Blocking IL-1β Induces a Healing-associated Wound Macrophage Phenotype
and Improves Healing in Type-2 Diabetes

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ABSTRACT

Diabetes is associated with persistent inflammation and defective tissue repair responses. The hypothesis of this study was that IL-1β is part of a pro-inflammatory positive feedback loop that sustains a persistent pro-inflammatory wound macrophage phenotype which contributes to impaired healing in diabetes. Macrophages isolated from wounds in diabetic humans and mice exhibited a pro-inflammatory phenotype, including expression and secretion of IL-1β. The diabetic wound environment appears to be sufficient to induce these inflammatory phenomena as in vitro studies demonstrated that conditioned medium of both mouse and human wounds upregulates expression of pro-inflammatory genes and downregulates expression of pro-healing factors in cultured macrophages. Furthermore, inhibiting the IL-1β pathway using a neutralizing antibody and macrophages from IL1 receptor knockout mice blocked the conditioned medium-induced upregulation of pro-inflammatory genes and downregulation of pro-healing factors. Importantly, inhibiting the IL-1β pathway in wounds of diabetic mice using a neutralizing antibody induced a switch from pro-inflammatory to healing-associated macrophage phenotypes, increased levels of wound growth factors and improved healing of these wounds. Our findings indicate that targeting the IL-1β pathway represents a new therapeutic approach for improving the healing of diabetic wounds.
INTRODUCTION

Chronic wounds associated with diabetes, venous insufficiency and/or pressure represent a major health problem with millions of patients afflicted and the associated treatment costing billions of dollars per year (1). Despite the socioeconomic impact of chronic wounds, the underlying causes of impaired healing are not well understood and effective treatments remain elusive. A common characteristic of these poorly healing wounds is a persistent inflammatory response, with prolonged accumulation of macrophages (Mp) and elevated levels of pro-inflammatory cytokines (2-5). Translational research on the dysregulation of inflammation associated with impaired healing in diabetes should provide insight into the development of new therapeutic approaches.

During normal wound healing in mice, inflammatory cells such as Mp promote healing indirectly by killing pathogens and clearing the wound of damaged tissue, but also promote healing directly by producing growth factors that induce angiogenesis, collagen deposition and wound closure (6-9). In contrast, during impaired healing of diabetic mice, wounds exhibit prolonged accumulation of Mp associated with elevated levels of pro-inflammatory cytokines and proteases and reduced levels of various growth factors, all of which mimic chronic wounds in humans (10-12). We recently demonstrated that, in wounds of diabetic mice, Mp exhibit a sustained pro-inflammatory phenotype with an impaired upregulation of healing associated factors that is observed in non-diabetic mice as healing progresses (13). However, the underlying causes of the dysregulation of Mp in diabetic wounds remain to be elucidated.

Multiple factors can influence Mp phenotype and the actual phenotypes expressed in chronic wounds are likely determined by the balance of the pro- and anti-inflammatory stimuli present in the wound environment. The pro-inflammatory environment observed in diabetic
wounds has the potential to sustain a pro-inflammatory Mp phenotype, which in turn, would contribute to sustaining the pro-inflammatory environment. In fact, hyperglycemia is known to induce expression of IL-1β in a number of different cell types, including Mp (14-16) and IL-1β, in turn, is known to induce a pro-inflammatory Mp phenotype in part by inducing itself (17). Thus, the IL-1β pathway may be part of a positive feedback loop that sustains inflammation in chronic wounds and contributes to impaired healing. However, little is known about the actual role of IL-1β in diabetic wounds.

The central hypothesis of this study is that sustained activity of the IL-1β pathway in diabetic wounds contributes to impaired healing of these wounds. The results of this study demonstrate that sustained IL-1β expression in wounds of diabetic humans and mice is associated with a pro-inflammatory Mp phenotype, and that inhibiting the IL-1β pathway in wounds of diabetic mice induces the switch from pro-inflammatory to healing-associated Mp phenotypes and improves healing of these wounds.
RESEARCH DESIGN AND METHODS

Human subjects. Five patients (2 male and 3 female) with chronic wounds provided informed consent. Patients ranged in age from 54 to 70, were diagnosed with type 2 diabetes and had non-healing wounds on either the sacral region or the lower limb lasting at least 3 months. Biopsies were taken from debridement tissue that was removed from 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 the Declaration of Helsinki Principles.

Animals. Diabetic db/db mice, non-diabetic db/+ controls, IL-1 receptor 1 (IL1R1) knockout mice and C57Bl/6 wild-type controls were obtained from Jackson Laboratories. 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 anesthetized with isoflurane and their dorsum shaved and cleaned with betadine and then alcohol swab. Four 8 mm excisional wounds were made on the back of each mouse with a dermal biopsy punch and wounds covered with Tegaderm (3M) to keep the wounds moist and maintain consistency with treatment of human wounds. For some mice, an IL-1β neutralizing antibody (R&D Systems) was administered as a single total dose of 20 µg per wound by intradermal injection at four equally spaced sites around the periphery of each wound; this treatment was applied 3 days post-injury to allow the initial inflammatory response to proceed normally. Controls were treated with non-specific rat IgG.

Cell isolation. Cells were dissociated from human chronic wound biopsies and mouse excisional wounds using an enzymatic digest with collagenase I, collagenase XI and hyaluronidase (13). 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 (HI98), anti-CD3 (UCHT1) and anti-CD19 (HIB19) for human cells (Biolegend, 1:10); these cells were depleted from the total cell population using anti-FITC magnetic beads following the manufacturer’s instructions (Miltenyi Biotec). The remaining myeloid cells, primarily cells of the monocyte/Mp lineage, were then isolated using CD11b magnetic beads following the manufacturer’s instructions. Previous studies indicated that > 90% of the cells thus isolated were positive for monocyte/Mp markers Ly6C and/or F4/80 by flow cytometry (13). These cells were then either incubated overnight to measure cytokine release or stored at -80°C for later RNA analysis.

RNA analysis. Total RNA was isolated from human or mouse cells using the RNeasy kit (Qiagen). cDNA was synthesized from 1 µg RNA using the Thermoscript RT-PCR System (Invitrogen). Real-time PCR was performed in a 7500Fast System (Applied Biosystems) using TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assay primer/probe sets (Applied Biosystems; Table S1). All reactions were performed in triplicate, and cycle threshold (CT) values were averaged over triplicates. Relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method, with GAPDH as the endogenous control gene.

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 incubated overnight with primary antibodies against CD68 (Y1/82A, 1:100, Biolegend) and IL-1β (CRM56, 1:100, EBioscience). Sections were then incubated with FITC- and TRITC-conjugated isotype specific secondary antibodies (1:200, Invitrogen). Negative controls included no primary antibody or isotype specific control antibodies (IgG1 and IgG2b; Biolegend) along with secondary antibodies. To visualize nuclei, slides were mounted with medium containing
DAPI (Vector Laboratories). Digital images were obtained using a Nikon Instruments Eclipse 80i microscope with a 40x/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. Re-epithelialization and granulation tissue thickness were measured by morphometric analysis of cryosections taken from the center of the wound (found by serial sectioning through the entire wound) and stained with hematoxylin and eosin. Digital images were obtained using a Nikon Instruments 80i microscope, a 2x/0.06 objective and a DS-Qi1 digital camera and analyzed using NIS Elements image analysis software. The percentage of re-epithelialization \[ \left( \frac{\text{distance traversed by epithelium over wound from wound edge}}{\text{distance between wound edges}} \right) \times 100 \] was calculated for two sections per wound and was averaged over sections to provide a representative value for each wound (9; 13). Average granulation thickness was measured in the same sections by dividing the wound bed area by wound length.

For angiogenesis, new blood vessels were identified by immunohistochemical staining for CD31 (390, 1:100; BD Biosciences) using our published procedure (9). Collagen deposition was assessed using Masson’s trichrome staining (IMEB). For each assay, digital images were first obtained covering the wound bed (2-3 fields using a 20x/0.50 objective). The percent area stained in each image was then quantified by counting the number of clearly stained pixels above a threshold intensity and normalizing to the total number of pixels. The software allowed the observer to exclude staining identified as artifact and areas deemed to be outside the wound bed. For both trichrome and CD31 staining, two sections per wound were analyzed and data averaged over sections to provide a representative value for each wound.

**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 macrophage colony stimulating factor (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 chronic wound conditioned medium. Human wound conditioned medium was generated by incubating chronic wound 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 knockout mice as described (18). Briefly, bone marrow cells were flushed from femurs and tibias and cultured in Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 10% FBS, 10% L-929 cell-conditioned medium (source of mouse M-CSF), 2mM L-glutamine and 1% penicillin/streptomycin at 10% CO2 and 37C. Mp were then 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 or control IgG (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 37C.

**ELISA.** Mouse wounds were homogenized in cold PBS (10 µl of PBS per mg wound tissue) supplemented with protease inhibitor cocktail (Sigma) using a dounce homogenizer and then sonicated and centrifuged. Supernatants were used for enzyme-linked immunoassay (ELISA) of IL-1β, IL-6, IL-10, TGF-β1, TNF-α (eBioscience) and IGF-1 (R&D Systems). For cell culture studies, culture medium was centrifuged and supernatants used for ELISA assays. 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, re-epithelialization, granulation tissue thickness, trichrome staining and CD31 staining data were compared using ANOVA. 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

Pro-inflammatory Mp phenotype in diabetic human wounds. Although Mp are known to populate chronic wounds in diabetic patients (2), little is known about their phenotype. We isolated Mp from biopsies of chronic wounds in type 2 diabetic patients and phenotyped these cells by real time PCR. Compared to non-activated blood monocyte-derived Mp, chronic wound Mp expressed high levels of the pro-inflammatory molecules IL-1β, MMP-9 and TNF-α (Figure 1a-d) and low levels of the healing-associated phenotype markers CD206, IGF-1, TGF-β and IL-10 (Figure 1e-h). In addition, immunofluorescence analysis of chronic wound biopsy cryosections showed that IL-1β protein colocalized with the Mp marker CD68, indicating that chronic wound Mp produce IL-1β protein (Figure 2). Overall, the phenotype of chronic wound Mp was remarkably similar to that of “classically activated” blood monocyte-derived Mp stimulated with IFN-γ and TNF-α in vitro, although IL-6 expression was lower in wound Mp than classically activated Mp.

To provide insight into whether the chronic wound environment can induce the pro-inflammatory wound Mp phenotype observed, we cultured non-activated blood monocyte-derived Mp with conditioned medium of chronic wounds. Compared to non-activated blood monocyte-derived Mp, chronic wound conditioned medium increased expression of pro-inflammatory markers IL-1β, MMP-9 and TNF-α (Figure 1a-d) and decreased expression of non-pro-healing markers IGF-1, TGF-b and IL-10 (Figure 1e-h). Expression of IL-6 and CD206 were not significantly altered by wound conditioned medium. Selected cytokines were also assessed at the protein level – chronic wound conditioned medium increased release of IL-1β and TNF-α into the cell culture medium and decreased release of IGF-1 and TGF-β (Figure 1i-l).
Thus, chronic wound conditioned medium induced a pro-inflammatory Mp phenotype similar to that of classically activated Mp.

*Sustained pro-inflammatory Mp phenotype in diabetic mouse wounds.* We reported previously that, whereas wound Mp in non-diabetic mice exhibit a switch from pro-inflammatory to pro-healing phenotypes from days 5 to 10 post-injury, diabetic mice exhibit a persistent pro-inflammatory phenotype similar to that observed in the human chronic wound Mp in the present study. To determine whether the differences observed in Mp phenotype between db/+ and db/db mice translated to changes in protein secretion by these cells, we isolated cells from wounds of db/+ and db/db mice and measured release of pro-inflammatory, anti-inflammatory and pro-healing cytokines. In db/+ mice, Mp release of IL-1β and TNF-α was high on day 5 post-injury but decreased on day 10 (Figure 2a,b). In contrast, Mp release of the pro-healing growth factors IGF-1 and TGF-β were low on day 5 post-injury but increased on day 10 (Figure 2c,d). These data are consistent with downregulation of the pro-inflammatory phenotype and upregulation of a healing-associated phenotype of wound Mp as wound healing progresses in non-diabetic mice (13). On the other hand, in db/db mice, Mp release of IL-1β and TNF-α was sustained at high levels on both days 5 and 10 post-injury, and the level on day 10 was significantly higher than that produced by db/+ wound Mp (Figure 2a,b). In addition, Mp release of IGF-1 and TGF-β was maintained at low levels on days 5 and 10 post-injury, which were all significantly lower than levels produced by db/+ wound Mp on day 10 (Figure 2c,d). These data support the notion that wound Mp in diabetic mice exhibit a persistent pro-inflammatory phenotype and fail to upregulate healing-associated factors.

To determine whether the wound environment in diabetic mice can induce the pro-inflammatory wound Mp phenotype observed, we cultured bone marrow-derived Mp from non-
diabetic mice with day 5 or day 10 wound conditioned medium from non-diabetic db/+ or diabetic db/db mice. Conditioned medium from day 5 wounds of both db/+ and db/db mice induced a pro-inflammatory phenotype of cultured mouse bone marrow-derived Mp with upregulation of IL-1β, MMP-9, TNF-α and IL-6 (Figure 2e-h) and downregulation of IGF-1, TGF-β and IL-10 compared with non-activated control Mp (Figure 2i-l). Conditioned medium from day 10 wounds of db/+ mice induced significantly less upregulation of pro-inflammatory genes and no longer downregulated healing-associated factors (Figure 2e-l). In contrast, conditioned medium from day 10 wounds of db/db mice induced significantly higher pro-inflammatory gene expression and significantly lower healing-associated gene expression than conditioned medium from db/+ wounds (Figure 2e-l). One exception to this pattern was that expression of CD206 was not significantly altered by conditioned medium from db/+ wounds but was significantly reduced by conditioned medium from db/db compared to db/+ wounds. Selected cytokines were also assessed at the protein level and the differences in mRNA expression were paralleled by differences in protein levels. For example, IL-1β and TNF-α secretion was higher and IGF-1 and TGF-β secretion was lower in Mp stimulated with day 10 db/db wound conditioned medium compared to cells stimulated with day 10 db/+ wound conditioned medium (Figure 2m-p). Taken together, these in vitro data indicate that the diabetic wound environment is sufficient to induce a pro-inflammatory Mp phenotype and to suppress the healing-associated phenotype.

Blocking IL-1β activity downregulates pro-inflammatory Mp phenotype and upregulates pro-healing factors in cultured cells. IL-1β is a potent pro-inflammatory cytokine and is known to induce itself in Mp and produce a pro-inflammatory phenotype (17). To determine whether IL-1β in the wound environment thus contributes to such a pro-inflammatory positive feedback
loop, we blocked IL-1β signaling in cultured Mp treated with wound conditioned medium using an IL-1β neutralizing antibody or using Mp isolated from IL-1R1 knockout mice. The IL-1β blocking antibody inhibited the induction of pro-inflammatory markers IL-1β, MMP-9, TNF-α and IL-6 by day 10 db/db wound conditioned medium (Figure 3a-d). Interestingly, the blocking antibody also increased expression of healing-associated markers CD206, IGF-1, TGF-β and IL-10 (Figure 3e-h). Differences in protein secretion of selected cytokines, namely IL-1β, TNF-α, IGF-1 and TGF-β, paralleled the changes in mRNA levels (Figure 3i-l). IL-1β in the culture medium were undetectable when the neutralizing antibody was present; this finding could be the result of interference of the blocking antibody with detection by the ELISA assay as well as a potential reduction in the amount released by the macrophages (NB: however, see data from IL-1R1 null macrophages in the next paragraph).

The IL-1β neutralizing data were corroborated in experiments using Mp cultured from IL-1R1 knockout mice; wound conditioned medium treated IL-1R1 knockout Mp exhibited blunted upregulation of IL-1β, MMP-9, TNF-α and IL-6 expression (Figure 4a-d) and maintained higher level expression the healing-associated markers IGF-1, TGF-β and IL-10 (Figure 4e-h), compared to wild-type Mp. In contrast, there was no significant difference in expression of CD206 between wild-type and IL-1R1 knockout Mp. Again, the mRNA data were consistent with protein secretion data, which showed that IL-1β and TNF-α release was reduced and release of both IGF-1 and TGF-β was higher in wound conditioned medium treated IL-1R1 knockout Mp compared with wild-type Mp (Figure 3m-p). These data indicate that IL-1R1 knockout Mp are less sensitive to wound conditioned medium-induced upregulation of pro-inflammatory markers and downregulation of healing-associated markers than wild-type Mp and
are consistent with the hypothesis that IL-1β in the diabetic wound environment induces a pro-inflammatory Mp phenotype.

_Feeling IL-1β activity downregulates pro-inflammatory Mp phenotype in wounds, upregulates pro-healing phenotype and improves healing in db/db mice._ We next performed in vivo experiments to determine whether treating wounds in db/db mice with an IL-1β neutralizing antibody could downregulate the pro-inflammatory wound Mp phenotype, upregulate a healing-associated phenotype and improve healing of these wounds. Mice whose wounds were treated with blocking antibody showed no change in blood glucose (459±92 mg/dl) compared with mice treated with control IgG (439±106 mg/dl).

Mp isolated on days 5 and 10 post-injury from wounds treated with the IL-1β neutralizing antibody exhibited progressively lower levels of expression of pro-inflammatory genes IL-1β, MMP-9, TNF-α and IL-6 over time compared to Mp isolated from IgG treated wounds (Figure 5a-d). Interestingly, Mp did not yet show upregulation of healing-associated factors IGF-1, TGF-β or IL-10 on day 5 post-injury but did show upregulation of these factors on day 10 post-injury compared to Mp isolated from IgG treated wounds (Figure 5e-h). In contrast, CD206 did not show upregulation at either time point. Thus, treatment with the IL-1β blocking antibody downregulated the pro-inflammatory Mp phenotype by day 5 post-injury and upregulated a healing-associated phenotype by day 10 in wounds of diabetic mice.

Wounds treated with the IL-1β neutralizing antibody also showed accelerated re-epithelialization as well as granulation tissue formation assessed in hematoxylin and eosin stained cryosections on day 10 post-injury (Figure 6a-d). Wounds treated with blocking antibody also showed increased collagen deposition assessed in trichrome stained cryosections (Figure 5e). The improved healing was not associated with increased CD31 staining, indicating little to
no effect on angiogenesis (Figure 6f). Furthermore, wounds treated with the blocking antibody showed reduced levels of IL-1β and TNF-α and increased levels of IGF-1 and TGF-β (Figure 4e-g), paralleling the changes in wound Mp phenotype and indicating a more pro-healing cytokine environment.
DISCUSSION

Chronic wounds are common in diabetic patients and the associated morbidity and mortality are high (19-21). Chronic wounds are typically associated with a persistent inflammatory response that involves accumulation of Mp (2-5); however little is known about the regulation and function of these cells. The major novel finding of this study is that the pro-inflammatory cytokine IL-1β is part of a key regulatory pathway in this chronic inflammatory response. Our studies indicate that IL-1β participates in a pro-inflammatory positive feedback loop that sustains the pro-inflammatory Mp phenotype observed in poorly healing wounds of both humans and mice and blocks the induction of a healing-associated Mp phenotype observed during normal healing. Importantly, inhibiting IL-1β in vivo downregulates the pro-inflammatory Mp phenotype and upregulates expression of pro-healing factors in wounds of diabetic mice and improves healing of these wounds.

IL-1β is a potent pro-inflammatory cytokine that plays a role in the pathophysiology of many inflammatory diseases, including diabetes (17; 22). Recent studies have shown that blocking IL-1β systemically can improve glycemic control and β-cell function in mice and humans (23; 24). In our studies, we applied the IL-1β blocking antibody locally to wounds and observed no changes in blood glucose levels. Thus, the downregulation of the pro-inflammatory Mp phenotype and improved wound healing observed is likely due to direct effects in the wound. Our data thus indicate that targeting the IL-1β pathway in chronic wounds may provide a novel approach for improving wound healing in diabetic patients.

IL-1 appears to play homeostatic roles in non-injured skin as well as roles in skin inflammation. IL-1 produced by keratinocytes is thought to play a role in epithelial-mesenchymal signaling required for normal skin homeostasis (25). In addition, IL-1 is thought to
contribute to skin inflammation in autoinflammatory diseases and skin diseases such as psoriasis and scleroderma (26-28). During skin wound healing in healthy mice, levels of keratinocyte-derived chemokine and macrophage inhibitory protein-1α were reduced by IL-1 receptor antagonist treatment (29). However, inflammatory and healing responses were not altered significantly in IL-1 receptor knockout mice in one study (30), although these mice exhibited reduced fibrosis in skin wounds in another study (31), suggesting that IL-1 may play ancillary roles in normal healing. In contrast, IL-1β levels are sustained at high levels in chronic wounds of humans and in wounds of diabetic mice (3; 5; 11; 13; 32; 33) and our findings demonstrate that these elevated levels of IL-1β promote the pro-inflammatory wound environment and impaired healing associated with diabetes.

The phenotype adopted by Mp during tissue repair may be influenced both by cell lineage and by the microenvironment of the Mp during the healing process (34; 35). For example, following ischemic damage to the heart, a switch from pro-inflammatory and proteolytic Ly6C<sup>hi</sup> monocytes (Mo) to non-inflammatory, pro-healing Ly6C<sup>lo</sup> Mo/Mp appeared to be the result of separate waves of Mo infiltration, indicating that cell lineage may play an important role in determining cell phenotype during tissue repair (36). In contrast, after toxin-induced injury to skeletal muscle, the switch from Ly6C<sup>hi</sup> Mo to Ly6C<sup>lo</sup> Mo/Mp appeared to be the result of differentiation of the Ly6C<sup>hi</sup> subset within the muscle, indicating environmental control of this phenotype switch (37). Although we have not yet investigated the role of cell lineage in the phenotypes of wound Mp observed in our studies, data from both our in vitro and our in vivo experiments support a role for the wound environment in regulating Mp phenotype and, in particular, sustaining a pro-inflammatory phenotype in diabetic wound Mp. For our studies, we compared the phenotypes of Mp isolated from human and mouse wounds to the phenotypes of
cultured blood monocyte-derived (human) or bone marrow-derived (mouse) Mp. These cultured Mp may exhibit different phenotypes than tissue Mp and may respond differently to changes in the cytokine environment. However, this limitation should not detract from our finding that blocking IL-1β in wounds of diabetic mice in vivo or in the modeled wound environment in vitro downregulates the pro-inflammatory Mp phenotype and promotes a healing-associated phenotype.

Mp are critical orchestrators in the healing of many tissues, including skin, skeletal and cardiac muscle, and liver (9; 37-39). In addition to their roles in host defense, Mp promote tissue repair by clearing the wound of damaged tissue and by producing growth factors that regulate the activity of other cells required for healing. Despite the positive role of Mp in promoting wound healing in non-diabetic mice, the prolonged presence of Mp in wounds of diabetic mice (33; 40) and chronic wounds of humans (2; 4) suggests the potential for Mp dysfunction to contribute to impaired healing. In fact, Mp isolated from chronic venous ulcers (41) exhibit a similar pro-inflammatory phenotype to that observed for Mp isolated from diabetic wounds in the present study. This pro-inflammatory phenotype in chronic venous ulcers was attributed to phagocytosis of erythrocytes that had been extravasated due to underlying venous hypertension and subsequent iron overloading. Since this series of events is likely specific to chronic venous ulcers, other pathways likely induce the pro-inflammatory phenotype in diabetic wounds. Our data indicate that the IL-1β plays a key role in sustaining the pro-inflammatory Mp phenotype and impairing the healing of diabetic wounds. Our data further suggest that the IL-1β pathway may be a therapeutic target for improving the healing of these wounds.
AUTHOR CONTRIBUTIONS

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

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REFERENCES


FIGURE CAPTIONS

Figure 1. Macrophages isolated from chronic wounds in diabetic patients exhibit a pro-inflammatory phenotype that may be induced by the wound environment. Macrophages were isolated from chronic wound biopsies and expression of pro-inflammatory markers IL-1β, MMP-9, TNF-α and IL-6 (a-d) and healing-associated/anti-inflammatory markers CD206, IGF-1, TGF-β and IL-10 (e-h) were assessed by real time PCR. For comparison, blood-derived macrophages from healthy volunteers were either left non-stimulated (Non), stimulated with TNF-α and IFN-γ (classically activated, CA) or with chronic wound conditioned medium (WCM). For the in vitro experiments, release of IL-1β, TNF-α, IGF-1 and TGF-β (i-l) into cell culture medium was measured by ELISA. In addition, chronic wound biopsy cryosections were immunostained for IL-1β and the macrophage marker CD68, images taken at 20x (m-o, scale bar = 100 μm) and at 40x (p-r, scale bar = 50 μm); location of 40x images shown in box on 20x images. Nuclei stained with DAPI. For all graphs, bars = mean ± SD, n = 5 for both in vivo and in vitro experiments. *mean value significantly different from that for non-stimulated controls, p < 0.05.

Figure 2. Macrophages isolated from wounds in diabetic mice exhibit a persistent pro-inflammatory phenotype that may be induced by the wound environment. Macrophages were isolated from wounds of non-diabetic (db/) and diabetic (db/db) mice on days 5 and 10 post-injury, cultured overnight, and release of (a-d) IL-1β, TNF-α, IGF-1 and TGF-β was measured using ELISA. Also, bone marrow-derived macrophages from wild-type mice were cultured with conditioned medium (WCM) of day 5 (5d) or 10 (10d) wounds from non-diabetic db/ or diabetic db/db mice, expression of pro-inflammatory markers (e-h) IL-1β, MMP-9, TNF-α and IL-6 and healing-associated/anti-inflammatory markers (i-l) CD206, IGF-1, TGF-β, and IL-10.
were assessed by real time PCR. In addition, release of (m-p) IL-1β, TNF-α, IGF-1 and TGF-b was measured using ELISA. For all graphs, bars = mean ± SD. For in vivo experiments, n = 6-7 mice for each strain and time point. For in vitro experiments, a separate set of bone marrow derived macrophages (each harvested from a different mouse) was used for each of 2 experiments and wound conditioned medium was generated from 3 mice per experiment, totaling n = 6 for each strain and time point. *mean value significantly different from that for same strain on day 5 post-injury, **mean value for db/db significantly different from that for db/+ at same time point, p < 0.05.

Figure 3. IL-1β neutralizing antibody downregulates diabetic wound conditioned medium-induced pro-inflammatory phenotype and upregulates healing-associated phenotype in cultured macrophages. Bone marrow-derived macrophages from wild-type mice either left non-stimulated (Non) or stimulated with day 10 db/db wound conditioned medium (WCM) along with control IgG or IL-1β neutralizing antibody (IL1ab), expression of pro-inflammatory markers (a-d) IL-1β, MMP-9, TNF-α and IL-6 and healing-associated/anti-inflammatory markers (e-h) CD206, IGF-1, TGF-β, and IL-10 measured by real-time PCR. In addition, release of (i-l) IL-1β, TNF-α, IGF-1 and TGF-b measured using ELISA. For all graphs, bars = mean ± SD. For these experiments, a separate set of bone marrow derived macrophages (each harvested from a different mouse) was used for each of 2 experiments and wound conditioned medium was generated from 3 mice per experiment, totaling n = 6 for each condition. *mean value significantly different from that for non-stimulated controls, **mean value significantly different from that for conditioned medium + IgG treated samples, p < 0.05.
Figure 4. Cultured macrophages from IL-1R1 knockout mice are less sensitive to diabetic wound condition medium than macrophages from wild-type mice. Bone marrow-derived macrophages from wild-type (WT) and IL-1 receptor 1 knockout (IL-1R1 ko) mice stimulated with day 10 db/db wound conditioned medium, expression of pro-inflammatory markers (a-d) IL-1β, MMP-9, TNF-α and IL-6 and healing-associated/anti-inflammatory markers (e-h) CD206, IGF-1, TGF-β, and IL-10 measured by real-time PCR. In addition, release of (i-l) IL-1β, TNF-α, IGF-1 and TGF-b measured using ELISA. For all graphs, bars = mean ± SD. For these experiments, a separate set of bone marrow derived macrophages (each harvested from a different mouse) was used for each of 2 experiments and wound conditioned medium was generated from 3 mice per experiment, totaling n = 6 for each condition. *mean value significantly different from that for non-stimulated controls of same strain, **mean value significantly different from that for wound conditioned medium treated wild-type macrophages, p < 0.05.

Figure 5. IL-1β neutralizing antibody downregulates pro-inflammatory Mp phenotype and upregulates healing-associated phenotype in wounds of diabetic mice. Wounds in db/db mice were treated with control IgG or IL-1β blocking antibody (IL1ab) on day 3 post-injury and macrophages were isolated on days 5 and 10 post-injury. Expression of pro-inflammatory markers (a-d) IL-1β, MMP-9, TNF-α and IL-6 and healing-associated/anti-inflammatory markers (e-h) CD206, IGF-1, TGF-β, and IL-10 were measured by real-time PCR. For all graphs, bars = mean ± SD, n = 6. *mean value significantly different from that for IgG treated mice at same time point, p < 0.05.
Figure 6. IL-1β neutralizing antibody improves healing of wounds in diabetic mice. Images showing cryosections stained with trichrome for (a) control IgG treated and (b) IL-1β neutralizing antibody treated wounds on day 10 post-injury. Note the increased re-epithelialization and granulation tissue in the IL-1β antibody treated wounds. Scale bar = 0.5 mm. Arrows indicate ends of migrating epithelial tongues. gt: granulation tissue, mm: deep muscle. The muscle layer observed underneath the wound in some sections is likely a part of the deep tissue collected that helps to maintain the integrity of the fragile wounds particularly in untreated and IgG treated diabetic mice. For wounds in db/+, untreated db/db, IgG-treated db/db and IL-1ab treated db/db mice on day 10 post-injury, quantification of (c,d) re-epithelialization and granulation tissue thickness measured in hematoxylin and eosin stained cryosections, (e) trichrome staining measured as percent area stained blue for collagen and (f) CD31 staining measured as percent area stained for this endothelial cell marker. In addition, wounds from each group of mice were homogenized and levels of (g-j) IL-1β, TNF-α, IGF-1 and TGF-β measured using ELISA. For all graphs, bars = mean ± SD, n = 6-8. *mean value significantly different from that for wounds in db/+ mice, **mean value significantly different from that for control IgG treated wounds.
SUPPLEMENTAL FIGURE CAPTIONS

Figure S1. Larger images of IL-1β and CD68 staining in cryosections of chronic wound biopsy along with isotype controls. Top: chronic wound biopsy cryosections immunostained for IL-1β and the macrophage marker CD68. Bottom: isotype controls. Nuclei stained with DAPI. Images taken at 20x (scale bar = 100 µm).
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