



Published in final edited form as:

J Invest Dermatol. 2022 March ; 142(3 Pt A): 692–704.e14. doi:10.1016/j.jid.2021.08.428.

IL-10 Dysregulation Underlies Chemokine Insufficiency, Delayed Macrophage Response, and Impaired Healing in Diabetic Wound

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Abstract

Persistent inflammation is a major contributor to healing impairment in diabetic chronic wounds. Paradoxically, diabetic wound environment during the acute phase of healing is completely different in that it exhibits reduced macrophage response due to inadequate expression of CCL2 proinflammatory cytokine. What causes reduction in CCL2 expression in diabetic wound early after injury remains unknown. Here, we report that in contrast to prolonged exposure to high

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AUTHOR CONTRIBUTIONS: Conceptualization: SHS; Formal Analysis: SHS, RR, MB; Funding Acquisition: SHS; Investigation: RR, JZ, MFM, AA, KD; Methodology: RR, JZ, MFM, AA, KD, JW; Resources: SHS, TMK, JR; Supervision: SHS; Validation: RR, JZ, MFM, AA, KD; Visualization: RR, JZ, MFM, AA, KD; Writing - Original Draft Preparation: SHS, RR; Writing - Review and Editing: SHS, RR, JZ, MFM, AA, KD, MB, TMK, JR.

CONFLICT OF INTEREST: Rush University Medical Center has filed a patent on these findings. Drs. Sasha Shafikhani and Ruchi Roy are the listed inventors on this application.

Dr. Reiser reports personal fees from Biomarin, grants from National Institute of Health, grants from Nephcure, other from TRISAQ, grants from Thermo BCT, personal fees from Astellas, personal fees from Massachusetts General Hospital, personal fees from Genentech, personal fees from Up to Date, personal fees from Merck, personal fees from Inceptiosci, personal fees from GLG, Personal fees from Visterra outside the submitted work; In addition, Dr. Reiser has a patent US20110212083- Role of soluble uPAR in the Pathogenesis of Proteinuric Kidney Disease with royalties paid to TRISAQ, a patent US9867923-Reducing Soluble rokinase Receptor in the Circulation with royalties paid to Miltenyi, a patent JP2016530510-Non-Glycosylated suPAR Biomarkers and Uses thereof with royalties paid to TRISAQ, a patent US20160296592-Methods/Compositions for the Treatment of Proteinuric Diseases with royalties paid to TRISAQ, a patent US9144594-Dynamin Mediated Diseases with royalties paid to TRISAQ, and a patent US8809386-Dynamin Ring Stabilizers with royalties paid to TRISAQ.

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glucose which transforms monocytes proinflammatory, short-term exposure to high glucose causes a rapid monocyte reprogramming, manifested by increased expression and secretion of IL-10 which in an autocrine/paracrine fashion, reduces glucose uptake and transforms monocytes into anti-inflammatory phenotype by dampening signaling through toll-like receptors (TLRs). We show that IL-10 expression is significantly increased in diabetic wound during the acute phase of healing, causing significant reductions in TLR signaling and proinflammatory cytokines production, delaying macrophage and leukocyte responses, and underlying healing impairment in diabetic wounds. Importantly, blocking IL-10 signaling during the acute phase of healing improves TLR signaling, increases proinflammatory cytokines production, enhances macrophage and leukocyte responses, and stimulates healing in diabetic wound. We posit that anti-IL-10 strategies have therapeutic potential if added topically after surgical debridement process which resets chronic wounds into acute fresh wounds.

INTRODUCTION

Diabetic foot ulcers are the leading cause of lower extremity amputations in the United States (US), accounting for ~67% of all amputations and are responsible for more hospitalizations than any other complications of diabetes (Brem and Tomic-Canic, 2007, Sen et al., 2009). Persistent non-resolving inflammation, characterized by increased proinflammatory cytokines and leukocytes (e.g., macrophages), is a major contributing factor to impaired healing in diabetic foot ulcers (Bjarnsholt et al., 2008, Blakytyn and Jude, 2006). Paradoxically, diabetic wound environment is completely different during the acute phase of healing in that it suffers from inadequate macrophage response, due to dampened expression of CCL2 proinflammatory cytokine (Ishida et al., 2019, Wood et al., 2014). In line with these findings, diabetic monocytes extracted from diabetic patients also exhibit impaired CCL2 expression (Abke et al., 2006). What causes reductions in CCL2 expression and macrophage trafficking in diabetic wound during the acute phase of healing remains unknown, prompting us to revisit this issue.

RESULTS

Proinflammatory cytokine expression is substantially reduced during the acute phase of wound healing early after injury.

CCL2 is one of several chemokines that can recruit monocytes into the wound and promote their differentiation into macrophages (Snyder et al., 2016). The fact that macrophage influx is substantially reduced in diabetic wounds early after injury (Wood et al., 2014) suggested that the expression of other proinflammatory cytokines may be similarly affected in these wounds early after injury. We generated full-thickness excisional wounds in C57B (normal) and db/db (type 2 obese diabetic) mice, as described (Goldufsky et al., 2015, Wood et al., 2014), and assessed them for their IL-1 α and TNF- β proinflammatory cytokines - which are expressed early after injury in normal wound (Hubner et al., 1996, Ridiandries et al., 2018) - by ELISA.

Mirroring the macrophage response delay (Wood et al., 2014), IL-1 α and TNF- β levels were significantly lower in db/db diabetic wounds early after injury on days 1 and 3 but increased

over time reaching their highest levels in day 10 (Figure S1a). Supporting these data, the mRNA levels of IL-1 and TNF, as assessed by RT-PCR (after normalizing the data to 18S to account for reduced macrophage response), were also significantly reduced in day 1 db/db wounds as compared to normal wounds (Figure S1b). Moreover, the expression of CCL2, G-CSF, and GM-CSF - other cytokines that are produced early in wounds (Dipietro et al., 2001, Huang et al., 2017, Mann et al., 2006) - were also significantly reduced in day 1 db/db wounds, as assessed by RT-PCR and Western blotting (Figure S1b-d). Collectively, these data indicated that diabetic wounds suffer from insufficient chemokines during the acute phase of wound healing early after injury. However, it remained unclear why the production of chemokines is dampened in diabetic wounds early after injury.

Toll-like receptor signaling is significantly diminished during the acute phase wound healing in diabetic wound.

Toll-like receptors (TLRs) are major factories for the production of proinflammatory cytokines, including CCL2, and play critical roles in wound healing (Akhter et al., 2018, Chen and DiPietro, 2017, Kluwe et al., 2009, Lin et al., 2011, Macedo et al., 2007, Sato et al., 2010, Serbina et al., 2012, Suga et al., 2014). Diabetic chronic wounds show elevated TLR signaling (Dasu et al., 2010a), but little is known about TLR signaling in diabetic wound early after injury. Data indicated that the expression of representative TLRs (TLR1, TLR2, and TLR4) and TLR signaling components, (MyD88, TRAF6, and NF- κ B (Kawasaki and Kawai, 2014)) were all significantly reduced in day 1 diabetic wound as compared to normal wound (Figure 1a, and Figure S2a). Corroborating these data, mRNA analyses and histological assessments of representative TLR signaling components (TLR4 and MyD88) also showed significant reductions in their expressions in day 1 diabetic wounds (Figure 1b-e). These data indicated that TLR signaling is dampened in diabetic wound early after injury. However, it remained unclear what was causing the reduction in the TLR signaling in diabetic wound early after injury.

Short-term exposure to high glucose leads to impaired TLR signaling and reduced chemokine expression in human and murine monocytes.

Circulating monocytes migrate into wound tissues after injury (Schulz et al., 2012). They are generated primarily in the bone marrow and released into the circulation rapidly within 1-3 hours, and injury and/or infection further accelerate their release from bone marrow and their trafficking into injured tissue and/or toward infection (Goto et al., 2003, Zhao et al., 2018). In the absence of injury and/or infection, these classical monocytes circulate in the blood with a lifespan of ~24h (Daley et al., 2010, Patel et al., 2017). Leukocytes (including monocytes/macrophages) extracted from the blood of diabetic patients or diabetic animals have functional impairments, including in their chemotaxis, and these impairments have been attributed to prolonged exposure to HG (Delamare et al., 1997, Hill et al., 1983). In contrast, leukocytes extracted from bone marrow of diabetic animals appear to be normal (Park et al., 2009, Repine et al., 1980, Scully et al., 2017, Sima et al., 1988), suggesting that bone marrow environment may be protective against the harmful effects of prolonged exposure to HG. Because of chemotaxis impairment in circulating monocytes, we posited that newly released monocytes from bone marrow would likely be the monocytes migrating into the wound early after injury and these monocytes would be exposed to

HG for short time (~1-3 hours). Long-term (24-72 hours) exposure to HG transforms monocytes/macrophages proinflammatory (Hotamisligil et al., 1995, Yaghini et al., 2011), as we confirmed (Figure S2b-c), but the impact of short-term acute exposure on inflammatory responses in monocytes has not been assessed.

We purified human Peripheral Blood Monocytes (hPBMCs) from non-diabetic individuals and Bone Marrow Derived Monocytes (mBMDMs) from C57B mice, exposed them to glucose levels in the normal range (90mg/dL) or diabetic range (300mg/dL) for 1h, and assessed the impact of HG on the expression of TLR signaling components and proinflammatory cytokines. Acute (1h) exposure to HG caused significant reductions in the mRNA levels of all indicated genes in hPBMCs and mBMDMs (Figure 1f and Figure S2e). Similarly, the corresponding proteins were also reduced in mBMDMs and hPBMCs, except MyD88 which was significantly reduced in mBMDMs but not in hPBMCs, suggesting that MyD88 protein stability/turnover may be differentially regulated in hPBMCs and mBMDMs (Figure 1g, and Figure S2d, S2f, and S2g). Collectively, these data indicated that, in complete contrast to long-term exposure to HG which transforms monocytes proinflammatory, short-term acute exposure to HG causes reductions in TLR-mediated signaling and proinflammatory cytokines. However, what mediated the reduction in TLR expression and in proinflammatory cytokines production in monocytes after short-term exposure to HG remained unknown.

IL-10 expression is significantly increased during the acute phase of healing in diabetic wounds and in response to short-term exposure to high glucose.

IL-10 is a potent immunosuppressive cytokine that inhibits TLR signaling and proinflammatory cytokines' production through multiple mechanisms (Curtale et al., 2013, Knödler et al., 2009, Murray, 2005, Wang et al., 1995). Interestingly, IL-10 also inhibits glucose uptake in lipopolysaccharide (LPS)-stimulated macrophages (Ip et al., 2017), linking IL-10 to glucose metabolism. Moreover, long-term (1-5 days) exposure to HG causes mitochondrial damage and culminates in proinflammatory responses (Devi et al., 2013, Park and Park, 2013). We postulated that in diabetic environment where glucose is in excess, IL-10 may be upregulated as a protective measure to prevent excess glucose uptake and to protect monocytes from HG-induced cellular damage.

We first measured IL-10 levels in normal and diabetic wounds. IL-10 levels were significantly higher in diabetic wounds early after injury on days 1 and 3, but significantly lower on day 10 (Figure 2a). IL-10 mRNA and protein level assessments corroborated the ELISA data, showing that IL-10 was significantly upregulated in day 1 diabetic wounds (Figure 2b-d). Further corroborating these data, 1h exposure to HG also caused significant increases in IL-10 mRNA and IL-10 protein levels in both cell lysates and culture supernatants of monocytes (Figure 2e-g and Figure S3a-c). Interestingly, IL-10 could not be detected in monocytes or in their culture supernatant after 24h exposure to HG (Figure S2b), suggesting that IL-10 expression is transient and elevated IL-10 in the supernatant of monocytes acutely exposed to HG, is consumed or degraded during prolonged exposure to HG.

Consistent with our hypothesis, glucose uptake, as determined by 2-NBDG fluorescent D-glucose analog, was significantly reduced in monocytes after 1h exposure to HG (Figure 2h), while blocking IL-10 signaling by anti-IL-10 antibody (α -IL10) increased glucose uptake in monocytes acutely exposed to HG (Figure 2i). Moreover, blocking IL-10 signaling by α -IL10 antibody also significantly increased the expression of representative TLR signaling components, and enhanced proinflammatory cytokines' production in hPBMCs and mBMDMCs under 1h exposure to HG (Figure 3 and Figure S4), but not in primary epidermal keratinocytes or dermal endothelial cells (Figure S5), which do not express IL-10 (Fiorentino et al., 1989, Li et al., 1999, Moore et al., 2001, Park and Barbul, 2004), indicating that IL-10 autocrine/paracrine signaling is responsible for reducing glucose uptake and dampening TLR signaling in monocytes exposed acutely to HG.

Importantly, blocking IL-10 signaling by α -IL10 antibody significantly reduced IL-10 levels in diabetic wounds (Figure 4a), and increased the expression of indicated TLR components in diabetic wounds early after treatment, albeit with varying kinetics (Figure 4b and Figure S6). We further corroborated these data by assessing the expression of representative TLR signaling components (TLR4 and MyD88) in day 1 wounds treated with α -IL10 by mRNA analysis and by histological analysis (Figure 4c-e).

Blocking IL-10 stimulates healing in diabetic wound.

As compared to α -IgG-treated wounds, diabetic wounds treated with α -IL-10 antibody showed significant increases in IL-1 β and TNF- α early after treatment within the first 5h, but significantly lower levels in day 10 older wounds (Figure 5a-b). CCL2 expression showed similar pattern, except that it also remained higher in day 1 db/db wounds treated with α -IL-10 but by day 10, CCL2 expression was also significantly diminished in these wounds (Figure 5c). Similarly, leukocyte numbers - as assessed by immunohistochemistry, using Hematoxylin Eosin (H&E) staining (Goldufsky et al., 2015, Wood et al., 2014) - increased significantly early after injury but decreased in day 10 wounds in α -IL10-treated diabetic wounds (Figure 5d-e and Figure S7).

Macrophages are generally classified as either classically activated proinflammatory macrophages (M1) or alternatively activated anti-inflammatory and reparative macrophages (M2), although other M2 macrophage subtypes have also been described (Ferrante and Leibovich, 2012, Loegl et al., 2016). In normal healing, M1 macrophages predominate early after injury and contribute to inflammatory responses, whereas M2 macrophages predominate the wound as it transitions into the proliferation phase and play critical roles in new tissue regeneration and remodeling phases (Krzyszczuk et al., 2018, Mirza et al., 2009). We assessed the impact of IL-10 inhibition on the dynamics and distribution of M1 and M2 macrophages by co-staining the wound tissues with monocyte/macrophage marker F4/80 either with M1 macrophage marker iNOS (inducible nitric oxide synthase), or with M2 macrophage marker MMR/CD206 (Bastian et al., 2018, Klar et al., 2018). Treatment with α -IL-10 significantly increased both M1 and M2 macrophages in diabetic wounds on days 1 and 3 (Figure 5f-i and Figure S8). However, M1 macrophages were significantly reduced in α -IL-10-treated day 10 diabetic wounds and the remaining macrophages were of the M2 type, as assessed by M1/M2 ratios (Figure 5i-j).

Importantly, treatment with α -IL-10 antibody significantly improved healing in diabetic wounds, as assessed by digital photography and histological analyses of re-epithelization and epidermal thickening assessment using H&E staining (Figure 6a-d and Figure S9a). Persistent inflammatory environment in diabetic chronic wounds adversely affects fibroblast and myofibroblasts functions resulting in reduced collagen and elastin extracellular matrix deposition in diabetic chronic wounds (Augustine et al., 2014, Diegelmann and Evans, 2004, Yue et al., 1986). α -IL-10-treated diabetic wounds showed significant increases in fibroblast, myofibroblast, elastin, and collagen healing markers in day 10 diabetic wounds (Figure 6e-f and Figure S9b), as assessed by IHC, using their respective markers Vimentin, α -SMA, Elastin, and Masson's Trichrome staining (Goldufsky et al., 2015, Hinz, 2006, Wilgus et al., 2008).

Corroborating these data, blocking IL10 signaling by treating diabetic wounds with anti-IL10 receptor antibody (α -IL10R) also yielded similar results, showing significant increases in representative TLR components (TLR4 and MyD88) in day 1 diabetic wound (Figure S10a-b), and higher levels of CCL2 in day 1 and day 3 wounds, but significantly lower levels of CCL2 in day 6 and day 10 old wounds (Figure S10c). Diabetic wounds treated with α -IL10R antibody also contained significantly more leukocytes, as assessed by H&E staining, and more macrophages as assessed by IHC using macrophage marker anti-CD68 antibody (Wood et al., 2014), on days 1 and 3 (Figure S10d-g) and healed significantly better than (α -IgG-treated diabetic wounds (Figure S11). Collectively, these data indicated that diabetic wounds will not develop persistent non-resolving inflammation and heal significantly better, if IL-10 signaling is disrupted in these wounds early after injury.

DISCUSSION

Here we report that acute exposure (1h) to HG causes a rapid transformation of human and mouse monocytes into an anti-inflammatory phenotype, manifested by increased expression and secretion of IL-10, which in an autocrine/paracrine fashion, causes reduction in TLR signaling and proinflammatory cytokines production in hPBMCs and mBMDMs. We posit that the rapid rise in the IL-10 expression and secretion in response to acute exposure to HG is a protective measure taken by monocytes to reduce glucose uptake (as our data show) in order to prevent cellular and organelle damage which has been shown to occur after prolonged exposure to HG (Allen et al., 2003, Kumar and Sitasawad, 2009, Vanhorebeek et al., 2009).

Our data indicate that inadequate inflammatory responses in diabetic wound during the acute phase of healing early after injury is a major contributing factor to impaired healing in diabetic wound. Consistent with this notion, prospective studies involving diabetic individuals with healing and non-healing foot ulcers have found elevated proinflammatory characteristics, (e.g., higher M1/M2 macrophage and enhanced NF- κ B signaling network), at initial visit (early after injury) as prognostic markers for healing DFUs, whereas these markers were lower at the initial visit but continued to increase in non-healing DFUs (Nassiri et al., 2015, Theocharidis et al., 2020).

Our data further indicate that increased IL-10 expression in diabetic wound early after injury is a major factor, contributing to impaired healing in diabetic wound. IL-10 knockout mice exhibit accelerated healing, albeit healing is associated with increased extracellular deposition and scarring (Eming et al., 2007, Liechty et al., 2000), suggesting that IL-10 is a negative regulator of scarring during wound healing. Interestingly, diabetic chronic wounds exhibit inadequate extracellular matrix deposition and reduced scarring (Augustine et al., 2014, Diegelmann and Evans, 2004, Yue et al., 1986), suggesting that reduced scarring in DFUs may be due to increased IL-10.

There appears to be a disconnect between our data showing increased IL-10 expression and reduced proinflammatory cytokine production resulting from short-term exposure to HG and the clinical reports indicating decreased IL-10 levels and increased proinflammatory cytokines in the blood of diabetic individuals, and proinflammatory phenotype in monocytes/macrophages after prolonged exposure to HG (Hotamisligil et al., 1995, Keane et al., 2017, Yaghini et al., 2011). Circulating classical monocytes have 24h lifespan (Daley et al., 2010, Patel et al., 2017), thus they have prolonged exposure to HG in diabetic individuals. We also find that 24h exposure to HG transforms monocytes proinflammatory but interestingly, no IL-10 could be detected in these cells or in their culture supernatants. We propose that despite initial increases in IL-10 expression and secretion in monocytes exposed to HG, prolonged exposure to HG allows for gradual increases in cytosolic glucose levels, which in turn causes mitochondrial damage (Devi et al., 2013, Freerman et al., 2014, Ip et al., 2017, Park and Park, 2013), which eventually shuts down IL-10 expression and drives monocytes/macrophages toward proinflammatory phenotype. More studies are needed to determine the underlying causes of monocyte's biphasic and opposite responses to short-term versus long-term exposure to HG, and how these responses may lead to insufficient inflammatory responses in diabetic wounds early after injury, and persistent non-resolving inflammation as these become chronic.

Previous reports by Dasu *et al.*, (Dasu and Jialal, 2013, Dasu et al., 2010b) have shown increased expression of TLR2, TLR4, TLR6 and proinflammatory cytokines in streptozotocin (STZ)-induced type 1 diabetic mice at day 10 post-wounding. They also provided bar graphs to show accelerated healing in STZ-induced TLR2^{-/-} or TLR4^{-/-} diabetic mice. Disappointingly, in these reports there were no histological or photographic data to corroborate their accelerated healing data. Our data support their finding with respect to the heightened inflammatory environment in day 10 diabetic wounds. However, our data highlight the need for proinflammatory responses during the acute phase of healing for proper healing in diabetic wounds. Our findings are in line with the preponderance of evidences that point to a central role for inflammatory responses and TLR signaling in mediating effective wound healing (Chen and DiPietro, 2017, Goren et al., 2009, Leibovich and Ross, 1975, Lucas et al., 2010, Maruyama et al., 2007, Mirza et al., 2009, Portou et al., 2015).

Our data indicates that blocking IL-10 signaling resulted in significant increases in both M1 and M2 macrophages early after injury, as assessed by F4/80 staining in combination with iNOS or MMR/CD206 respectively. It remains unclear whether the monocytes trafficking into diabetic wounds after IL-10 signaling blockade originated from circulation

(hematopoietic origin) or from skin resident monocytes (yolk-sac origin). Previously, circulating monocytes/macrophages from hematopoietic origin were shown to be F4/80^{lo} whereas skin resident monocytes originating from yolk sac were found to be F4/80^{high} (Schulz et al., 2012), thus suggesting that these macrophages may be originating from skin. However, more recent studies have shown that circulating monocytes from hematopoietic origin populate wound initially after injury and these monocytes/macrophages also express F4/80 (Castela et al., 2017, Crane et al., 2014, Hopkinson-Woolley et al., 1994, Li et al., 2008, Maruyama et al., 2007, Wood et al., 2014). Interestingly, Francke *et al.*, reported that while bone marrow monocytes are initially F4/80^{lo}, they become F4/80^{high} after adhering to the surface in the presence of M-CSF (Francke et al., 2011), suggesting that bone marrow/hematopoietic monocytes have the capacity to become F4/80^{high} after populating the wound. More detailed studies are needed to determine the source(s) of monocytes populating the diabetic wound after IL-10 signaling blockade.

Encouragingly, we found that one-time topical treatment with anti-IL-10 or anti-IL-10R antibodies significantly enhanced inflammatory responses during the acute phase of healing and substantially improved healing in diabetic wounds. Given that diabetic foot ulcers suffer from persistent inflammation, one might question the therapeutic value of anti-IL-10 treatments in diabetic foot ulcers. We propose that anti-IL-10 strategies may have therapeutic potential in diabetic wound care, (at least in a subset of obese type 2 diabetic individuals which our model represents), if applied topically after the surgical wound debridement, which is performed as a standard of care weekly or biweekly to reset a chronic non-healing ulcer into an acute fresh wound (Cardinal et al., 2009, Golinko et al., 2008, Lebrun et al., 2010), which we propose to be more similar to the day 1 wound environment in diabetic mice.

MATERIALS AND METHODS (Additional experimental details are provided in Supplementary Materials & Methods)

Animals:

We have an approval from the Rush University Medical Center Institutional Animal Care and Use Committee (IACUC No: 18-037), which allow us to conduct research as indicated. We obtained 8-10 weeks old C57BL/6 (normal) and their diabetic littermates, C57BLKS-m *leprdb* (db/db), mice from the Jackson Laboratories (Bar Harbor, ME). Wounding was carried out as we described previously (Goldufsky et al., 2015, Wood et al., 2014).

MONOCYTES ISOLATION FROM HUMAN AND MOUSE:

We have an Institutional Review Board (IRB)- approved protocol (ORA #: 16120704-IRB02) from Rush University Medical Center which allows us to collect blood samples from volunteers with their informed written consents for these studies. Human monocytes from healthy subjects (both male and female) were purified from peripheral blood using the EasySep™ Human Monocytes Enrichment Kit (STEMCELL Technologies), according to manufacturer's protocol. Mouse monocytes were extracted from either bone marrow using the EasySep™ Mouse Monocytes Enrichment Kit (STEMCELL Technologies), as per manufacture's protocol.

HISTOPATHOLOGICAL EVALUATION by H&E Staining:

Leukocytes in wounds were identified by their rounded or polymorphonuclear morphology as described previously (Goldufsky et al., 2015, Kroin et al., 2016). Macrophages were assessed by immunohistochemical (IHC) analysis using monocyte/macrophage markers F4/80 with iNOS (M1 macrophages) or F4/80 with MMR/CD206 (M2 macrophages), or macrophage marker CD68 as described (Lucas et al., 2010, Wood et al., 2014). Wound tissues' contents of IL-1 β , TNF- α and CCL2 were assessed by ELISA, as described (Gupta et al., 2017). Wound healing was assessed by digital photography, by re-epithelization assessment using H&E staining, and by histological assessments of elastin, vimentin, α -SMA, and collagen matrix deposition, as described (Almine et al., 2012, Goldufsky et al., 2015, Wilgus et al., 2008, Wood et al., 2014).

GLUCOSE UPTAKE:

Glucose uptake in monocytes was assessed by EZCell™ Glucose Uptake Assay Kit (Cat. No. K924 from BioVision) according to the manufacturer's protocol.

WESTERN BLOTTING:

We performed Western immunoblotting on cell lysates or on tissue lysates, using the indicated antibodies, as we described previously (Kroin et al., 2016, Shafikhani and Engel, 2006).

GENE EXPRESSION ANALYSIS BY REAL-TIME POLYMERASE CHAIN REACTION (RT-PCR):

Gene expression at transcriptional level in wound tissues was assessed by mRNA analysis of indicated genes using RT-PCR, as described (Gupta et al., 2017, Wood et al., 2014).

STATISTICAL ANALYSIS

was performed as we described previously (Goldufsky et al., 2015, Gupta et al., 2017, Wood et al., 2014). Briefly, we applied One-way ANOVA with Bonferroni post hoc testing for statistical analyses involving multiple groups. For pair-wise comparisons between any two groups, we used unpaired Student's *t*-test. Tabulated data were presented as the Mean \pm SEM. *P*-values less than or equal to 0.05 were taken as significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS:

We would like to thank Dr. Luisa DiPietro (University of Illinois Chicago) and Dr. Aristatis Veves (Beth Israel Deaconess Medical Center) for their feedbacks and insights into these projects. We would like to thank the rest of Shafikhani lab for their valued opinions on these studies. This work was supported by the National Institutes of Health (NIH) grants RO1DK107713 to (S.H.S.), F31DK118797 to (J.Z.), and the institutional NIH training grant R25109421.

DATA AVAILABILITY STATEMENT:

No datasets were generated or analyzed during this study.

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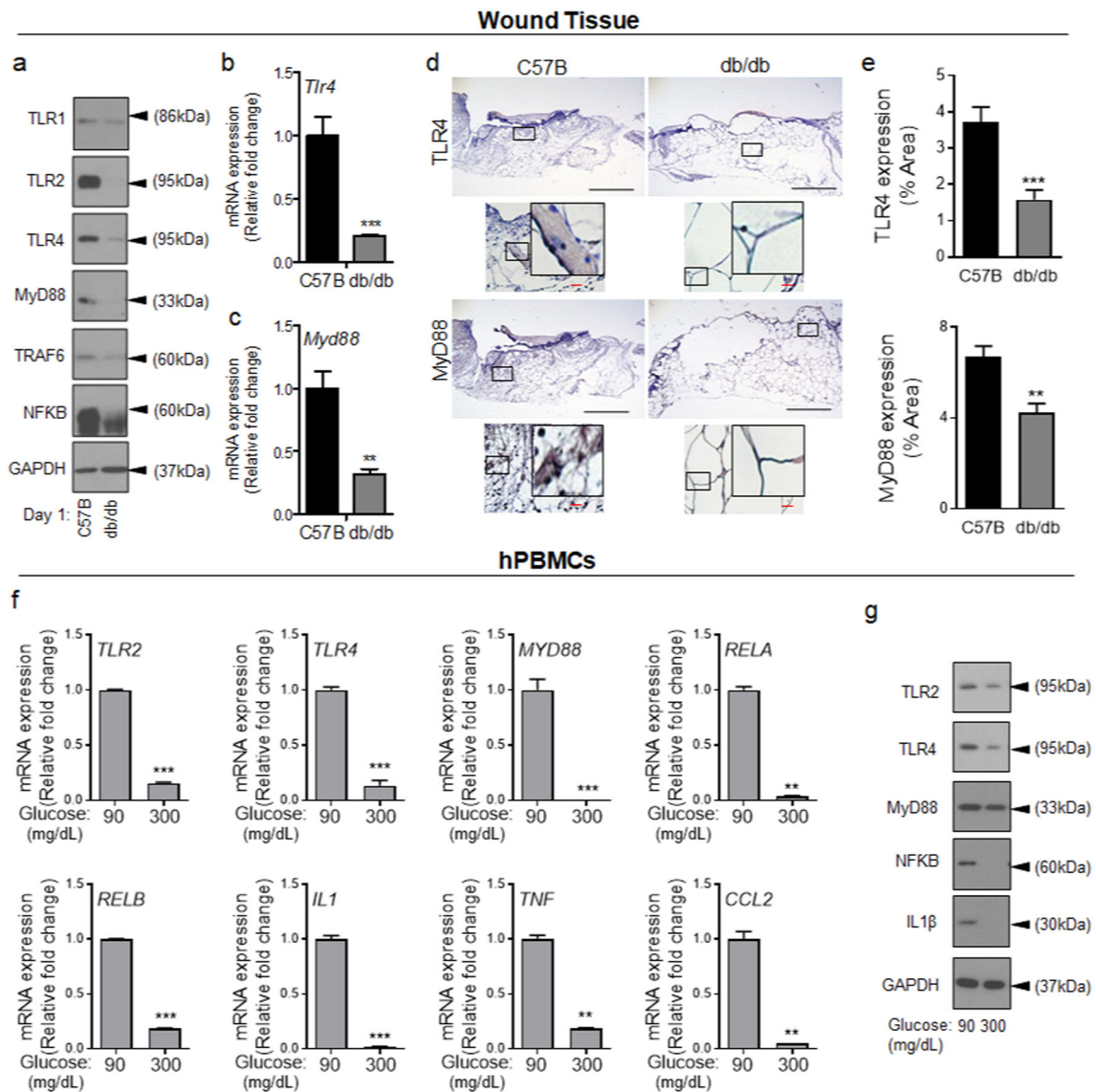


Figure 1. Toll-like receptor signaling is diminished during the acute phase wound healing in diabetic wound and in response to short-term exposure to HG.

C57B and db/db wounds were collected 24h after wounding and assessed for the expression of indicated genes by Western blotting (a), by mRNA analysis using RT-PCR (b-c), and by immunohistochemistry (d-e). Representative images are shown in (d) and the corresponding data in (e). (N=5 mice/group, 9 random fields/wound/mouse. Black scale bars=500 μ m; Red scale bars=50 μ m). (e-f) hPBMCs were exposed to 90mg/dl or 300 mg/dl for 1h and assessed for the expression of indicated genes by mRNA (f), or by Western blotting (g). Data are plotted as mean \pm SEM. (Each experiment was repeated at least 2 times for (e) and at least 3 times for (f). *p<0.05, **p<0.01, ***p<0.001).

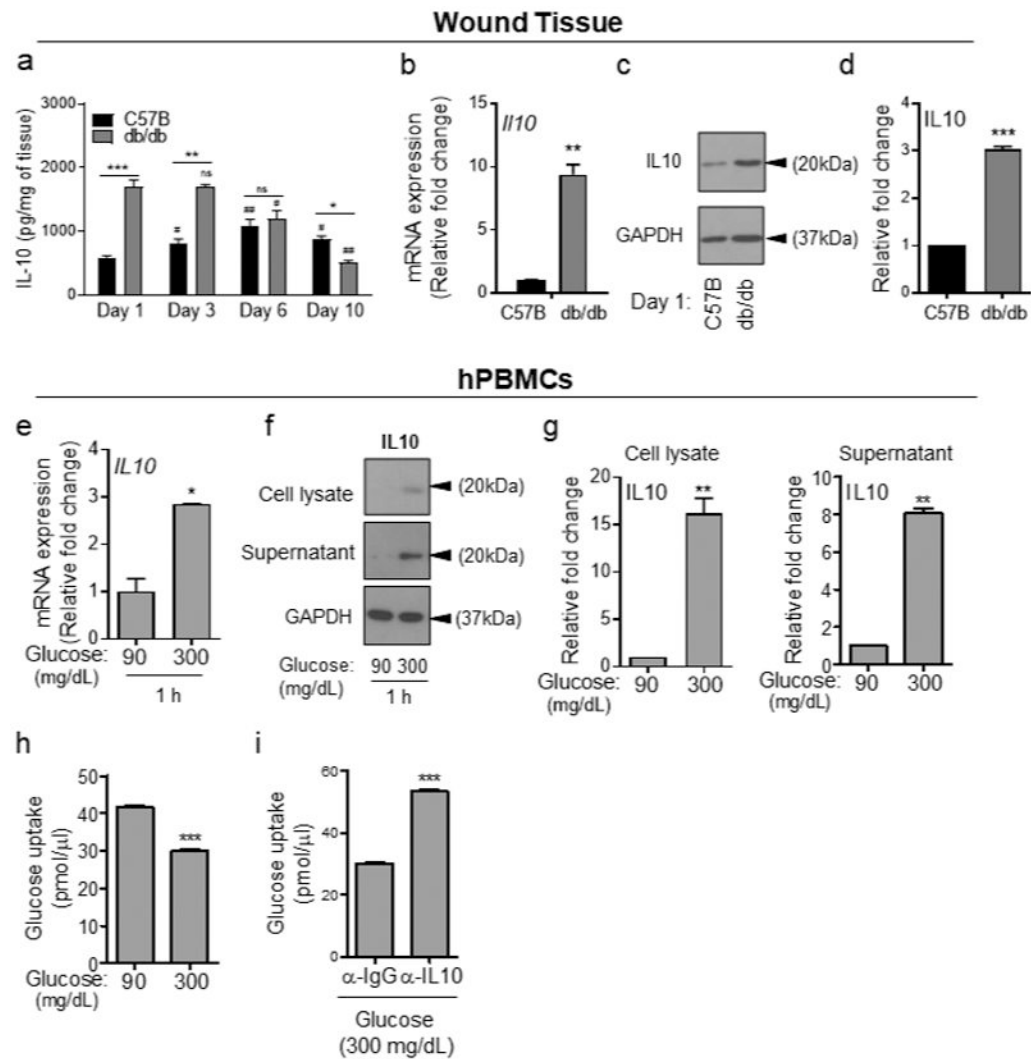


Figure 2. IL-10 expression is significantly increased during the acute phase of healing in diabetic wounds and in response to short-term exposure to high glucose.

(a) C57B and db/db wounds at indicated times were assessed for IL10 levels by ELISA. (b-d), day 1 wounds were assessed for IL10 by mRNA (b), and by Western blotting (c) and corresponding densitometer of Western blot is shown in (d). (e-h) hPBMCs were exposed to 90 or 300 mg/dl glucose for 1h and analyzed for IL10 expression by mRNA (e), by Western blotting (f-g), or for glucose uptake using 2-NBDG assay in the absence or presence of 5 μ g/ml anti-IL-10 antibody (α -IL-10) (h-i). Data are plotted as the mean \pm SEM. (N 3 mice/ group (a-d); for (e-h), N 3 independent times; *p<0.05; **p<0.01; ***p<0.001).

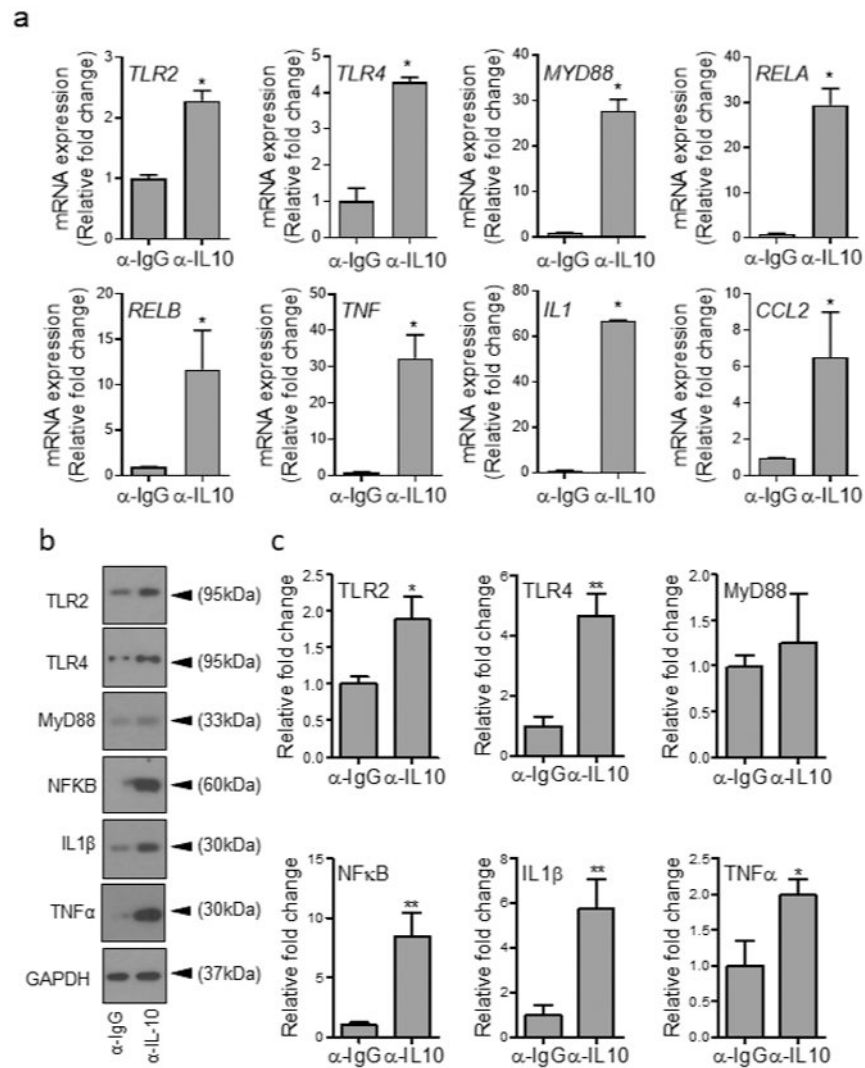


Figure 3. Blocking IL-10 signaling enhances TLR signaling and proinflammatory cytokines' production in PBMCs in the presence of high glucose.

(a-c) hPBMCs were exposed to high glucose (300 mg/dl) for 1h in the presence of either α -IL-10 or α -IgG antibodies at 5 μ g/ml and assessed for mRNA levels of indicated genes using RT-PCR (a), or by protein analyses using Western blotting (b) and corresponding densitometer tabulated data for Western blots are in (c). All data are plotted as the mean \pm SEM. (Each experiment was repeated at least 2 times for (a) and at least 3 times for (b-c); * p <0.05, ** p <0.01, *** p <0.001. Pair-wise statistical analyses between groups were performed by unpaired Student's t -test).

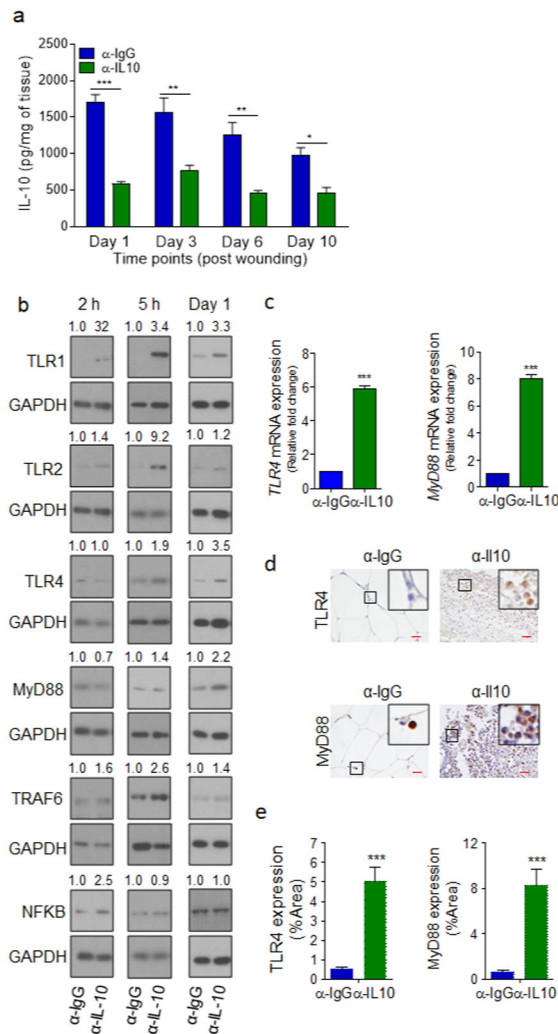


Figure 4. Blocking IL-10 signaling dampens IL-10 and enhances TLR signaling and proinflammatory cytokines production in diabetic wound.

(a-e) db/db wounds were treated topically with α -IgG or α -IL10 antibodies (10 μ g/wound), immediately after wounding. Wound tissues were analyzed at indicated timepoints for IL-10 by ELISA (a), for the expression of indicated genes by Western blotting (b), by RT-PCR (c), by IHC (d), and the corresponding data for (d) are plotted as the mean \pm SEM in (e). (N=5 mice/group for all experiments. For (d-e) 9 random fields/wound/mouse, red scale bars=50 μ m. Data were then normalized per wound area and plotted as mean \pm SEM, *p<0.05, ** p<0.01, ***p<0.001, Statistical analyses between groups were performed by One-way ANOVA, and pair-wise comparisons within groups were performed or by unpaired Student's *t*-test).

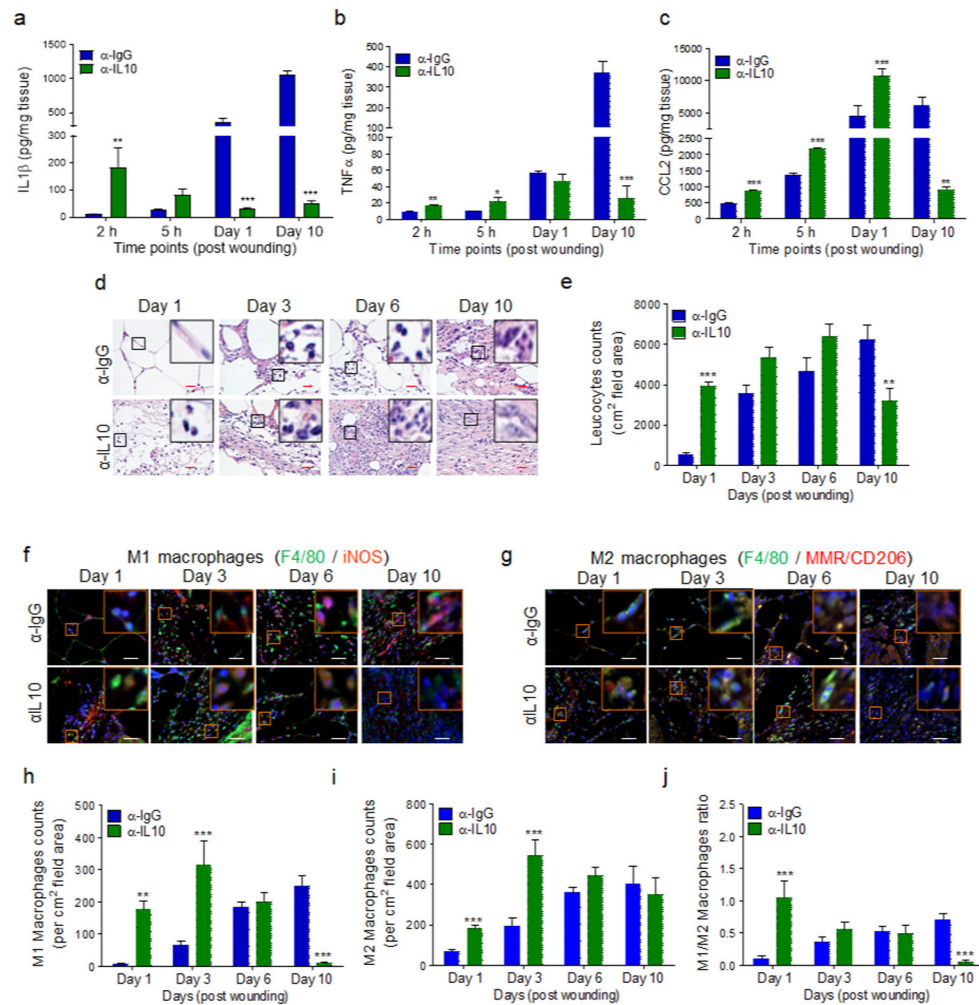


Figure 5. Blocking IL-10 signaling enhances inflammatory responses during the acute phase of wound healing.

db/db wounds were treated with α -IgG or α -IL10 (10 μ g/wound) after wounding and assessed at indicated timepoints for indicated proinflammatory cytokines (a-c), for leukocytes by H&E staining (d-e), and for M1 and M2 macrophages, by IF microscopy using F4/80/iNOS or F4/80/MMR/CD206 respectively (f-g). The corresponding data of (d, f, & g) are plotted as mean \pm SEM (Red scale bars=50 μ m, White scale bar= 50 μ m) in (e, h, & i) respectively. M1/M2 macrophage ratios are shown in (j). (N=5 mice/group; 9 random fields/wound/mouse). The number of leukocytes and macrophages were normalized per wound area. *p<0.05, ** p<0.01, ***p<0.001. Statistical analyses between groups were performed by One-way ANOVA, and pair-wise comparisons within groups were performed or by unpaired Student *t*-test).

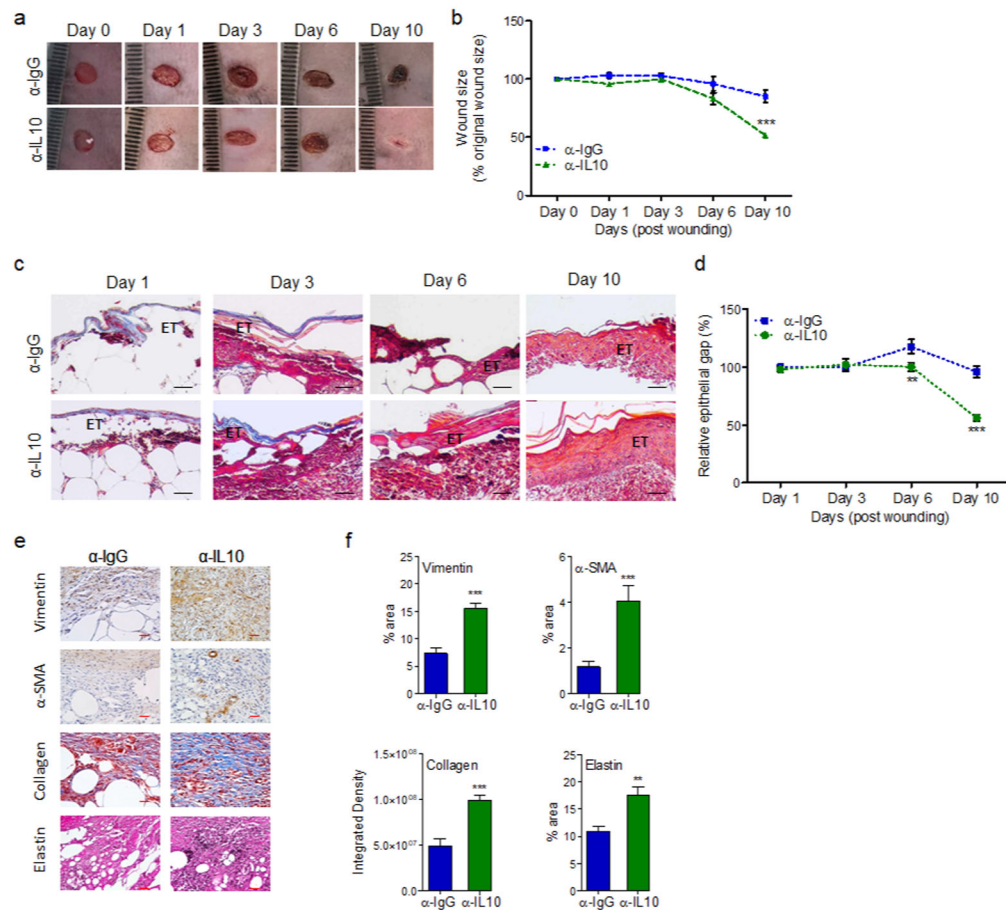


Figure 6. Blocking IL-10 signaling stimulates wound healing in diabetic wound.

db/db wounds were treated with either α -IgG or α -IL10 (10 μ g/wound) and assessed for wound healing by digital photography (a-b); by the histochemical assessment of re-epithelization and epidermal thickening, using H&E staining (c & d); and by histochemical assessments of the Vimentin, α -SMA, Masson's Trichrome staining, and Elastin healing markers (e-f). Representative images are shown in (a, c, & e) and the corresponding data are plotted as mean \pm SEM in (b, d, & f). Black scale bars=100 μ m, red scale bars=50 μ m. (N 5 mice/group, 9 random fields/wound/mouse, *p<0.05, ** p<0.01, ***p<0.001. Statistical analyses between groups were performed by One-way ANOVA, and pair-wise comparisons within groups were performed or by unpaired Student's *t*-test).

RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
NF- κ B p65 (D14E12) XP® Rabbit mAb	Cell Signaling Technology	Cat. No. 8242
IL-1 β (3A6) Mouse mAb	Cell Signaling Technology	Cat. No. 12242
F4/80 (D2S9R) XP® Rabbit mAb	Cell Signaling Technology	Cat. No. 70076
Mouse (G3A1) mAb IgG1 Isotype Control	Cell Signaling Technology	Cat. No. 5415
GAPDH Antibody Rabbit Polyclonal	Proteintech	Cat. No. 1094-I-AP
MCP-1 Antibody (Mouse Specific)	Cell Signaling Technology	Cat. No. 2029
TLR1 Rat anti-Mouse, Clone: 285923	R & D systems	Cat. No. MAB1475
TLR2 Goat anti-Mouse, Polyclonal	R & D systems	Cat. No. AF1530
TLR4 Antibody (25)	Santa Cruz Biotechnology	Cat. No. 293072
IL-10 Antibody (E-10)	Santa Cruz Biotechnology	Cat. No. 8438
TLR2 Antibody (TL2.1)	Santa Cruz Biotechnology	Cat. No. 21759
TNF alpha Antibody	Santa Cruz Biotechnology	Cat. No. 52746
Anti-MyD88 antibody	Abcam	Cat. No. ab135693
Anti-IL-10 antibody [JES5-2A5]	Abcam	Cat. No. ab33471
InVivoMab anti-mouse IL-10R (CD210), clone: 1B1.3A	InVivoMab Antibodies	Cat. No. BE0050
Recombinant Anti-G-CSF antibody [EPR3203(N)(B)]	Abcam	Cat. No. ab181053
TRAF6 Polyclonal Antibody	Invitrogen	Cat. No. 38-0900
GM-CSF Monoclonal Antibody (22E9)	Invitrogen	Cat. No. MM500C
IL-10 Monoclonal Antibody (JES3-9D7)	Invitrogen	Cat. No. AHC0102
Mouse (MOPC-21) mAb IgG1 Isotype Control	Cell Signaling Technology	Cat. No. 4097
InVivoMab anti-mouse IL-10, Clone: JES5-2A5	InVivoMab Antibodies	Cat. No. BE0049
anti-horseradish peroxidase, Clone: HRPN	InVivoMab Antibodies	Cat. No. BE0088
F4/80 (D2S9R) XP® Rabbit mAb	Cell Signaling Technology	Cat. No. 70076
MMR/CD206/Mannose Receptor Antibody	Novus Biologicals	Cat no. NBP1-90020
iNOS Antibody	Novus Biologicals	Cat no. NBP2-22119
F4/80 Antibody (C-7)	Santa Cruz Biotechnology	Cat no. 377009
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594	Thermo Fisher	Cat no. A11-012
Alexa Fluor® 488 AffiniPure Goat Anti-Mouse IgG (H+L)	Jackson Immuno Research	Cat no. 115-545-062
Anti- α -SMA antibody	Abcam	Cat. No. ab5694
Anti-vimentin antibody	Abcam	Cat. No. ab92547
Reagents and kits		
Hematoxylin	Thermo Fisher	Cat. No. 7111L
Eosin Y	Thermo Fisher	Cat. No. 7211L
Bluing Reagent	Thermo Fisher	Cat. No. 7301L
Masson's Trichrome stain	Abcam	Cat. No. ab150686
EasySep Human Monocytes Enrichment Kit	STEMCELL Technologies	Cat. No. 19359

REAGENT or RESOURCE	SOURCE	IDENTIFIER
EasySep Mouse monocytes Enrichment Kit	STEMCELL Technologies	Cat. No. 19861
EasySep Buffer	STEMCELL Technologies	Cat. No. 20144
SYBR™ Green PCR Master Mix	Thermo Fisher	Cat. No. 4309155
SuperScript™ III First-Strand Synthesis System	Thermo Fisher	Cat. No. 18080051
Elastic connective tissue stain	Abcam	Cat. No. ab150667
Critical Commercial Assays		
CCL2 ELISA kit	Thermo Fisher	Cat. No. 88-7391-22
IL-1 β ELISA kit	Thermo Fisher	Cat. No. 88-7013-88
TNF- α ELISA kit	Thermo Fisher	Cat. No. 88-7324-88
EZCell™ Glucose Uptake Assay kit	BioVision	Cat. No. K924
Experimental Models: Organisms		
Mouse: C57BL/6J	Jackson laboratories	000664
Mouse: C57BLKS-m <i>leprdb</i> (db/db)	Jackson laboratories	000642
Oligonucleotides		
Human:		
Tlr2 Forward: GAAGAGTGAGTGGTCAAGTAT, Reverse: AATGGGCTCCAGAAGAATGAG;	Integrated DNA Technologies	N/A
Tlr4 Forward: TTTCAGCTCTGCCTTCACTAC, Reverse: GACACCACAACAATCACCTTTC	Integrated DNA Technologies	N/A
MYD88 Forward: CTGTGTCTGGTCTATTGCTAGTG, Reverse: TTCCTTGCTCTGCAGGTAATC	Integrated DNA Technologies	N/A
IL1 Forward: CAAAGGCGGCCAGGATATAA, Reverse: CTAGGGATTGAGTCCACATTCAG	Integrated DNA Technologies	N/A
TNF Forward: GCAGGTCTACTTTGGGATCATT, Reverse: AGAAGAGGTTGAGGGTGTC	Integrated DNA Technologies	N/A
RelA Forward: CTGTCCTTTCTCATCCCATCTT, Reverse: TCCTCTTTCTGCACCTTGTC	Integrated DNA Technologies	N/A
RelB Forward: CTGCGGATTTGCCGAATTAAC, Reverse: ACACCACTGATATGTCCTCTTTC	Integrated DNA Technologies	N/A
Ccl2 Forward: TCATAGCAGCCACCTTCATTC, Reverse: CTCTGCACTGAGATCTTCTATTG	Integrated DNA Technologies	N/A
Ccl3 Forward: GGCAGATTCCACAGAATTCATAG, Reverse: TCGCTTGTTAGGAAGATGAC	Integrated DNA Technologies	N/A
IL10 Forward: TCCTTGCTGGAGGACTTTAAGGGT, Reverse: TGTCTGGGTTTGGTTCTCAGCTT	Integrated DNA Technologies	N/A
18s Forward: CACGGACAGGATTGACAGATT, Reverse: GCCAGAGTCTCGTTCGTTATC	Integrated DNA Technologies	N/A
GAPDH Forward: GGTGTGAACCATGAGAAGTATGA, Reverse: GAGTCCTTCCACGATACCAAAG	Integrated DNA Technologies	N/A
Mice:		
granulocyte (Csf3) Forward: TGTTCCCAAACCTGGGTTCTT, Reverse: TGGCTGCCACTGTTTCTT	Integrated DNA Technologies	N/A
GM-CSF Forward: AGCTCTGAATCCAGCTTCTC, Reverse: CCACATCTCTGGTCCCTTFA	Integrated DNA Technologies	N/A
Ccl2 Forward: CTCACCTGCTGCTACTCATT, Reverse: ACTACAGCTTCTTTGGGACAC	Integrated DNA Technologies	N/A

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Ccl3 Forward: TCACTGACCTGGAACTGAATG, Reverse: CAGCTTATAGGAGATGGAGCTATG	Integrated DNA Technologies	N/A
Rela Forward: CCGACTTGTTGGGTGATCT, Reverse: TCCGTCTCCAGGAGGTTAAT	Integrated DNA Technologies	N/A
Relb Forward: TGCCGAATCAACAAGGAGAG, Reverse: TGCTGAACACCACGGATATG	Integrated DNA technologies	N/A
Tlr2 Forward: CACTATCCGAGGTTGCATATC, Reverse: GGAAGACCTTGCTGTTCTCTAC	Integrated DNA Technologies	N/A
Tlr4 Forward: GAGCAAACAGCAGAGGAAGA, Reverse: CCAGGTGAGCTGTAGCATTTA	Integrated DNA Technologies	N/A
Myd88 Forward: AGCAACTAGGACTGCCTTTC, Reverse: GAACTCTTCCACTCAGCTATCC	Integrated DNA Technologies	N/A
IL-1 Forward: GCACTACAGGCTCCGAGATGAAC; Reverse: TTGTCGTTGCTTGGTTCTCCTTGT	Integrated DNA Technologies	N/A
Tnf Forward: TTGTCTACTCCCAGGTTCTCT, Reverse: GAGGTTGACTTCTCCTGGTATG	Integrated DNA Technologies	N/A
GAPDH Forward: TTGGGTGTACATCCAAGCA, Reverse: CAAGAAACAGGGGAGCTGAG	Integrated DNA Technologies	N/A
Cell lines		
Mouse Primary Epidermal Keratinocytes	Cell Biologics	Cat No: C57-6066K
Mouse Primary Dermal Lymphatic Endothelial Cells	Cell Biologics	Cat No: C57-6064L
Software and Algorithms		
GraphPad Prism	GraphPad	https://graphpad.com/scientific-software/prism/