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CD301b+ Macrophages Are Essential for Effective Skin Wound Healing

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Regeneration of skin's barrier function after injury requires temporally coordinated cellular interactions between multiple cell types. Macrophages are essential inflammatory cells in skin wound regeneration. These cells switch their phenotype from inflammatory in the early regenerative stages to anti-inflammatory in the midstages of healing to coordinate skin repair. However, little is known about how different subsets of anti-inflammatory macrophages contribute to skin wound healing. Here, we characterize midstage macrophages ($CD45^+/CD11b^+/F4\text{-}80^+$) and identify two major populations: $CD206^+/CD301b^+$ and $CD206^+/CD301b^-$. The numbers of $CD206^+/CD301b^+$ macrophages increased concomitantly with repair, when the anti-inflammatory phenotype switch occurs in midstage healing. Using diphtheria toxin–mediated depletion models in mice, we show that selective depletion of midstage CD301b-expressing macrophages phenocopied wound healing defects observed in mice where multiple myeloid lineages are depleted. Additionally, when FACS-isolated subpopulations of myeloid cells were transplanted into 3-day wounds of syngeneic mice, only $CD206^+/CD301b^+$ macrophages significantly increased proliferation and fibroblast repopulation. These data show that the CD301b-expressing subpopulation of macrophages is critical for activation of reparative processes during the midstage of cutaneous repair.

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INTRODUCTION

Restoration of skin after injury is essential for life and involves sequential regenerative phases involving immune, epithelial, and mesenchymal cells to reform the epidermis and its underlying supportive dermis. Early stage repair is defined by inflammation, wherein myeloid cells, including neutrophils and then macrophages, are recruited to the injury site to clear pathogens and debris (Delavary et al., 2011; Eming et al., 2007). The midstage of healing consists of a proliferative phase in which macrophages promote robust migration and proliferation of keratinocytes to reseal the epidermal barrier and dermal regeneration via fibroblast and blood vessel restoration (Lucas et al., 2010; Mirza et al., 2010). During late stage wound healing, newly regenerated tissue is pruned and remodeled to resemble the cellular arrangement of nonwounded tissue (Brancato and Albina, 2011; Delavary et al., 2011).

The immune cell repertoire within skin wounds evolves with each repair stage and promotes distinct aspects of regeneration. After injury, $CD11b^+/F4\text{-}80^+$ macrophages are recruited to skin in a CCR2-dependent manner and express high levels of Ly6C ($Ly6C^{hi}$) (Ramachandran et al., 2012; Rodero et al., 2012; Willenborg et al., 2012) and inflammatory cytokines (Eming et al., 2007; Werner and Grose, 2003). Early stage macrophages are similar to classically activated or the M1 macrophages described by others (Brancato and Albina, 2011; Ferrante and Leibovich, 2012). Throughout healing, macrophage phenotype changes as macrophage cell surface protein and cytokine messenger RNA expression changes (Auffray et al., 2009; Brancato and Albina, 2011; Ferrante and Leibovich, 2012; Mirza and Koh, 2014). As regeneration proceeds into midstage healing, $CD11b^+/F4\text{-}80^+/Ly6C^{hi}$ macrophages decline, and the macrophage pool expresses mannose receptor (CD206), Fizz1, IL-10, transforming growth factor (TGF)-β1 and vascular endothelial growth factor (Daley et al., 2010; Mirza and Koh, 2014; Werner and Grose, 2003). These midstage macrophages, referred to as alternatively activated or M2 macrophages (Gordon, 2003; Martinez et al., 2008), mediate epithelial and dermal repair (Delavary et al., 2011; Knipper et al., 2015; Lucas et al., 2010). In the late phase of cutaneous repair, wound macrophages up-regulate metalloproteases to prune excess extracellular matrix to prevent scar formation (Duffield et al., 2005). The progression of macrophage phenotype after injury suggests that multiple populations of myeloid cells likely regulate specific aspects of regeneration.

Macrophages are essential for skin repair in adult mammals. Human chronic nonhealing venous leg ulcers or diabetic wounds in mice and humans display alterations in monocyte-derived cells (Goren et al., 2003; Mirza et al., 2013; Mirza and Koh, 2011; Wetzler et al., 2000). Furthermore, delayed healing in adult mice occurs when macrophages are depleted using anti-sera (Leibovich and Ross, 1975) or diphtheria toxin

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Abbreviations: DC, dendritic cell; DT, diphtheria toxin; DTR, diphtheria toxin receptor; EdU, 5-ethynyl-2′-deoxyuridine; GFP, green fluorescent protein; iDTR, simian diphtheria toxin receptor; TGF, transforming growth factor

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(DT)-induced death of monocyte-derived cells in genetic mouse models wherein DT receptor (DTR) is selectively expressed in these lineages (Goren et al., 2010; Lucas et al., 2010; Mirza et al., 2010). In the later experiments, DTR expression was induced by Cre recombinase activity under the *Lysozyme M* (*LysM*) promoter, which is expressed by monocytes, dendritic cells (DCs), and macrophages, or *CD11b*, which is expressed by macrophages and DCs (Auffray et al., 2009; Tamoutounour et al., 2013). Although these studies support the role of macrophages in skin repair, the precise cell type controlling skin regeneration within this heterogeneous and versatile lineage is not understood.

Here, we investigated the contribution of specific macrophage subsets to reparative processes during skin regeneration by transplanting specific myeloid cells into skin wounds and depleting specific myeloid cells during wound healing in mice. Consistent with other groups (Knipper et al., 2015; Novak and Koh, 2013), we found that the macrophage phenotype switches from inflammatory to anti-inflammatory before re-epithelialization, fibroblast repopulation, and revascularization of skin wounds and that depleting midstage myeloid cells severely impairs multiple processes of cutaneous repair. Interestingly, we find that CD301b marks a portion of midphase macrophages and that depletion of CD301b-expressing macrophages is sufficient to phenocopy skin repair defects observed by depletion of myeloid cells more broadly. Transplanting CD301b$^+$ macrophages is sufficient to enhance re-epithelialization, dermal proliferation, and fibroblast repopulation during midstage repair. Additionally, we showed that CD301b-expressing macrophage gene expression is enriched for growth factors and cytokines involved in skin regeneration. Therefore, our results identify a subset of CD301b$^+$ macrophages critical for activating cutaneous repair during midstage wound healing.

RESULTS

Altered macrophage phenotype precedes skin regeneration

Because macrophage phenotype varies in different mouse backgrounds and wound paradigms, we sought to define the timing of myeloid cell plasticity compared with cutaneous regeneration after full-thickness excision of murine dorsal skin. We have previously shown in our wound paradigm that the early stage of healing in mice (1.5 days) corresponds to the inflammatory phase of injury (Daley et al., 2010; Eming et al., 2007), and that 3–5 days postinjury is the midstage or proliferative phase of repair when re-epithelialization, fibroblast repopulation, and revascularization occurs (McGee et al., 2012; Schmidt and Horsley, 2013) (see Supplementary Figure S1a and b online). Analysis of immune subsets showed that the number of viable macrophages (Sytox$^-$/CD45$^+$/CD11b$^+$/F4-80$^+$) increased 5 and 7 days after wounding compared with 1.5- and 3-day wounds (Figure 1a–c).

To assess broad macrophage classes in early versus midstage repair, we examined macrophage Ly6C and CD206 levels throughout healing using flow cytometry (Figure 1d). Although 1.5-day wounds were enriched for Ly6C^{hi} macrophages, most 3-day wound macrophages were CD206$^+$ (Figure 1e). The percentage of CD206$^+$ macrophages remained high 5 and 7 days after injury, similar to nonwounded skin, and these macrophages were enriched for cytokines and growth factors that promote repair (Figure 1f). These data are consistent with previous studies (Daley et al., 2010; Yin et al., 2013) and indicate that a wound bed macrophage phenotype switch precedes regeneration during the midstage of epidermal and dermal repair.

Midstage myeloid cells are required for cutaneous repair

To further examine the relationship between macrophage phenotype and cutaneous repair, we used a previously

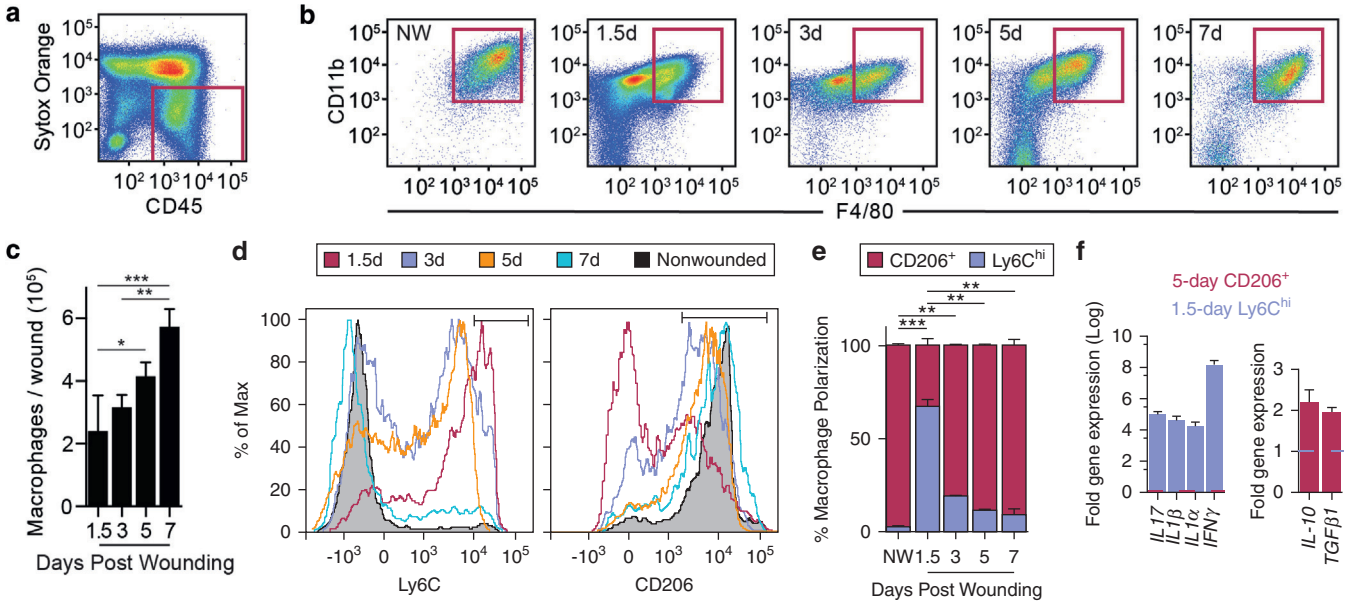

Figure 1. Macrophage phenotypic switch during cutaneous repair. (**a, b**) Representative FACS dot plots of (**a**) Sytox$^-$/CD45$^+$ gating for analysis of CD11b$^+$/F4-80$^+$ macrophages (red box) in nonwounded and indicated time points after injury. (**c**) Quantification of **b**. (**d**) Representative FACS histogram of Ly6C and CD206 on wound bed macrophages. (**e**) Quantification of the percentage of Ly6C^{hi} or CD206$^+$ CD11b$^+$/F4-80$^+$ macrophages within wound beds at indicated time points. (**f**) Fold change in messenger RNA for cytokines in 1.5-day Ly6C^{hi} wound macrophages (purple bar and line) versus 5-day CD206$^+$ wound macrophages (red bar and line) macrophages. $n = 3$–4 mice for each time point. All data are mean ± standard error of the mean. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. d, day; NW, nonwounded.