Diabetic Wound Keratinocytes Induce Macrophage JMJD3-Mediated Nlrp3 Expression via IL-1R Signaling

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Macrophage (Mφ) plasticity is critical for normal wound repair; however, in type 2 diabetic wounds, Mφs persist in a low-grade inflammatory state that prevents the resolution of wound inflammation. Increased NLRP3 inflammasome activity has been shown in diabetic wound Mφs; however, the molecular mechanisms regulating NLRP3 expression and activity are unclear. Here, we identified that diabetic wound keratinocytes induce *Nlrp3* gene expression in wound Mφs through IL-1 receptor–mediated signaling, resulting in enhanced inflammasome activation in the presence of pathogen-associated molecular patterns and damage-associated molecular patterns. We found that IL-1α is increased in human and murine wound diabetic keratinocytes compared with nondiabetic controls and directly induces Mφ *Nlrp3* expression through IL-1 receptor signaling. Mechanistically, we report that the histone demethylase, JMJD3, is increased in wound Mφs late post-injury and is induced by IL-1α from diabetic wound keratinocytes, resulting in *Nlrp3* transcriptional activation through an H3K27me3-mediated mechanism. Using genetically engineered mice deficient in JMJD3 in myeloid cells (*Jmjd3*$^{f/f}$*lyz2*$^{Cre+}$), we demonstrate that JMJD3 controls Mφ-mediated *Nlrp3* expression during diabetic wound healing. Thus, our data suggest a role for keratinocyte-mediated IL-1α/IL-1R signaling in driving enhanced NLRP3 inflammasome activity in wound Mφs. These data also highlight the importance of cell cross-talk in wound tissues and identify JMJD3 and the IL-1R signaling cascade as important upstream therapeutic targets for Mφ NLRP3 inflammasome hyperactivity in nonhealing diabetic wounds.

ARTICLE HIGHLIGHTS

- The molecular mechanism regulating macrophage (Mφ) NLRP3 inflammasome activity in diabetic wounds remains unclear.
- Diabetic wound keratinocytes induce *Nlrp3* gene expression and enhance inflammasome activation in wound Mφs through IL-1 receptor–mediated signaling.
- IL-1α is increased in human and murine wound diabetic keratinocytes compared with nondiabetic controls and directly induces Mφ *Nlrp3* expression via histone demethylase, JMJD3.
- JMJD3 controls Mφ-mediated *Nlrp3* expression during wound healing.

Wound healing is a complex process involving coordination through the stages of homeostasis, inflammation, re-epithelization, and resolution for proper healing to occur. Macrophage (Mφ) plasticity is essential during the inflammation phase of tissue repair for wounds to transition into the resolution state (1,2). During the initial inflammatory phase, Mφs demonstrate increased production of inflammatory cytokines and inflammasome activation, while late inflammatory-phase Mφs produce other

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mediators important in the transition to the proliferative and resolution phases of wound healing; however, this has been shown to not occur in type 2 diabetes (T2D) wounds. In the diabetic state, wound Mφs display a prolonged inflammatory phenotype and promote low-grade inflammation associated with enhanced inflammasome activity that contributes to poor wound healing (1–6). Although keratinocytes can regulate Mφ phenotypes within the wound environment (7), the molecular mechanisms regulating Mφ activity, and, in particular, the increase in inflammasome activity, in diabetic wounds have not been explored.

Activation of the NOD-like receptor protein 3 (NLRP3) inflammasome is closely linked to driving skin inflammation in diabetic wounds (8–12). NLRP3 inflammasome activation consists of two steps, where signal 1 is the priming step and involves the induction of gene transcription of NLRP3 inflammasome components (i.e., NLRP3, caspase 1, pro-IL1β) (13). Signal 2 is the activation step; this entails the cleavage of proform caspase-1, which then cleaves and activates proinflammatory cytokines IL-1β and IL-18 (14). Elevated levels of IL-1β have been found in human and murine diabetic wound Mφs, which correlates with increased NLRP3 inflammasome activity (15–17), yet the mechanism driving the inflammasome activity has remained ill defined. Studies have identified that increased priming of Mφs, resulting in elevated inflammasome gene transcription, can lead to enhanced inflammasome activation (18,19); therefore, the priming step may play a critical role in driving the enhanced Mφ NLRP3 inflammasome activation and IL-1β release seen in pathologic diabetic wounds.

Mediators in diabetic wounds can induce increased NLRP3 inflammasome activity in monocytes (16); however, the reasons for this were not well explored. Keratinocytes are the primary cell type in the epidermis, and are involved via cross-talk with immune cells in the dermis to regulate inflammation during wound healing (20). Following cutaneous injury, keratinocytes release chemokines and inflammatory cytokines that can activate and recruit immune cells to the injury site (7,21–25). In particular, interleukin 1α (IL-1α) is constitutively expressed and induced by keratinocytes following skin injury (26,27). The IL-1α precursor, unlike IL-1β, is biologically active and can bind to the IL-1 receptor (IL-1R) to induce skin inflammation after being released from cells (28,29). IL-1R signaling can regulate Mφ *Nlrp3* expression; however, whether IL-1α is dysregulated in keratinocytes in the context of diabetic wounds and the mechanism by which it can regulate Mφ *Nlrp3* expression are unknown.

Our group and others have shown that epigenetic regulation plays an essential role in Mφ phenotype during wound healing by suppressing or promoting specific genes important for inflammation (30). Jumanji domain-containing protein-3 (JMJD3), a histone demethylase that regulates the trimethylation of histone H3 on lysine 27 (H3K27me3), has been shown to regulate *Nlrp3* expression in Mφs during colitis (31), but this has not been explored in the context of diabetes or wound repair. Further, our group recently identified that JMJD3 is upregulated in diabetic wound Mφs (32,33); however, the influence of keratinocytes on this pathway as well as upstream regulation and the downstream gene expression patterns related to NLRP3 were not examined.

Here, we demonstrate that diabetic wound keratinocytes induce Mφ JMJD3-mediated *Nlrp3* expression via IL-1R signaling. We found that stimulation of Mφs with diabetic wound keratinocyte-conditioned media (KCM) induced increased *Nlrp3* expression and enhanced inflammasome activation through an IL1-R signaling pathway. Further, we found that IL-1α is increased in human and murine diabetic wound keratinocytes and drives increased *Nlrp3* expression in Mφs. Using an epigenetic PCR array on Mφs treated with diabetic wound KCM, we identified that JMJD3 was the most significantly altered enzyme, and pharmacologic inhibition of JMJD3 in Mφs decreased *Nlrp3* expression following IL-1α stimulation and treatment with diabetic wound KCM. We then examined this in vivo using our Mφ-specific JMJD3-deficient mice ($Jmjd3^{f/f}lyz2^{Cre+}$) and found that JMJD3 regulates *Nlrp3* expression and inflammasome activation in wound Mφs following injury. Altogether, our data highlight the keratinocyte-Mφ cross-talk in wounds and suggest a role for IL-1α/IL-1R signaling in driving increased JMJD3-mediated *Nlrp3* expression and enhanced inflammasome activation in diabetic wound Mφs.

RESEARCH DESIGN AND METHODS

Mice

Male C57BL/6J mice were purchased from The Jackson Laboratory and maintained in breeding pairs on a normal chow diet (13.5% kcal fat; LabDiet) in the Unit for Laboratory Animal Medicine facilities. To initiate a "diabetic" state, male C57BL/6J mice were fed a high-fat diet (60% kcal fat; Research Diets) for 12–16 weeks to generate the DIO model. $Jmjd3^{f/f}Lyz2^{Cre}$ mice on a C57BL/6J background were created as previously reported by our group to obtain male mice deficient in *Jmjd3* in Mφs, monocytes, and granulocytes (32). All mouse protocols were approved by and complied with the guidelines established by the Institutional Animal Care and Use Committee at the University of Michigan, Ann Arbor, MI.

Wound Creation and Healing

Four-millimeter punch biopsy wounds were created on mouse dorsal skin as previously described (7). In local injection experiments, Anakinra, anti-IL1α, or control were injected subcutaneously at two points along the wound edge, as previously described by our group (7). For the acute wound healing model, photographs were taken following injury, and the wound area was calculated, as described previously (7). The 6-mm wound biopsies (2-mm wound edge) were collected at the indicated time points and prepared for keratinocyte or Mφ isolation.

Wound Keratinocyte Isolation

Primary keratinocytes were isolated from the wounds of mice as previously described (7). Following isolation and culture of primary wound keratinocytes, cells were harvested for RNA, and KCM was collected for Mφ stimulation and protein studies.

Isolation of Wound Monocytes/Mφ

For monocyte/Mφ isolation, wounds were digested, and monocyte/Mφs (CD3-CD19-Ly6G-NK1.1-CD11b+) were isolated as described previously by our group (32). Cells were immediately saved or cultured ex vivo for RNA, cDNA, or protein studies.

Bone Marrow–Derived Mφ Culture and Stimulation

Bone marrow–derived Mφs (BMDMs) were grown as detailed previously (34). On day 6, the cells were replated and stimulated with control or DIO wound KCM, IL-1α, GSK-J4, IgG, or anti-IL1α. For siRNA experiments, cells underwent transfection using Lipofectamine RNAimax (Invitrogen), as previously described (35). Briefly, 20 pmol of the nontargeting siRNA or JMJD3 pooled siRNA were added to BMDM cultures prior to KCM stimulation. siRNA information is provided in the Supplementary Material. After 5 h of stimulation, the cells were placed in Trizol (Invitrogen) for RNA analysis. After 24 h of stimulation, cells were either immediately saved for western and chromatin immunoprecipitation (ChIP) analysis or continued in culture for IL-1β ELISA experiments.

ELISA

Wound Mφs and BMDMs were cultured for 4 h in RPMI with lipopolysaccharide (LPS) (100 ng/mL) and 2 h with ATP (5 mmol/L). After stimulation, the cell-free supernatant was collected and analyzed by a specific enzyme immunoassay kit for IL-1β. Primary wound KCM was analyzed by a specific enzyme immunoassay kit for IL-1α or IL-1β. All ELISA kits are from R&D Systems and were performed according to the manufacturer's instructions.

Quantitative PCR

RNA was isolated from BMDMs, wound Mφs, and keratinocytes using chloroform, isopropanol, and ethanol. Superscript III Reverse Transcriptase (Thermo Fisher Scientific) kits were then used to reverse transcribe RNA to cDNA. Real-time PCR was performed with 2 × TaqMan Fast PCR mix via the 7500 Real-Time PCR System. Primers are listed in the Supplementary Material. Data were examined in a relative quantification analysis to 18S ($2^{-\Delta\Delta Ct}$). All samples were performed in triplicate.

Microarray

RNA was extracted from BMDMs treated with control or DIO KCM using an RNeasy Micro kit (Qiagen), and then column DNAase digestion was used to eliminate genomic DNA contamination. After digestion, the RT2 First Strand kit (Qiagen) was used to prepare cDNA from RNA. Next, the RT2 Profiler PCR array (PAMM-085Z; Qiagen) was used for gene expression analysis of chromatin-modifying enzymes. Data were analyzed using the GeneGlobe web portal (Qiagen).

ChIP

ChIP was performed on cells using an anti-H3K27me3 antibody or isotype control, as detailed previously by our group (32). H3K27me3 deposition was measured by quantitative PCR (qPCR) using 2× SYBR PCR mix (Invitrogen), and primers are listed in the Supplementary Material.

Western Blot

BMDM protein suspensions in lysis buffer were standardized for protein concentrations and subjected to gel electrophoresis and wet transfer as previously described (32). Nitrocellulose membranes were probed with β-actin primary antibody (8H10D10; Cell Signaling) or NLRP3 primary antibody (D4D8T; Cell Signaling) diluted to 1:1,000 and 1:500, respectively, in 5% BSA in Tris-buffered saline with Tween buffer, followed by incubation with anti-mouse IgG or anti-rabbit IgG horseradish peroxidase–conjugated secondary antibody (Cell Signaling, Inc.) and then visualized with chemiluminescence (Thermo Fisher Scientific). ImageJ (National Institutes of Health) was used to calculate densitometry.

Histology

Six-millimeter punch biopsy whole wounds were excised from mice. Wound sections were fixed overnight in 10% formalin before embedding the wound in paraffin. Five-micrometer sections were stained with hematoxylin-eosin as described previously (7). Images were taken using a Zeiss Axioskop 2 microscope at 4× magnification.

Single-Cell RNA Sequencing Analysis

Single-cell RNA sequencing (scRNA-seq) was performed on 8-mm punch biopsy samples obtained from normal and diabetic wounds, as previously described (36). The data accession numbers include GSE154557 and GSE179162 (Gene Expression Omnibus). Patient consent for collecting wound tissue was exempt by the institutional review board because the tissue was obtained from discarded surgical material (protocol no. HUM00060733).

Statistics

All data were analyzed and graphed with GraphPad Prism software. Data comparing differences between two groups were obtained using a two-tailed Student *t* test for normally distributed groups, and Welch correction was applied for data with unequal variances. Analysis between more than two groups was done using one-way ANOVA. All *P* values less than 0.05 were considered significant.

Detailed methods are provided in the Supplementary Material.

Data and Resource Availability

All data needed to support the conclusions of the paper are present in the paper. All materials are available by request, restricted by institutional material transfer agreements.

RESULTS

Diabetic Wound Keratinocytes Drive Increased Mφ NLRP3 Inflammasome Activity via IL-1R Signaling

Given that increased priming of *Nlrp3* gene transcription activation in Mφs is important for enhanced inflammasome activation and keratinocytes in the skin regulate Mφ phenotype through inflammatory cytokine production following injury (7,19), we examined the role of diabetic wound keratinocytes in driving increased *Nlrp3* expression and enhanced inflammasome activation in Mφs. First, to establish the kinetics of *Nlrp3* expression in Mφs from diabetic wounds, we generated a diet-induced obesity (DIO) murine model of T2D. C57BL/6J DIO mice were wounded with a 4-mm punch biopsy, and *Nlrp3* expression was assessed in sorted wound Mφs (CD3-CD19-Ly6G-NK1.1-CD11b+) isolated on days 1, 3, and 5 following injury. We found that *Nlrp3* is upregulated by day 5 in DIO wound Mφs, confirming the increased regulation of *Nlrp3* gene expression within the diabetic wound environment takes place late following injury (Fig. 1*A*). Further, *Nlrp3* expression is significantly increased in Mφs isolated from DIO wounds compared with control on day 5 post-injury (Fig. 1*B*). A trending increase in *Nlrp3* expression was detected in spleen Mφs (Supplementary Fig. 1). To understand the translational relevance, we examined human Mφs previously analyzed by our group using scRNA-seq from non-T2D and T2D chronic wounds (7). Similar to others, we see that *NLRP3* was increased in Mφs from chronic wounds of T2D patients (Fig. 1*C*). To determine the relevance of keratinocytes in inducing the increased Mφ *Nlrp3* expression seen in diabetic wounds, total wound keratinocytes were isolated and cultured on day 5 following injury from control and DIO mice. This period is consistent with the day *Nlrp3* expression is elevated in DIO wound Mφs. Following the culture of the wound keratinocytes, the KCM were collected and added to wound Mφs or BMDMs. We identified a significant increase in *Nlrp3* expression in wound Mφs and BMDMs stimulated with DIO wound KCM compared with controls (Fig. 1*D* and *E*). NLRP3 protein was also increased in BMDMs stimulated with DIO wound KCM compared with control (Fig. 1*F*). These data suggest that keratinocytes play a role in priming Mφs for increased *Nlrp3* gene expression levels within the diabetic wound environment late following injury.

Since IL-1R signaling can activate *Nlrp3* transcription in Mφs, we then examined the extent to which diabetic wound keratinocyte induction of Mφ *Nlrp3* expression relied on the IL-1R signaling pathway. To determine this, we stimulated BMDMs from IL-1R knockout (KO), MyD88 KO, and wild-type (WT) mice with DIO wound KCM. We observed that KO of the IL-1R and MyD88 in BMDMs resulted in decreased *Nlrp3* expression following stimulation with DIO wound KCM compared with WT, suggesting the induction of *Nlrp3* expression in Mφs occurs in part via IL-1R/MyD88 signaling (Fig. 1*G*). We next examined whether this increased priming of *Nlrp3* gene transcription in Mφs resulted in enhanced inflammasome activation. To activate the inflammasome, Mφs were stimulated with LPS and ATP following the addition of wound KCM, and the release of IL-1β was used to track inflammasome activation changes. We noted the enhanced release of IL-1β by BMDMs stimulated with DIO wound KCM compared with control and media alone. Additionally, IL-1R KO BMDMs displayed decreased IL-1β release following stimulation with DIO wound KCM, indicating that the enhanced inflammasome activation occurs via IL-1R signaling (Fig. 1*H*). To further evaluate the role of IL-1R signaling in regulating Mφ *Nlrp3* expression and enhanced inflammasome activation in diabetic wounds, DIO mice were subcutaneously injected with PBS or Anakinra (an IL-1R antagonist) following injury. We observed that local injection of Anakinra resulted in decreased Mφ *Nlrp3* expression and inflammasome activity, indicated by decreased release of IL-1β (Fig. 1*I* and *J*). However, local injection of Anakinra did not result in improved healing. This may be due to Anakinra's shortened half-life and bioactivity following injection (Supplementary Fig. 2). Together, these data show that *Nlrp3* is increased late following injury in DIO wound Mφs, and keratinocytes within the diabetic wound environment can induce *Nlrp3* expression and enhance inflammasome activation in part via IL-1R signaling.

IL-1α Is Increased in Diabetic Wound Keratinocytes and Induces Mφ Nlrp3 Expression Through a JMJD3-Mediated Mechanism

Since IL-1α is constitutively expressed by keratinocytes and signals through the IL-1R to induce tissue inflammation (26,27), we investigated the differences in IL-1α levels between keratinocytes from normal and diabetic wounds. We noted that *Il1a* was increased in DIO keratinocytes isolated from day 5 wounds (Fig. 2*A*). These cells also demonstrated increased release of IL-1α, but not IL-1β, into the KCM compared with control (Fig. 2*B* and Supplementary Fig. 3*A*). Using scRNA-seq from non-T2D and T2D chronic wounds, we found that *IL1A* was increased in keratinocytes from chronic wounds of T2D patients (Fig. 2*C*). While *IL1B* was increased in T2D chronic wound keratinocytes compared with control, a smaller percentage of keratinocytes in T2D chronic wounds express *IL1B* (Supplementary Fig. 3*B*). Since IL-1α is increased in DIO wound KCM, we then examined the ability of IL-1α to prime *Nlrp3* transcriptional activation in Mφs. We found that stimulation of BMDMs with IL-1α increased *Nlrp3* expression (Fig. 2*D*). To directly examine the effects of IL-1α in DIO KCM on Mφ *Nlrp3* expression, we added anti-IL1α or IgG control antibodies to BMDMs following stimulation with DIO KCM. We observed a significant decrease in *Nlrp3* expression in BMDMs after the addition of anti-IL1α antibodies following stimulation

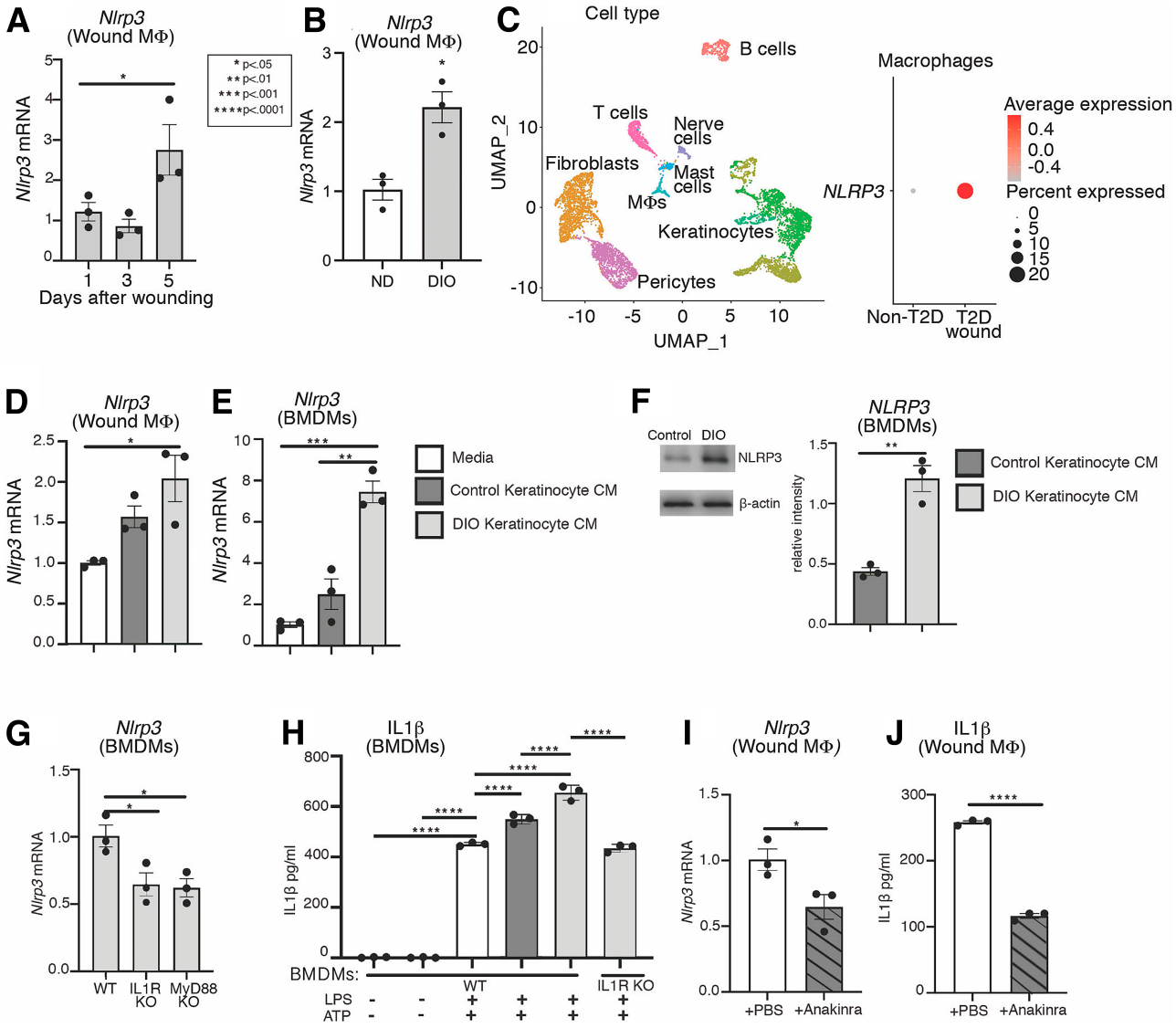

Figure 1—Diabetic wound keratinocytes enhance Mφ Nlrp3 inflammasome activity. *A*: Wound monocytes/Mφs ($CD3^-CD19^-NK1.1^-Ly6G^-CD11b^+$) were isolated from DIO mice on days 1, 3, and 5, $n = 4$ mice per group, wounds pooled, repeated in triplicate. Gene expression of *Nlrp3* was measured via qPCR. *B*: *Nlrp3* expression was examined via qPCR in wound monocytes/Mφs isolated from DIO and control mice on day 5 post-injury; $n = 4$ mice per group, wounds pooled, repeated in triplicate. *C*: UMAP cluster analysis of single-cell sequencing from non-T2D ($n = 2$) and T2D wound ($n = 1$) samples. Dot plot demonstrating *NLRP3* expression within Mφs in human T2D and non-T2D samples. The dot size represents the proportion of cells within the group expressing *NLRP3*, and the dot color corresponds to the level of expression. *D* and *E*: *Nlrp3* expression was examined via qPCR in WT wound monocyte/Mφs and BMDMs isolated from non-DIO mice following stimulation with and without DIO or control day 5 wound KCM for 5 h; $n = 4$ mice per group for KCM, wounds pooled, repeated in triplicate. *F*: Protein levels of NLRP3 were assayed in WT BMDMs isolated from non-DIO mice stimulated with and without DIO or control wound KCM for 24 h. Representative blot shown (β-actin served as loading control). Protein band density of NLRP3 was normalized to β-actin, as digitally quantified by ImageJ; $n = 4$ mice per group for KCM, wounds pooled, repeated in triplicate. *G*: BMDMs were harvested from non-DIO WT, IL-1R KO, and MyD88 KO mice and stimulated with DIO wound KCM for 5 h. *Nlrp3* expression was examined via qPCR; $n = 4$ mice per group for KCM, wounds pooled, repeated in triplicate. *H*: BMDMs from non-DIO WT or IL-R KO mice were treated with DIO or control KCM overnight; 100 ng/mL LPS and 5 mmol/L ATP were added before harvesting to activate the NLRP3 inflammasome and release mature IL-1β. Supernatants were collected, and IL-1β was measured by ELISA; $n = 4$ mice per group for KCM, wounds pooled, repeated in triplicate. *I*: Four-millimeter punch biopsy wounds were created on DIO mice, and wounds were injected daily post-injury with Anakinra (0.75 μg/100 μL) or PBS control. Wound monocytes/Mφs ($CD3^-CD19^-NK1.1^-Ly6G^-CD11b^+$) were isolated from Anakinra or PBS control mice on day 5; $n = 5$ mice per group, wounds pooled, repeated in triplicate. *Nlrp3* gene expression was measured via qPCR; $n = 5$ mice per group, wounds pooled, repeated in triplicate. *J*: IL-1β was measured by ELISA from day 5 wound monocyte/Mφs isolated from Anakinra-treated and PBS control mice; $n = 4$ mice per group, wounds pooled, repeated in triplicate. Data were analyzed for variances, and a one-way ANOVA or two-tailed Student *t* test was performed. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. Data are presented as mean and SEM.

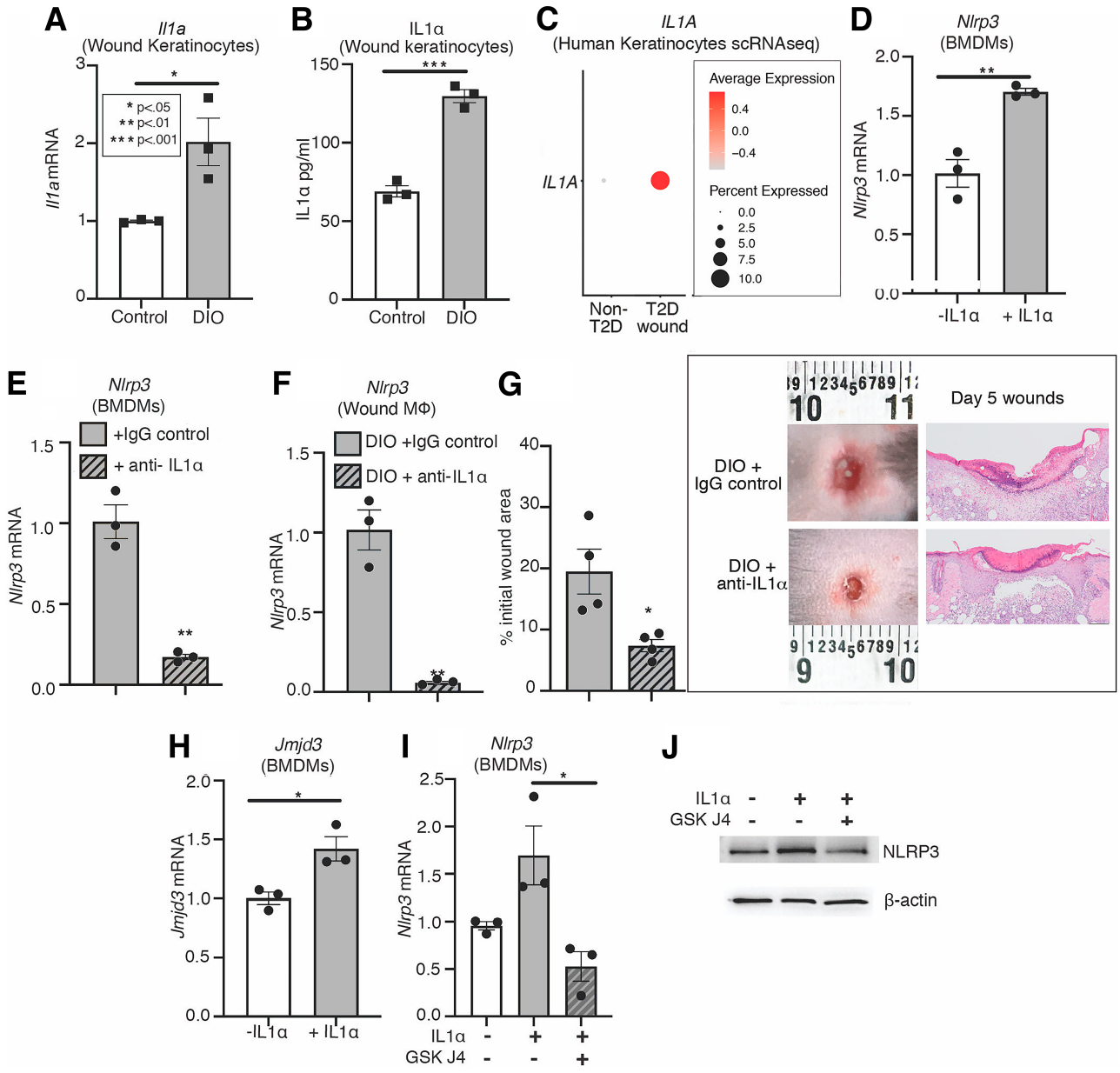

Figure 2—IL-1α is increased in human diabetic wound keratinocytes and induces Mφ *Nlrp3* expression via JMJD3. *A*: Four-millimeter punch biopsy wounds were created on DIO and control mice. Wound keratinocytes were isolated on day 5. *Il1a* expression was measured via qPCR; $n = 4$ mice per group, wounds pooled, repeated in triplicate. *B*: IL-1α production by DIO and control day 5 wound keratinocytes were measured by ELISA; $n = 4$ mice per group, wounds pooled, repeated in triplicate. *C*: Dot plot demonstrating *IL1A* expression within keratinocytes in human T2D and non-T2D samples. The dot size represents the proportion of cells within the group expressing *IL1A*, and the dot color corresponds to the level of expression. *D*: *Nlrp3* expression was assayed via qPCR in non-DIO WT BMDMs stimulated with and without 3 pg/mL IL-1α for 5 h, repeated in three independent experiments. *E*: Non-DIO WT BMDMs stimulated with DIO KCM with IgG control or anti-IL1α for 5 h. *Nlrp3* gene expression was assayed via qPCR, repeated in three independent experiments. *F* and *G*: Four-millimeter punch biopsy wounds were created on DIO mice, and wounds were injected daily post-injury with 1 mg/kg IgG control or anti-IL1α. *F*: Wound monocytes/Mφs were isolated from IgG control or anti-IL1α–treated mice on day 5. *Nlrp3* gene expression was measured via qPCR; two wounds per mouse, $n = 4$ mice per group, pooled, repeated in triplicate. *G*: The changes in wound area were recorded daily and analyzed using ImageJ software. Displayed is the percent of initial wound area on day 5 post-injury. Representative photographs of the wounds and hematoxylin-eosin stained representative images (magnification, 4×) were taken on day 5; two wounds per mouse, $n = 4$ mice per group. *H*: *Jmjd3* expression was assayed via qPCR in non-DIO WT BMDMs stimulated with and without 3 pg/mL IL-1α for 5 h, repeated in three independent experiments. *I*: *Nlrp3* gene expression was assayed via qPCR in non-DIO WT BMDMs stimulated with and without 3 pg/mL IL-1α and 10 μmol/L GSK-J4 for 5 h, repeated in three independent experiments. *J*: NLRP3 protein levels were examined in non-DIO WT BMDMs stimulated with and without 3 pg/mL IL-1α and 10 μmol/L GSK-J4 for 24 h. Representative blot shown (β-actin served as loading control). Data were analyzed for variances, and a one-way ANOVA or two-tailed Student *t* test was performed. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. Data are presented as mean and SEM.

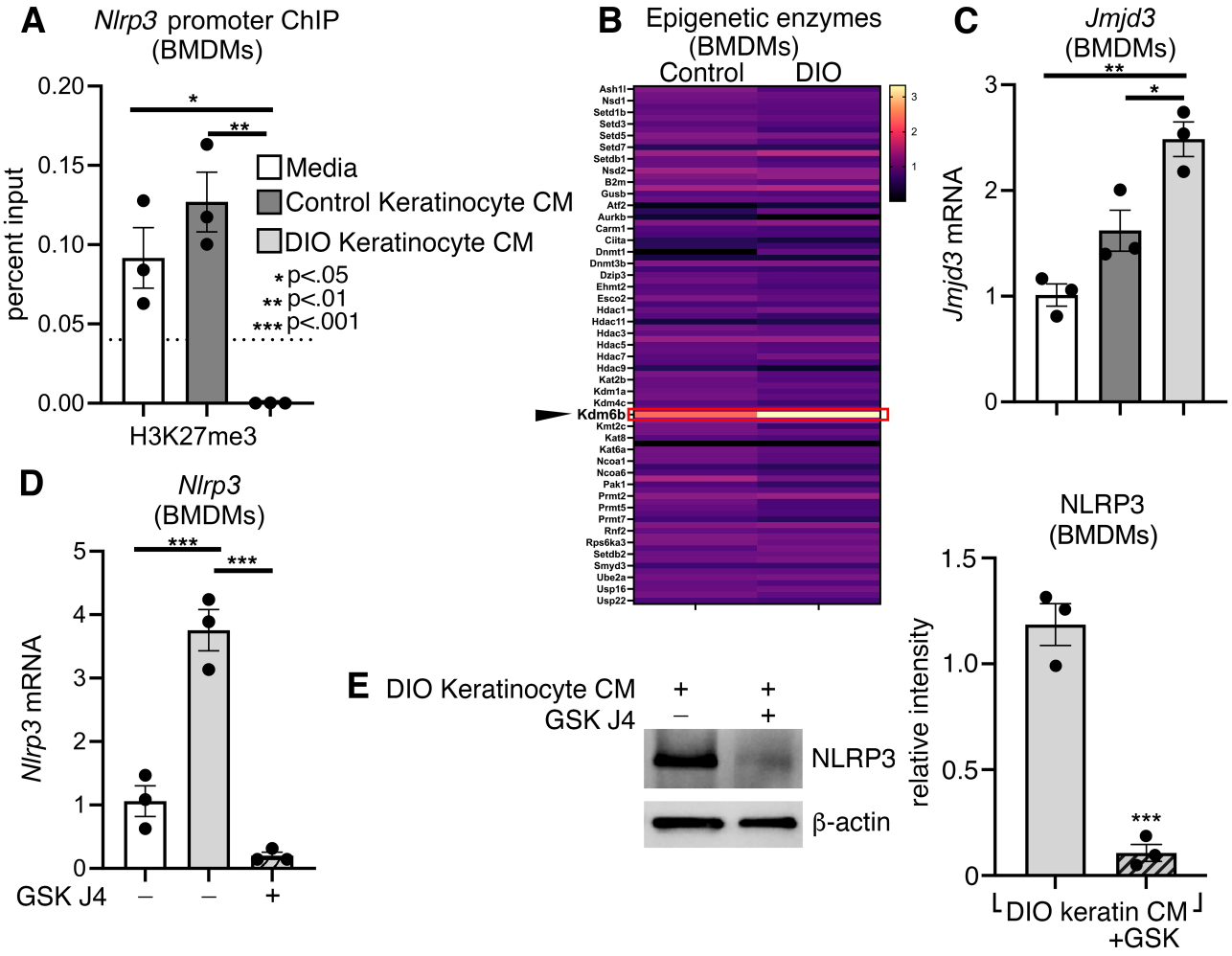

Figure 3—Diabetic wound keratinocytes induce Mφ *Nlrp3* expression via JMJD3. *A*: BMDMs were harvested from non-DIO WT mice and stimulated with and without DIO or control KCM for 24 h. ChIP analysis for H3K27me3 at the *Nlrp3* promoter; $n = 4$ mice per group for KCM, wounds pooled, repeated in triplicate. Dotted line represents IgG controls. *B*: An epigenetic array on non-DIO WT BMDMs stimulated with DIO or control KCM for 5 h. Color corresponds to the level of expression. The red box outlines *Kdm6b* (*Jmjd3*). *C*: *Jmjd3* expression assayed by qPCR in BMDMs isolated from non-DIO mice stimulated with and without DIO or control KCM for 5 h; $n = 4$ mice per group for KCM, wounds pooled, repeated in triplicate. *D*: *Nlrp3* expression was examined via qPCR in BMDMs isolated from non-DIO mice stimulated with and without DIO KCM and 10 μM GSK-J4 for 5 h; $n = 4$ mice per group for KCM, wounds pooled, repeated in triplicate. *E*: NLRP3 protein levels were assayed in BMDMs isolated from non-DIO mice stimulated with DIO KCM with and without 10 μM GSK-J4 for 24 h. A representative blot is shown (β-actin served as loading control). Protein band density of NLRP3 normalized to β-actin, as digitally quantified by ImageJ; $n = 4$ mice per group for KCM, wounds pooled, repeated in triplicate. Data were analyzed for variances, and a one-way ANOVA or two-tailed student *t* test was performed. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Data are presented as mean and SEM.

with DIO KCM compared with control (Fig. 2*E*), suggesting that keratinocyte IL-1α production may contribute to increased Mφ *Nlrp3* expression in chronic diabetic wounds. To further evaluate the role of IL-1α signaling in regulating Mφ *Nlrp3* expression in diabetic wounds, DIO mice were subcutaneously injected with anti-IL1α or IgG control following injury. We observed that local injection of anti-IL1α in DIO mice resulted in decreased Mφ *Nlrp3* expression and improved healing compared with control (Fig. 2*F* and *G*).

Since JMJD3 has previously been identified as one of the most relevant epigenetic enzymes in driving the diabetic Mφ inflammatory profile late post-injury and has been shown to regulate *Nlrp3* expression in Mφs during colitis (31–33), we sought to determine the extent to which IL-1α induced Mφ *Nlrp3* expression via a JMJD3-mediated mechanism. Following stimulation of BMDMs with IL-1α, we identified that *Jmjd3* expression was significantly increased (Fig. 2*H*). Furthermore, addition of GSK-J4, a JMJD3 inhibitor, to BMDMs following IL-1α stimulation resulted in decreased *Nlrp3* gene expression and protein levels (Fig. 2*I* and *J*), suggesting that increased IL-1α produced by DIO wound keratinocytes late post-injury contributes to priming *Nlrp3* gene transcriptional activation in Mφs via a JMJD3-mediated mechanism.

Inhibition of JMJD3 Decreases Keratinocyte-Induced Nlrp3 Expression in Mφs

Since diabetic wound keratinocytes produce increased IL-1α, and JMJD3 plays a central role in IL-1α–mediated

Nlrp3 expression in Mϕs, we examined whether diabetic wound keratinocytes induced *Nlrp3* transcriptional activation via a JMJD3-mediated mechanism. JMJD3 regulates gene expression by demethylating H3K27, so we first examined Mϕs following stimulation with DIO and control wound KCM for changes in H3K27me3 levels at the *Nlrp3* promoter by ChIP. We found a significant decrease in the repressive H3K27me3 mark at the *Nlrp3* promoter in BMDMs stimulated with DIO wound KCM compared with control and media only (Fig. 3*A*). Since several methyltransferases and demethylases are specific for H3K27, we performed an epigenetic PCR array on BMDMs stimulated with control or DIO wound KCM. We observed *Jmjd3* (*Kdm6b*) was the main epigenetic enzyme that was increased following stimulation with DIO wound KCM (Fig. 3*B*). Quantitative PCR analysis also revealed a significant increase in *Jmjd3* expression in DIO wound KCM-stimulated BMDMs (Fig. 3*C*). Interestingly, we found that pharmacologic inhibition (GSK-J4) or siRNA knockdown of JMJD3 following the addition of DIO wound KCM to BMDMs resulted in a significant decrease in *Nlrp3* expression (Fig. 3*D* and Supplementary Fig. 4). Concurrently, this decrease was also noted at the protein level (Fig. 3*E*). Taken together, these data demonstrate that diabetic wound keratinocytes late post-injury can induce *Nlrp3* gene transcription in Mϕs via a JMJD3/H3K27me3-mediated mechanism.

Mϕ-Specific JMJD3 KO Decreases NLRP3 Inflammasome Activity Late Post-Injury

Our group has demonstrated that Mϕ-specific inhibition of JMJD3 improves diabetic wound healing (32). Additionally, as previously identified, we see increased *Jmjd3* expression in DIO wound Mϕs late following injury (Fig. 4*A*). To identify whether JMJD3 could regulate Mϕ *Nlrp3* gene expression in vivo, we isolated Mϕs from DIO and control mice from day 5 wounds, and examined changes in H3K27me3 at the *Nlrp3* promoter by ChIP. We found a significant decrease in the H3K27me3 mark at the *Nlrp3* promoter in DIO wound Mϕs (Fig. 4*B*). Next, to confirm the relevance of JMJD3 in regulating *Nlrp3* transcriptional activation in Mϕs during wound repair, we utilized the Cre recombinase Lox-P

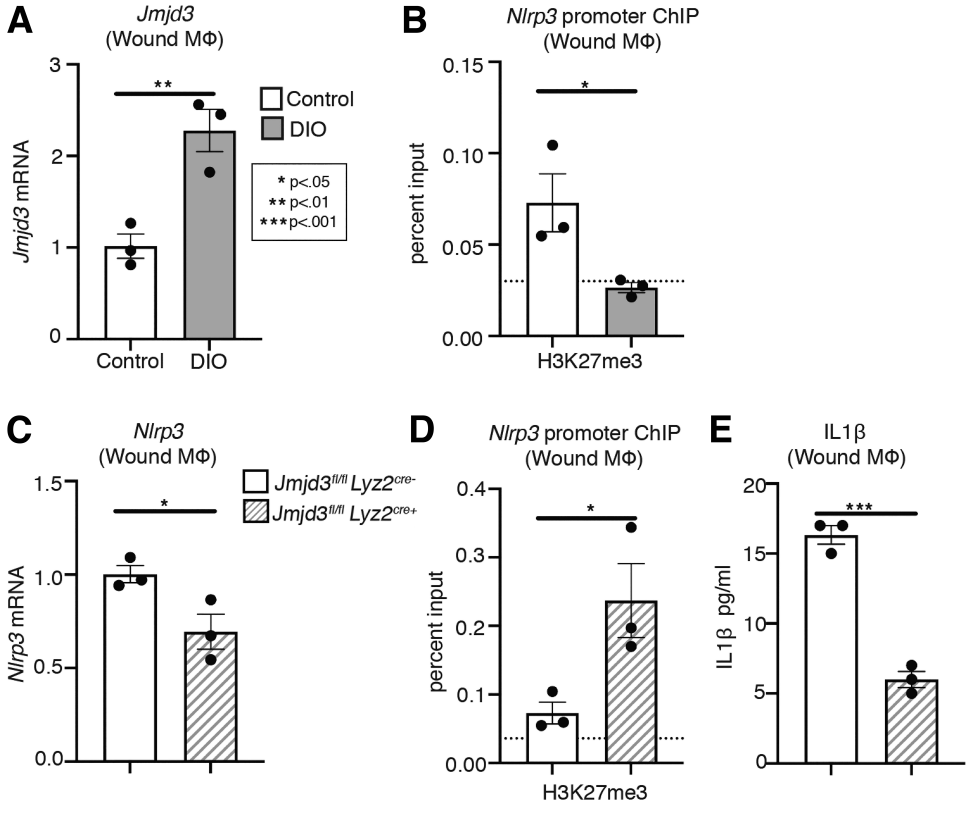

Figure 4—Mϕ-specific KO of JMJD3 decreases NLRP3 inflammasome activity. *A*: Four-millimeter punch biopsy wounds were created on DIO and control mice. Wound monocytes/Mϕs were isolated from day 5 wounds. Gene expression of *Jmjd3* was measured via qPCR; n = 5 mice per group, wounds pooled, repeated in triplicate. *B*: ChIP analysis for H3K27me3 on the *Nlrp3* promoter from monocyte/Mϕs isolated from DIO and control day 5 wounds; n = 5 mice per group, wounds pooled, repeated in triplicate. Dotted line represents IgG controls. *C*: *Nlrp3* expression in *Jmjd3*$^{f/f}$ *Lyz2*$^{Cre+}$ and littermate control wound monocyte/Mϕs harvested on day 5; n = 4 mice per group, wounds pooled, repeated in triplicate. *D*: Monocyte/Mϕs were isolated from *Jmjd3*$^{f/f}$ *Lyz2*$^{Cre+}$ and littermate control day 5 wounds. ChIP analysis for H3K27me3 at the *Nlrp3* promoter; n = 4 mice per group, wounds pooled, repeated in triplicate. Dotted line represents IgG controls. *E*: IL-1β release measured by ELISA. n = 4 mice per group, wounds pooled, repeated in triplicate. Data were analyzed for variances, and a one-way ANOVA or two-tailed student t test was performed. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. Data are presented as mean and SEM.

system to generate a myeloid-specific, JMJD3-deficient mouse (*Jmjd3*$^{f/f}$ *Lyz2*$^{Cre+}$). Wound Mφs isolated on day 5 following injury from *Jmjd3*$^{f/f}$ *Lyz2*$^{Cre+}$ mice exhibited a decrease in *Nlrp3* expression compared with the control mice (Fig. 4*C*), and ChIP analysis of the *Nlrp3* promoter showed an increase in H3K27me3 levels in these Mφs (Fig. 4*D*). These data indicate that the increased *Nlrp3* expression observed in DIO wound Mφs late following injury is mediated by JMJD3/H3K27me3 activity. Finally, we explored the effect of Mφ specific loss of JMJD3 on inflammasome activation during wound repair by examining IL-1β release from wound Mφs on day 5 and found decreased release of IL-1β by *Jmjd3*$^{f/f}$ *Lyz2*$^{Cre+}$ mice compared with control (Fig. 4*E*). Taken together, these data suggest a central role for JMJD3 in regulating Mφ NLRP3 inflammasome hyperactivity noted in chronic diabetic wounds.

DISCUSSION

NLRP3 inflammasome activity is elevated in diabetic wound Mφs and contributes to impaired wound healing (5,6); however, the molecular mechanism regulating this is unclear. In this study, we showed that Mφ *Nlrp3* expression is increased late post-injury in diabetic wounds, and stimulation of wound Mφs with conditioned media from keratinocytes isolated from diabetic wounds late following injury results in both increased *Nlrp3* expression and enhanced inflammasome activation that is regulated in part by IL-1R signaling. Next, we identified that IL-1α is increased by human and murine diabetic wound keratinocytes, and neutralization of IL-1α inhibits induction of Mφ Nlrp3 expression by diabetic wound keratinocytes. Furthermore, we demonstrated that diabetic wound keratinocytes induce *Nlrp3* expression through a JMJD3/H3K27me3-mediated mechanism. Significantly, Mφ-specific KO of JMJD3 (*Jmjd3*$^{f/f}$*lyz2*$^{Cre+}$) reduced *Nlrp3* expression and inflammasome activity in wound Mφs late following injury. Together, our data suggest a role for keratinocyte IL-1α/IL-1R signaling in driving the JMJD3-mediated increased *Nlrp3* expression and enhanced inflammasome activity seen in chronic diabetic wound Mφs (Fig. 5).

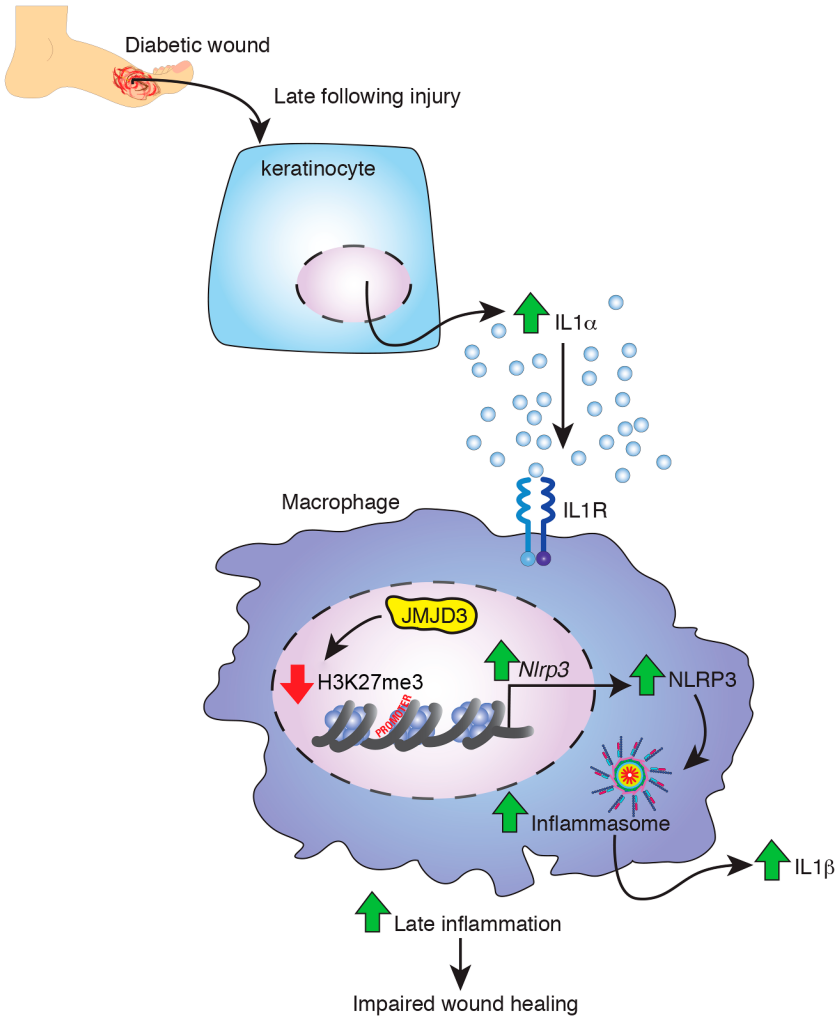

Figure 5—NLRP3 inflammasome activity regulation in diabetic wound Mφs late post-injury.

Current literature has demonstrated an important role for epigenetic modifications, including histone modification, DNA methylation, and RNA interference in regulating proper wound healing through controlling structural and immune cell phenotype (2,30,37). In particular, our group and others have examined the role of histone demethylase JMJD3 on Mφ phenotype and keratinocyte re-epithelization during wound healing (32,33,38–40). In normal wound healing, JMJD3 drives Mφs toward an inflammatory phenotype early after injury, whereas, in diabetic wounds, this occurs late following injury contributing to impaired wound healing. In this work, we found that JMJD3 is important for regulating the increased *Nlrp3* expression and the resulting enhanced inflammasome activation seen in chronic diabetic wounds. In diabetic wound Mφs, *Jmjd3* expression can be regulated by janus kinase/signal transducer and activator of transcription and MyD88 signaling (32,33). This pathway differs depending on the ligand and the downstream targeted genes of interest. This study suggests a role for IL-1α/IL-1R signaling via MyD88 in driving Mφ JMJD3-mediated NLRP3 inflammasome activity in diabetic wounds.

Similar to others, we show Mφs in diabetic wounds have increased *Nlrp3* expression and inflammasome activity, suggesting these cells are primed for enhanced inflammasome activation. Our data identify that keratinocytes within diabetic wounds late after injury can drive increased *Nlrp3* transcriptional activation in BMDMs and wound Mφs. While BMDMs display a stronger response, this difference may occur because BMDMs are stimulated with KCM from baseline. This increased priming of Mφs by diabetic wound keratinocytes results in enhanced inflammasome activation in the presence of pathogen-associated molecular patterns. To this end, diabetic patients exhibit increased polymicrobial colonization, which is associated with impaired healing (41). Neutrophils are also associated with contributing to Mφ NLRP3 inflammasome activation at the posttranslational modification level through the induction of reactive oxygen species in Mφs by the secretion of neutrophil extracellular traps (42), which are increased in diabetic injured skin (43).

Following injury, keratinocytes secrete NFkB-regulated cytokines and type I IFNs (T1IFN) that can regulate immune cell phenotype (7,21–25). Our group has previously shown that IFNκ, a keratinocyte-produced T1IFN, is increased early during normal wound healing and is decreased in diabetic wound keratinocytes (7). While this decreased keratinocyte IFNκ contributes to the impaired Mφ inflammatory phenotype seen initially in diabetic wounds, the factors driving the delayed chronic increase in Mφ inflammatory phenotype have remained undefined. This study demonstrates that IL-1α is increased in diabetic wound keratinocytes late following injury, which is significant since IL-1α is associated with driving skin inflammation (26,27,44). Our study suggests IL-1α drives inflammation through priming the Mφ NLRP3 inflammasome in diabetic wounds.

Although this study provides insight into the mechanisms behind the dysregulation of Mφ NLRP3 inflammasome activity in diabetic wounds, a few limitations must be addressed. First, we acknowledge that IL-1α is produced by other cell types, such as activated Mφs; however, nonhematopoietic cells are considered the primary source of pathogenic IL-1α, and we show it is elevated in diabetic wound keratinocytes. Second, while this paper focuses on changes in Mφ *Nlrp3* expression in diabetic wounds, we see a trending increase in *Nlrp3* expression in spleen Mφs; however, it is unclear whether keratinocytes are the source of this systemic effect. Third, we acknowledge that no cre-specific transgene line is perfect for Mφs, and that using the Lyz2Cre system generates a myeloid-specific KO of JMJD3. While this paper focused on Mφs, other cell interactions could also be affected within the Lyz2Cre system. Fourth, even though JMJD3 appears to regulate Mφ *Nlrp3* expression via an H3K27-mediated mechanism, we recognize that other epigenetic enzymes may also regulate *Nlrp3* gene expression. Finally, although IL-1α influences Mφ *Nlrp3* expression, further studies assessing the role of IL-1α in Mφs using ChIP sequencing and RNA sequencing would be useful to determine other genes influenced by IL-1α.

In summary, our study suggests an important role for keratinocytes within the diabetic wound environment in inducing Mφ NLRP3 inflammasome activity in chronic diabetic wounds. To our knowledge, this study is the first to examine keratinocyte-produced IL-1α in both normal and diabetic wounds. It provides important mechanistic information that IL-1R signaling regulates JMJD3-mediated *Nlrp3* transcriptional activation. Given that JMJD3 plays a central role in regulating NLRP3 inflammasome activity during wound healing, Mφ-specific targeting of JMJD3 as well as IL-1α/IL-1R signaling may be a viable therapeutic for decreasing NLRP3 inflammasome-mediated inflammation in chronic diabetic wounds.

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Author Contributions. S.J.W. designed the experiments, collected research data, and wrote, reviewed, and edited the manuscript. C.O.A., J.Y.M., A.D.J., W.J.M., E.C.B., K.M., G.S.d.J., S.R., S.B., Z.A., and R.W. contributed to research data, and reviewed and edited the manuscript. J.M.K. contributed to designing experiments, and reviewed and edited the manuscript. L.C.T. and J.E.G. contributed to research data, and reviewed and edited the manuscript. K.A.G. contributed to the experiment design, and wrote, reviewed, and edited the manuscript. S.J.W. and K.A.G. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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