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Full Title
Epigenetic Changes in Bone Marrow Progenitor Cells Influence the Inflammatory Phenotype and Alter Wound Healing in Type 2 Diabetes

Short Running Title
Epigenetics Impair Diabetic Wound Healing

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Abstract

Classically-activated (M1) macrophages are known to play a role in the development of chronic inflammation associated with impaired wound healing in Type 2 diabetes (T2D); however, the mechanism responsible for the dominant pro-inflammatory (M1) macrophage phenotype in T2D wounds is unknown. Since epigenetic enzymes can direct macrophage phenotypes, we assessed the role of histone methylation in bone marrow (BM) stem/progenitor cells in the programming of macrophages towards a pro-inflammatory phenotype. We have found that a repressive histone methylation mark, H3K27me3, is decreased at the promoter of the \( IL12 \) gene in BM progenitors and this epigenetic signature is passed down to wound macrophages in a murine model of glucose intolerance (diet-induced obese [DIO]). These epigenetically ‘pre-programmed’ macrophages result in poised macrophages in peripheral tissue and negatively impact wound repair. We found that in diabetic conditions, the H3K27 demethylase, Jmjd3, drives \( IL12 \) production in macrophages and that \( IL12 \) production can be modulated by inhibiting Jmjd3.

Using human T2D tissue and murine models, we have identified a previously unrecognized mechanism by which macrophages are programmed towards a pro-inflammatory phenotype, establishing a pattern of unrestrained inflammation associated with non-healing wounds. Hence, histone demethylase inhibitor-based therapy may represent a novel treatment option for diabetic wounds.
**Introduction**

According to the CDC, 9.6% of the population over the age of 20 suffers from diabetes with an estimated economic cost of 200 billion dollars with a third of the burden due to peripheral wounds (1). Impaired cutaneous wound healing is the most prevalent cause of hospitalization in patients with Type 2 diabetes (T2D) and the most common cause of lower extremity amputation in the US. Wound repair is a complex process that involves the local tissue environment, resident cells that proliferate and migrate, and systemic mobilization and differentiation of bone marrow (BM)-derived cells (2, 3). In normal wound healing, myeloid cells are mobilized from the BM into the circulation and migrate into the peripheral tissues where they differentiate into macrophages, a key immune cell that drives wound inflammation (4, 5). Classically activated macrophages (M1) express a defined set of pro-inflammatory mediators, in contrast to alternatively activated macrophages (M2), which perform a more regulatory role (6, 7). The pro-inflammatory macrophages promote an inflammatory immune response through the production of cytokines, including IL12 (8, 9). Under normal wound healing conditions, macrophages initially secrete pro-inflammatory mediators (i.e., IL12) performing antimicrobial functions (10, 11). This is followed by conversion to an anti-inflammatory macrophage phenotype (M2) promoting tissue repair (12). Although transient inflammation is an integral part of successful wound healing, it is necessary that the inflammatory phase resolves in a timely fashion in order to allow the healing cascade to progress. Clinically in T2D, this macrophage functional/phenotypic switch does not readily occur and the macrophages remain predominantly in a pro-inflammatory, M1 activation state and chronic inflammation ensues (11, 13, 14).

Evidence suggests that epigenetic regulation (e.g., DNA methylation, histone modification) of gene expression plays a key role in influencing immune cell phenotypes (15). Epigenetic
modifications have been documented in inflammation, and have been shown to regulate downstream immune-mediator expression in monocyte-derived macrophages (16, 17). Although the underlying mechanisms are being actively investigated, the notion that gene expression patterns can be maintained over a period of time and are heritable arises because specifically modified histones within nucleosomes can act as templates to initiate identical modifications during replication. Thus, these specific histone modifications can be transferred to more differentiated cells (18).

The contributions of epigenetic-based mechanisms on the regulation of macrophage phenotypes in diabetic wounds are unknown. While histone methylation changes have been shown to influence macrophage phenotypes, the effects in T2D or in wounds have not been addressed. Histone methylation is important as it plays a key role in maintaining active or suppressed gene expression depending on the methylation site. Methylation of lysine 27 (K27) of histone 3 (H3) keeps the chromatin in a confirmation such that the promoter for specific genes is unavailable for transcription and thus genes are silenced (17, 19, 20). In contrast to this, methylation of lysine 4 on H3 opens up the chromatin and allows for gene expression (16). In this study we hypothesized that histone methylation changes are induced in BM cells and set a ‘metabolic memory’ phenomenon resulting in peripheral macrophages that are predisposed towards a pro-inflammatory phenotype in the setting of the glucose intolerance. We further hypothesized that this macrophage phenotype contributes to the chronic inflammation observed in T2D peripheral wounds. These results validate our hypothesis and show for the first time that histone methylation influences macrophages towards a pro-inflammatory response in diabetic wounds and plays an essential role in the initiation and maintenance of chronic wound inflammation.
Research Design and Methods

Mice. Mice were maintained in the University of Michigan pathogen-free animal facility and all protocols were approved by and in accordance with the guidelines established by the Institutional Animal Care and Use Committee (UCUCA). Male C57BL/6 mice maintained on a normal chow diet (ND) (13.5% kcal fat; LabDiet) or high fat diet (HFD) (60% kcal fat; Research Diets, Inc.) were purchased at 20 weeks from The Jackson Laboratory (Bar Harbor, ME). Animals underwent all procedures at 22-26 weeks of age. Body weights and insulin levels were determined prior to experimentation. Wounds were induced on the back using a 4mm punch biopsy. Full-thickness skin was removed, exposing the underlying muscle. Two wounds per mouse were made in such a manner. A 6mm punch biopsy was used to harvest wounds.

Assessment of wound healing. Initial wound surface area was recorded and digital photographs were obtained daily using an Olympus digital camera. Photographs contained an internal scale to allow for standard measurement calibration. Wound area was quantified using ImageJ software (NIH, Bethesda, MD) and was expressed as the percentage of original wound size over time.

Flow cytometry. Human or mouse cells were harvested and Fc receptors of cells were blocked with anti-CD16/32 (BioLegend) or human IgG (Sigma-Aldrich) for 10 minutes followed by staining of cells for 15 minutes at RT. For intracellular staining, cells were fixed in 2% formaldehyde and then permeabilized using the Perm/Wash buffer kit (BD Biosciences) followed by antibody incubation. Monoclonal antibodies used for flow cytometry include: anti-c-Kit, anti-Sca1, anti-CD48, anti-CD150, anti-Flt3 ligand, anti-CD3, anti-TER119, anti Gr-1, anti-B220 (BioLegend) for mouse studies and anti-CD68 (BioLegend), anti-CD163 (Biolegend), and anti-206 (eBioscience) for human studies. Rabbit anti-trimethylated H3K4 (Abcam) and anti-
trimethylated H3K27 (Millipore) were used to detect histone marks, followed by secondary stain with FITC-AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch). All populations were routinely back-gated to verify purity and gating. Samples were analyzed on an LSR II (BD Biosciences). One million viable cells were analyzed. Data were analyzed using FlowJo software version 9.0 (Tree Star, Inc.) and compiled using Prism (GraphPad Software).

Cell culture and cytokine analysis. BM cells were collected by flushing mouse femurs and tibias with RPMI. BM macrophages were cultured as previously detailed (21). On day 6, the cells were replated and after resting for 24 hours, they were incubated with or without IFNγ (100 ng /mL) and LPS (100 ng/ml) for 6 to 48 hours. GSK-J4 (R&D Systems) was used as described (20).

Microscopy. Wounds were harvested; paraffin embedded and examined using HRP-DAB staining kit (R&D Systems). The slides were stained with anti-CD86 (BD Biosciences) overnight at 4ºC followed by secondary antibody stain for 1h at RT. Cells were observed under Olympus microscope and images were captured using cellSens software (Center Valley, PA).

Chromatin Immunoprecipitation Assay (ChIP). The ChIP assay was carried out as previously described by our group (21, 22). Purified DNA was used in a PCR reaction with oligonucleotide primers to the promoter region of the following genes: IL12p35 ('CAGACTCAGTGCCACGAT') and ('GCCAGTGCCCTTCTAAAGT'); Jmjd3 ('CAAGCACATTTCACGTACAGATGA') and ('CCACAAAGCCAGAAGGGGT'); Arginase-1 ('GTCAGAGAGCAGAAGGCTTTG') and ('GGAAGTCTGAACAATGCCTCA'); Mannose Receptor ('CCGCCCTTACAGTACAGATGA') and ('TCCCTTTTAAAGGACTCCTCA'). Primers to detect H3K37me3 along the promoter region of IL12 are listed in Table 1.
Quantitative RT-PCR. Total RNA extraction was performed using TRIzol per manufacturer’s instructions. Total RNA was reverse-transcribed to cDNA with MMLV (Invitrogen, Carlsbad, CA). RT-PCR was performed with 2XTaqman PCR mix using the 7500 Real-Time PCR System. Primers for NOS2, IL-1β, IL12, Jmjd3 were purchased (Applied Biosystems). Gapdh was used as the internal control. All standards and samples were assayed in triplicate. The threshold cycle values were used to plot a standard curve.

ELISA. Mouse IL12 concentration was measured by the Quantikine mouse IL12 Elisa kit (R&D Systems) per the manufacturer’s protocol. Color intensity was measured at 492 nm. Culture supernatants were obtained at 24 and 48 hours. The ELISA kit has a detection limit of 15 pg/mL.

Bioplex. Levels of IL6, IL10, IL12, and TNF-α were determined using the Luminex/Bioplex assay 200 system (Bio-Rad) per manufacturer’s protocol. The limit of detection for each cytokine was <5 pg/ml. The cytokine levels were normalized to protein present in cell-free preparation of each sample measured by Bradford Assay (Bio-Rad).

PCR Array Analysis. Wounds from HFD and control mice were digested in Collagenase A (Roche, Indianapolis, IN) and passed through 100 µM cell strainer to get a single cell suspension. CD11b+ cells were isolated using a MACS column. RNA extracted from CD11b+ cells was concentrated and cleaned using RNAeasyMinElute Cleanup kit (Qiagen, Germantown, MD). RT² First Strand kit (Qiagen) was used to reverse transcribe RNA. Gene expression was analyzed using PCR array PAMM-085Z (Qiagen), which includes PCR primers for 84 chromatin modifying enzymes. Data was analyzed using PCR Array Data Analysis Web Portal.

Genetic silencing using siRNA. Lipofectamine RNAiMAX (Invitrogen) was used to transfect murine BMDM according to manufacturer’s protocol. On-TARGET plus Jmjd3siRNA and ON-
TARGET plus Non-Targeting pool siRNA was purchased from GE Healthcare. Cells were incubated in serum free medium (Acell, GE Healthcare) with or without Lipofectamine-siRNA complexes for 48h after which the monolayer was washed and stimulated for 24h using LPS/IFNγ (100 ng/mL) as described above. Jmdj3 and IL12 expression was analyzed by RT-PCR. IL12 production was examined using Bioplex.

*Isolation of human macrophages from tissue/BM.* A 6mm punch biopsy of human wound tissue and 30cc of BM was obtained from T2D and non-diabetic patients (with toe-brachial index [TBI] > 0.3) following amputation under University of Michigan IRB approved protocols. Wound biopsies contained no healthy, non-wounded skin. Patients were male, with a mean age of 63. There were no statistical differences between the groups. Co-morbid conditions including CHF, CAD, CRI, smoking status and obesity (BMI > 35) were similar between the groups. Immune cell isolations were performed immediately after surgery. Bones were flushed with RPMI-supplemented with 10%FBS/Pen/Strep. Tissue was incubated in 0.16% collagenase at 37°C. Cells underwent RBC lysis followed by Ficoll separation. Leukocytes were harvested from the buffy coat. Macrophages were cultured from human BM in the presence of 25 ng/ml of M-CSF, 2.5 ng/ml of GM-CSF, 50 ng/ml of SCF and 20 ng/ml of IL3 (R&D Biosystem) as described previously (23).

*Statistical analysis.* Data were analyzed using GraphPad Prism software version 6. We expressed the results as means ± S.E. except when multiple independent groups were used where we expressed results as mean ±S.D. The statistical significance of differences between two groups was determined using Student’s t-tests; while differences between more than two groups were evaluated by ANOVA followed by Newman-Keuls post-test. p-Values ≤0.05 were considered significant.
Results

Macrophages mount a distinct pro-inflammatory phenotype in human Type 2 diabetes (T2D) wounds. To determine the peripheral macrophage phenotype in human diabetic wound tissue, macrophages were isolated from chronic non-healing wounds immediately following lower extremity amputation from T2D and non-diabetic patients. Human macrophages are often phenotypically and functionally defined as M1 based on the absence of M2 cell surface markers and the expression of soluble proteins (24). These human wound-derived macrophages (CD68) were analyzed by flow cytometry for M2 surface markers (CD206 and CD163). Macrophages isolated from T2D human wounds showed a significant decrease in M2 scavenger receptors compared to wound macrophages in non-diabetic individuals (Fig. 1A). Functionally, upon stimulation with LPS/IFNγ, human T2D BM-derived macrophages (BMDM) demonstrated exaggerated production of IL12, a key pro-inflammatory cytokine, compared to non-diabetic controls (Fig. 1B).

Delayed wound healing in DIO mice is associated with increased pro-inflammatory macrophages. We used our established wound healing model in glucose intolerant, diet-induced obese (DIO) mice and wildtype (WT) controls to examine macrophage phenotypes and their effect on wound T2D healing (25). Data detailing the body weights, glucose tolerance test (GTT) results and plasma insulin levels are shown in supplemental Fig. 1. We hypothesized that our findings in a mouse model of metabolic syndrome and glucose intolerance would mimic our results seen with human T2D macrophages. Wound healing in DIO mice was significantly delayed when compared to controls (Fig. 2A). Both immunohistochemical and flow cytometry analysis of wound tissue from DIO mice demonstrated increased numbers of pro-inflammatory macrophages compared to WT (Fig. 2B/C). CCR7+ was used as a cell surface marker for M1
polarization based on our published data (23). Perhaps more important than the actual cytokine levels in the tissue is the balance between the pro- and anti-inflammatory cytokines in the wound at a given time point. Consistent with this surface marker, functional analysis based on cytokine production as detected by Bioplex revealed increased ratios of IL12/IL10 levels in DIO wounds at days 3 and 7 post-wounding (Fig. 2D). These findings are consistent with an altered balance of macrophages in DIO wounds favoring an increase in pro-inflammatory macrophages.

**Bone marrow-derived macrophages (BMDM) are predisposed towards an M1, pro-inflammatory phenotype in T2D murine model.** To examine macrophages derived from the BM in our T2D murine model, BM from DIO and control mice was grown in vitro via standard techniques to culture macrophages. The DIO BMDM exhibit increased pro-inflammatory activation as compared to controls as shown by increased inducible nitric oxide synthase (NOS2), interleukin 1β (IL-1β), interleukin 6 (IL6), tumor necrosis factor-α (TNF-α) and interleukin 12 (IL12) (Fig. 3). This suggests that similar to peripheral tissue macrophages, DIO mice BMDM display pronounced pro-inflammatory activity.

**DIO BM stem/progenitor cells and peripheral macrophages display decreased histone lysine trimethylation (H3K27me3), at the promoter region of a key pro-inflammatory macrophage gene, IL12.** Since our data suggest that DIO wound macrophages are poised towards a pro-inflammatory phenotype, probably set in the BM, we examined BM (lin-/-c-Kit+) (LK) cells for histone lysine methylation changes that could result in ‘programmed’ changes in peripheral macrophage subsets. We first examined multiple histone methylation marks in murine BM lin-/-Sca1+/c-Kit+ (LSK) cells and macrophages (CD11b+/F4/80-). In Figure 4A, both LSK cells and macrophages from DIO mice have significantly decreased levels of H3K27me3, a
methylation mark causing transcriptional repression. The H3K27me3 methylation mark maintains the chromatin in a conformation so specific genes are effectively silenced (17).

Since H3K27me3 was decreased in BM LSK and macrophages, we postulated that this repressive mark was decreased on the *IL12* promoter, a pro-inflammatory cytokine primarily produced by macrophages, that we found was increased in both human T2D and murine DIO wounds. Importantly, IL12 has been shown to be an important cytokine produced in excess by T2D macrophages in adipose tissue (26). Using chromatin immunoprecipitation (ChIP) assays, we found H3K27me3 was significantly decreased on the *IL12* gene promoter in DIO BM stem/progenitor cells (LK), peripheral wound macrophages (CD11b⁺) and EWAT macrophages (CD11b⁺) compared to controls (Fig. 4B-D). We further examined multiple sites along the *IL12* promoter in BMDM and found that DIO mice have decreased H3K27me3 methylation at multiple sites along the *IL12* promoter (Fig. 4E). This likely corresponds to the increased IL12 protein expression seen in the DIO macrophages. These changes in histone methylation on the *IL12* promoter detected by ChIP in BM LK cells, BMDM and CD11b⁺ cells from wounds were also seen in *db/db* mice, a genetic model of T2D (supplemental Fig. 2A-D). ChIP analysis of the promoter of two M2-associated genes demonstrated no differences in H3K27 methylation in DIO BMDM compared to controls (supplemental Fig. 3A/B). These data suggest that histone methylation changes may affect gene expression in multiple T2D models and that IL12 gene expression may be altered by H3K27me3 methylation.

The JumanjiC (JmjC) domain-containing protein, Jmjd3 is responsible for the decrease in the H3K27me3 repressive methylation mark seen in BM (lin⁻/c-Kit⁺) (LK) cells and bone marrow-derived macrophages (BMDM). The JmjC family of histone demethylases are essential components of transcriptional chromatin complexes and act to demethylate lysine
residues on histones in a methylation-state and sequence specific fashion. Jmjd3, specifically
removes methyl groups from H3K27me3, effectively eliminating the repressive function of
H3K27me3 and thus, allows for increased gene expression (27, 28). Previous studies from our
lab and others have shown that Jmjd3 plays a role in macrophage polarization, and that LPS
treated macrophages incubated with a Jmjd3 inhibitor demonstrated a reduction in pro-
inflammatory cytokines, including IL12 (20, 21, 27, 29). A superarray was performed on
CD11b⁺ magnetic associated cell sorting (MACS) isolated cells from peripheral wounds at day 3
post-wounding, which demonstrated over a 23 fold increase in Jmjd3 in CD11b⁺ cells from DIO
wounds compared to controls (Fig. 5A). Since H3K27me3 was decreased in the DIO
CD11b⁺ wound cells and the BM LK cells, we investigated the relationship between Jmjd3 and
H3K27me3 in the DIO BM. The amount of Jmjd3 transcript present in vivo in BMLK cells and
BMDM is significantly increased in the DIO cells compared to controls (Fig. 5B). Further,
Jmjd3 levels are significantly increased in BM isolates from human T2D patients compared to
non-diabetic controls (Fig. 5C). In addition, ChIP of H3K4me3 at the Jmjd3 promoter in DIO
LK cells demonstrated a significant increase in H3K4 trimethylation resulting in an open
chromatin conformation and increased gene expression. Further, a trend towards decreased gene
repression (H3K27me3) was observed in the DIO LK cells at the Jmjd3 promoter, allowing for
increased Jmjd3 gene expression (Fig. 5D). A ChIP analysis of Jmjd3 levels on the IL12
promoter showed significantly increased Jmjd3 on the IL12 promoter in DIO macrophages as
opposed to controls (Fig. 5E). To assess the biological relevance of Jmjd3-directed histone
remodeling in the DIO BM cells and macrophages, we used the newly generated H3K27
demethylase inhibitor, GSK-J4, the first selective inhibitor shown to prevent the Jmjd3-induced
loss of H3K27me3 (20). In DIO BMDM, GSK-J4 increased H3K27me3 mediated repression at
the IL12 promoter, suggesting that modulation of Jmjd3 may alter the epigenetic signature at the IL12 promoter in the DIO setting (Fig. 5F). Importantly, when BMDM were treated with LPS/IFNγ, GSK-J4 decreased IL12 production in a dose-dependent fashion (Fig. 5G). Similar trends were observed in two other inflammatory cytokines (supplemental Fig. 4A/B). In order to more specifically examine the effects of Jmjd3, we targeted Jmjd3 using siRNA. Following specific reduction in Jmjd3 levels in DIO macrophages, we observed a significant reduction in IL12 in the DIO macrophages treated with Jmjd3-specific siRNA as compared to the DIO macrophages treated with a scrambled sequence (Fig. 5H). Thus, both the chemical inhibitor and specific Jmjd3-targeted siRNA were able to effectively reverse IL12 expression in DIO macrophages.

**Bone marrow (BM) chimeras demonstrate that the peripheral wound M1-dominant macrophage phenotype is set at the BM level.** To evaluate whether the peripheral macrophage phenotype is controlled by BM stem/progenitor cells, BM chimeric mice were created. We created 2 groups of chimeric mice: 1) GFP+DIO (mice on a high fat diet [HFD]) BM donors into normal diet (ND) C57BL/6 recipients, and 2) GFP+ND BM donors into ND C57BL/6 recipients (30). Degree of chimerism was found to be 96.7% at 8 weeks post-transplant (Fig. 6A) using peripheral blood flow cytometry (31). Wound healing was significantly delayed in the WT mice receiving DIO BM (GFP+HFD BM donors into ND C57BL/6 recipients), compared to controls (Fig. 6B). Expression of Jmjd3 and IL12 in CD11b+ wound macrophages was also increased in the chimeric mice receiving DIO BM, suggesting that H3K27 trimethylation in BM cells may play a key role in promoting the exaggerated pro-inflammatory response seen in diabetic macrophages (Fig. 6C/D).
Discussion

The role of macrophages in chronic inflammation development associated with obesity and T2D has been well studied; however, how macrophages maintain a pro-inflammatory environment and promote chronic inflammation in T2D wounds has not been defined (32). In these studies, we used the DIO model to evaluate the origins of dysfunctional wound healing. The DIO mouse has progressive obesity, dyslipidemia and hyperglycemia and poor wound healing, paralleling that seen in humans and do not have the potential for T-cell dysfunction seen in db/db mice and ob/ob mice due to deficient leptin signaling (28, 33-35). Here, we demonstrate that macrophages in DIO wounds are poised, via an epigenetic mechanism, toward an unrestrained ‘M1-like’ or pro-inflammatory activation state and produce significantly higher IL12 levels. This prolongs the inflammatory phase and complicates the healing process by slowing resolution of wound site inflammation. The precise molecular and cellular mechanisms leading to alterations in the epigenetic signature remain to be determined, but could reflect additional targets for directed therapy.

Our findings represent the first report on the role of epigenetics in BM cells and macrophages in obese, insulin resistant T2D conditions and the resultant effects on modulating peripheral macrophage function/phenotype in wound healing. If monocytes mobilized to circulation from the BM are already poised towards a pro-inflammatory phenotype, then clinical treatments aimed at changing the local environment become less efficacious as they fail to address the systemic problem with the BM cells and macrophage polarization in T2D. Recent studies aimed at improving diabetic wound healing have focused on improving stem cell mobilization, which has resulted in only a modest improvement in angiogenesis and wound healing. These studies have failed to address the excessive number of inflammatory monocyte-macrophages that perpetuate
chronic inflammation in T2D wounds, and hence, T2D patients have not seen significant benefits from stem cell therapies directed at wound healing (36, 37). Thus strategies aimed at altering these epigenetic changes at the BM level could significantly influence immune cell function in peripheral tissues and improve diabetic wound healing and potentially other secondary complications of T2D. Our findings provide a rationale for further studies targeting key epigenetic enzymes at the BM and local levels in order to promote an anti-inflammatory, repair macrophage phenotype and alter the inflammatory cascade maintained by unrestrained pro-inflammatory activation in the setting of diabetic wounds. Hence, these data provide the foundation for significant improvements in existing clinical protocols for this significant unresolved epidemic.

Although the H3K27 demethylase, Jmjd3, has been shown to influence polarization of macrophages in vitro, the role of this epigenetic enzyme in a pathological condition such as T2D is unknown (21). It stands to reason that the epigenetic signature differs during chronic inflammatory disease states. In this study, we found a potential new role for Jmjd3/KDM6B in modulating IL12 expression by macrophages in the setting of T2D. This is in agreement with a recent study where treatment of macrophages with a selective Jmjd3 inhibitor led to alterations in pro-inflammatory cytokines (20). As mentioned earlier, the DIO mouse may better reflect human physiology than some genetic models. However, it is not possible to determine the degree to which the observed changes in histone methylation are due to nutrient over consumption and obesity, insulin resistance or hyperglycemia. Further work is needed to investigate, how changes in epigenetic signatures in metabolic diseases affect gene expression and how these chromatin modifications can be altered to achieve therapeutic benefit.
Since a large proportion of macrophages present at a wound site are recruited from the circulation and are not resident skin macrophages, we chose to focus on day 3 post-wounding, so that circulating monocytes had adequate time to enter the tissues, transform into macrophages and perform a function (2, 38). This is consistent with previous work that has demonstrated that at day 3 post-wounding, recruited macrophage numbers are at their highest levels (39). Although controversy exists regarding the most accurate markers to define macrophage subsets, surface markers in isolation do not fully differentiate subpopulations of macrophages (40, 41). Rather, the functional activity of macrophages plays an important role in defining potential macrophages subtypes (42, 43). Among the many differences between activation of macrophages, the ratio of IL12 to IL10 appears important for distinguishing macrophage phenotypes (44). ‘M1-like’ macrophages promote an inflammatory immune response through, among other cytokines, the production of IL12. Thus, we focused on the production of IL12 by macrophages as a defining characteristic of the pro-inflammatory (M1) macrophages (45). Although IL10 and IL12 are largely produced by macrophages, these cytokines can be affected by the local environment including other immune cell types, including T lymphocytes and dendritic cells, that are present in smaller numbers following wounding (46).

While we have examined how changes in the BM affect macrophage phenotype, other factors undoubtedly play a role in the complex process of wound healing. The function of macrophages during wound repair is likely influenced by both changes in cell programming and by the local microenvironment present at the wound site. Several studies have supported a role for the local wound environment in regulating macrophage function (47). These studies found that blocking the pro-inflammatory cytokine, IL-1β at the local level, down-regulates pro-inflammatory macrophages and promotes wound healing (48). From a clinical standpoint, in order to fully
restore wound healing in T2D, therapies will need to promote an appropriate M1/M2 balance throughout the course of healing. Initially, pro-inflammatory macrophages are needed in the wound to clear debris and infection; however, conversion to an M2 anti-inflammatory response is needed in order for collagen deposition to occur at the appropriate time in the wound healing cascade (47). In this sense, therapies that target systemic changes in the BM stem cells, influence peripheral phenotypes, and restore a more ‘normal’ macrophage balance are more attractive than just blocking the pro-inflammatory phenotype at the local wound site.

Macrophages are key orchestrators in the wound healing of many tissue types, including peripheral wounds. The prolonged presence of macrophages in wounds of both diabetic mice and human wounds suggests they are critical for restoring healing in T2D. Our group has recently examined macrophage polarization and found that macrophage phenotypes behave functionally similar in both murine models and humans (23). Macrophages from human venous ulcers have been shown to exhibit a similar pro-inflammatory phenotype to that observed in our human T2D wounds (49). The mechanism proposed for the M1 phenotype in the venous ulcers was due to excess iron, since hemosiderin deposits are clinically apparent in chronic venous ulcers. Thus, this is likely a specific pathway in the venous ulcer disease process, as diabetic wounds do not contain hemosiderin (49). In diabetic arterial wounds, other pathways are likely involved in promoting the pro-inflammatory macrophage phenotype.

In summary, we find that the insulin resistant, hyperglycemic environment results in epigenetic changes in the bone marrow that persist in peripheral wounds. These changes, triggered in part by the signaling-independent activation of the transcription factor Jmjd3, result in the expression of inflammatory cytokines (IL12) and lead to persistent wound inflammation. The identification of epigenetic changes in the BM stem/progenitor cells as an inciting event in the complex
pathological process of inflammation has relevance to wound healing and may also play a role in persistent inflammation seen in other tissues in insulin resistance and T2D, such as adipose tissue and arterial walls (50, 51). The T2D-related factors that trigger these epigenetic changes remain uncertain, but understanding them may provide a path to preventing macrophage dysfunction and associated pathologies.
Author Contributions
Katherine A. Gallagher, M.D. performed the research, analyzed data and wrote the manuscript. Amrita Joshi, Ph.D., performed the research, analyzed data and assisted writing the manuscript. William F. Carson, Ph.D. performed the research, reviewed and edited the manuscript. Ronald Allen, Ph.D. performed the research. Eva Feldman, M.D., Ph.D., Matthew Schaller, Ph.D., Peter K. Henke, M.D., Cory Hogaboam, Ph.D., Charles F. Burant, M.D., Ph.D., Sumanta Mukerjee, Ph.D., Nico Kittan, M.D., and Steven L. Kunkel, Ph.D., reviewed and edited the manuscript.

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References


Figure Legends

Figure 1. Human wound and bone marrow-derived macrophages (BMDM) exhibit decreased anti-inflammatory, (M2-like) and increased pro-inflammatory, ‘M1-like’ characteristics in Type 2 diabetes (T2D). (A) Flow cytometry analysis of human macrophages (CD68⁺) isolated from non-ischemic, non-diabetic and T2D chronic wound tissue. Cells were stained for CD163 and mannose receptor (CD206). Percentage of CD68⁺ cells expressing either CD206 (top right) or CD163 (bottom right) are shown. (n=6) (B) RT-PCR quantification of IL12 (M1 cytokine) expression in human BMDM 6 hours following stimulation with LPS/IFNγ. BMDM from T2D patients are compared to BMDM from non-diabetic patients. (n=6) Data are expressed as mean ± SE.

Figure 2. Delayed wound healing in diet induced obese (DIO) mice is associated with increased pro-inflammatory macrophages. Punch biopsies (4mm) were performed on the back of DIO and control mice. Change in wound area was recorded daily using Image J software (NIH) until complete healing was observed. (A) Wound healing curves in DIO and control mice. (n=10/time point) Data is pooled from three experiments and is expressed as mean ± S.D. Representative images are shown of wounds at day zero and 3 days post-wounding. (B) Immunohistochemical analysis of mononuclear cells (CD86⁺) in wounds at day 3 (cells/high powered field [hpf]) in DIO compared to controls. (n=6) Representative examples of DIO and control wounds are shown. (C) Flow cytometry of DIO and control wounds at day 3. Pro-inflammatory macrophages were defined as CD11b⁺/F4/80⁻ cells that co-expressed CCR7. (n=6, experiment replicated 1x.) (D) Ratio of IL12 (M1) to IL10 (M2) cytokine levels in wounds at
days 3 and 7 analyzed by Bioplex. (n=4, experiment replicated 1x.) Data are expressed as mean ± SE.

**Figure 3.** Macrophages are predisposed towards a pro-inflammatory (M1) phenotype in a T2D murine model. (A/B) NOS2 and IL-1β expression levels quantified by RT-PCR in DIO and control BM-derived macrophages (BMDM) at 6 and 24 hours after stimulation with LPS/IFNγ. (n=4, replicated 2x.) (C) Levels of IL12 in supernatants from DIO and control BMDM following treatment with LPS/IFNγ at 24 and 48 hours as measured by ELISA. (n=4, plated in triplicate.) (D/E) Levels of +IL6 and TNF-α in supernatants from DIO and control BMDM following treatment with LPS/IFNγ at 24 and 48 hours as measured by Bioplex. Protein levels are expressed as pg/ml. (n=4, plated in triplicate.) Data are expressed as mean ± SE.

**Figure 4.** DIO bone marrow (BM) stem/progenitor cells and peripheral macrophages display decreased trimethylation of histone3 lysine27 (H3K27me3) on the IL12 promoter. (A) H3K27me3 levels in BM stem/progenitor cells (lin⁻/Sca1⁺/c-Kit⁺) and BM-derived macrophages (BMDM) (CD11b⁺/F4/80⁺) from DIO and control mice measured by flow cytometry. (n=4) (B-D) BM stem/progenitor cells (lin⁻/c-Kit⁺), wound and EWAT macrophages (CD11b⁺) were isolated *in vivo* using magnetic associated cell sorting (MACS) and analyzed by chromatin immunoprecipitation (ChIP) for H3K27me3 levels on the promoter of IL12. (n=3/group, plated in triplicate.) (E) H3K27me3 methylation was measured along the IL12 promoter in BMDM. (n=3, replicated 2x.) Data are expressed as mean ± SE.
Figure 5. Increased JumanjiC (JmJC) demethylase, Jmjd3, production in bone marrow (BM) lin⁻/⁻/c-Kit⁺ (LK) cells and macrophages. (A) Gene expression of chromatin modifying enzymes were analyzed using PCR array plates in CD11b⁺ MACS isolated cells from DIO and control wounds at day 3. Enzymes that were upregulated are shown. The threshold was set to a 4-fold difference. Data are expressed as fold over control. (n=3/group) (B) RT-PCR quantification of Jmjd3 levels in DIO and control BM progenitor cells (lin⁻/⁻/c-Kit⁺) (isolated by MACS) and BMDM (following stimulation with LPS/IFNγ). (n=4/group) (C) RT-PCR analysis of human BM isolated from non-diabetic and T2D patients undergoing amputations at the femur level. (n=2/group) (D) ChIP analysis of H3K4me3 and H3K27me3 on the Jmjd3 promoter of in vivo MACS isolated BM LK cells from DIO and control mice. (n=5/group) (E) ChIP analysis for Jmjd3 on the IL12 promoter in DIO BMDM compared to controls. (n=3/group, replicated 1x.) (F) BMDM from DIO and control mice were treated with the H3K27me3 demethylase inhibitor, GSK-J4 (10µM) for 6 hours and ChIP analysis of H3K27me3 on the IL12 promoter was performed. (n=3/group) (G) BMDM from DIO and control mice were stimulated with LPS/IFNγ in the presence or absence of GSK-J4 (3µM, 10 µM) for 6 hours and analysis of IL12 transcription was performed. (n=3/group) Data are expressed as mean ± SE. (H) DIO BMDM were transfected with Jmjd3 siRNA or non-targeting siRNA or lipofectamine alone in triplicate wells. 48 hours later cells were stimulated with LPS/IFNγ for 24 hours. Transcript levels of Jmjd3 and IL12 in cells treated with Jmjd3 siRNA or non-targeting siRNA are shown. Following stimulation, cell supernatants were collected and used for IL12 Bioplex. Results are expressed as mean +/- SE. Statistical analysis was done using student t-tests and p value < .05 was considered significant.
Figure 6. Wound healing is impaired and macrophage function altered in GFP\(^{+}\)DIO BM chimeras. BM chimeras were created using GFP\(^{+}\) mice on a C57BL/6 background. GFP\(^{+}\) mice were fed a high-fat diet (HFD, 60% fat) or ND (ND, 12% fat) for 14 weeks and BM from these mice was transferred into irradiated C57BL/6 recipients. (A) Peripheral blood analysis performed weekly and at 8 weeks confirmed 96.5% donor chimerism. (B) Change in wound area compared to initial wound size at day 3 post-wounding. (n=4/group) (C/D) Jmjd3 and IL12 expression in in vivo macrophages (CD11b\(^{+}\)) MACS isolated from wounds at day 3 were quantified by RT-PCR in ND→ND and HFD→ND GFP\(^{+}\) chimeric mice. (n=4/group) Data are expressed as mean ± SE.
### Table 1 – Oligonucleotide Primers on *IL12p35*

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<th>Primer#</th>
<th>Site</th>
<th>Forward</th>
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<tr>
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<td>ATCTCTCTGAGAGGAGCTAA</td>
</tr>
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<td>CTTGCCCAGGAGGTACAAT</td>
</tr>
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<td>TTATGGCTGAGGCAACAAGT</td>
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<td>9</td>
<td>-2821 to -2890</td>
<td>TTTCTCCTGGACGCTTGAAA</td>
<td>TAATGTCAGCAGACAGCAG</td>
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Figure 1. Human wound and bone marrow-derived macrophages (BMDM) exhibit decreased anti-inflammatory, (M2-like) and increased pro-inflammatory, 'M1-like' characteristics in Type 2 diabetes (T2D). (A) Flow cytometry analysis of human macrophages (CD68+) isolated from non-ischemic, non-diabetic and T2D chronic wound tissue. Cells were stained for CD163 and mannose receptor (CD206). Percentage of CD68+ cells expressing either CD206 (top right) or CD163 (bottom right) are shown. (n=6) (B) RT-PCR quantification of IL12 (M1 cytokine) expression in human BMDM 6 hours following stimulation with LPS/IFNγ. BMDM from T2D patients are compared to BMDM from non-diabetic patients. (n=6) Data are expressed as mean ± SE.

110x82mm (300 x 300 DPI)
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84x105mm (300 x 300 DPI)
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80x130mm (300 x 300 DPI)
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177x60mm (300 x 300 DPI)
Supplemental Figures

Supplemental Figure 1: Diet-induced obese mice (DIO) develop hyperglycemia and hyperinsulinemia. (A) Body weight in control and DIO mice on HFD from weeks 6-26 (n=6/group). (B) The glucose tolerance test was performed on control and DIO mice. Mice were fasted for 4h and then injected intraperitoneally with 1 mg/g body weight D-glucose. Blood glucose measurements (mg/dl) were obtained at 15 minute intervals for 1 hour and then at the 2 hour timepoint. (n=6/group) (C) Fasting plasma insulin levels in control and DIO mice were obtained in 26 week old animals (n=3/group). Data are expressed as mean ± SE.

Supplemental Figure 2: *db/db* mice have impaired wound healing and display decreased H3k27 methylation on the *IL12* promoter in bone marrow-derived macrophages (BMDM), bone marrow linε-Kit+ (LK) cells and wound macrophages (CD11b+). (A) Male 22-26 week old *db/db* and WT control (*db/+*) mice were wounded using a 4mm punch biopsy and wound area was measured at day 3. Data shown are percent initial wound area and are expressed as mean ± SE. (n=3/group) (B) H3k27me3 levels on the *IL12* promoter as analyzed by Chromatin Immunoprecipitation (ChIP) assay in BMDM. (n=3/group) (C) LK (linε-Kit+) cells were isolated in vivo from bone marrow using magnetic associated cell sorting (MACS) and ChIP analysis was performed for H3K27me3 on the *IL12* promoter. (N=3/group) (D) Macrophages (CD11b+) were isolated in vivo from wounds using MACS and ChIP analysis was performed for H3K27me3 on the *IL12* promoter. (n=3/group) Data are expressed as mean ± SE.
Supplemental Figure 3: Chemical inhibition of Jmjd3 by GSK-J4 in bone marrow derived macrophages (BMDM) results in decreased expression of M1-associated cytokine genes, *IL-1β* and *IL6*. (A)/(B) BMDM were stimulated with LPS/IFNγ in the presence of absence of GSK-J4 (3µM or 10µM) for 6 h and transcript levels of IL-1β and IL6 were analyzed by RT-PCR. (n=3/group) Data are expressed as mean ± SE.

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Diabetes
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107x70mm (300 x 300 DPI)