Cx3CR1 Expression Identifies Distinct Macrophage Populations That Contribute Differentially to Inflammation and Repair

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**ABSTRACT**

Bone marrow (BM)–derived classical monocytes are critical to wound repair, where they differentiate into macrophages and purge foreign materials and dead cells while also laying the framework for tissue repair and regeneration. A subset of this recruited population persists in the wound and acquires alternative activation states to promote cell proliferation and matrix remodeling. In diabetes, this phenotypic switch is impaired and inflammation persists in an elevated state, contributing to delayed wound healing. Long-term tissue-resident macrophages can also play a key role in the resolution of inflammation to varying degrees across different organs. In this study, we investigated different macrophage subpopulations in nondiabetic and diabetic wounds over time using Cx3CR1eGFP transgenic mice and BM transplants. We show Cx3CR1eGFP-hi macrophages in skin wounds are derived from long-term tissue-resident macrophages and predominantly exhibit an alternative activation state, whereas cells expressing low-intermediate Cx3CR1eGFP are derived from the BM, contribute to both early and later stages of wound healing, and show both classical and alternative activation states. Diabetic mice showed significant differences in the dynamics of these subpopulations, which likely contribute to elevated and persisting inflammatory states over time. In particular, failure of Cx3CR1int macrophages to mature into Cx3CR1hi links maturation to resolution of inflammation. Thus strategies to promote macrophage maturation may be effective therapeutic tools in chronic inflammatory environments. **ImmunoHorizons,** 2019, 3: 262–273.

**INTRODUCTION**

Diabetes is one of the most prevalent diseases worldwide, with patients developing a wide array of comorbidities, including the formation of inflammatory, nonhealing lesions (1). Current therapies for chronic diabetic wounds primarily target the symptoms of the diabetic changes in wound pathology, with poor healing outcomes in many patients (2). To better understand the etiology behind diabetic wounds there has been much focus upon the role of inflammatory cell populations within the wound environment and how they may be altered in diabetes (3–7).

The myeloid lineage is a major contributor to inflammatory populations but also plays a key role in the reparative stages of wound healing (reviewed in Ref. 8, roles of nonmyeloid lineages reviewed in Ref. 9). Wound-recruited neutrophils, monocytes, and macrophages release reactive oxygen species and inflammatory cytokines and phagocytose debris and dying cells (10). At the resolution of inflammation, a subset of macrophages phenotypically...
switches to prohealing, alternatively activated states, releasing cytokines and growth factors that suppress inflammation, promote neovascularisation, and stimulate granulation tissue deposition by fibroblasts (11–15).

Macrophages involved in wound healing originate from multiple sources. Hematopoietic stem/progenitor cells of the BM niche differentiate into classical monocytes (CD14hi CD16neg/lo in humans, Ly6Chi Cx3CR1neg/lo in mice) that circulate and infiltrate tissues in response to inflammatory signals (16). In addition, the spleen also contains a reservoir of monocytes that are released upon tissue injury. However, macrophages in most tissues can be derived from primitive and definitive embryonic hematopoietic stem/progenitor cells and colonize tissues during development (17). In skin, some dermal macrophages, as well as epidermal Langerhans cells, are established during fetal development (18–24).

Myeloid maturation defects are associated with altered inflammatory responses in myeloid cell populations (4, 6, 25, 26). Type 2 diabetes perturbs myeloid lineage phenotypes to the detriment of tissue homeostasis and wound healing (reviewed in Ref. 27); additionally, diabetic hyperglycemia promotes monopoiesis (28). Diabetic macrophages are hyperresponsive to inflammatory stimuli in vitro, are less able to switch to alternatively activated phenotypes, and exhibit a prolonged inflammatory phenotype in diabetic wounds (4, 11, 29). There is also evidence for diabetes altering the number or phenotype of nonclassical monocytes in humans (30–32). Together with diabetes-induced changes in other myeloid cell lineages such as neutrophils (7), macrophages drive the elevated, nonresolving inflammatory state of chronic nonhealing diabetic wounds (33–35).

The expression of the fractalkine receptor Cx3CR1 has been identified as a key regulator of macrophage function at sites of inflammation (36, 37). Cx3CR1 knockout mice display impaired cutaneous wound healing, which can be rescued by wild-type BM transplantation (36). The presence of inflammatory macrophages and the production of inflammatory cytokines in psoriasis-like inflammation is Cx3CR1 dependent, and fractalkine has been suggested as a potential target for inflammatory diseases (38, 39).

Increasing Cx3CR1 expression is also linked to monocytic maturation. Classical circulatory monocytes express low levels of Cx3CR1, whereas nonclassical monocytes have high Cx3CR1 expression (20). In many tissues a link between the macrophage Cx3CR1 phenotype and their activation state was also observed. Cx3CR1hi populations were proinflammatory, whereas the Cx3CR1hi populations produced anti-inflammatory factors (40–44).

To test if macrophage Cx3CR1 phenotype follows a similar pattern during cutaneous wound healing, we performed excisional wounds on the Cx3CR1GFP reporter mouse and tracked the expression of GFP in wound myeloid populations at various stages of wound healing. The same wounding studies were repeated in Cx3CR1GFP leptin receptor-deficient (Lepr−/−) diabetic mice to determine if Cx3CR1 cell recruitment and/or anti-inflammatory function was defective in the diabetic environment. These studies identify maturing myeloid populations within the wound and a GFP-high population that is long-term tissue-resident in the skin and predominantly anti-inflammatory/prohealing. Diabetes inhibits maturation of recruited GFPneg macrophages, but not GFPhi resident macrophages, while promoting classical activation and inhibiting alternative activation in both subpopulations.

**MATERIALS AND METHODS**

**Mice**

All animals were housed at the University of Manchester animal care facility. Procedures were approved by the local ethical review committee and the Home Office. C57BL/6 Lepr−− mice were purchased from Harlan/Envigo (Oxfordshire, U.K.). All animals were 8–16 wk of age and age matched to controls on the same C57BL/6 background. Cx3CR1GFP/− mice were a gift from Kathryn Else at the University of Manchester and bred with the Lepr−− mice in house. All mice were maintained on a 12-h light-dark cycle and fed a standard diet.

**BM chimeras**

Diabetic and nondiabetic mice 8 wk of age were irradiated (2 × 5 Gy, 4 h apart). BM was harvested from 10-wk-old Cx3CR1GFP/+ donor mice and 10 million donor BM cells injected into each recipient mouse with an insulin needle via tail vein ~6 h after irradiation. Mice were maintained in sterilized cages on antibiotic-treated water for 2 wk and given 6 wk for reconstitution before wounding procedures performed.

**Wound model**

Mice were anesthetized with 2.5% isoflurane, shaved on the dorsum, swabbed with ethanol, and given 0.05 mg/kg buprenorphine s.c. for analgesia. A 10-mm diameter full-thickness excisional wound was made on the dorsum, and excised skin was collected for analysis of unwounded (day 0) samples. Wounded animals were housed individually and wounds left undressed. Day 4 and day 7 Cx3CR1GFP/+ wounds (n = 4), day 7 nondiabetic and diabetic wounds (n = 5) and BM chimera day 7 wounds (n = 2) were collected under anesthesia, including a 2-mm perimeter, and quartered. Mice were then sacrificed. One quarter was fixed (4% neutral buffered formalin in PBS) and paraffin embedded and the remainder processed for cell isolation. Tibias and femurs were collected for BM isolation.

**BM-derived macrophage culture**

BM was resuspended to a concentration of 0.4 million cells/ml in macrophage growth medium (DMEM with 1000 mg/l glucose, 10% FBS, 1% penicillin/streptomycin (Thermo Fisher Scientific) with 20% L929 cell-conditioned medium), and 2 ml added per well of a six-well plate (Corning). Cells were cultured in a humidified incubator at 37°C, 5% (v/v) CO2. Each well received 1ml of growth medium after 4 d and had full media replaced after 6 d. Macrophages were washed with DMEM and then stimulated for activation in 1 ml of DMEM with 1000 mg/l glucose, 10% FBS, 1% penicillin/streptomycin, and either 100 ng/ml murine IFN-γ (Sigma-Aldrich) and 100 ng/ml Escherichia coli-derived LPSs (Sigma-Aldrich) for classical activation or 50 μg/ml anti-IFN-γ

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(Bio X Cell) and 20 ng/ml murine IL-4 (PeproTech) for alternative activation. Nonactivated controls received no supplements.

**Wound cell isolation**
Excised wounds were weighed, cut into <2 mm cubes and digested overnight at 4°C on rocking roller in HBSS (20 μl/mg wound tissue) with 3% FBS, 1 mg/ml dispase (Life Technologies), and 10 mg/ml G418 (Melford Biolabs). Tissue was transferred to a fresh tube containing HBSS (80 μl/mg wound tissue) with 1 mg/ml collagenase D (Roche) and 75 U/ml DNase 1 (Qiagen) and digested for 2 h in a rotary shaker at 37°C, 300 rpm. Digestion was inhibited with 5% FBS. Tissue was dissociated by passing through a 19-gauge needle then both solutions passed through a 70-μm filter.

**Flow cytometry**
Cells were resuspended in 3% FBS in PBS without Ca2+ and Mg2+. Samples were incubated with FcBlock (anti-mouse CD16/CD32, 0.5 μg per 1 million cells; eBioscience) before incubation with isotype controls or the following Abs: anti-CCR2 APC (0.6 μg/ml, BD Biosciences), anti-Ly6C Alexa Fluor 700 (0.06 μg/ml, BD Biosciences), and secondary Abs: donkey anti-rabbit Alexa Fluor555 (1:500, A31572; Thermo Fisher Scientific), and secondary Abs: donkey anti-chicken Alexa Fluor647 (1:500, A11055; Invitrogen). All sections were mounted in Prolong Gold Antifade with DAPI (Invitrogen) and imaged at 200× on a confocal microscope (Olympus FLUOVIEW FV1000).

**Cytology**
Cells were resuspended in PBS with a Cytospin 4 (Thermo Fisher Scientific) and transferred to microscopy slides. Slides were left to dry and then fixed by methanol before treatment with Giemsa stain and Modified Solution (48900; Fluka Analytical) prior to mounting and coverslipping. Cells were imaged on an Olympus BX51 upright microscope using 100×/1.40 Plan Apo objective and captured using Axioskop color CCD camera. Images were processed and analyzed using ImageJ (http://rsb.info.nih.gov/ij) and Photoshop (Adobe).

**RNA isolation and quantitative RT-PCR**
Cells were washed in PBS then resuspended in TRIzol (Invitrogen) for RNA isolation. Any DNA contamination was removed by DNaseI treatment and RNA concentration analyzed by Nanodrop (ND-1000; Thermo Fisher Scientific). One microgram of RNA was used in reverse transcription reactions using Bioscript (Bioline) and random primers (Bioline) in a total volume of 25 μl. Quantitative RT-PCRs were performed using 20 ng of template cDNA. TaqMan probe primers (for Adgre1, Csflr, Nos2, Tnf, Ccl2, Arg1, Tgfb1, and Mrc1) and reference genes Hist2h2aa1 and Hsp90ab; Thermo Fisher Scientific), and FAST master mix (Applied Biosystems) were used on a StepOnePlus machine according to the manufacturer’s instructions. Data were analyzed with StepOnePlus software and Microsoft Excel.

**Immunohistochemistry**
Harvested wounds were incubated in formalin for 24 h, processed, and embedded in paraffin. Tissue was then sectioned at 5-μm thickness, and sections were adhered onto slides, dried for at least 24 h, and then stored at room temperature prior to staining. Sections were dewaxed through three changes of xylene, a xylene/ethanol mix, two ethanol washes, one methanol wash, and then gradual rehydration. Ag retrieval was performed by microwaving sections in sodium citrate buffer (10 mM, pH 6) for 20 min followed by gradual cooling and then dilution of the buffer with dH₂O. Immunofluorescent staining was performed with the following primary Abs: chicken anti-GFP (1:1000, ab13970; Abcam), rabbit anti-Nos2 (1:50, sc-651; Santa Cruz Biotechnology), goat anti-Arg1 (1:200, sc-18354; Santa Cruz Biotechnology), and secondary Abs: donkey anti-rabbit Alexa Fluor555 (1:500, A31572; Thermo Fisher Scientific), donkey anti-chicken Alexa Fluor647 (1:500, 43R-ID060AF; Fitzgerald), donkey anti-goat Alexa Fluor488 (1:500, A11055; Invitrogen). All sections were mounted in Prolong Gold Antifade with DAPI (Invitrogen) and imaged at 200× on a confocal microscope (Olympus FLUOVIEW FV1000).

**Statistical analysis**
Statistical significance was determined using a Student t test for experiments where control and experimental groups were being compared. ANOVA was used to compare between the isolated populations. A p value <0.05 was considered significant unless otherwise noted.

**RESULTS**

**BM monocytes show dynamic expression of Cx3CRI<sup>GFP</sup> during wound healing**
CCR2 and Cx3CRI cell surface expression on BM monocytes positively and negatively regulate recruitment to the BM sinusoidal periphery in response to chemokine signaling and subsequent release into circulation (45). We used Cx3CRI<sup>GFP<sup>+/−</sup></sup> mice (20) to analyze expression levels of CCR2 and Cx3CRI<sup>GFP</sup> before injury and at 4 and 7 d postwounding. Cx3CRI<sup>GFP<sup>+/−</sup></sup> mice have functional myeloid recruitment and comparable wound closure rates to Cx3CRI<sup>GFP<sup>+/+</sup></sup> mice (data not shown) (43, 46). Day 4 represents the peak of inflammation in this wound model, whereas day 7 represents the peak of the proliferative phase in wound repair (4, 47). Monocytes/macrophages freshly isolated from BM, as shown in Fig. 1A and Supplemental Fig. 1A, were CD11b<sup>+</sup>, Ly6G<sup>−</sup>, Ly6C<sup>lo/hi</sup>, CCR2<sup>−</sup>, and GFP<sup>lo/hi</sup>. The cell surface expression of CCR2 did not change during the response to injury (Fig. 1B); however, GFP expression diminished significantly during the peak inflammatory phase (day 4, Fig. 1C). At day 7 these levels were comparable to day 0 (day 7, Fig. 1C).

**Wound-derived macrophages can be divided into three subpopulations on the basis of Cx3CRI<sup>GFP</sup> expression**
Macrophages in the skin are composed of embryonically deposited resident macrophages and BM-derived macrophages that are continuously recruited under homeostatic conditions. This process is amplified following injury (5, 36). To examine how Cx3CRI<sup>GFP</sup> expression aligns with these populations during wound healing.

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CD11b⁺, Ly6Gneg, Ly6Clo-hi, and CCRlo-hi-expressing macrophages were isolated from day 0, day 4 and day 7 wounds (Fig. 2Ai–iii, Supplemental Fig. 1B) and further analyzed for GFP fluorescence. Three subpopulations based on GFP expression were identified at days 0, 4, and 7 (Fig. 2Bi–iii). Isolated cells from each population, GFPneg/lo cells, GFPint, and GFPhi cells were stained with Giemsa and revealed distinct phenotypes in each subpopulation (Fig. 2C). The GFPneg/lo cells were mostly monocytes (Fig. 2Ci, 59%), with a few neutrophils (Fig. 2Cii, 23%) and some small macrophages (Fig. 2Ciii, 12%) also present, whereas GFPint and GFPhi cells were mostly monocytes (Fig. 2Civ, vi, 51 and 26% respectively) and large macrophages (Fig. 2Cv, vii, 30, and 67%; data not shown). Changes in these populations were quantified over time and show different dynamics with healing progression. GFPneg/lo cells are rare in unwounded skin (day 0, Fig. 2D, left panel), but increase significantly following injury (day 4, Fig. 2D, left panel), and then diminish by inflammatory resolution (day 7, Fig. 2D, left panel). The GFPint population, the most prevalent population, is already present at a low level at day 0 and shows a trend toward increasing slightly at day 4, and then dramatically increases by day 7 (Fig. 2D, middle panel). In contrast, the GFPhi population increases sharply at day 4 and remains elevated at day 7 (Fig. 2D, right panel). Analysis of expression of cell surface maturation markers Adgre1 (encoding F4/80), Csf1r

FIGURE 1. Ly6C⁺ BM monocytes downregulate Cx3CR1-GFP during inflammation.

Cells isolated from bone marrow flushes of unwounded mice, and 4 or 7 d post cutaneous wounding. (A) Isolation of the CCR2⁺ bone marrow monocyte population from the CD11b⁺Ly6G⁻ myeloid population, day 7. (B) Distribution of CCR2 surface expression intensity in the bone marrow monocyte population. MFI is presented. (C) Distribution of GFP expression intensity (MFI) in the bone marrow monocyte population (*p < 0.05, n = 5).
(encoding the M-CSF receptor), and CD11b suggests GFP\textsuperscript{int} and GFP\textsuperscript{hi} macrophages are more mature than GFP\textsuperscript{neg/lo} cells. Despite showing the highest proportion of macrophages by morphology (data not shown), GFP\textsuperscript{hi} cells had a lower relative expression level of \textit{Adgre1} and mean fluorescence intensity (MFI) of CD11b than Cx3CR1\textsuperscript{int} suggestive of a Langerhans cell contribution.

FIGURE 2. Cx3CR1-GFP expression identifies myeloid subpopulations in the cutaneous wound.

Cells harvested from enzymatically digested murine unwounded skin (Ai, Bi, D0), day 4 postwounding (Aii, Bii, D4) and day 7 postwounding (Aiii, Biii, D7). (A) Isolation of the CD11b\textsuperscript{+}CCR2\textsuperscript{+}Ly6C\textsuperscript{+} population by flow cytometry. (B) The CD11b\textsuperscript{+}Ly6C\textsuperscript{+}CCR2\textsuperscript{+} myeloid population separates into three subsets of GFP expression, negative-low (neg-lo), intermediate (int), and high (hi). (C) Representative Giemsa stains for the GFP subsets: neg/lo (Ci neutrophils, Cii monocytes, Ciii macrophages); int (Civ monocytes, Cv macrophages); and hi populations (Cvi monocytes, Cvii macrophages), day 7. Scale bar, 10 \textmu m. (D) Population counts of the GFP subsets at the indicated time points (*p < 0.05). (E) Expression of \textit{Adgre1} and \textit{Csf1r} in GFP neg, int, and hi populations relative to Hist2h2 mRNA levels. MFI of CD11b surface expression (***p < 0.01, n = 5).
The **GFP**<sup>hi</sup> population in wounds is derived from long-term skin-resident macrophages

To investigate the ontogeny of the GFP<sup>hi</sup> population, BM transplants from Cx3CR1<sup>GFP+/+</sup> mice into nontransgenic recipients were used to trace the origins of these cells in cutaneous wounds over time.

The composition of the BM was similar at day 7 in wounded transplant recipient mice compared with nontransplanted Cx3CR1<sup>GFP+/+</sup> mice with respect to CD11b, Ly6G, Ly6C, CCR2, and GFP (data not shown). In addition, the wound-derived cell populations were also similar with respect to CD11b, Ly6G, Ly6C, and CCR2 (data not shown). However, although the GFP<sup>neg/lo</sup> and GFP<sup>int</sup> populations were similar, the wound-derived macrophage population showed significantly fewer GFP<sup>hi</sup> cells in the transplanted mice, demonstrating that these cells are derived from long-term tissue-resident macrophages (Fig. 3A, 3B), and thus their sharp increase in numbers in wound healing is at least partially due to local proliferation.

**Recruited and resident macrophages contribute differentially to classically and alternatively activated populations during inflammation and resolution**

To determine the activation state of the GFP<sup>int</sup> and GFP<sup>hi</sup> macrophage populations during wound healing, activation marker expression was assessed during the peak inflammatory (day 4) and proliferative (day 7) phases of wound healing. The classical activation markers Ccl2, Nos2, and Tnf were all significantly elevated in the GFP<sup>int</sup> population compared with the GFP<sup>hi</sup> macrophage population during the peak inflammatory phase at day 4 (Fig. 4A), but by day 7, when the inflammatory phase has switched to the proliferative phase, the GFP<sup>int</sup> population was significantly reduced and comparable to the GFP<sup>hi</sup> cells.

Analysis of alternative activation marker expression showed a mixed response in the two populations (Fig. 4B). Arg1 was downregulated between day 4 and day 7 in the GFP<sup>int</sup> population, whereas Tgfβ was upregulated from day 4 to day 7 in both GFP<sup>int</sup> and GFP<sup>hi</sup> macrophages. Expression of Mrc1 showed no change in either population. Overall the data suggest that the recruited GFP<sup>int</sup> population is the predominant contributor of the classically activated phenotype during inflammation. Upon the transition to the proliferative phase, both the resident and recruited populations contribute to alternatively activated macrophages although the recruited cells are present in greater number. Interestingly, BM-derived macrophages from day 7 wounded mice showed higher expression of both GFP and CCR2 in classically activated samples compared with alternatively activated samples in vitro (Supplemental Fig. 2A, 2B).

**Diabetes alters myeloid cell differentiation in the BM and wound tissue**

The Cx3CR1<sup>GFP+</sup> transgene was crossed into Lepr<sup>+/-</sup> mice to investigate how monocyte/macrophage subpopulations and wound recruitment dynamics are altered in a model of type 2 diabetes. The CD11b<sup>+</sup> Ly6G<sup>-</sup> Ly6C<sup>lo-hi</sup> GFP<sup>lo-hi</sup> myeloid compartment (monocytes/macrophages) was analyzed in the diabetic BM compared with the nondiabetic equivalent population. This monocyte/macrophage population appeared less uniform than the nondiabetic counterpart and consisted of small cells with high apparent nuclear to cytoplasmic ratio, indicative of immaturity and larger monocytes/macrophages with weaker Geimsa staining and a vacuolated appearance (Fig. 5A). BM-derived macrophages were cultured in vitro, classically or alternatively activated, and levels of CCR2 and GFP measured. These cells showed no change in CCR2 levels but a significant decrease in levels of GFP in nonactivated as well as both activation conditions (Supplemental Fig. 3).

Analyses at days 4 and 7 following wounding showed a decrease of both CCR2 and GFP on day 4 in diabetic mice compared

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**FIGURE 3. BM transplants identify Cx3CR1-GFP hi cells as resident macrophages.**

Cx3CR1<sup>GFP+</sup> BM was transplanted into irradiated wild-type recipients (BMT) and CD11b<sup>+</sup>Ly6C<sup>-</sup>CCR2<sup>+</sup> cells isolated from day 7 wounds as before. (A) GFP populations of Cx3CR1<sup>GFP+</sup> (Ai) and BMT (Aii) animals. (B) Proportion of GFP neg/lo, int. and hi populations in day 7 wounds from non-BMT (Ai) and BMT (Aii) animals (*p < 0.05, n = 4).
with nondiabetic (Fig. 5B), whereas CCR2 levels were also decreased on BM monocytes/macrophages at day 7 in diabetic mice compared with nondiabetic.

Giemsa staining of wound myeloid populations at day 4 showed that the GFPneg/lo, GFPint, and GFPhi subpopulations appeared less vacuolated than the nondiabetic samples (Fig. 5C, compare with Fig. 2C). Flow cytometry analysis showed a significant increase in the GFPneg/lo and GFPint populations during the inflammatory phase (day 4), whereas the GFPphi-resident macrophage population was not significantly changed (Fig. 5D). There were no significant differences during the proliferative phase (day 7).

Macrophage maturation markers Adgre1 and Csf1r were reduced in the diabetic wound-derived GFPneg/lo and GFPint subpopulations at Day 7 (Fig. 5E), supporting previous observations that inflammatory macrophages appear to be less mature in the diabetic environment (4, 25). Giemsa counts showed no change in proportions of monocytes and macrophages between diabetic and non-diabetic samples (data not shown). However, CD11b levels were higher on GFPint and GFPphi subpopulations in diabetic mice, indicative of increased macrophage cell adhesion in the diabetic environment, as has been previously reported (4).

**GFPint population exhibits enhanced inflammatory activation in the diabetic environment**

In normal wound healing, Day 7 is a key time point when inflammation is resolving. The expression of classical activation markers was assessed in day 7 wound macrophage populations in nondiabetic and diabetic mice. The recruited GFPint population showed greatly elevated classical activation marker expression compared with nondiabetic equivalent cells (Fig. 6A), whereas the GFPphi population showed a trend in increased Nos2 expression in diabetic-derived cells and a trend toward a decrease in Tnf expression.

Changes to alternative activation in diabetic wounds also showed a significant difference. Arg1 was expressed at a higher level in both diabetic GFPint and GFPphi subpopulations, but Mrc1 was significantly reduced in diabetic-derived cells (Fig. 6B). We then assessed GFP+ macrophages in day 7 wound tissue sections and analyzed the number of GFP+Nos2+ (classically activated) and GFP+Arg1+ (alternatively activated) cells (Fig. 6C). We found there were significantly more GFP+Arg1+ cells in nondiabetic wounds at this time point, whereas in diabetic wounds there was a similar number of classically activated versus alternatively activated (Fig. 6D). Taken together, the data suggest that the recruited GFPint diabetic macrophages were more proinflammatory, as has been previously reported for other macrophage subsets (4, 27, 34) but that the long-term skin-resident macrophages responded to injury with a mixed phenotype.

**DISCUSSION**

In this study we investigated the contribution and dynamics of different Cx3CR1GFP-expressing subsets of macrophages to the inflammatory and proliferative phases of wound healing in both healthy and diabetic backgrounds. We found that the majority of wound macrophages in both backgrounds are BM-derived,
recruited to wounds, classically activated, and express low to intermediate levels of Cx3CR1GFP, whereas a smaller population is comprised of long-term resident macrophages, expressing high levels of Cx3CR1GFP, whose numbers significantly increase in response to wounding. GFPlo/int cells are initially predominantly inflammatory but can switch phenotype at later stages in wound healing to become alternatively activated, whereas GFPhi are predominantly anti-inflammatory. These phenotypes are altered by diabetes.

Our results support previous analyses of classically activated and alternatively activated macrophages in skin in which CCR2+ macrophages recruited from BM were the predominant macrophage subtype present and initially showed a classically activated phenotype, playing a critical role during the early inflammatory stages of wound healing (23) but later switched to an alternatively activated phenotype and promoted repair and angiogenesis. By day 7 a significant number of both the GFPint and GFPhi populations expressed alternative activation markers.

Previous studies have shown that depletion of resident macrophage populations in tissues, including the peritoneum, gastrointestinal tract, liver, kidney, and skin has no effect on inflammatory monocyte recruitment in a range of inflammatory conditions.

FIGURE 5. Diabetes perturbs the myeloid cell composition in wounded mice.

CD11b+Ly6G+CCR2+ BM monocytes isolated from diabetic (Db) and non-diabetic (NDb) mouse BM flushes of animals at days 4 and 7 post-wounding. (A) BM monocytes from day 7 wounded nondiabetic and diabetic mouse BM were stained for Geimsa. Scale bar, 10 μm. (B) MFI of CCR2 and GFP on BM monocytes from nondiabetic and diabetic animals at days 4 and 7 postwounding (*p < 0.05, **p < 0.01, n = 3). Ly6C+CCR2+ cells isolated from diabetic and nondiabetic cutaneous wounds as before. (C) Representative Geimsa stains for the GFP subsets: neg/lo (Ci neutrophils, (Cii) monocytes, (Ciii) macrophages); int [(Civ) monocytes, (Cv) macrophages]; and hi populations [(Cvi) monocytes, (Cvii) macrophages] from day 7 wounded diabetic mice. Scale bar, 10 μm. (D) Cell counts for the GFP neg/lo, GFP int, and GFP hi populations (*p < 0.05, n = 3). (E) Relative expression of Adgre1 and Csf1r to Hist2h2 mRNA levels, and MFI for CD11b (*p < 0.05, n = 3).

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FIGURE 6. Activation marker expression in macrophage subsets.

(A) Classical activation markers Ccl2, Nos2, and Tnf in GFP int and hi wound populations at day 7 postwounding (*p < 0.05, **p < 0.01, n = 3).

(B) Alternative activation markers Arg1, Mrc1, and Tgfb1 in GFP int and hi wound populations at day 7 postwounding (*p < 0.05, n = 4).

(C) Immunofluorescent staining for GFP (green), Nos2 (red), and Arg1 (blue) in day 7 nondiabetic and diabetic wounds. Scale bar, 25 μm.

(D) Distribution of Nos2 GFP double-positive and Arg1 GFP double-positive cells (**p < 0.01, n = 3).
pathologies, including tissue repair (43). In other tissues, such as the lung, alternatively activated macrophages could be entirely derived from local tissue-resident macrophages when blood-derived monocytes were ablated (48), demonstrating the importance of the tissue environment to macrophage phenotype and function. Langerhans cells are highly resistant to irradiation depletion, so may account for the reduction of the GFp subset following BM transplants (49). This is further supported by the lower average Adgre1 expression of the GFp subset. The GFp cells that are repopulated in the BM transplants are likely dermal macrophages that are rapidly replenished in depletion models (50).

Cx3CR1 has been reported to inhibit monocyte release from the BM, as knockout mice show reduced accumulation in the BM, faster recovery in blood, and faster recruitment to peripheral tissues (45). Our observed downregulation of Cx3CR1GFP in BM cells following injury may facilitate monocyte release and may contribute to the elevated release in diabetes, as it was further downregulated in the diabetic environment. However, the downregulation of CCR2 in diabetic wound-recruited macrophages is counter-intuitive to the enhanced accumulation of these cells in wounds (4), as this receptor has been shown to be important in both release from the BM and recruitment to inflamed tissues (51–53). It is possible that the partial downregulation we observed is not enough to ablate the migratory potential of these cells, particularly because the elevated levels of the CCR2 ligand, CCL2/MCP-1, in diabetic wounds is likely to overcome any reduction in CCR2 expression in these cells (54).

The subdivision, or spectrum, of alternative activation and the potential differing roles for resident and recruited macrophages have been of keen interest in inflammation research (55, 56). The recruited and resident populations in our experiments showed differing alternative activation gene expression profiles and likely represent different alternative phenotypes as has been described for the lung (57). As in the lung, the recruited macrophage population expressed significantly more Mrc1 (CD206) at days 4 and 7 postwounding than the resident population. This has been associated with IL-4-stimulated macrophages in multiple tissues that promote the recruitment of other immune cells as part of type 2 immune responses (58).

As has been previously reported for recruited macrophages (46), we found that diabetes significantly alters resident macrophage classical and alternative phenotypes. Resident-tissue macrophages are more proinflammatory and fail to switch effectively to an alternative activated phenotype. This difference is indicative of low-level chronic inflammation in the skin of diabetic individuals that leads to persistence in proinflammatory signaling and severely delayed wound healing. In recruited inflammatory cells this has been linked to an immaturity phenotype (6), but our results suggest this does not appear to be the case with the tissue-resident macrophages. However, prepriming of resident macrophages from continuous exposure to inflammatory cytokines prior to injury may underlie their altered responses as it does for BM-derived myeloid cells (7). Further investigation into the molecular mechanisms contributing to altered resident macrophage responses in diabetes should address these issues.

DISCLOSURES

The authors have no financial conflicts of interest.

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