



# The Histone Methyltransferase MLL1 Directs Macrophage-Mediated Inflammation in Wound Healing and Is Altered in a Murine Model of Obesity and Type 2 Diabetes

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*Diabetes* 2017;66:2459–2471 | <https://doi.org/10.2337/db17-0194>

Macrophages are critical for the initiation and resolution of the inflammatory phase of wound repair. In diabetes, macrophages display a prolonged inflammatory phenotype in late wound healing. Mixed-lineage leukemia-1 (MLL1) has been shown to direct gene expression by regulating nuclear factor- $\kappa$ B (NF- $\kappa$ B)-mediated inflammatory gene transcription. Thus, we hypothesized that MLL1 influences macrophage-mediated inflammation in wound repair. We used a myeloid-specific *Mll1* knockout (*Mll1<sup>fl/fl</sup>Lyz2<sup>Cre+</sup>*) to determine the function of MLL1 in wound healing. *Mll1<sup>fl/fl</sup>Lyz2<sup>Cre+</sup>* mice display delayed wound healing and decreased wound macrophage inflammatory cytokine production compared with control animals. Furthermore, wound macrophages from *Mll1<sup>fl/fl</sup>Lyz2<sup>Cre+</sup>* mice demonstrated decreased histone H3 lysine 4 trimethylation (H3K4me3) (activation mark) at NF- $\kappa$ B binding sites on inflammatory gene promoters. Of note, early wound macrophages from prediabetic mice displayed similarly decreased MLL1, H3K4me3 at inflammatory gene promoters, and inflammatory cytokines compared with controls. Late wound macrophages from prediabetic mice demonstrated an increase in MLL1, H3K4me3 at inflammatory gene promoters, and inflammatory cytokines. Prediabetic macrophages treated with an MLL1 inhibitor demonstrated reduced inflammation. Finally, monocytes from patients with type 2 diabetes had increased *Mll1* compared with control subjects without diabetes. These results define an important role for MLL1 in regulating macrophage-mediated inflammation in wound

repair and identify a potential target for the treatment of chronic inflammation in diabetic wounds.

Impaired peripheral wound healing is a major clinical problem in type 2 diabetes (T2D) and represents the leading cause of lower-extremity amputation in the U.S. Amputations are associated with substantial morbidity and a 3-year mortality rate of 50% (1,2). Wound healing consists of integrated and overlapping phases of hemostasis/coagulation, inflammation, proliferation, and resolution/remodeling. These phases must occur at prescribed times and continue for a specific duration, or pathological wound healing ensues (3). Chronic wounds, like those seen in T2D, fail to progress through these discrete stages because of dysregulated inflammation that occurs during the inflammatory phase of wound healing (4–8).

A common characteristic of these poorly healing wounds is an impaired initial immune response to injury. In normal wound healing, the early innate inflammatory response is critical for establishing the healing cascade (9–11). During the first part of the inflammatory phase of wound healing, macrophages exist in an inflammatory phenotype where they release inflammatory cytokines and mediators, recruit additional leukocytes, and promote tissue and pathogen destruction (11). After this early inflammatory phase in normal wound healing, macrophages undergo a phenotype switch and begin secreting anti-inflammatory mediators as

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Received 14 February 2017 and accepted 19 June 2017.

This article contains Supplementary Data online at <http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db17-0194/-/DC1>.

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well as growth factors to promote tissue repair and wound resolution (11). Prior studies in diabetic murine models have demonstrated that this critical early macrophage-mediated inflammatory response is impaired in diabetic wounds (9,10). In addition, the proinflammatory-to-anti-inflammatory macrophage phenotype switch is impaired in multiple models of diabetes wherein a persistent hyperinflammatory macrophage phenotype ensues (4,12,13).

The gradual switch in wound macrophage response from proinflammatory to anti-inflammatory is a key component of normal healing and is necessary for effective wound closure (13). The ability to fully understand and control the initiation and resolution of inflammation in wound macrophages is critical to advancing the field of wound healing. At present, what specifically drives changes in wound macrophage phenotype throughout the course of healing is unknown (14–17). Thus, the examination of the molecular mechanisms underlying macrophage plasticity in wounds is necessary to address the pathology seen in diabetes.

Accumulating evidence suggests that epigenetic regulation of gene expression influences immune cell phenotypes (12,18). At present, a paucity of data exists on epigenetic-based mechanisms that regulate wound macrophage plasticity. Mixed-lineage leukemia 1 (MLL1) is a histone methyltransferase with site specificity for lysine 4 on histone H3 (H3K4) (14,19). H3K4 trimethylation (H3K4me3) of gene promoter regions is associated with active gene expression (16). In mammals, H3K4me3 is controlled by the SET1-MLL family of enzymes (20). Although the role of MLL1 in oncogenesis has been investigated, few studies have examined the role of MLL1 in innate immunity (17,19,21). Furthermore, the function of MLL1 in normal physiology, particularly in wound healing, is unknown. MLL1 has been shown to be recruited by nuclear factor- $\kappa$ B (NF- $\kappa$ B) to the promoters of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and matrix metalloproteinase-9, suggesting a role for MLL1 in NF- $\kappa$ B-mediated signaling (20,22). This is likely relevant in human tissues because MLL1 expression in human monocyte-derived macrophages has been shown to influence inflammatory gene transcription (12,23). Because the NF- $\kappa$ B pathway is critical for inflammatory gene transcription in wound macrophages, we hypothesized that MLL1-dependent chromatin remodeling affects macrophage-mediated inflammation in wound healing (24).

On the basis of previous work suggesting that MLL1 influences immune cell function, we investigated the role of MLL1 in directing macrophage-mediated inflammation during both normal and pathological wound healing (17,19,20,25). Specifically, by using a myeloid-specific MLL1 knockout (*Mll1<sup>ff</sup>/Lyz2<sup>Cre+</sup>*), we demonstrate that MLL1 is necessary for an adequate early inflammatory response in normal wound healing and that the absence of MLL1 delays wound healing. We show that the decreased inflammatory response seen in *Mll1<sup>ff</sup>/Lyz2<sup>Cre+</sup>* mice correlates directly with the low levels of MLL1, H3K4me3, and inflammatory cytokine production seen in early wound macrophages in the

diet-induced obese (DIO) model of glucose intolerance/insulin resistance. Furthermore, we demonstrate that in late wound healing, DIO wound macrophages display increased MLL1, increased H3K4me3 at inflammatory gene promoters, and increased inflammatory mediator production compared with control animals, suggesting a potential mechanism for dysregulated inflammation in diabetes. Of note, DIO macrophages treated with an MLL1 inhibitor, MI-2, demonstrated decreased inflammatory cytokine transcription. As a translational corollary, monocytes from patients with T2D expressed an increased MLL1 transcript, suggesting that MLL1 is a viable therapeutic target to regulate macrophage inflammation in human wounds.

## RESEARCH DESIGN AND METHODS

### Mice

Male C57BL/6 mice were fed a normal chow diet (13.5% kcal fat; LabDiet) or high-fat diet (60% kcal fat; Research Diets) for 10–12 weeks to generate the DIO model of glucose intolerance/insulin resistance (26). *Mll1<sup>+/-</sup>* mice on a C57BL/6 background were obtained from Y. Dou (University of Michigan). Mice with the *Mll1* gene deleted in myeloid cells were generated by mating *Mll1<sup>ff</sup>* mice with *LysM-Cre* mice (The Jackson Laboratory) (27). All animal procedures were approved under the University of Michigan Institutional Animal Care and Use Committee.

### Wound Healing Assessment and Tissue Harvest

Before wounding, mice were anesthetized, hair was removed with Veet (Reckitt Benckiser), and skin was cleaned with sterile water. Full-thickness back wounds were created by 4-mm punch biopsy. An 8-megapixel iPad camera with an internal scale was used to record wound size. Wound area was calculated by using ImageJ software (National Institutes of Health). Wound tissue was harvested postinjury by 6-mm punch biopsy. Wounds were digested at 37°C for 30 min with Liberase (50  $\mu$ g/mL; Roche) and DNaseI (20 units/mL; Sigma-Aldrich). Samples were filtered over a 100- $\mu$ m cell strainer to produce a single-cell suspension.

### Histology

Wounds were prepared for histological analysis in a standard fashion. Analysis of staining was performed with conventional methods. Details are provided in the Supplementary Data.

### Wound Macrophage Isolation and Magnetic-Activated Cell Sorting

Wounds were digested as described above. Single-cell suspensions were incubated with fluorescein isothiocyanate-labeled anti-CD3, anti-CD19, and anti-Ly6G (BioLegend) followed by anti-fluorescein isothiocyanate microbeads (Miltenyi Biotec). Flow-through was then incubated with anti-CD11b microbeads (Miltenyi Biotec) to isolate the nonneutrophil, nonlymphocyte, CD11b<sup>+</sup> cells. Cells were saved in TRIzol (Invitrogen) for quantitative

PCR (qPCR) or fixed for chromatin immunoprecipitation (ChIP).

### RNA Isolation

RNA isolation was performed by using conventional qPCR methods. Details are provided in the Supplementary Data.

### ChIP Assay

ChIP assay was performed as described previously (18). Detailed methods and primer sequences are provided in the Supplementary Data.

### Bone Marrow–Derived Macrophage Culture

For all in vitro studies, femurs/tibias of mice were flushed with cold RPMI medium. Cells were plated to yield a 99.99% homogenous population as confirmed by flow cytometry (12). Additional details are provided in the Supplementary Data. For MLL1 inhibitor experiments, bone marrow–derived macrophages (BMDMs) were treated for 24 h with MI-2.

### Nitric Oxide Measurement

For extracellular nitric oxide (NO) measurement, BMDMs were replated in triplicate and stimulated with lipopolysaccharide (LPS) (100 ng/mL) for 24 h. Nitrite levels were measured by using a Measure-iT High-Sensitivity Nitrite Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

### Adoptive Transfer

CD3<sup>-</sup>CD11c<sup>-</sup>CD19<sup>-</sup>Ly6G<sup>-</sup>NK1.1<sup>-</sup>CD11b<sup>+</sup> single-cell suspensions were isolated by magnetic-activated cell sorting (MACS) from spleens of *Mll1<sup>ff</sup>Lyz2<sup>Cre+</sup>* and *Mll1<sup>ff</sup>Lyz2<sup>Cre-</sup>* mice as described above. One million cells were injected intravenously in wounded (day 1) *Mll1<sup>ff</sup>Lyz2<sup>Cre-</sup>* mice, and wound closure was measured.

### Flow Cytometry

Before flow cytometry, single-cell suspensions were either surface stained directly or placed in ex vivo culture for 2 h with GolgiStop (1:2,000 dilution) with or without LPS (100 ng/mL). Staining details are provided in the Supplementary Data. Cells were acquired on a three-laser NovoCyte Flow Cytometer (ACEA Biosciences), and data were analyzed with FlowJo version 10.0 software (Tree Star). All populations were routinely backgated to verify consistency and purity.

### Immunoblotting

Conventional methods were used to detect interleukin-1 $\beta$  (IL-1 $\beta$ ) by Western immunoblotting. Details are provided in the Supplementary Data.

### Isolation of Human Monocytes From Peripheral Blood

Thirty milliliters of peripheral blood were obtained from patients with T2D and control subjects without diabetes under the direction of institutional review board protocol (HUM#00060733). After red blood cell lysis and Ficoll separation, monocytes were isolated by using human anti-CD14 magnetic beads.

### Statistical Analysis

Data were analyzed with GraphPad Prism 6.0 software. The statistical significance of differences between groups was determined by Student *t* test. Differences among more than two groups were evaluated by ANOVA followed by Newman-Keuls post hoc test. Data are expressed as the mean  $\pm$  SEM. *P*  $\leq$  0.05 was considered statistically significant.

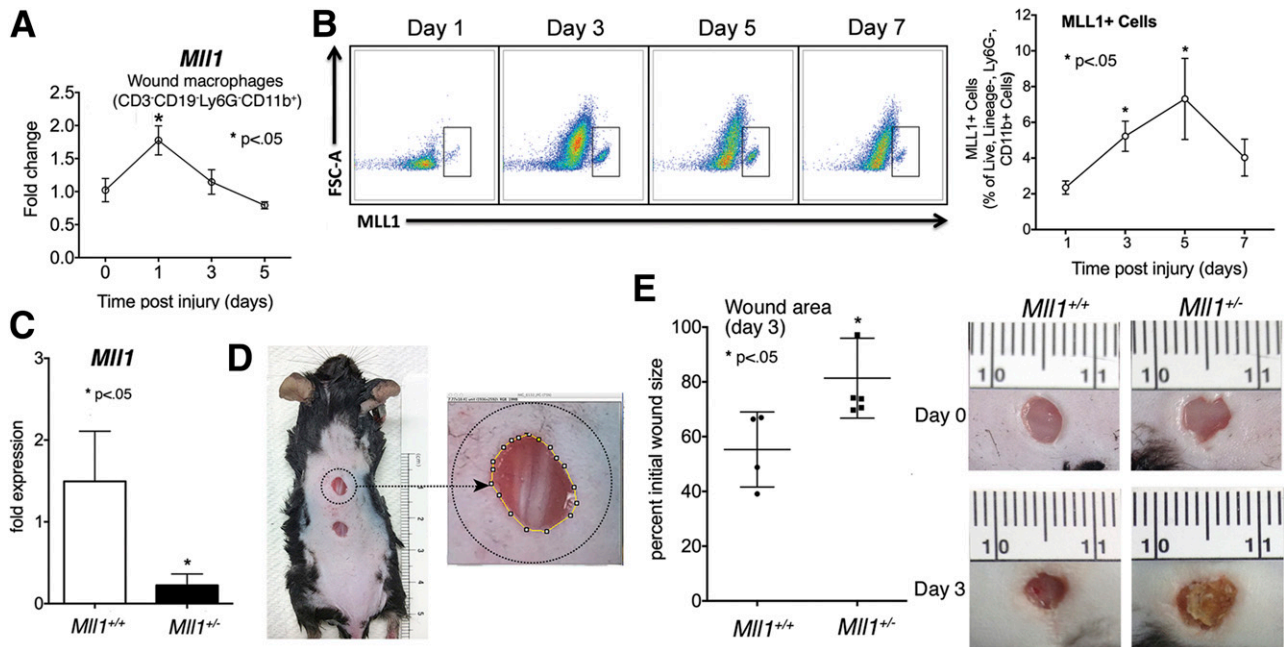
## RESULTS

### MLL1 Is Upregulated in Macrophages in the Early Inflammatory Phase of Wound Healing

Increasing evidence suggests that proper wound healing requires the establishment of a regulated inflammatory response mediated by macrophages (9,12,28). Because previous studies suggested that the histone methyltransferase MLL1 drives inflammatory gene expression in myeloid cells (23), we examined the role of MLL1 in macrophages during wound repair. C57BL/6 mice were subjected to 4-mm full-thickness wounds as previously described (12), and macrophages (CD11b<sup>+</sup>[CD3<sup>-</sup>CD19<sup>-</sup>Ly6G<sup>-</sup>]) were isolated from wounds through MACS at early time points (days 1–5) postwounding. We first examined *Mll1* gene expression and found it significantly increased in macrophages early after injury (Fig. 1A). To determine whether changes in *Mll1* transcript resulted in altered MLL1 protein, we analyzed wound macrophages by flow cytometry for MLL1 at days 1, 3, 5, and 7 postinjury (Fig. 1B). We found significant increases in MLL1 in wound macrophages at days 3 and 5, suggesting that MLL1 plays an important role in regulating early macrophage-mediated wound inflammation. To determine whether this early increase in MLL1 in wound macrophages is necessary for healing, we used *Mll1<sup>+/-</sup>* heterozygote mice (*Mll1<sup>-/-</sup>* are embryonically lethal) to determine whether wounds with decreased MLL1 had impaired early healing (21). We first confirmed that wounds isolated from the *Mll1<sup>+/-</sup>* mice expressed less *Mll1* transcript than littermate controls (Fig. 1C). *Mll1<sup>+/-</sup>* mice and controls were wounded, and wound closure was measured at day 3 by using ImageJ software (Fig. 1D). We found that *Mll1<sup>+/-</sup>* mice had impaired healing at day 3 compared with littermate controls (Fig. 1E). These findings suggest that upregulation of MLL1 is necessary for normal wound closure.

### Wound Healing Is Impaired in Macrophage-Specific MLL1-Deficient Mice

Because *Mll1* was upregulated early in normal wound macrophages and mice with insufficient *Mll1* demonstrated impaired early healing, we generated mice deficient in *Mll1* in cells of the myeloid lineage with lysosomes (monocytes, macrophages, granulocytes) by using the Cre-lox system. Myeloid-specific depletion of *Mll1* was confirmed by examining MACS splenic and wound monocyte/macrophages from *Mll1<sup>ff</sup>Lyz2<sup>Cre+</sup>* mice and littermate controls (*Mll1<sup>ff</sup>Lyz2<sup>Cre-</sup>*) (Fig. 2A). This was also confirmed with flow cytometry where MLL1 could be detected in wound macrophages from littermate controls but not from *Mll1<sup>ff</sup>Lyz2<sup>Cre+</sup>* mice. Moreover, no differences in



**Figure 1**—MLL1 is upregulated in macrophages in the early inflammatory phase of wound healing. **A:** Wounds were created by 4-mm punch biopsy on the back of C57BL/6 mice. Wounds were harvested on days 1–5 postwounding. Wound macrophages CD11b<sup>+</sup>[CD3<sup>+</sup>CD19<sup>+</sup>Ly6G<sup>+</sup>CD11b<sup>+</sup>] were isolated by MACS. *Mll1* gene expression was quantified by qPCR over time ( $n = 16$ , repeated two times). **B:** Wounds were created by 4-mm punch biopsy on the back of C57BL/6 mice. Wounds were harvested on days 1, 3, 5, and 7 postinjury; cells were isolated; and single-cell suspensions were processed for flow cytometry. Pseudocolor plots and data analysis of MLL1<sup>+</sup> cells as a percentage of live, lineage<sup>-</sup>, Ly6G<sup>-</sup>, CD11b<sup>+</sup> cells ( $n = 20$ , repeated one time). **C:** *Mll1*<sup>+/-</sup> heterozygote and *Mll1*<sup>+/+</sup> wild-type mice were wounded, and *Mll1* expression was examined by qPCR ( $n = 10$ , repeated one time). **D:** Wound healing model. Wound closure was monitored daily with photographs of the mice by using an internal scale, and wound areas were measured with ImageJ software. **E:** *Mll1*<sup>+/-</sup> heterozygote and *Mll1*<sup>+/+</sup> wild-type mice were wounded and images recorded until day 3 postinjury. Scatter plot of *Mll1*<sup>+/-</sup> heterozygote and *Mll1*<sup>+/+</sup> wild-type wound area at day 3 and representative photographs of wounds at days 0 and 3 ( $n = 10$ , repeated one time). Statistical analysis was by Student *t* test. Data are mean  $\pm$  SEM. FSC-A, forward scatter area.

MLL1 were detected in lineage<sup>+</sup> nonmyeloid cells (Fig. 2B). Wounds were generated in the *Mll1*<sup>ff</sup>*Lyz2*<sup>Cre+</sup> mice and their littermate controls, and wound closure was analyzed daily for 7 days. Wound closure was markedly delayed at early time points in the *Mll1*<sup>ff</sup>*Lyz2*<sup>Cre+</sup> mice (Fig. 2C). Histological assessment showed that the wounds from *Mll1*<sup>ff</sup>*Lyz2*<sup>Cre+</sup> mice had significantly less reepithelialization than littermate controls. Furthermore, less inflammatory infiltrate was seen on hematoxylin-eosin-stained sections, and trichrome staining showed less collagen deposition in *Mll1*<sup>ff</sup>*Lyz2*<sup>Cre+</sup> mice than in controls (Fig. 2D). To determine whether MLL1-deficient macrophages would alter healing in wild-type mice, we performed an adoptive transfer with macrophages from *Mll1*<sup>ff</sup>*Lyz2*<sup>Cre+</sup> and *Mll1*<sup>ff</sup>*Lyz2*<sup>Cre-</sup> spleens and injected them into the peripheral blood of wounded (day 1) *Mll1*<sup>ff</sup>*Lyz2*<sup>Cre-</sup> mice. Wound healing was significantly impaired in the mice that received *Mll1*<sup>ff</sup>*Lyz2*<sup>Cre+</sup> macrophages (Fig. 2E). Taken together, these results suggest that MLL1 is important in wound macrophages in the inflammatory phase of wound healing.

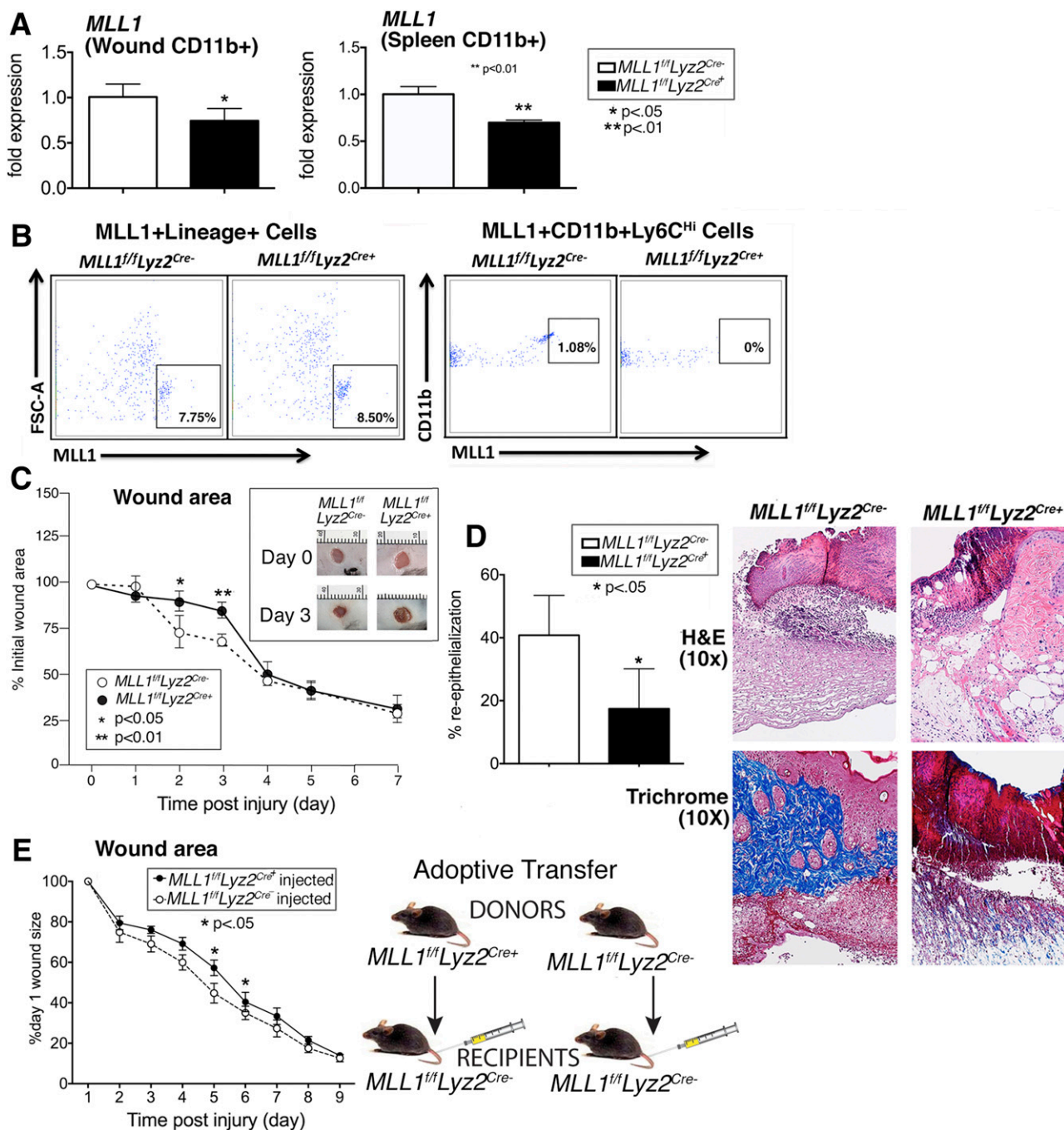
#### BMDMs From Mice Deficient in MLL1 Demonstrate Decreased Inflammatory Cytokine Expression

Because MLL1 is important for NF- $\kappa$ B-mediated initiation of the inflammatory response in other models, we

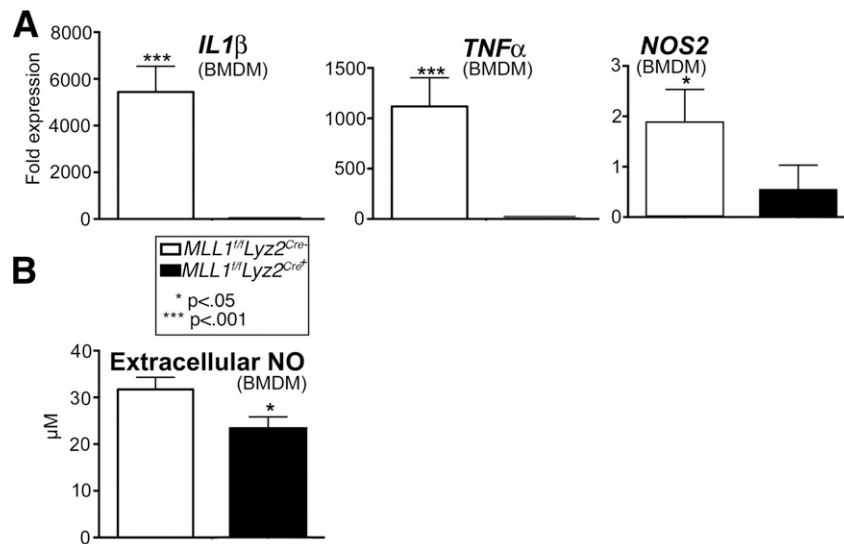
hypothesized that the absence of MLL1 in macrophages impairs healing by altering the early inflammatory response (20–22,24). To study this in vitro, BMDMs from *Mll1*<sup>ff</sup>*Lyz2*<sup>Cre+</sup> mice and their littermate controls (*Mll1*<sup>ff</sup>*Lyz2*<sup>Cre-</sup>) were stimulated with LPS and analyzed by qPCR for inflammatory genes important in wound healing, including *IL1 $\beta$* , *NOS2*, and *TNF $\alpha$*  (12). The current analysis demonstrated significantly less *IL1 $\beta$* , *NOS2*, and *TNF $\alpha$*  transcript produced by the *Mll1*<sup>ff</sup>*Lyz2*<sup>Cre+</sup> BMDMs compared with controls (Fig. 3A). Furthermore, when extracellular NO was measured in cell supernatants, less NO was made by *Mll1*<sup>ff</sup>*Lyz2*<sup>Cre+</sup> BMDMs than by those in controls (Fig. 3B). These data may indicate that inflammatory gene transcription in macrophages is controlled, at least partially, by MLL1.

#### Macrophages Isolated From Wounds of *Mll1*<sup>ff</sup>*Lyz2*<sup>Cre+</sup> Mice Display Decreased Inflammatory Cytokine Gene Expression and Protein Production

To determine whether our in vitro findings translate to in vivo wound macrophages, we isolated wound macrophages through MACS from *Mll1*<sup>ff</sup>*Lyz2*<sup>Cre+</sup> and littermate controls. We examined inflammatory cytokines known to play a major role in healing and found that *IL1 $\beta$*  and *TNF $\alpha$*  were significantly reduced in the *Mll1*<sup>ff</sup>*Lyz2*<sup>Cre+</sup> wound



**Figure 2**—Wound healing is impaired in macrophage-specific MLL1-deficient mice (*MLL1<sup>fl/fl</sup>Lyz2<sup>Cre+</sup>*). We generated mice deficient in *MLL1* in cells of myeloid lineage with lysosomes (monocytes, macrophages, granulocytes) by using the Cre-lox system. **A**: Myeloid depletion of *MLL1* was examined by qPCR in MACS splenic and wound macrophages CD11b<sup>+</sup>[CD3<sup>-</sup>CD19<sup>-</sup>Ly6G<sup>-</sup>] from *MLL1<sup>fl/fl</sup>Lyz2<sup>Cre+</sup>* mice and littermate controls (*MLL1<sup>fl/fl</sup>Lyz2<sup>Cre-</sup>*) ( $n = 10$ , repeated one time). **B**: Depletion of MLL1 was examined by flow cytometry in nonmyeloid Lin<sup>+</sup> cells (CD3, CD19, NK1.1, Ter-119)<sup>+</sup> and CD11b<sup>+</sup>, Ly6C<sup>Hi</sup> cells from spleens of *MLL1<sup>fl/fl</sup>Lyz2<sup>Cre+</sup>* mice and littermate controls (*MLL1<sup>fl/fl</sup>Lyz2<sup>Cre-</sup>*). **C**: Wounds were created by 4-mm punch biopsy on the backs of *MLL1<sup>fl/fl</sup>Lyz2<sup>Cre+</sup>* mice and littermate control mice. The change in wound area was recorded daily with ImageJ software until complete healing was observed. Representative photographs of the wounds of *MLL1<sup>fl/fl</sup>Lyz2<sup>Cre+</sup>* mice and littermate controls on days 0 and 3 postinjury are shown ( $n = 20$ , repeated three times). **D**: Wounds were created by 4-mm punch biopsy on the backs of *MLL1<sup>fl/fl</sup>Lyz2<sup>Cre+</sup>* mice and littermate control mice. Wounds were harvested on day 2, paraffin embedded, and sectioned. Sections (5  $\mu$ mol/L) were stained with hematoxylin-eosin (H&E) and Masson’s trichrome. Percent reepithelialization was calculated by measuring the distance traveled by epithelial tongues on both sides of the wound divided by total distance for full reepithelialization. Representative images are shown ( $n = 10$ , repeated one time). **E**: CD3<sup>-</sup>CD11c<sup>-</sup>CD19<sup>-</sup>Ly6G<sup>-</sup>NK1.1<sup>-</sup>CD11b<sup>+</sup> single-cell suspensions were isolated from *MLL1<sup>fl/fl</sup>Lyz2<sup>Cre+</sup>* and *MLL1<sup>fl/fl</sup>Lyz2<sup>Cre-</sup>* spleens by MACS. Cells ( $1 \times 10^6$ ) were injected intravenously in wounded (day 1) *MLL1<sup>fl/fl</sup>Lyz2<sup>Cre-</sup>* mice, and wound closure was measured daily with ImageJ software ( $n = 15$ ). Statistical analysis was by Student *t* test. Data are mean  $\pm$  SEM. FSC-A, forward scatter area.



**Figure 3**—BMDMs from mice insufficient for the histone methyltransferase MLL1 demonstrated decreased inflammatory cytokine expression in vitro. Cultured BMDMs harvested from *Mll1<sup>f/f</sup>Lyz2<sup>Cre+</sup>* mice and littermate controls were stimulated with LPS (100 ng/mL) for 6 h and then collected for analysis. *A*: *IL1β*, *TNFα*, and *NOS2* gene expression was quantified by qPCR ( $n = 9$ , repeated two times in triplicate). *B*: NO assay in BMDM supernatants ( $n = 16$ , repeated one time in triplicate). Statistical analysis was by Student *t* test. Data are mean  $\pm$  SEM.

macrophages (12,29) (Fig. 4A). To examine whether these altered inflammatory transcript levels correlated with decreased cytokine production by wound macrophages, we performed flow cytometry on day 2 wounds and found that the macrophages (live, lineage<sup>-</sup>, Ly6G<sup>-</sup>, CD11b<sup>+</sup>, Ly6C<sup>Hi</sup>) from *Mll1<sup>f/f</sup>Lyz2<sup>Cre+</sup>* wounds made significantly less IL-1β and TNF-α protein than controls (Fig. 4B). In addition, we performed a Western blot on wound macrophage lysates to determine IL-1β levels. There was significantly reduced IL-1β made by the *Mll1<sup>f/f</sup>Lyz2<sup>Cre+</sup>* wound macrophages compared with controls (Fig. 4C). Finally, similar to that performed above, wound macrophages were isolated from *Mll1<sup>f/f</sup>Lyz2<sup>Cre+</sup>* mice and controls at a later time point (day 5) and analyzed by flow cytometry for IL-1β production, which demonstrated a similar MLL1-dependent reduction in IL-1β (Supplementary Fig. 1). These data support that MLL1 is important in vivo for macrophage-mediated inflammation in wounds.

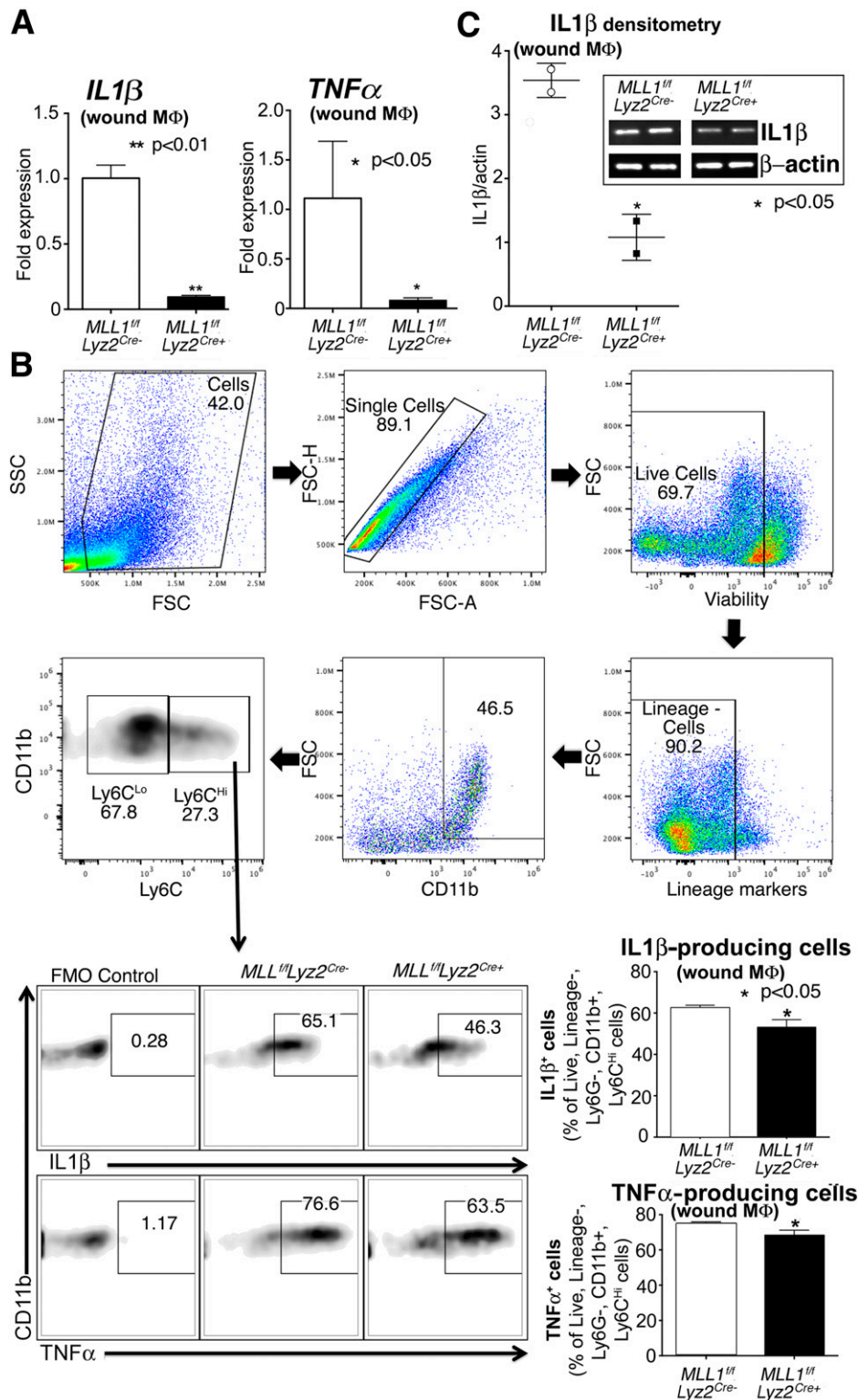
#### MLL1 Mediates Wound Macrophage Inflammation by Increasing H3K4me3 at the NF-κB Transcription Factor Binding Site

To determine the underlying mechanism for changes in inflammatory cytokine expression in MLL1-deficient macrophages, we cultured *Mll1<sup>f/f</sup>Lyz2<sup>Cre+</sup>* and littermate control BMDMs and performed ChIP analysis of the NF-κB binding sites of the *IL1β* and *TNFα* gene promoters. As stated previously, MLL1 has substrate specificity for H3K4. H3K4me3 is well established to result in gene activation and transcription (16,30). We found that H3K4me3 was significantly decreased at the NF-κB binding site on the *IL1β* and *TNFα* promoter regions in *Mll1<sup>f/f</sup>Lyz2<sup>Cre+</sup>* BMDMs compared with controls (Fig. 5A). To confirm this finding in vivo, we isolated macrophages by MACS from *Mll1<sup>f/f</sup>Lyz2<sup>Cre+</sup>* wounds

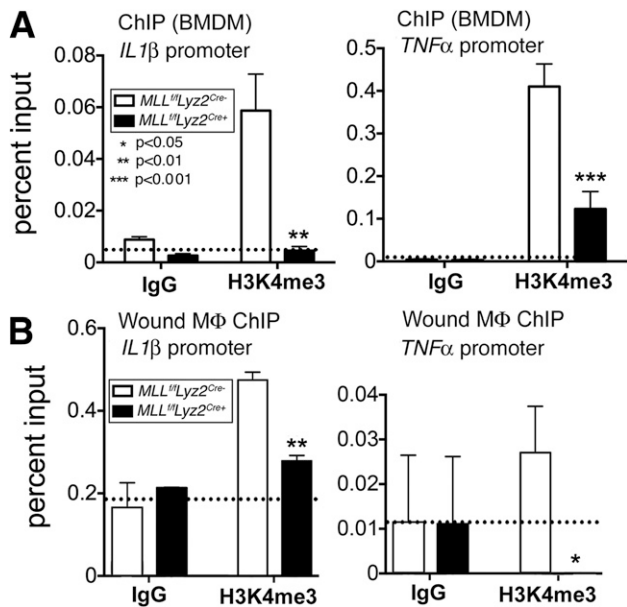
and controls for ChIP analysis. We found significant decreases in H3K4me3 at the NF-κB binding sites on the *IL1β* and *TNFα* promoters in the *Mll1<sup>f/f</sup>Lyz2<sup>Cre+</sup>* mice (Fig. 5B). Non-NF-κB binding sites on the *IL1β* and *TNFα* promoters for H3K4me3 showed no differences between the *Mll1<sup>f/f</sup>Lyz2<sup>Cre+</sup>* mice and controls (Supplementary Fig. 2). These findings suggest an NF-κB-associated epigenetic mechanism for the impaired inflammatory response seen in MLL1-deficient mice.

#### Wound Macrophages Isolated From DIO Mice Display Decreased Early Inflammatory Cytokine Production, MLL1, and H3K4me3 at Inflammatory Gene Promoters

Although a significant amount of literature details chronic inflammation in diabetic wounds, less is known about the effects of diabetes on the early inflammatory phase after acute injury. Prior studies have suggested that an early coordinated inflammatory response by macrophages is essential for normal wound healing (9). Because we demonstrated that MLL1 is necessary for an appropriate early inflammatory response in normal wound healing, we examined MLL1 in a murine model of glucose intolerance/insulin resistance to determine whether MLL1 plays a role in the dysregulated inflammation seen in diabetic wounds. We used the DIO mouse as a model of prediabetes because it physiologically mirrors human disease and does not have the genetic leptin manipulations that have been shown to alter innate immunity (31,32). We first examined DIO and control wounds by flow cytometry for TNF-α and IL-1β production 2 days after injury. We found that IL-1β and TNF-α were decreased in macrophages (live, lineage<sup>-</sup>, Ly6G<sup>-</sup>, CD11b<sup>+</sup>, Ly6C<sup>Hi</sup>) from the DIO wounds at this early time point, similar to the *Mll1<sup>f/f</sup>Lyz2<sup>Cre+</sup>* mice in Fig. 4B (Fig. 6A). Of note, no statistical differences in macrophage



**Figure 4**—Macrophages isolated from wounds of  $Mll1^{fl/fl}Lyz2^{Cre/+}$  mice display decreased inflammatory cytokine gene expression and protein levels. **A:** Wound macrophages were isolated from  $Mll1^{fl/fl}Lyz2^{Cre/+}$  mice and littermate controls at day 2 postinjury by MACS for  $CD11b^+CD3^-CD19^-Ly6G^-$  cells.  $TNF\alpha$  and  $IL1\beta$  gene expression in isolated macrophages was measured by qPCR ( $n = 12$ , repeated two times). **B:**  $Mll1^{fl/fl}Lyz2^{Cre/+}$  and littermate control wound cell isolates were processed for ex vivo intracellular flow cytometry after stimulation with LPS (100 ng/mL) for 2 h. The gating strategy used for ex vivo intracellular flow cytometry selecting live, lineage<sup>-</sup>, Ly6G<sup>-</sup>, CD11b<sup>+</sup>, Ly6C<sup>Hi</sup> cells is shown. Flow cytometry quantification of IL-1 $\beta$  and TNF- $\alpha$  in wounds ( $n = 10$ , repeated one time). **C:** IL-1 $\beta$  protein levels in wound cell lysates from  $Mll1^{fl/fl}Lyz2^{Cre/+}$  mice and littermate controls by Western blot ( $n = 20$ , repeated one time). Statistical analysis was by Student *t* test. Data are mean  $\pm$  SEM. FMO, fluorescence minus one; FSC, forward scatter; FSC-A, forward scatter area; FSC-H, forward scatter height; M $\Phi$ , macrophage; SSC, side scatter.



**Figure 5**—MLL1 mediates wound macrophage inflammation by increasing H3K4me3 at the NF- $\kappa$ B transcription factor binding site. **A**: ChIP analysis for H3K4me3 at the NF- $\kappa$ B binding site of the *TNF $\alpha$*  and *IL1 $\beta$*  promoters in *MLL1<sup>fl/fl</sup>Lyz2<sup>Cre+</sup>* and littermate control BMDMs ( $n = 10$ , repeated two times in triplicate). **B**: Wound macrophages were isolated from *MLL1<sup>fl/fl</sup>Lyz2<sup>Cre+</sup>* mice and littermate controls at day 2 postinjury by MACS for CD11b<sup>+</sup>[CD3<sup>-</sup>CD19<sup>-</sup>Ly6G<sup>-</sup>] cells. ChIP analysis for H3K4me3 at the NF- $\kappa$ B binding site of the *TNF- $\alpha$*  and *IL-1 $\beta$*  promoters in macrophages isolated from the wounds of *MLL1<sup>fl/fl</sup>Lyz2<sup>Cre+</sup>* and littermate controls was performed ( $n = 10$ , repeated two times). For all ChIP experiments, isotype control antibody to IgG was run in parallel. Statistical analysis was by Student *t* test. Data are mean  $\pm$  SEM. M $\Phi$ , macrophage.

numbers between DIO and control wounds were observed (Supplementary Fig. 3). These minor differences in cell numbers are a result of a greater tendency for apoptosis in DIO wound macrophages (Supplementary Fig. 4). In addition, MLL1 was found to be decreased in DIO wound macrophages by both qPCR and flow cytometry at day 2 postinjury, correlating with *MLL1<sup>fl/fl</sup>Lyz2<sup>Cre+</sup>* wounds (Fig. 6B and C). Given the decrease in MLL1 in DIO macrophages, we performed a ChIP analysis on DIO and control wound macrophages at day 2 for H3K4me3 at the NF- $\kappa$ B binding sites on the *IL1 $\beta$*  and *TNF $\alpha$*  promoters. NF- $\kappa$ B binding sites on the *IL1 $\beta$* , *TNF $\alpha$* , and *NOS2* promoters demonstrated a significant decrease in H3K4me3 in the DIO wound macrophages compared with controls (Fig. 6D and E and Supplementary Fig. 5). These findings suggest a direct correlation between decreased MLL1 and an impaired early inflammatory response in DIO wound macrophages.

#### Upregulation of MLL1 in DIO Wound Macrophages at Late Time Points Is Associated With Increased Inflammatory Mediator Production and Is Reversible With a Chemical Inhibitor

Given that a decrease in MLL1 in early wound macrophages is associated with an impaired early inflammatory response, the data suggest a possible mechanism for epigenetic regulation

of DIO wound macrophage inflammatory disposition. Because DIO wounds have previously been shown by our group and others to have impaired wound healing secondary to a prolonged inflammatory response, we examined whether MLL1 and H3K4me3 were increased in DIO wound macrophages at late time points (12,33). Wound macrophages were analyzed by flow cytometry or isolated by MACS from DIO and control wounds on day 5 postinjury. MLL1 was found to be significantly increased in day 5 DIO wound macrophages by qPCR and flow cytometry (Fig. 7A and B). Intracellular flow cytometry analysis of wounds at day 5 showed increased IL-1 $\beta$  and a trend toward increased TNF- $\alpha$  in the DIO wound macrophages compared with controls (Fig. 7C). ChIP analysis of the NF- $\kappa$ B binding site on the *IL1 $\beta$* , *TNF $\alpha$* , and *NOS2* promoters demonstrated increased H3K4me3 in the day 5 DIO wound macrophages compared with controls (Fig. 7D and E and Supplementary Fig. 5). Finally, to examine the effect of MLL1 in DIO macrophages, we cultured BMDMs from DIO mice and treated for 24 h with the MLL1 inhibitor MI-2 and found decreased expression of *IL1 $\beta$*  and *TNF $\alpha$*  after stimulation with LPS (Fig. 7F). These findings suggest that MLL1-mediated H3K4me3 at the inflammatory cytokine promoters may be responsible, at least in part, for the increased inflammation seen in DIO wounds.

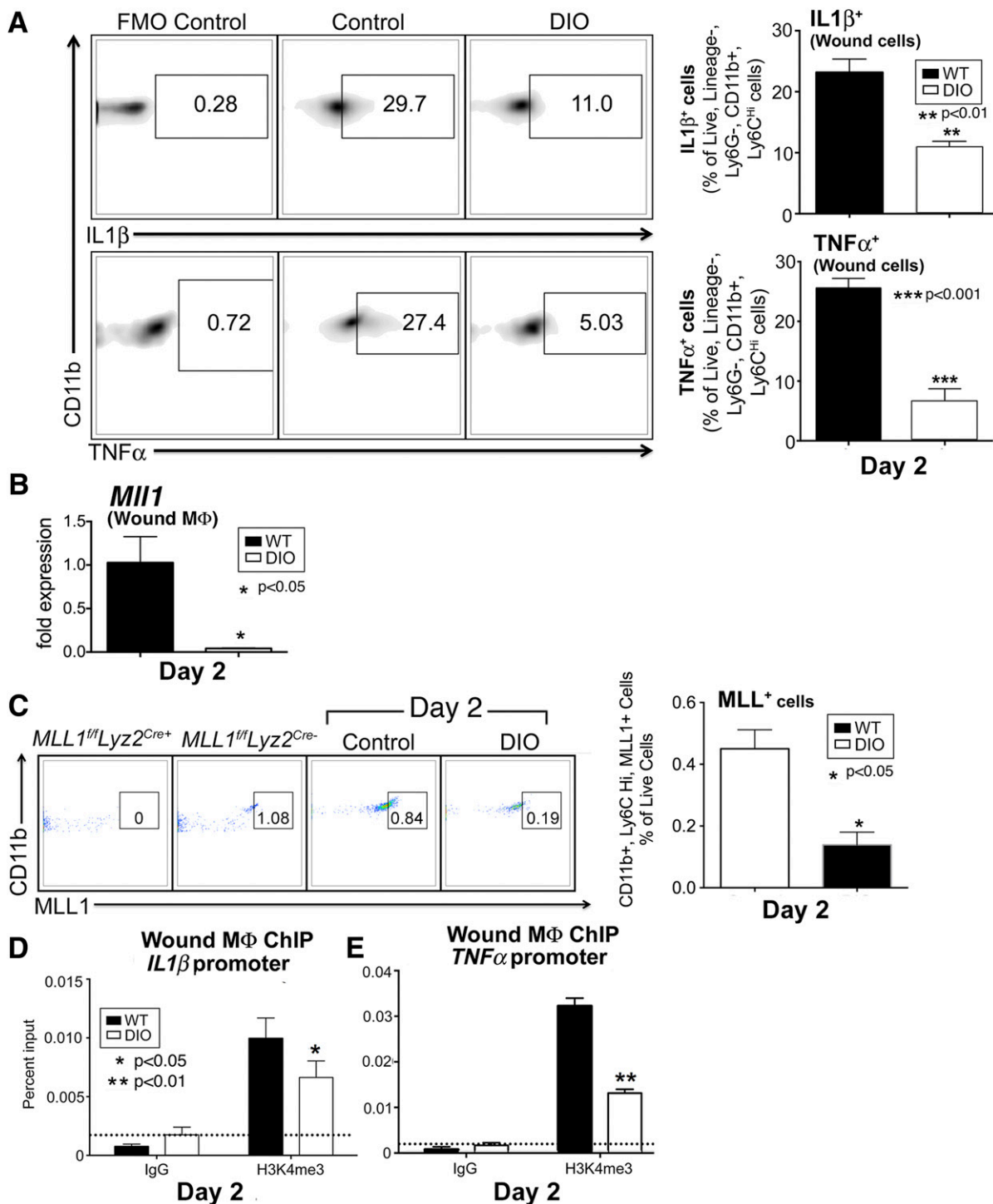
#### Human Monocytes From Patients With T2D Display Increased *MLL1*

To evaluate whether our findings are translatable to humans, we isolated peripheral blood monocytes (CD14<sup>+</sup>) from patients with T2D and healthy volunteers without diabetes. *MLL1* expression was increased in T2D blood monocytes compared with nondiabetic control monocytes (Fig. 8), which may indicate that in patients with diabetes, monocytes recruited from the blood to peripheral wounds exist in a hyperinflammatory state, given that the *MLL1* transcript is already present, and provides for increased inflammation through H3K4me3 of inflammatory cytokine promoters. These findings may partially explain the chronic inflammatory state of nonhealing T2D wounds.

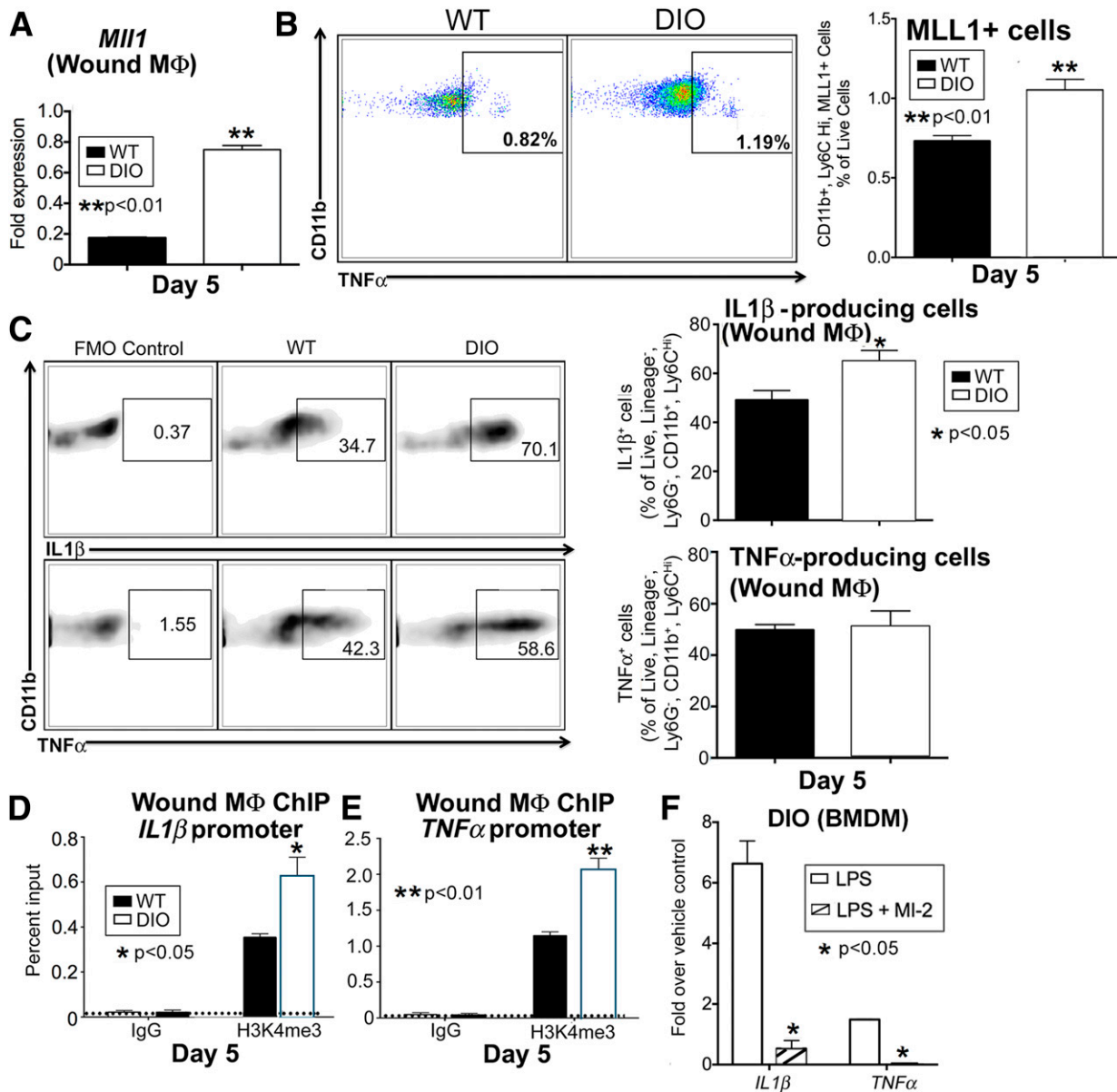
#### DISCUSSION

It is well established that macrophages drive increased inflammation in obesity and T2D and contribute to the chronic inflammation seen in diabetic wounds; however, the etiology of this increased inflammatory state is unclear (5,12,29,33,34). We have previously shown that the histone demethylase JMJD3 contributes to the inflammatory macrophage phenotype seen in diabetic wounds (12). In this study, we identify MLL1-mediated mechanisms that alter macrophage activity in both normal and prediabetic wound healing. To our knowledge, this is the first study to report that the histone methyltransferase MLL1 regulates macrophage-mediated inflammation in peripheral wounds. Specifically, MLL1 increases H3K4me3 at NF- $\kappa$ B binding sites on inflammatory cytokine gene promoters in wound macrophages, which correlates with increased inflammatory





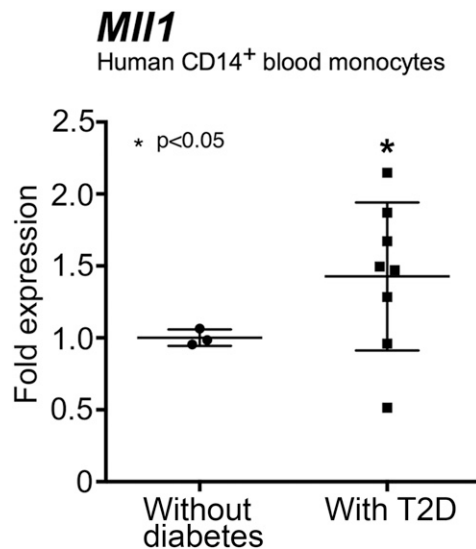
**Figure 6**—Macrophages isolated from DIO mice display decreased early inflammatory cytokine production, MLL1, and H3K4me3 at inflammatory gene promoters. **A:** DIO and control murine wounds were harvested 2 days after injury, and wound cells were isolated. Single-cell suspensions were processed for flow cytometry. Density plots and associated data for IL-1 $\beta$  and TNF- $\alpha$  staining in live, lineage<sup>-</sup>, Ly6G<sup>-</sup>, CD11b<sup>+</sup>, Ly6C<sup>Hi</sup> cells (as gated in Fig. 4) from DIO and control wounds ( $n = 12$ , repeated one time). **B:** DIO and control wounds were harvested 2 days after injury for cell isolation and macrophage selection for CD11b<sup>+</sup>[CD3<sup>-</sup>, CD19<sup>-</sup>, Ly6G<sup>-</sup>] cells through MACS. *MLL1* expression as measured by qPCR from wound macrophages ( $n = 14$ , repeated one time). **C:** Pseudocolor plots and data analysis of wound MLL1<sup>+</sup>, CD11b<sup>+</sup>, Ly6C<sup>Hi</sup> cells as a percentage of live cells in control and DIO mice at day 2 postinjury. *Mll1*<sup>f/f</sup>*Ly2*<sup>Cre/+</sup> and littermate control spleens were used as staining controls for MLL1 ( $n = 10$ ; repeated one time). **D** and **E:** DIO and control wounds were harvested 2 days after wounding for cell isolation and macrophage selection for CD11b<sup>+</sup>[CD3<sup>-</sup>, CD19<sup>-</sup>, Ly6G<sup>-</sup>] cells through MACS. ChIP analyses of wound macrophages for H3K4me3 at the NF- $\kappa$ B binding sites of the promoters of *IL1β* (**D**) and *TNFα* (**E**) were performed ( $n = 12$ , repeated two times). Statistical analysis was by Student *t* test. Data are mean  $\pm$  SEM. FMO, fluorescence minus one; MΦ, macrophage; WT, wild type.



**Figure 7**—MLL1 upregulation in DIO mice and consequent epigenetic changes are associated with increased inflammation that is reversible with an MLL1 inhibitor. **A**: DIO and control wounds were harvested 5 days after injury for cell isolation and macrophage selection for CD11b<sup>+</sup>[CD3<sup>-</sup>, CD19<sup>-</sup>, Ly6G<sup>-</sup>] cells through MACS. *Mll1* expression as measured by qPCR from wound macrophages ( $n = 12$ , replicated two times). **B**: DIO and control murine wounds were harvested 5 days after injury, and wound cells were isolated and processed for flow cytometry. Pseudo-color plots and data analysis of MLL1<sup>+</sup>, CD11b<sup>+</sup>, Ly6C<sup>Hi</sup> cells as a percentage of live cells in DIO and control wounds at day 5 postinjury. *Mll1<sup>f/f</sup>Ly2z<sup>Cre+</sup>* and littermate control spleens were used as staining controls for MLL1 ( $n = 10$ ). **C**: DIO and control wound cell isolates were collected at day 5 postinjury and processed for ex vivo intracellular flow cytometry after 2 h of LPS (100 ng/mL) stimulation. Density plots and associated data for IL-1 $\beta$  and TNF- $\alpha$  staining in live, lineage<sup>-</sup>, Ly6G<sup>-</sup>, CD11b<sup>+</sup>, Ly6C<sup>Hi</sup> cells (as gated in Fig. 4) ( $n = 10$ , repeated one time). **D** and **E**: DIO and control wounds were harvested 5 days after injury for wound cell isolation and macrophage selection for CD11b<sup>+</sup>[CD3<sup>-</sup>, CD19<sup>-</sup>, Ly6G<sup>-</sup>] cells through MACS. ChIP analyses of wound macrophages for H3K4me3 at the NF- $\kappa$ B binding sites of the promoters of *IL1 $\beta$*  (**D**) and *TNF $\alpha$*  (**E**) were performed ( $n = 15$ , repeated two times). **F**: BMDMs from DIO mice were cultured in the presence of an MLL1 inhibitor, MI-2, for 24 h followed by stimulation with LPS (100 ng/mL) for 6 h. qPCR was performed for expression of *IL1 $\beta$*  and *TNF $\alpha$*  ( $n = 6$ , repeated two times in triplicate). Statistical analysis was by Student *t* test. Data are mean  $\pm$  SEM. FMO, fluorescence minus one; MΦ, macrophage; WT, wild type.

cytokine expression and results in increased wound inflammation. Furthermore, the early deficit and late overexpression of MLL1 and the corresponding changes in H3K4me3 at inflammatory gene promoters in wound macrophages from our glucose intolerant/insulin resistant mice directly

correlate with early impaired inflammation and late increased inflammation in this prediabetic model. Hence, these findings define a potential therapeutic target to correct impairments in the inflammatory program in diabetic wound macrophages that contribute to dysregulated inflammation.



**Figure 8**—Human monocytes from patients with T2D display increased *Mll1*. Peripheral blood (30 mL) was collected from patients with T2D and control subjects without diabetes. No statistical differences were found between groups with respect to sex, age, or comorbid conditions. Peripheral blood mononuclear cells underwent red blood cell lysis followed by Ficoll separation. CD14<sup>+</sup> monocytes were then positively selected by MACS, and *Mll1* gene expression was measured by qPCR ( $n = 11$ ). Statistical analysis was by Student *t* test. Data are mean  $\pm$  SEM.

Because macrophages exhibit different functional phenotypes as tissue repair progresses, the ability to modulate macrophage phenotype at a particular time after injury is an attractive therapeutic strategy (11). For example, an MLL1 agonist may augment early macrophage-mediated inflammation and help the wound healing cascade to occur in a programmed fashion. This early introduction of MLL1 in wound macrophages may prevent the subsequent late overexpression of MLL1 that contributes to increased inflammation in diabetic wounds. The second and most translatable strategy would be MLL1 small-molecule inhibitors that are currently approved by the U.S. Food and Drug Administration for use in cancer (35,36). MLL1 inhibitors are attractive for use in wounds because they can be locally administered, negating many of the toxic effects of systemic administration (36). One currently available inhibitor, MI-2, inhibits MLL1 activity by blocking binding of its cofactor and preventing cell proliferation in leukemia (36). In the current study, we demonstrate that prediabetic macrophages treated with MI-2 express less inflammatory transcript, suggesting that modulation of macrophage-mediated inflammation with this drug may be possible. MLL1 small-molecule inhibitors administered at the appropriate time postinjury may be an ideal therapy to decrease chronic inflammation in diabetic wounds.

The current findings support the theory that the diabetic milieu alters immune cell phenotypes through epigenetics (37,38). These epigenetic changes can contribute to a phenomenon known as metabolic memory where

chromatin modifications persist even after a normal physiological environment is restored (37). For example, hyperglycemia has been shown to stimulate the histone methyltransferase SET7, which monomethylates H3K4 at NF- $\kappa$ B binding sites, leading to increased inflammatory mediator production (39). Likewise, in the current study, we show that prediabetic wound macrophages have aberrant expression of MLL1; which in turn affects H3K4me3 at inflammatory gene promoters, inflammatory mediator expression, and ultimately, macrophage function. Because current treatments to abrogate chronic inflammation in diabetic wounds have been only marginally effective (5), the current findings provide a rationale for targeting MLL1 at the local level to reduce chronic inflammation and potentially improve rates of wound closure.

Although this study produces insight into the mechanism behind dysregulated inflammation in prediabetic wound healing, some limitations must be addressed. First, our finding of an early deficit and late overexpression of MLL1 in prediabetic murine wound macrophages associated with decreased early and increased late inflammation is correlative. Although the H3K4me3/MLL1 upregulation in these prediabetic mice suggests a potential mechanism for increased late inflammation, it does not prove that MLL1 is the sole contributor to macrophage inflammatory cytokine production. Furthermore, although MLL1 appears to influence wound macrophage phenotype, we recognize that other epigenetic enzymes may play a role in aberrant macrophage function in pathological states (12,40–43). Finally, although MLL1 clearly influences macrophage inflammatory disposition, what other off-target effects it may have on macrophage function that further affect wound healing are unclear. For instance, MLL1 has been shown in tumor angiogenesis to upregulate hypoxia-inducible factor 1 $\alpha$  and vascular endothelial growth factor (44,45). Thus, further studies assessing the role of MLL1 in wound macrophages that use ChIP sequencing with parallel RNA sequencing would be useful to determine other genes influenced by MLL1.

In summary, we have established that the histone methyltransferase MLL1 alters macrophage-mediated inflammation in wounds. An MLL1-mediated increase in inflammatory mediators is necessary for normal wound healing, and loss of MLL1 in macrophages results in an impaired inflammatory response that delays wound closure. An early deficit and late overexpression of MLL1 in prediabetic wound macrophages directly correlates with the decreased early and increased late inflammation in our murine model of prediabetes. These findings suggest that MLL1 plays a significant role in dictating wound macrophage phenotype; furthermore, it may have significant relevance to macrophage-mediated inflammation in other secondary complications of diabetes (40,46–48). Although the exact molecular mechanisms that contribute to epigenetic enzyme alterations in hyperglycemia and insulin resistance remain largely obscure, understanding how MLL1 contributes to dysregulated macrophage function increases our

comprehension of the complex regulation of chronic inflammation in diabetic wounds.

**Acknowledgments.** The authors thank Robin G. Kunkel, research associate in the Pathology Department, University of Michigan, for the artwork.

**Funding.** This work was supported in part by National Institutes of Health grants DK-102357 and T32-HL076123 and the Taubman Scholars Foundation.

**Duality of Interest.** No potential conflicts of interest relevant to this article were reported.

**Author Contributions.** A.S.K. performed the research, analyzed the data, and assisted in writing the manuscript. A.J. performed the research. W.F.C. performed the research and reviewed and edited the manuscript. A.E.B., M.S., R.A., J.B., F.M.D., P.K.H., C.F.B., and S.L.K. reviewed and edited the manuscript. K.A.G. performed the research, analyzed data, and wrote the manuscript. K.A.G. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Prior Presentation.** Parts of this study were presented at the 76th Scientific Sessions of the American Diabetes Association, New Orleans, LA, 10–14 June 2016.

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