRP-3 inflammasome may be part of a
positive feedback loop that sustains inflammation in chronic wounds and contributes to impaired healing. Thus, the central hypothesis of this study is that sustained activity of the inflammasome in diabetic wounds contributes to impaired early healing responses of these wounds.
MATERIALS AND METHODS

**Human subjects.** Five patients (2 male and 3 female) with chronic wounds provided informed consent. Patients were diagnosed with type 2 diabetes and had non-healing wounds on the sacral region or the lower limb lasting at least 3 months. During a sharp debridement, biopsies were taken from tissue located near the center of the wound. All procedures involving human subjects were approved by the Institutional Review Board at the University of Illinois at Chicago according to Declaration of Helsinki Principles.

**Animals.** Diabetic db/db mice, non-diabetic db/+ controls and C57Bl/6 wild-type controls were obtained from Jackson Laboratories. Breeding pairs of NLRP-3 knockout mice were provided by Genentech and caspase-1 knockout mice were provided by Drs. Mihai Netea and Leo Joosten, Radboud University Nijmegen Medical Center. Experiments were performed on 12-16 week-old mice. All procedures involving animals were approved by the Animal Care Committee at the University of Illinois at Chicago.

**Excisional wounding and treatment.** Mice were subjected to excisional wounding with an 8 mm biopsy punch as described previously (14; 15). Wounds were treated with the caspase-1 inhibitor Tyr-Val-Ala-Asp (YVAD) or the inflammasome inhibitor glyburide as indicated; treatment was initiated 3 days post-injury to allow the initial inflammatory response to proceed normally. Ac-YVAD-cmk (Cayman Chemical; 20 µM) and glyburide (InvivoGen; 200 µM) were applied topically to wounds every other day in F-127 pluronic gel (50 µl of a 25% gel in saline) (22; 23). Controls were treated with DMSO vehicle-loaded gel.

**Bone marrow transfer.** Bone marrow recipient mice (8-10 week-old db/db mice) were subjected to lethal irradiation by 2 doses of 5 Gy at 1.02 Gy/min with 3 hours between doses. Bone marrow cells were collected from donor 8-10 week-old C57Bl/6 wild-type, caspase-1 null
and NLRP-3 null mice and injected retro-orbitally (5 x 10^6 cells per mouse in 200 µl saline) into recipient mice at 1 day after lethal irradiation. Mice were allowed to recover for 30 days and then subjected to excisional wounding. Engraftment was verified in preliminary experiments using congeneric mice and was found to be > 85% by flow cytometry.

**Cell isolation.** Cells were dissociated from human chronic wound biopsies and mouse excisional wounds using an enzymatic digest (14). Neutrophils, T cells and B cells were marked for depletion by incubating cells for 15 minutes with FITC-conjugated anti-Ly6G (1A8), anti-CD3 (17A2) and anti-CD19 (6D5) for mouse cells and FITC-conjugated anti-CD15 (H198), anti-CD3 (UCHT1) and anti-CD19 (H19) for human cells (all from Biolegend, diluted 1:10), and then depleted from the total cell population using anti-FITC magnetic beads (Miltenyi Biotec). Cells of the monocyte/Mp lineage were then isolated using CD11b magnetic beads.

**RNA analysis.** Total RNA was isolated from human or mouse cells using the RNeasy kit (Qiagen). cDNA was synthesized using the Thermoscript RT-PCR System (Invitrogen). Real-time PCR was performed using TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assay primer/probe sets (Applied Biosystems). Relative gene expression was determined using the 2^(-ΔΔCT) method, with GAPDH as the endogenous control gene.

**Flow cytometry.** Cells were dissociated from mouse wounds and neutrophils, T cells and B cells were depleted by magnetic sorting as described above. The remaining cells were stained using PE-conjugated anti-CD11b (clone M1/70, diluted 1:10) and active caspase-1 was labeled using the FLICA detection reagent (Immunochemistry Technologies). Mp containing active caspase-1 were identified as CD11b^+FLICA^+ cells.

**Immunofluorescence.** Cryosections (10 µm thick) were cut from human chronic wound biopsies, fixed in cold acetone and blocked with buffer containing 3% BSA. Sections were then
incubated overnight with primary antibodies against CD68 (clone Y1/82A, 1:100, Biolegend) and caspase-1 (Millipore). Sections were then incubated with FITC- and TRITC-conjugated isotype specific secondary antibodies (1:200, Invitrogen). Negative controls included non-stained slides and isotype specific control antibodies (IgG1 and IgG2b; Biolegend) along with secondary antibodies. Digital images were obtained using a Nikon Instruments Eclipse 80i microscope with a 20x/0.75 objective, a DS-Fi1 digital camera, and NIS Elements software.

Wound healing assays. For mouse wounds, healing was assessed on day 10 post-injury using our previously published assays of granulation tissue formation and re-epithelialization using hematoxylin and eosin stained cryosections (4; 14). In addition, angiogenesis, myofibroblast accumulation and collagen deposition were measured in CD31, α-smooth muscle actin and Trichrome stained cryosections, respectively (4; 14). For all assays, digital images were obtained using a Nikon Instruments 80i microscope and DS-Q11 digital camera and analyzed using NIS Elements image analysis software.

Cell culture. To generate cultures of human Mp, peripheral blood mononuclear cells from normal volunteers (Zen-Bio) were plated in RPMI supplemented with 10% FBS, 2mM L-glutamine, 1% penicillin/streptomycin and 20 ng/ml recombinant human M-CSF (Peprotech). After 7 days in culture, cells were stimulated for 18 hours with IFN-γ and TNF-α (20 ng/ml each, Peprotech) or 20% human wound conditioned medium along with DMSO vehicle, YVAD or glyburide. Wound conditioned medium was generated by incubating biopsies in DMEM + 10% FBS (1 ml/100 mg tissue) for 2 hours at 37C.

Bone marrow-derived mouse Mp were cultured from wild-type C57Bl/6 mice and IL1R1, NLRP-3 and caspase-1 knockout mice as described (24). Mp were stimulated for 18 hours with IFN-γ and TNF-α (20 ng/ml each, R&D Systems) or 20% mouse wound conditioned medium
with IL-1β blocking antibody, YVAD, glyburide, the antioxidant N-acetyl cysteine (NAC) or the NADPH oxidase inhibitor diphenyliodonium (DPI) or appropriate controls (see Figures for details). Mouse wound conditioned medium was generated by incubating excised wounds in DMEM + 10% FBS (1 ml/100 mg tissue) for 2 hours at 37°C.

For measurement of ROS, cells were incubated in 5 µM 5(6)-Carboxy-2′,7′-dichlorofluorescein diacetate (DCF-DA; Invitrogen) for 10 minutes at 37°C and stimulated with wound conditioned medium with 25 mM NAC, 25 µM DPI or vehicle for 1 hour at 37°C. Controls were loaded with DCF-DA but not treated with conditioned medium. Median DCF fluorescence was measured by flow cytometry (Accuri C6) and expressed relative to controls.

**ELISA.** Mouse wounds were homogenized in cold PBS (10 µl of PBS per mg wound tissue) supplemented with protease inhibitor cocktail (Sigma). Supernatants of wound homogenates or cell culture medium were used for enzyme-linked immunoassay (ELISA) for IL-1β, IL-6, IL-10, TGF-β1, TNF-α (eBioscience), IL-18 (MBL International) and IGF-1 (R&D Systems). When wound conditioned medium was used as a cell culture supplement, cytokine release was measured as the difference between levels achieved in wells with cultured cells and levels in blank wells that contained identical medium composition but no cells.

**Statistics.** Values are reported as means ± standard deviation. Measurements of Mp gene expression, cytokine and growth factor levels, ROS production, re-epithelialization, granulation tissue thickness, Trichrome staining and CD31 staining data were compared using ANOVA. ANOVA on ranks was used if data sets did not pass tests of normality and equal variance. The Student-Newman-Keuls post hoc test was used when ANOVAs demonstrated significance. Differences between groups were considered significant if \( P \leq 0.05 \).
RESULTS

*Inflammasome components in macrophages from chronic diabetic human wounds.*

Subjects recruited for this study were obese and diabetic (Table S1). Mp isolated from chronic wounds expressed high levels of inflammasome components NLRP-3 and caspase-1, and inflammasome targets IL-1β and IL-18, but only low levels of proteinase inhibitor-9 (PI-9) and caspase-12, which are negative regulators of inflammasome activity (Figure 1a-f). Overall, expression of inflammasome components in chronic wound Mp was remarkably similar to that of blood-derived Mp stimulated with IFN-γ and TNF-α in vitro, with the striking exception of PI-9 and caspase-12 which were expressed at low levels in wound Mp. Immunofluorescent staining of chronic wound cryosections confirmed protein expression of the end effector of the inflammasome, caspase-1, in the majority of CD68+ wound Mp (Figure 1g-i).

*Sustained inflammasome activity in macrophages from diabetic mouse wounds.* As expected, db/db mice were obese and diabetic (Table S2). Mp expression and release of IL-1β and IL-18 was high on day 5 post-injury and then downregulated on day 10 post-injury in db/+ mice. In contrast, expression and release of these inflammasome targets was maintained at high levels on day 10 in db/db mice (Figure 2a,b and e,f). Comparing different wound cell populations, Mp were the dominant producers of IL-1β and IL-18 following wounding in both db/+ and db/db mice (Figure 2c,d). These data indicate that inflammasome activity is downregulated as wound healing progresses in db/+ mice, but is sustained at high levels in db/db mice.

High level activation of caspase-1 was confirmed in db/db versus db/+ wound Mp by flow cytometry using labeled YVAD which binds to active caspase-1 (Figure 2g,h); active caspase-1 was detected in 4.8 ± 1.1% of db/+ wound Mp and in 9.9 ± 1.4% of db/db wound Mp.
(mean ± SD). Interestingly, although no difference was observed in Mp expression of NLRP-3 or caspase-1 on days 5 and 10 post-injury in either db/+ and db/db mice (Figure 2i,j), expression of the inflammasome inhibitors PI:9 and caspase:12 was upregulated from day 5 to day 10 post-injury in db/+ mice (Figure 2k,l) associated with the downregulation of inflammasome activity. In contrast, expression of these inflammasome inhibitors was maintained at low levels in db/db mice, associated with sustained high levels of inflammasome activity. These data indicate that, during wound healing, activity of the inflammasome may be regulated at least in part by expression of its endogenous inhibitors.

*Diabetic wound environment is sufficient to upregulate inflammasome activity.* To provide insight into whether soluble factors in the biochemical milieu of the wound can activate the inflammasome in both human and mouse Mp, we cultured non-activated Mp with conditioned medium of wounds from diabetic individuals of each species. First, blood-derived Mp from normal human subjects were stimulated with chronic wound conditioned medium from diabetic patients. Wound conditioned medium increased release of IL-1β and IL-18 into the medium surrounding cultured Mp (Figure 3a,b). Activation of the inflammasome was confirmed as both the caspase-1 inhibitor YVAD and the inflammasome inhibitor glyburide reduced the conditioned medium-induced release of IL-1β and IL-18. Activation of caspase-1 was also assessed in conditioned medium-treated cells by flow cytometry (Figure 3c,d); active caspase-1 was detected in 2.9 ± 0.7% of non-stimulated control cells and in 10.5 ± 1.3% of conditioned medium stimulated cells.

Next, bone marrow-derived Mp from wild-type (C57Bl/6) mice were stimulated with conditioned medium of wounds from db/db mice. Wound conditioned medium from db/db mice induced IL-1β and IL-18 release and both YVAD and glyburide reduced cytokine release (Figure
As a control, we cultured Mp with conditioned medium from non-injured db/db skin samples and found that non-injured skin conditioned medium did not induce IL-1β or IL-18 release from cultured Mp (data not shown). In addition, whereas db/db wound conditioned medium induced IL-1β and IL-18 release from wild-type Mp, cytokine release was absent from NLRP-3 and caspase-1 null Mp (Figure 3g,h). Thus, wound conditioned medium-induced IL-1β and IL-18 release appears to be mediated by the NLRP-3 inflammasome.

**Blocking ROS reduces inflammasome activity in cultured Mp.** Whereas a number of different danger signals, including ATP, uric acid crystals, cholesterol crystals and glucose can activate the inflammasome (16; 17; 25), a common downstream mediator may be increased production of reactive oxygen species (ROS) (25). To determine whether wound conditioned medium induces ROS production from cultured Mp, we used the ROS-sensitive fluorescent dye DCF-DA. Conditioned medium from day 5 wounds of both db/+ and db/db mice increased DCF fluorescence in cultured Mp above medium-only controls (Figure 4a). Conditioned medium from day 10 wounds of db/+ mice did not increase DCF fluorescence but conditioned medium from db/db mice increased DCF fluorescence to a level similar to that of day 5 conditioned medium. Thus, ROS induction by wound conditioned medium paralleled IL-1β and IL-18 production from cultured Mp. We next inhibited ROS production using the antioxidants NAC and DPI (Figure 4b-d), and found that such treatment reduced IL-1β and IL-18 release. In total, these data indicate that the diabetic wound environment activates the inflammasome at least partly by increasing ROS production.

**Blocking IL-1β activity reduces inflammasome activity in cultured Mp.** Inflammasome activation is thought to be a two-step process in which the initial signal induces expression of IL-1β and IL-18 and a second stimulus induces cleavage of IL-1β and IL-18 (16; 17; 25). To
determine whether IL-1β itself contributes to the initial signal in this process, we treated cultured Mp with day 10 db/db wound conditioned medium and blocked IL-1β signaling with an IL-1β blocking antibody. Wound conditioned medium upregulated expression and release of IL-1β and IL-18, and the IL-1β blocking antibody inhibited the induction and release of these cytokines (Figure 5a,b and g,h). Interestingly, wound conditioned medium downregulated expression of NLRP-3, caspase-1, PI-9 and caspase-12 but the blocking antibody only inhibited the downregulation of PI-9 and caspase-12 (Figure 5c-f). One should note that, in this experiment, the IL-1β blocking antibody may interfere with ELISA assessment of IL-1β release, but the measurement of IL-18 release should be reflective of inflammasome activity.

The IL-1β blocking antibody data were corroborated in experiments using Mp cultured from IL-1 receptor 1 (IL-1R1) knockout mice; compared to wild-type Mp, wound conditioned medium-treated IL-1R1 knockout Mp exhibited blunted upregulation of IL-1β and IL-18 expression and release (Figure 5i,j and o,p) and blunted downregulation of PI-9 and caspase-12 (Figure 5m,n). These data indicate that IL-1R1 knockout Mp are less sensitive to wound conditioned medium-induced inflammasome activity than wild-type Mp. Taken together, these data indicate that IL-1β in the diabetic wound environment may induce expression of IL-1β and IL-18 and contribute to sustained inflammasome activity in wound Mp.

Blocking inflammasome activity improves healing in db/db mice. To determine whether inhibiting inflammasome activity can improve healing of wounds in db/db mice, we applied the NLRP-3 inflammasome inhibitor glyburide or the irreversible caspase-1 inhibitor YVAD topically to wounds of db/db mice. Mice whose wounds were treated with glyburide or YVAD showed no change in body weight or blood glucose levels compared with vehicle treated mice (Table S2). Treatment with glyburide or YVAD accelerated re-epithelialization and increased
granulation tissue formation (Figure 6a-d) and glyburide but not YVAD treatment increased collagen deposition on day 10 post-injury (Figure 6e). Whereas neither glyburide nor YVAD treatment influenced angiogenesis as assessed by CD31 labeling (Figure 6f), both treatments led to increased α-smooth muscle actin staining, indicating increased myofibroblasts (Figure S1).

As expected, treatment with either glyburide or YVAD reduced levels of IL-1β and IL-18 in wound homogenates (Figure 6g,h), consistent with reduced activity of the NLRP-3 inflammasome and caspase-1. In addition, there were non-significant trends of decreased TNF-α and IL-6 in glyburide and YVAD treated wounds (Figure 6i,j). In contrast, wound levels of IGF-1 and TGF-β were increased both by glyburide and YVAD treatment (Figure 6k,l), indicating a more pro-healing wound environment.

To determine whether the change in wound environment and improved healing were associated with a change in Mp phenotype, we isolated Mp from wounds treated with DMSO vehicle or glyburide. Compared to Mp from DMSO treated wounds, Mp from glyburide treated wounds exhibited lower level expression of IL-1β, IL-18 and TNF-α (Figure 6o-q), although expression of IL-6 was increased (Figure 6r) as was expression of IGF-1, TGF-β, IL-10 and CD206 (Figure 6s-v; note that changes in Mp phenotype with YVAD treatment were not measured). These data indicate that blocking inflammasome activity downregulated Mp expression of pro-inflammatory cytokines measured and upregulated expression of pro-healing factors, which in turn, was associated with a more pro-healing wound environment and improved healing in db/db mice.

*Bone marrow transfer between NLRP-3 null and db/db mice improves healing in db/db mice.* To more specifically target the inflammasome in Mp, we used bone marrow transfer to determine the role of bone marrow cell-derived inflammasome activity in impaired healing of
wounds in diabetic mice. Bone marrow transfer from wild-type to db/db mice resulted in wound healing that was similar to db/db mice that had not received bone marrow transfer (compare Figure 7a-d with Figure 6a-d and (14; 15)), indicating that impaired healing is not a result of intrinsic defects in db/db macrophages. In contrast, transfer of NLRP-3 or caspase-1 null bone marrow resulted in accelerated re-epithelialization and increased granulation tissue formation, mimicking the effects of the inflammasome inhibitors (Figure 7a-d). In addition, transfer of NLRP-3 null but not caspase-1 null bone marrow resulted in increased collagen deposition (Figure 7e). Although transfer of bone marrow from either NLRP-3 or caspase-1 null mice did not influence angiogenesis (Figure 7f), in both cases, α-smooth muscle actin staining was increased (Figure S2).

Furthermore, bone marrow transfer from NLRP-3 null or caspase-1 null mice to db/db mice resulted in reduced levels of IL-1β and IL-18 in wound homogenates (Figure 7g,h), indicating that bone marrow-derived NLRP-3 inflammasome produces the majority of IL-1β and IL-18 in these wounds. These bone marrow transfers also reduced levels of TNF-α and IL-6 (Figure 7i,j), indicating additional downregulation of the pro-inflammatory wound environment. In contrast, wound levels of IGF-1 and TGF-β were increased both by transfer of NLRP-3 and caspase-1 null bone marrow (Figure 7k,l), indicating the induction of a more pro-healing wound environment.
DISCUSSION

Chronic wounds are typically associated with a persistent inflammatory response that involves accumulation of Mp (19; 26-28); however little is known about the regulation and function of Mp in non-healing wounds. We and others have observed a persistent pro-inflammatory wound Mp phenotype in diabetic humans and mice (14; 29) and in chronic venous ulcers (30). The major novel finding of this study is that the NLRP-3 inflammasome is a key regulatory pathway in the chronic inflammatory response in wounds of diabetic humans and mice. In vitro studies indicated that the diabetic wound environment is sufficient to activate the inflammasome in cultured Mp via a ROS-mediated pathway and that its downstream target IL-1β participates in a pro-inflammatory positive feedback loop that contributes to the persistent inflammatory response (Figure 8). Importantly, inhibiting the inflammasome in vivo either using topical application of pharmacological inhibitors, or via bone marrow transfer from NLRP-3 or caspase-1 null mice to db/db mice, promotes the early healing response in part by downregulating the pro-inflammatory phenotype characteristic of Mp in diabetic wounds and increasing production of pro-healing factors.

The NLRP-3 inflammasome plays a role in the pathophysiology of many inflammatory diseases, including obesity, insulin resistance and diabetes (21; 31-35). Although previous studies have demonstrated that PDGF, FGF, VEGF or erythropoietin can improve healing in diabetic mice (13; 36; 37), our data indicate that targeting the inflammasome may provide a novel target for improving healing in diabetic patients. Glyburide is a commonly used sulfonylurea drug for the treatment of type 2 diabetes and was recently shown to inhibit the NLRP-3 inflammasome through a mechanism that appears to be different from that involved in its ability to promote insulin secretion from pancreatic beta cells (38). In our studies, topical
glyburide improved wound healing in diabetic mice, and thus this FDA approved drug may represent a convenient and effective therapeutic approach for targeting this pathway.

Much has been learned recently about the pathways involved in activating the inflammasome; however, far less is known about the negative regulators that act as a brake on inflammasome activity. Following activation signals, NLRP-3 monomers are thought to oligomerize, the caspase-1 adaptor protein ASC is recruited through pyrin domain (PYD) interactions, and then caspase-1 is recruited to ASC through the caspase recruitment domain (CARD) interactions. CARD-only proteins (COPs) such as Iceberg, Pseudo-ICE and caspase-12 and PYD-only proteins (POPs) such as pyrin, POP1 and POP2 are thought to inhibit inflammasome activity by blocking inflammasome assembly and subsequent caspase-1 cleavage (16; 39). In addition, other inhibitors such as PI-9 act by directly binding and inhibiting caspase-1 (40; 41). Interestingly, humans express a broad array of COPs and POPs, whereas rodents only express a limited subset (16; 39). We found that PI-9 and caspase-12 are upregulated in Mp as they switch from a pro-inflammatory to a pro-healing phenotype in non-diabetic mice but that both the upregulation of these inflammasome inhibitors and the Mp phenotypic switch is impaired in diabetic mice. In addition, Mp isolated from chronic human diabetic wounds exhibited only low level expression of PI-9 and caspase-12. Better understanding of the regulation of these inflammasome inhibitors may lead to additional targets for limiting inflammasome activity in inflammatory pathologies.

Components of the inflammasome can be expressed in cell types other than Mp that are involved in wound healing (42). For example, both murine and human keratinocytes express inflammasome components NLRP-3, ASC, caspase-1, IL-1β and IL-18 (43; 44). In addition, UV irradiation-induced IL-1β secretion from human keratinocytes in vitro appears to be mediated by
the NLRP-3 inflammasome (43) and contact hypersensitivity appears to be mediated by the inflammasome/IL-1β pathway (44; 45). Thus, topical application of glyburide and YVAD in our studies could block inflammasome activity in keratinocytes as well as Mp. However, our studies also indicate that Mp are the dominant producers of IL-1β in wounds of diabetic mice, and our bone marrow transfer experiments indicate that loss of bone marrow cell-derived inflammasome activity is sufficient to reduce IL-1β and IL-18 levels in the wounds of diabetic mice. A limitation of our study is that the caspase-1 null mice were also found to be deficient in caspase-11 (46; 47) and so our bone marrow transfer experiments do not distinguish the role of these caspases in the impaired healing of wounds in db/db mice. Nonetheless, our experiments indicate that the NLRP-3 inflammasome plays an important role in the impaired healing of these wounds.

Accumulating evidence indicates that Mp are critical orchestrators in healing of many tissues, including skin (3; 4; 7; 48). Recent studies have shown that Mp phenotypes change over the different phases of tissue repair and that the sequential appearance of these phenotypes may be required for efficient repair (11; 14; 29; 49; 50). In fact, either premature changes in phenotype or delayed/impaired changes in phenotype have been associated with impaired healing (14; 29; 49). Thus, further understanding of the regulation of Mp phenotype, particularly in the setting of chronic wounds, could lead to improved therapeutic options for controlling inflammation in these wounds. Our data indicate that targeting the inflammasome may be an appealing candidate in this regard.
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AUTHORSHIP

R.M. contributed to study design, researched data, wrote manuscript. M.F. researched data, reviewed/edited manuscript, E.W. researched data, reviewed/edited manuscript, W.E. contributed to study design, reviewed/edited manuscript. T.K. designed study, wrote manuscript.
REFERENCES


FIGURE CAPTIONS

Figure 1. Macrophages from chronic wounds of diabetic patients express components of the NLRP-3 inflammasome. (a-f) Macrophages isolated from chronic wound biopsies and expression of IL-1β, IL-18, NLRP-3, caspase-1, PI-9 and caspase-12 assessed by real time PCR. For comparison, blood-derived macrophages from healthy volunteers were either left non-stimulated (Non) or stimulated with TNF-α and IFN-γ (Classical) and gene expression assessed along wound macrophages. (g-i) Wound biopsy cryosections immunostained for caspase-1 and the macrophage marker CD68; scale bar = 100 µm. For all graphs, bars = mean ± SD, n = 5. Data compared between groups using ANOVA on ranks. *mean value significantly different from that for non-stimulated controls, **mean value significantly different from that for classically activated cells, p < 0.05.

Figure 2. Macrophages exhibit sustained inflammasome activity in wounds of diabetic mice. (a,b) Macrophages isolated from wounds of non-diabetic (db/+ ) and diabetic (db/db) mice on days 5 and 10 post-injury and expression of IL-1β and IL-18 assessed by real time PCR. (c,d) Neutrophils (Ly6G+/B cells (CD19+)/T cells (CD3+)/ combined NBT+ cells), macrophages (NBT-CD11b+ cells) and remaining cells (NBT-CD11b- cells) isolated from day 5 db/+ and db/db wounds and incubated overnight, release of IL-1β and IL-18 measured using ELISA. (e,f) Macrophages (NBT-CD11b+ cells) isolated from day 5 and 10 db/+ and db/db wounds and incubated overnight, release of IL-1β and IL-18 measured using ELISA. (g,h) Representative flow cytograms of active caspase-1 in macrophages (NBT-CD11b+ cells) isolated from day 10 db/+ and db/db wounds. (i-l) Macrophages isolated from db/+ and db/db wounds on days 5 and 10 post-injury and expression of NLRP-3, caspase-1, PI-9 and caspase-12 assessed by real time...
PCR. For all graphs, bars = mean ± SD, n = 6-8. Data compared between groups using ANOVA. *mean value significantly different from that for same strain on day 5 post-injury. **mean value for db/db mice significantly different from that for db/+ mice at same time point, #mean value significantly different from those for other cell types, p < 0.05.

Figure 3. Wound conditioned medium activates the inflammasome in both human and mouse macrophages in vitro. (a,b) Blood-derived macrophages from healthy human volunteers either left non-stimulated (N) or stimulated with chronic wound conditioned medium (CM) along with vehicle (V), the caspase-1 inhibitor YVAD (Y: 20 µM) or the inflammasome inhibitor glyburide (G: 200 µM) and release of IL-1β and IL-18 measured by ELISA. (c,d) Representative flow cytograms of active caspase-1 in human blood-derived macrophages, macrophages either left non-stimulated or stimulated with chronic wound CM. (e,f) Bone marrow-derived macrophages from wild-type (C57BL/6) mice either left non-stimulated or stimulated with day 10 db/db wound CM along with vehicle or same inhibitors as used for human macrophages and release of IL-1β and IL-18 measured by ELISA. (g,h) Bone marrow-derived macrophages from wild-type (WT), caspase-1 and NLRP-3 knockout (ko) mice either left non-stimulated (Non), stimulated with TNF-α and IFN-γ (20 ng/ml each, CA) or stimulated with day 10 db/db wound CM. For all graphs, bars = mean ± SD, n = 5-6 in 2 separate experiments. Data compared between groups using ANOVA on ranks. *mean value significantly different from that for non-stimulated controls, **mean value significantly different from that for CM + vehicle treated cells, #mean value significantly different from that for non-stimulated controls, ###mean value significantly different from that for CM treated WT macrophages, @mean value significantly different from
that for same strain on day 5 post-injury, \(^{\#}\) mean value for db/db CM significantly different from that for db/+ CM at same time point, \(p < 0.05\).

Figure 4. ROS inhibitors block conditioned medium-induced inflammasome activity in cultured macrophages. (a) Bone marrow-derived macrophages from wild-type mice were cultured with conditioned medium (CM) of day 5 or 10 wounds from non-diabetic db/+ or diabetic db/db mice and ROS generation was measured using DCF-DA and flow cytometry. DCF fluorescence measured as the difference in median fluorescence intensity (MFI) between CM treated cells and non-stimulated controls. (b-d) Bone marrow-derived macrophages from wild-type mice treated with day 10 db/db wound CM along with vehicle (V), NAC (N: 25 mM) or DPI (D: 25 \(\mu\)M), ROS measured as change in DCF MFI and IL-1\(\beta\) and IL-18 release measured using ELISA. For all graphs, bars = mean ± SD, \(n = 6\) in 2 independent experiments. Data compared between groups using ANOVA on ranks. *mean value significantly different from that for CM of same strain on day 5 post-injury, **mean value for db/db CM significantly different from that for db/+ CM at same time point, # mean value significantly different from that for non-stimulated controls, ##mean value significantly different from that for CM + vehicle treated samples, \(p < 0.05\).

Figure 5. Blocking IL-1\(\beta\) signaling blunts conditioned medium-induced inflammasome activity in vitro. (a-f) Bone marrow-derived macrophages from wild-type (WT) mice either left non-stimulated (Non) or stimulated with day 10 db/db wound conditioned medium (CM) along with control IgG or IL-1\(\beta\) blocking antibody (IL1ab) and expression of IL-1\(\beta\), IL-18, PI-9 and caspase-12 measured by real-time PCR. (g,h) Bone marrow-derived macrophages from wild-type mice either left non-stimulated or treated with day 10 db/db wound CM along with control IgG
or IL-1β blocking antibody and IL-1β and IL-18 release measured using ELISA. (i-n) Bone marrow-derived macrophages from WT and IL-1 receptor 1 knockout (IL-1R1 ko) mice stimulated with day 10 db/db wound CM and expression of IL-1β, IL-18, PI-9 and caspase-12 measured by real-time PCR. (o,p) Bone marrow-derived macrophages from WT and IL-1R1 ko mice stimulated with day 10 db/db wound CM and IL-1β and IL-18 release measured using ELISA. For all graphs, bars = mean ± SD, n = 5-6 in 2 independent experiments. Data compared between groups using ANOVA on ranks. *mean value significantly different from that for non-stimulated controls, **mean value significantly different from that for CM + IgG treated samples, #mean value significantly different from that for non-stimulated controls, ##mean value significantly different from that for CM treated WT macrophages at same time point, p < 0.05.

Figure 6. Blocking inflammasome activity downregulates the pro-inflammatory wound environment, upregulates a pro-healing environment and improves healing in diabetic mice. (a,b) Excisional wounds in db/db mice whose wounds were treated topically with DMSO vehicle or glyburide (Glyb) harvested on day 10 post-injury, sectioned and stained with hematoxylin and eosin. Note the increased re-epithelialization in glyburide treated wounds. ep: epithelium, gt: granulation tissue, arrows indicate ends of epithelial tongues, scale bar = 0.5 mm. (c,d) Re-epithelialization and granulation tissue thickness measured in hematoxylin and eosin stained cryosections of wounds in db/db mice treated with DMSO, Glyb or acYVAD-cmk (YVAD). (e) Trichrome staining measured as pixels stained blue for collagen and (f) CD31 staining measured as pixels stained for this endothelial cell marker. (g-n) Levels of cytokines in wound homogenates measured using ELISA, including pro-inflammatory cytokines IL-1β, IL-18, TNF-α, IL-6 and healing-associated cytokines IGF-1, TGF-β, IL-10 and VEGF. (o-v) Macrophages
isolated from wounds in db/db mice treated with DMSO or Glyb and expression of pro-inflammatory cytokines IL-1β, IL-18 TNF-α, IL-6 and healing-associated cytokines IGF-1, TGF-β, IL-10 and CD206 assessed by real-time PCR. For comparison, bone marrow-derived macrophages from wild-type mice were left non-stimulated (Con) and gene expression assessed along with wound macrophages. For all graphs, bars = mean ± SD, n = 6-8. Data compared between groups using ANOVA. *mean value significantly different from that for DMSO treated wounds, # mean value significantly different from that for non-stimulated in vitro control macrophages, ## mean value significantly different from that for macrophages isolated from DMSO treated wounds, p < 0.05.

Figure 7. Bone marrow transfer from NLRP-3 and caspase-1 null mice to db/db mice is sufficient to downregulate the pro-inflammatory wound environment, upregulate a pro-healing environment and improve healing in diabetic mice. (a,b) Db/db mice were subjected to lethal irradiation and then bone marrow from wild-type (WT) or NLRP-3 (N3) null mice transferred 24 hours later. Four weeks later, mice were subjected to excisional wounding and wounds were harvested on day 10 post-injury, sectioned and stained with hematoxylin and eosin. Note the increased re-epithelialization in db/db mice receiving N3 bone marrow. ep: epithelium, gt: granulation tissue, arrows indicate ends of epithelial tongues, scale bar = 0.5 mm. (c,d) Re-epithelialization and granulation tissue thickness measured in hematoxylin and eosin stained cryosections of db/db mice receiving bone marrow from WT (WT-db), NLRP-3 null (N3-db) or Caspase-1 null (C1-db) mice. (e) Trichrome staining measured as pixels stained blue for collagen and (f) CD31 staining measured as pixels stained for this endothelial cell marker. (g-n) Levels of
cytokines in wound homogenates measured using ELISA, including pro-inflammatory cytokines IL-1β, IL-18, TNF-α, IL-6 and healing-associated cytokines IGF-1, TGF-β, IL-10 and VEGF. For all graphs, bars = mean ± SD, n = 6-8. Data compared between groups using ANOVA.

*mean value significantly different from that for mice receiving WT bone marrow, p < 0.05.

Figure 8. Model of the role of the inflammasome/IL-1β pathway in impaired wound healing in type 2 diabetes. Our data indicate that IL-1β can act as an upstream signal (Signal 1) for sustaining inflammasome activity in chronic wound macrophages and that reactive oxygen species (ROS) is involved in the second signal (Signal 2) required for inflammasome activity. Since the inflammasome, in turn, activates IL-1β, this cytokine appears to be part of a pro-inflammatory positive feedback loop that promotes a bias towards a pro-inflammatory macrophage phenotype and inhibits upregulation of the pro-healing phenotype and healing in diabetic wounds.
Diabetes
Diabetes
## TABLE S1. Human Subject data

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<th>Value</th>
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<td>Age (years)</td>
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<td>Blood glucose (mg/dl)</td>
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<td>Wound area (cm²)</td>
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<td>Insulin (n/N)</td>
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<td>Aspirin (n/N)</td>
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Values reported are mean (S.D.) or number of patients taking class of drug.

NB: none of the subjects were taking glyburide.
TABLE S2. Mouse data

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<th>Db/db glyburide</th>
<th>db/db YVAD</th>
<th>db/db BMT WT</th>
<th>db/db BMT NLRP-3</th>
<th>Db/db BMT caspase-1</th>
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SUPPLEMENTAL FIGURE CAPTIONS

Figure S1. Topical treatment with inflammasome inhibitors results in increased α-smooth muscle actin staining in wounds of db/db mice. Excisional wounds in db/db mice were treated topically with DMSO vehicle, glyburide (Glyb) or YVAD. Top: note that α-smooth muscle actin staining in DMSO treated wounds was primarily associated with vessels, and that there was more staining in Glyb or YVAD treated wounds both associated with vessels and throughout granulation tissue. Bottom: staining in wound cryosections quantified using image analysis and normalized to DMSO condition. Bars = mean ± SD, n = 6-8. Data compared between groups using ANOVA. *mean value significantly different from that for mice receiving WT bone marrow, p < 0.05.

Figure S2. Bone marrow transfer from NLRP-3 and caspase-1 null mice to db/db mice results in increased α-smooth muscle actin staining in wounds of db/db mice. Db/db mice were subjected to lethal irradiation and then bone marrow was transferred from wild-type (WT-db), NLRP-3 null (N3-db) or Caspase-1 null (C1-db) mice. α-smooth muscle actin staining in wound cryosections normalized to WT-db condition. Bars = mean ± SD, n = 6-8. Data compared between groups using ANOVA. *mean value significantly different from that for mice receiving WT bone marrow, p < 0.05.
$\alpha$-SMA staining (nrm to WT-db)

WT-db  N3-db  C1-db

*  *

39x30mm (600 x 600 DPI)