Chronic Inflammation in Response to Injury: Retention of Myeloid Cells in Injured Tissue Is Driven by Myeloid Cell Intrinsic Factors

Tanja Torbica¹, Kate Wicks¹, Takahiro Umehara¹, Lale Gungordu¹, Salma Alrdahe¹, Kelly Wemyss¹, John R. Grainger¹ and Kimberly A. Mace¹

Chronic inflammation is a hallmark of impaired healing in a plethora of tissues, including skin, and is associated with aging and diseases such as diabetes. Diabetic chronic skin wounds are characterized by excessive myeloid cells that display an aberrant phenotype, partially mediated by stable intrinsic changes induced during hematopoietic development. However, the relative contribution of myeloid cell—intrinsic factors to chronic inflammation versus aberrant signals from the local environmental was unknown. Moreover, identification of myeloid cell intrinsic factors that contribute to chronic inflammation in diabetic wounds remained elusive. Here we show that Gr-1⁺CD11b⁺ myeloid cells are retained specifically within the presumptive granulation tissue region of the wound at a higher density in diabetic mice and associate with endothelial cells at the site of injury with a higher frequency than in nondiabetic mice. Adoptive transfer of myeloid cells demonstrated that aberrant wound retention is due to myeloid cell intrinsic factors and not the local environment. RNA sequencing of bone marrow and wound-derived myeloid cells identified Selplg as a myeloid cell intrinsic factor that is deregulated in chronic wounds. In vivo blockade of this protein significantly accelerated wound healing in diabetic mice and may be a potential therapeutic target in chronic wounds and other chronic inflammatory diseases.

INTRODUCTION

Impaired wound healing in elderly people and patients with diabetes mellitus is a significant burden on the health care system, and was recently estimated to cost nearly £5 billion in the United Kingdom and $50 billion in the United States annually, totaling about 5% of the health care budget in each nation (Fife et al., 2010; Guest et al., 2015). The number of people older than age 60 years will increase from 901 million globally in 2015 to nearly 2 billion over the next few decades (United Nations, 2015), and the number of people with diabetes is projected to rise from 415 million in 2015 to more than 640 million by 2040 (Diabetes UK, 2016). Chronic wound development occurs in 15% of people with diabetes, with 84% of cases resulting in lower limb amputation, significant morbidity, and diminished quality of life (Brem and Tomic-Canic, 2007). Understanding the underlying mechanisms contributing to chronic wound development is critical to developing effective therapis for this unmet medical need.

Over the last 2 decades, the role of dysregulated inflammation associated with aging and diabetes has become apparent. In particular, myeloid cells, comprised predominantly of monocytes and granulocytes, display a hyperpolarized pro-inflammatory phenotype during wound healing, and fail to transition to the pro-healing phenotype at the appropriate time (Bannon et al., 2013; Mirza and Koh, 2011; Wicks et al., 2015). Although many previous studies suggest that the activation state of mature myeloid cells is dictated by their local environment, the diabetic environment may prime hematopoietic stem and progenitor cells toward a pro-inflammatory phenotype during early differentiation, and commit cells to this phenotype via epigenetic changes at loci involved in the inflammatory response (Bannon et al., 2013; Gallagher et al., 2015; Wicks et al., 2015) and, therefore, have important implications for treating diabetic patients with impaired wound healing.

However, the relative contributions of the microenvironment versus cell intrinsic factors, including potential epigenetic changes, on myeloid cell behavior have not been previously measured in vivo during wound healing. In this study, we address this using adoptive transfer of Gr-1⁺CD11b⁺ myeloid cells, the “first responders” to injured tissue that critically function to establish the inflammatory response. We identify a key cell intrinsic factor, PSGL-1/Selplg, using RNA sequencing, and show both epigenetic changes to the promoter, as well as aberrant NF-kB and STAT pathway activation, contribute to deregulation of this gene. Importantly, in vivo blockade of PSGL-1/Selplg significantly improves wound healing in a diabetic mouse model. These data show PSGL-1/Selplg is an important mediator of chronic inflammation in the diabetic environment, and may provide an important therapeutic target in treating excessive inflammation and delayed healing in future.
RESULTS
Gr-1<sup>+</sup> CD11b<sup>+</sup> cells show increased cell density in the granulation tissue region of wounds of diabetic mice

We have previously shown that Gr-1<sup>+</sup>CD11b<sup>+</sup> myeloid cells accumulate with significantly higher numbers in diabetic wounds in the inflammatory phase of wound healing (days 1–4) (Mahdipour et al., 2011), and are composed of granulocytes (neutrophils), inflammatory monocytes, and their progenitors. Once in the wound, they can differentiate into inflammatory macrophages. To better understand the kinetics of the myeloid cell subtypes within wounds over time, we analyzed nondiabetic and diabetic wounds at days 0, 1, 3, and 5 after wounding with additional markers (Figure 1, flow gating strategy shown in Supplementary Figure S1 online). Dendritic cells showed no significant differences at any time point between nondiabetic and diabetic wounds, however, in both backgrounds their number was significantly reduced at day 5 (Figure 1a). At day 1, neutrophil accumulation trended toward a decrease, and monocytes showed a significant decrease in diabetic wounds (Figure 1b, 1c), while macrophages showed no difference (Figure 1d). However, by days 3 and 5, these trends reversed, with more monocytes and neutrophils in diabetic wounds, and by day 5, diabetic wounds showed significantly more macrophages.

However, flow cytometry cannot provide spatial information and is a less sensitive method for detection of some markers (Saravanan and Juneja, 2010), as cell cluster removal from population analyses can skew results, particularly in sclerotic tissues such as wounds (Dunphy, 2004). Therefore, we performed immunofluorescence for Gr-1 and CD31, an endothelial cell marker, to analyze their co-localization at days 2, 3, and 5, as these time points represent the height of the inflammatory phase of healing and the onset of chronic inflammation in the diabetic wound.

Gr-1<sup>+</sup> cells predominantly localized to wound sites (α-regions) and peri-wound tissue (β-regions), with low-frequency localization to normal tissue (γ-regions) in both nondiabetic and diabetic wounds (Figure 2a–2e). We noted a significant increase in the accumulation of Gr-1<sup>+</sup> cells over time in the α-region in both nondiabetic and diabetic wounds, while remaining more constant in β-regions, where peri-wound blood vessels are localized, supporting an influx of Gr-1<sup>+</sup> cells to α-regions. Gr-1<sup>+</sup> cell density at all three time points was significantly increased in the α-region, containing the presumptive granulation tissue, in diabetic mice compared to nondiabetic controls, whereas there was only a significant increase in Gr-1<sup>+</sup> cell density in the β-region of wounds at days 2 and 5 in diabetic compared to nondiabetic mice (Figure 2a–2e).

Gr-1<sup>+</sup> cells show increased association with microvascular endothelial cells in wounds of diabetic mice

We previously demonstrated that Gr-1<sup>+</sup> cells from diabetic mice were more adherent to activated endothelial cells in vitro compared to nondiabetic-derived Gr-1<sup>+</sup> cells (Mahdipour et al., 2011). We therefore wanted to investigate Gr-1<sup>+</sup>-endothelial cell interactions in wounds.

Analysis of Gr-1<sup>+</sup> cell and CD31<sup>+</sup> endothelial cell co-localization within day-3 wounds showed an increased association in diabetic mice (Figure 2f, 2g), suggesting that diabetic Gr-1<sup>+</sup> cells may be more adherent to endothelial cells in vivo, possibly resulting in increased recruitment or longer retention of Gr-1<sup>+</sup> cells in diabetic wound tissue.

Retention of Gr-1<sup>+</sup> cells in diabetic wounds is primarily driven by cell intrinsic factors

Increased accumulation of Gr-1<sup>+</sup> cells in diabetic wounds might be due to either excessive recruitment or aberrant retention of Gr-1<sup>+</sup> cells, or both. In order to distinguish these possibilities, we performed adoptive transfer assays of nondiabetic-derived and diabetic-derived Gr-1<sup>+</sup> cells into nondiabetic and diabetic wounds, at day 2 following injury, followed by analysis at day 3.

To assess recruitment, GFP-expressing Gr-1<sup>+</sup> cells, freshly isolated from nondiabetic or diabetic mouse bone marrow (BM), were injected into wounded mice via the tail vein. After 24 hours, only a small proportion of injected cells (<1%) were detected in wounds. Although there was no significant difference in the recruitment of cells to wounds of nondiabetic and diabetic mice in this assay (data not shown), these results should be interpreted with caution, due to the lack of wound recruitment resulting from either rehoming of injected Gr-1<sup>+</sup> cells to BM and/or accumulation in peripheral tissues such as the lungs, as has been consistently observed for other BM-derived cell populations (De Becker and Riet, 2016).
To assess wound retention, adoptive transfer of RFP<sup>+</sup> nondiabetic and GFP<sup>+</sup> diabetic BM-derived Gr-1<sup>+</sup> cells directly to wounds was performed (Figure 3a). After 24 hours, labeled cells from both nondiabetic- and diabetic-derived Gr-1<sup>+</sup> cells were readily detectable in both wound environments. Diabetic-derived Gr-1<sup>+</sup> cells showed 5- to 10-fold significantly elevated retention in wounds of both nondiabetic and diabetic mice (Figure 3b) compared to nondiabetic-derived cells, suggesting that aberrant retention of Gr-1<sup>+</sup> cells is primarily dependent on cell intrinsic factors, rather than microenvironmental factors.
Gene expression profiling of BM-derived and wound-derived Gr-1<sup>+</sup> cells identifies candidate cell intrinsic factors

Gr-1<sup>+</sup> cell intrinsic factors that result in prolonged retention and chronic inflammation in the diabetic wound following injury may arise during differentiation in the BM, and are maintained in Gr-1<sup>+</sup> cells once they have been recruited to the wound. Therefore, in order to identify these factors, we performed RNA-sequencing analysis on Gr-1<sup>+</sup> cells from both BM and day-3 wounds of nondiabetic and diabetic mice. Cross comparisons of each of these conditions allowed identification of individual genes as well as biochemical pathways that might contribute to the chronic inflammatory state in diabetic wounds.

Differentially expressed gene lists were generated by comparing mapped reads from nondiabetic versus diabetic, BM-derived versus wound-derived Gr-1<sup>+</sup> cells to obtain BM and wound differentially expressed lists (\(P < 0.05\), false discovery rate <0.1, fold-change >2, expression >1 fragment per kilobase per million reads). In the BM, 1,615 genes were identified, with 472 downregulated and 1,143 upregulated in diabetic-derived Gr-1<sup>+</sup> cells compared to nondiabetic (Figure 4a), while analysis of wound-derived Gr-1<sup>+</sup> cells revealed 1,995 genes, with 1,304 genes downregulated and 691 genes upregulated in diabetic-derived cells (Figure 4b). These lists were then analyzed using Ingenuity Pathway Analyses (Qiagen, Hilden, Germany) and DAVID (Huang da et al., 2009a, 2009b) to identify significantly altered biological pathways (Figure 4c, 4d) and gene ontology terms associated with specific biological functions (Supplementary Table S1 online).

Interestingly, the most significantly deregulated canonical pathways in both lists showed some overlap in the same or similar pathways (eg, neuroinflammation and JAK/STAT signaling), but were significantly activated (z-score >2) in diabetic BM and significantly inhibited (z-score <−2) in diabetic wound Gr-1<sup>+</sup> cells. DAVID analyses do not take into consideration the direction of change in gene expression, but significantly enriched gene ontology terms from BM-derived and wound-derived Gr-1<sup>+</sup> cells also showed overlap of a number of terms (Supplementary Table S1, highlighted in yellow) relating to leukocyte activation. This is consistent with previous reports showing aberrant activation (both hyper- and hyporesponsiveness) in a variety of myeloid cell subsets in diabetic patients and animal models (Bannon et al., 2013; Khondkaryan et al., 2018; Miao et al., 2012; Mirza and Koh, 2011; Satoh et al., 2010).

We further analyzed the differentially expressed gene lists using Ingenuity Pathway Analysis for significant changes to predicted upstream regulators and again found that regulators associated with leukocyte activation were significantly activated in diabetic BM-derived Gr-1<sup>+</sup> cells and significantly inhibited in diabetic wound-derived Gr-1<sup>+</sup> cells compared to
their nondiabetic counterparts (Supplementary Figure S2a, S2b online). Interestingly, comparison of activation status of these pathways in nondiabetic- and diabetic-derived Gr-1\(^+\) cells showed that the nondiabetic-derived cells show a marked activation when they move from BM to wound, whereas the diabetic-derived cells show a duller response, lacking in the acute activation response observed in nondiabetic cells recruited to the wound (Supplementary Figure S2c). Altogether, these data suggest that diabetic myeloid cells are already in an activated state in the BM and undergo only minimal activation after arriving in the wound environment, reminiscent of a tolerance phenotype.

**Diabetes induces aberrant regulation of NF-κB and JAK/STAT pathway components**

Ingenuity Pathway Analysis and gene ontology process analysis identified the NF-κB and JAK/STAT pathways as potential mediators of chronic inflammation in diabetic wounds. Interestingly, RelA and STAT1/3 are known to act synergistically on promoters of inflammatory genes and interact with chromatin-modifying enzymes, such as p300 to acetylate these loci, thereby promoting their expression (Ivashkiv and Park, 2016; O’Brown et al., 2015). We therefore investigated the regulation of RelA and STAT1/3 pathways and their downstream targets in BM-derived Gr-1\(^+\) cells to try to identify mechanisms underlying chronic inflammation in diabetes.

RelA expression was not significantly altered in diabetic-derived Gr-1\(^+\) cells (not shown), and levels of phospho-RelA were not significantly changed in these cells (Figure 5a–5c). However, the RelA inhibitor, IκB\(\alpha\), encoded by the *Nikbia* gene, was significantly downregulated at the mRNA and protein level in diabetic-derived Gr-1\(^+\) cells (Figure 5d–5f), suggesting a potential mechanism for altered function of RelA.

STAT1 and STAT3 are the most highly expressed STATs in Gr-1\(^+\)CD11b\(^+\) cells (Figure 5g) and are phosphorylated by Janus kinase in response to a variety of stimuli, including IFNs, IL-6, and growth factor signaling. Both STAT1 and STAT3 have been shown to be significantly hyperactivated in the diabetic environment (Lan et al., 2012; Mashili et al., 2013). *Stat1* was significantly downregulated and *Stat3* was significantly upregulated in diabetic-derived Gr-1\(^+\) cells (Figure 5g), but we found higher overall levels of phosphorylated STATs in response to lipopolysaccharide/IFN-γ stimulation (pSTAT1) and IL-6 (pSTAT3) in diabetic-derived myeloid cells (Figure 5h–5i, 5k–5l), although the ratio of pSTAT1/total STAT1 and pSTAT3/total STAT3 were not significantly different (Figure 5j, 5m). Altogether these data support a role for RelA and STAT collaboration in promoting chronic inflammation in diabetic wounds.
Selplg is a target of RelA and STAT1/3 and blockade of Selplg protein accelerates wound healing in diabetic mice

We next sought to identify targets of RelA and STAT1/3 that were misexpressed in diabetic-derived Gr-1⁺ cells that might contribute to chronic inflammation in diabetic wounds. Our RNA-seq data identified PSGL-1/Selplg as significantly misexpressed in both BM- and wound-derived Gr-1⁺ cells. Selplg is a cell membrane protein expressed by myeloid cells that plays an important role in recruitment and retention of myeloid cells in injured tissue. It binds to P-selectin expressed on activated endothelial cells at the site of injury and mediates myeloid cell extravasation from peripheral blood. Thus, higher levels of this protein are associated with chronic inflammation (Carlow et al., 2009). Quantitative reverse transcriptase PCR and Western blot analyses demonstrated that Selplg was significantly upregulated in diabetic-derived Gr-1⁺ cells at the mRNA and protein level (Figure 6a—6c). Interrogation of chromatin immunoprecipitation data in the Gene Transcription Regulation Database (gtrd.biouml.org v17.04) (Yevshin et al., 2017) showed that the cis-regulatory region of Selplg is bound by both transcription factors in vivo in peritoneal and BM-derived macrophages (Supplementary Figure S3a, S3b online). Moreover, we found histone acetylation of the Selplg promoter at K27 of histone 3 was also significantly increased in BM-derived Gr-1⁺ cells from diabetic mice (Supplementary Figure S3c). Ep300, the histone acetyltransferase responsible for transferring this mark to histone 3, was predicted to be significantly activated in diabetic-derived myeloid cells (Supplementary Figure S2), suggesting an underlying epigenetic mechanism promoting upregulation of this gene in the diabetic environment.

Previous studies have shown that in vivo blockade of PSGL-1/SELPGL significantly reduces neutrophil and monocyte recruitment to injured tissues (Borges et al., 1997; Xu et al., 2008). Therefore, we sought to test whether in vivo blockade of SELPLG in diabetic wounds could reduce chronic inflammation and accelerate wound healing. Injection of 50 μg of a function-blocking antibody against PSGL-1 directly into 1-cm dorsal wounds every other day on days 0, 2, and 4 post-wounding reduced the appearance of inflammation and significantly accelerated wound healing in diabetic mice, as measured by planimetry (Figure 6e, 6f). Analysis of wound tissue using a fluorescent secondary
Figure 6. PSGL-1 (Selplg) is associated with chronic inflammation and in vivo blockade accelerates wound healing in diabetic mice. (a) Selplg quantitative real-time reverse transcriptase PCR analysis of BM-derived and day 3 wound-derived Gr-1+ cells. (b) Selplg/PSGL-1 blot from BM-derived Gr-1+ cells. (c) Quantification of Selplg levels. (d) PBS and anti-PSGL-1-treated wounds (day 4). (e) Healing curves in PBS (red) and anti-PSGL-1-treated diabetic mice. Statistical comparisons performed with a t test, PBS: n = 6, anti-PSGL-1: n = 5, **P < 0.01. (f) Day-2 wounds injected with PBS (Cntl) or rat-α-PSGL1 showing DAPI: blue and Gr-1+ cells: green. Statistical comparisons performed with a t test, n = 2/group, scale bar = 50 μm. (g) Quantification of Gr-1+ cells as shown in (f). (h) Day 10 wounds from PBS-injected (Cntl) or rat-α-PSGL1-injected. Statistical comparisons performed with a t test, n = 5/group, *P < 0.05, scale bar = 500 μm. (i) Quantification of wound width and wound area as shown in (h). BM, bone marrow; Cntl, control; db, diabetic; ndb, non-diabetic; PBS, phosphate buffered saline.
antibody against rat IgG at day 2 following wounding showed clear antibody binding of the injected PSGL-1 function blocking antibody to Gr-1<sup>+</sup> cells (Supplementary Figure S4 online). Moreover, there was a significant reduction in Gr-1<sup>+</sup> cell accumulation in day 2 wounds (Figure 6g, 6h). Histological analysis of wounds collected on day 10 post-wounding showed a significant reduction in wound width, suggesting accelerated re-epithelialization, and wound area (Figure 6i, 6j). Altogether, these results suggest that inhibition of local myeloid cell—endothelial interactions is sufficient to abrogate inflammation-mediated delayed repair.

**DISCUSSION**

To date, chronic inflammation and the development of chronic nonhealing wounds has largely been attributed to the prolonged expression of pro-inflammatory cytokines at the wound site (Wetzler et al., 2000) and excessive recruitment of pro-inflammatory myeloid cells (Eming et al., 2007) in response to these extrinsic factors, altering the local wound environment. However, our current data suggest that myeloid cell intrinsic factors are a major driver of chronic inflammation, with their deregulation starting in the BM, suggesting altered myeloid cell development in the diabetic environment. We hypothesize hyperactivation of NF-κB and JAK/STAT pathways in diabetic BM may result in a diminished inflammatory response in the wound, promoting a chronic inflammatory state, possibly due to inadequate negative feedback, which may require a robust initial activation.

The requirement for a robust inflammatory response in efficient wound healing was clearly demonstrated by historical experiments performed by Leibovich and Ross (1975), in which they effectively removed macrophages from guinea pigs during wound healing. This led to a compromised inflammatory response and subsequent chronic inflammation (accumulation of neutrophils) with impaired wound healing. A more recent study in mice showed that genetic ablation of myeloid cells impaired wound healing, which again was associated with increased accumulation of neutrophils (Lucas et al., 2010; Mirza et al., 2009). Recent studies in human patients with diabetes showed an association with increased inflammatory cytokines in the circulation and a failure of myeloid cells isolated from these patients to respond to further stimulation (Khondkaryan et al., 2018). Altogether these studies and the data presented here support the idea that the diabetic environment induces a highly pro-inflammatory phenotype in myeloid cells during differentiation in the BM, inducing a tolerance-like phenotype, which upon challenge results in a reduced response, leading to a chronic inflammatory phenotype and impaired wound healing.

The underlying causes of the hyperinflammatory phenotype of myeloid cells in the BM remains elusive. Over-activation of the NF-κB and JAK/STAT pathways during myeloid cell development, in conjunction with aberrant epigenetic regulation of chromatin, may be the result of deregulation of additional intrinsic factors. Hox proteins are also important intrinsic factors directing normal myeloid cell development and have been found to suppress inflammatory pathways (Al Sadoun et al., 2016; Mace et al., 2009; Mahdipour and Mace, 2011). Moreover, Hox transcription factors interact with chromatin modifying enzymes and promote epigenetic changes to chromatin (Huang et al., 2012) that may influence NF-κB promoter occupancy. Importantly, several Hox transcription factors have been shown to be underexpressed in the diabetic environment (Hansen et al., 2003; Mace et al., 2005).

Hyperacetylation of the Selplg promoter sets up a cell intrinsic, preprimed activated phenotype that, upon recruitment of these cells to injured tissue, results in increased interaction with endothelial cells and, subsequently, increased retention of these cells in the wound. Inhibition of cell-surface Selplg with anti—PSGL-1 function-blocking antibody significantly accelerated wound healing in diabetic mouse wounds and may provide a potential therapeutic target for reducing chronic inflammation in a variety of settings.

**MATERIALS AND METHODS**

**Animals**

Mice were maintained in the University of Manchester animal housing facility with a 12-hour light/dark cycle and were fed a standard diet. Diabetic BKS.Cg-Dock7m<sup>Lepr<sup>db</sup>/OlaHsd and nondiabetic controls were originally purchased from Harlan/Envigo (Huntingdon, UK) and maintained as a colony. C57BL/6-Tg(CAG-EGFP)1Osb transgenic mice were obtained as a gift from Graham Morrissey (University of Manchester) and crossed into the diabetic colony. B6.Cg-Tg(CAG-mRFp1)Hadj transgenic mice were obtained as a gift from Berenika Plusa (University of Manchester). All procedures were approved by the University of Manchester Ethical Review Committee and the Home Office.

**Wounding model**

Mice 8–16 weeks of age (mixed sex, equal numbers of each in each group to avoid bias) were wounded. Diabetic mice and nondiabetic littermate controls were wounded on the dorsum as described previously (Mahdipour et al., 2011). Further details are provided in the Supplementary Materials online.

**Flow cytometry**

Single-cell suspensions of the skin/wounds were stained with CD11b (M1/70), Ly6C (HK1.4), CD64 (X54-5/7.1) from BioLegend (San Diego, CA), CD45 (30-F11) from eBioscience (San Diego, CA), Ly6G (1A8) from BD Biosciences (San Jose, CA), and CCR2 (475301) from R&D Systems (Minneapolis, MN). The lineage antibody cocktail for excluding lymphocytes included CD3 (17A2), NK1.1 (PK136), and B220 (RA3-6B2) from eBioscience. Cell acquisition was performed on an LSR Fortessa running FACSDIVA 8 software (BD Biosciences). Data were analyzed using FlowJo software (TreeStar, Ashland, OR).

**Gr-1<sup>+</sup> cell isolation from BM**

BM was flushed from mouse femurs and tibias with sterile phosphate buffered saline and single-cell suspensions were achieved by forcing cells through a 70-μm strainer (BD Biosciences). Gr-1<sup>+</sup> cells were labeled using biotinylated anti–Gr-1 (clone RB6-8C5; eBioscience), biotinylated using a DSB-X Biotin Protein Labeling Kit (Invitrogen, Carlsbad, CA), according to manufacturer’s instructions. Labeled Gr-1<sup>+</sup> cells were isolated using Dynabeads magnetic beads (Invitrogen) according to manufacturer’s instructions.
Adaptive transfer of Gr-1⁺ cells
For wound recruitment assays, Gr-1⁺ cells were injected intravenously. Day-3 wounds were harvested 24 hours later, dispersed, and analyzed by flow cytometry as described previously (Mahdipour et al., 2011) with the modification that 37.5 U/ml DNase I (Qiagen) was used instead of 150 U/ml. Additional details are provided in the Supplementary Materials.

For wound retention assays, Gr-1⁺ cells were injected into day-2 wounds at four sites around the perimeter of the wound as described previously (Mahdipour and Mace, 2012). Injected wounds were harvested for flow analysis 24 hours later. Additional details are provided in the Supplementary Materials.

Immunofluorescence
Frozen sections were stained with the following primary antibodies: rat anti–Gr-1 (clone RB6-8C5; eBioscience, 1:1,000) and goat anti-PECAM/CD31 (SC1506; Santa Cruz Biotechnology, Santa Cruz, CA, 1:200). Secondary antibodies: anti–rat-Alexa-488 (A-21208; Invitrogen, 1:500) and anti–goat-Cy5 (ab6566; Abcam, Cambridge, MA, 1:500). Sections were mounted with Prolong Gold Antifade Reagent with DAPI (Invitrogen). Imaging was performed using an Olympus FluoView FV1000 inverted confocal microscope and accompanying software (Olympus, Tokyo, Japan). Additional details are provided in the Supplementary Materials.

Histological staining and analyses
Wax sections were stained with hemotoxylin and eosin, mounted, and imaged (five per group). Wound width and wound area were measured using Adobe Photoshop (Adobe, San Jose, CA). Additional details are provided in Supplementary Materials.

Fluorescent image analyses
Gr-1⁺ cells in each region were counted using Adobe Photoshop. Means of two to five fields of view for each region were calculated using six individuals per group. The Gr-1⁺ cell to endothelial cell association ratio was calculated by counting the number of Gr-1⁺ cells in contact with or within one cell diameter of the nearest CD31⁺ endothelial cell.

RNA sequencing and gene expression analysis
Gr-1⁺ cells were isolated from BM and day-3 wound suspensions made from 10 nondiabetic and 10 diabetic mice, which were divided into two pools of five mice each. Wounds were enzymatically dissociated as described here and biotinylated anti–Gr-1 antibody and Dynabeads were used, as described in the section on Gr-1⁺ cell isolation from BM. Total RNA was isolated from freshly purified Gr-1⁺ cells and sent to GATC Biotech AG (Konstanz, Germany) for sequencing. Additional details are provided in the Supplementary Materials.

Genes showing an expression level higher than 1 fragment per kilobase per million reads and differential expression between nondiabetic- and diabetic-derived Gr-1⁺ cells of more than two fold-change, with a P < 0.05 (two-sample t test) and false discovery rate <0.1 (Benjamini-Hochberg) were further analyzed using Ingenuity Pathway Analysis (Ingenuity Systems; Qiagen) and DAVID (version 6.7, david.ncifcrf.gov). TaqMan gene expression assays (Applied Biosystems, Foster City, CA) were used to validate RNA-sequencing data. Additional details are provided in the Supplementary Materials. Gene expression data were deposited into the online public database Gene Expression Omnibus with accession number GSE123536.

Western blot and ELISA analyses
Protein was isolated from freshly purified BM-derived Gr-1⁺ cells from nondiabetic and diabetic mice (n = 3/group). Western blots were incubated with primary antibodies: rabbit anti–phospho-p65 (3037; Cell Signaling Technology, Danvers, MA, 1:500), rabbit anti–IκBα (ab32518; Abcam, 1:1,000), anti–PSGL-1/SELPLG (4RA10; BioXcell, West Lebanon, NH, 1:500), and rabbit anti–β-actin (NB600-532; Novus Biologicals, Centennial, CO, 1:5,000), followed by goat anti-rabbit horseradish peroxidase (ab6721; Abcam, 1:5,000).

For phospho-STAT1 and phospho-STAT3 analyses, BM cells from nondiabetic and diabetic mice (n = 3/group) were isolated and differentiated into macrophages. Cells were stimulated with lipopolysaccharide/IFN-γ or IL-6. Additional details are provided in the Supplementary Materials. Protein was isolated as described and Western blotting performed as described with the following primary antibodies: rabbit anti-phospho-STAT1 (11044; Signalway Antibody, College Park, MD, 1:500), rabbit anti–phospho-STAT3 (9131S; Cell Signaling Technology, 1:1,000), and rabbit anti–β-actin (NB600-532; Novus, 1:5,000). ELISAs for phospho-RelA (p65) and total RelA (p65; Abcam, ab176663), phospho-STAT1/total STAT1 (Abcam, ab126457), and phospho-STAT3/total STAT3 (Abcam, ab126459) in cell protein lysates from nondiabetic- and diabetic-derived myeloid cells were performed according the manufacturer’s instructions.

In vivo SELPLG blockade wound healing assay
Two groups of 7 diabetic mice (Lepr+/db/db), 8–16 weeks of age and sex-matched, received 1-cm diameter dorsal full-thickness excisional wounds. Each wound received either 50 μg of rat anti–PSGL-1/SELPLG (11 μl of 4RA10; BioXcell) in 50 μl phosphate buffered saline, or 50 μl phosphate buffered saline, via direct injection into wounds on days 0, 2, and 4. Animals were individually housed in standard cages and wounds measured every other day using planimetry. Images were recorded using a digital camera. Two wounds from each group were collected on day two, 6 hours after injection with either phosphate buffered saline or anti–PSGL-1, and fresh frozen in OCT for section analysis. The remaining wounds were collected on day 10, Zn-formalin fixed overnight, and embedded in paraffin.

Statistical analysis
All results shown are mean ± standard error of mean unless otherwise indicated. Student t tests and two-way analyses of variance were used to analyze data following confirmation of normal distribution. Statistical significance was set at P < 0.05 unless otherwise stated. Statistical analysis was performed using GraphPad Prism, version 6.0c (GraphPad, La Jolla, CA) or Microsoft Office Excel, version 14.3.8 (Microsoft, Redmond, WA).

ORCID
Kimberly A. Mace: http://orcid.org/0000-0002-3184-878X

CONFLICT OF INTEREST
The authors state no conflict of interest.

ACKNOWLEDGMENTS
The authors would like to thank Michael Jackson for help with flow cytometry and cell sorting, Marzieh Kamkoo for technical assistance, Peter Walker for help with histology, Leo Zeef and the Bioinformatics Facility for help with using Ingenuity Pathway Analysis tools, Chris Pickford for assistance with analysis of raw gene expression data and Matt Ronshaugen for helpful comments on the manuscript. This work was funded by The Healing
