Genetic Ablation of Mast Cells Redefines the Role of Mast Cells in Skin Wound Healing and Bleomycin-Induced Fibrosis

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Conclusive evidence for the impact of mast cells (MCs) in skin repair is still lacking. Studies in mice examining the role of MC function in the physiology and pathology of skin regenerative processes have obtained contradictory results. To clarify the specific role of MCs in regenerative conditions, here we used a recently developed genetic mouse model that allows conditional MC ablation to examine MC-specific functions in skin. This mouse model is based on the cell type–specific expression of Cre recombinase in connective tissue–type MCs under control of the Mcpt5 promoter and the Cre-inducible diphtheria toxin receptor–mediated cell lineage ablation by diphtheria toxin. In response to excisional skin injury, genetic ablation of MCs did not affect the kinetics of reepithelialization, the formation of vascularized granulation tissue, or scar formation. Furthermore, genetic ablation of MCs failed to prevent the development of skin fibrosis upon bleomycin challenge. The amount of deposited collagen and the biochemistry of collagen fibril crosslinks within fibrotic lesions were comparable in MC-depleted and control mice. Collectively, our findings strongly suggest that significant reduction of MC numbers does not affect skin wound healing and bleomycin-induced fibrosis in mice, and provide to our knowledge previously unreported insight in the long-debated contribution of MCs in skin regenerative processes.

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INTRODUCTION

Impaired wound healing and fibrosis of diverse tissues are leading causes of morbidity and mortality (Wadman, 2005; Sen *et al.*, 2009). The molecular and cellular events underlying these processes are not completely understood and require further analysis to design effective therapeutic approaches. The physiologic healing response upon excisional skin injury is initiated by a local, timely limited inflammatory response, followed by the formation of vascularized granulation tissue, myofibroblast differentiation, epithelialization, and a long-lasting phase of tissue maturation (Martin, 1997; Gurtner *et al.*, 2008). The inflammatory response has potential to cause severe tissue damage with permanent remodeling of the extracellular matrix if imbalanced, resulting in uncontrolled connective tissue deposition and function-impairing scarring (Eming *et al.*, 2007; Lucas *et al.*, 2010).

Tissue-resident mast cells (MCs) and MC precursors recruited from the circulation have been implicated in controlling the balance of inflammatory signals directing the quality of tissue repair and fibrosis. Yet, MC-specific functions in the physiology and/or pathology of the wound healing response are not clear. MC accumulation and activation at the site of skin injury and in fibrotic tissue in humans and mice is well documented (Yamamoto *et al.*, 1999a; Trautmann *et al.*, 2000; Weller *et al.*, 2006; Gabrielli *et al.*, 2009). Furthermore, a recent study in mice suggests that MCs mediate the transition from scarless to fibrotic healing during fetal development (Wulff *et al.*, 2012). To investigate the cell-specific impact of MCs during skin repair, the MC-deficient mouse line WBB6F1-Kit^{W}/Kit$^{W\text{-}v}$ (W/W^{v} mice) has been frequently used (Kitamura *et al.*, 1978). However, independent studies in tissue repair generated in W/W^{v} mice resulted in partially contradictory results. In particular, the impact of MCs on the inflammatory infiltrate, the kinetics of wound closure, induction of angiogenesis, and the development of fibrosis are under debate (Yamamoto *et al.*, 1999b; Egozi *et al.*, 2003; Nauta *et al.*, 2013). Notably, recent studies revealed that

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Abbreviations: APC, allophycocyanin; DT, diphtheria toxin; eYFP, enhanced yellow fluorescent protein; i.d., intradermal; i.p., intraperitoneal; iDTR, inducible diphtheria toxin receptor; MC, mast cell; Mcpt5, mast cell protease 5

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W/W^v mice are not only MC deficient but are also characterized by other complex alterations of their immune system that might contribute to the phenotypes observed in specific disease models (Nigrovic *et al.*, 2008). In addition to MC deficiency, W/W^v mice suffer from anemia and neutropenia, lack intraepithelial γδ T lymphocytes, and are prone to dermatitis and gastritis (Zhou *et al.*, 2007; Nigrovic *et al.*, 2008). In fact, recent studies reported on major differences in the outcome of contact hypersensitivity responses and antibody-induced arthritis between W/W^v mice and genetic mouse models of inducible and constitutive MC ablation (Dudeck *et al.*, 2011; Feyerabend *et al.*, 2011).

To determine the specific role of MCs in skin repair and fibrosis, here we used a recently developed genetic mouse model of inducible depletion of connective tissue MCs, in which no side effects have been described (Dudeck *et al.*, 2011). Our findings strongly suggest that severe reduction of MC numbers does not affect skin wound healing and bleomycin-induced fibrosis in mice.

RESULTS

Depletion of connective tissue–type MCs in wound tissue in Mcpt5Cre/iDTR mice

To analyze the functional impact of MCs during tissue repair in the skin, we generated mice in which MCs can be specifically and inducibly ablated by the application of diphtheria toxin (DT). This mouse line (Mcpt5Cre/iDTR) was generated by crossing Cre-inducible human DT-receptor-transgenic mice (iDTR) to recently generated Mcpt5Cre mice, shown to express Cre recombinase specifically in connective tissue–type MCs (Figure 1a) (Buch *et al.*, 2005; Scholten *et al.*, 2008).

To assess specificity and efficiency of Mcpt5Cre-mediated recombination in wound MCs, we first inflicted full-thickness excision wounds on the back of Mcpt5Cre/eYFP reporter mice, in which Cre-mediated excision of a STOP cassette leads to enhanced yellow fluorescent protein (eYFP) expression under control of the Rosa26 promoter (Figure 1b). Immunostainings of wound tissue for MCs ($c\text{-}kit^+$) and eYFP showed that eYFP expression within the granulation tissue was restricted to $c\text{-}kit^+$ cells, with most of $c\text{-}kit^+$ cells expressing eYFP (Figure 1c). MC accumulation at the wound site was sparse within the granulation tissue during the early (day 4) and mid (day 7) phase of repair (data not shown), and was detectable in significant numbers at the late stage (day 14) within the scar tissue (Figure 1c). FACS analysis of wound cell suspensions isolated from Mcpt5Cre/eYFP reporter mice at day 14 after injury showed that $\sim 0.9 \pm 0.1\%$ of all wound cells expressed eYFP and that $CD45^+$ cells represented the major fraction of $eYFP^+$ cells (Figure 1d). The majority of $eYFP^+$ $CD45^+$ cells were $c\text{-}kit^+IgE^+$ (90.5%), representing connective tissue MCs. Few $eYFP^+CD45^+$ cells were $c\text{-}kit^+IgE^-$ (7.2%), corresponding to MC precursors or immature MCs. These findings demonstrate that recombination within the leukocyte compartment is highly specific for MCs. We observed a minor fraction of $eYFP^+CD45^-$ cells (0.1%) (Figure 1d, lower panel) that failed to express c-kit or Fc epsilon receptor I and that were sporadically detected in the hyperproliferative epidermis of day 14 wounds. At this stage, we cannot explain this observation because keratinocytes are not known to express Mcpt5. In fact, we never detected $eYFP^+$ cells in the epidermis of uninjured Mcpt5/eYFP reporter mice. To investigate the efficiency of Mcpt5Cre-mediated recombination, we next analyzed eYFP expression within the population of $c\text{-}kit^+IgE^+$ cells. On average, $81.8 \pm 5.9\%$ of $c\text{-}kit^+IgE^+$ cells expressed eYFP (Figure 1e).

To induce MC depletion in Mcpt5Cre/iDTR mice, we followed a protocol for DT injections as previously described (Dudeck *et al.*, 2011). $c\text{-}kit^+IgE^+$ MCs in the peritoneal cavity (Figure 2a) and $Giemsa^+$ MCs in unwounded back skin (Figure 2b) in DT-injected Mcpt5Cre/iDTR mice were efficiently ablated. Consistent with the quantification of MCs ($eYFP/c\text{-}kit^+$) in wound tissue during the time course of healing in Mcpt5Cre/eYFP reporter mice, analysis of Giemsa-stained sections in control mice revealed a significant increase of MCs during the late phase of healing, thereby restoring the average MC density of unwounded skin (Figure 2d and e). In contrast, MCs within the granulation (day 7)/scar (day 14) tissue and in unwounded skin at wound edges in MC-depleted mice were significantly reduced over the entire time course of healing when compared with control mice (Figure 2d and e). Particularly, the combination of systemic (intraperitoneal (i.p.)) and local (intradermal (i.d.)) DT injections after injury (Figure 2c) leads to severe MC depletion in late-stage wounds. Reduced DT injections after injury ($1\times$ i.p. after injury) resulted in a mild MC depletion (MC reduction at day 14 after injury: $42.3 \pm 13.5\%$) (Supplementary Figure S1 online). Efficient ablation of MCs in wound tissue of DT-treated Mcpt5Cre/iDTR mice through the entire repair response was confirmed by immunohistochemical staining for c-kit and Toluidine blue staining (Supplementary Figure S2 online).

MC ablation does not affect the development of granulation tissue and epithelialization in skin wounds

To examine the functional impact of MCs during the diverse stages of repair, full-thickness skin wounds were inflicted on the back of Mcpt5Cre/iDTR and control mice, and at diverse time points after injury the healing response was assessed by macroscopical and histological morphometric analyses. MC depletion was performed following the DT injection regime outlined in Figure 2c. The macroscopic analysis of wound closure was comparable in MC-depleted and control mice (data not shown). Determining the distance between the two epithelial tips (histological measure for epithelialization) revealed that the rate of wound closure was not affected by MC ablation. Figure 3a outlines the key histological features of an early- and mid-stage wound (Figure 3b). Furthermore, the kinetics of reepithelialization and development of an early vascularized granulation tissue were independent of MCs (Figure 3b). The only significant difference between wound tissue of MC-depleted and control mice was the increased distance between the two edges of the panniculus carnosus at day 3 after injury, indicating a temporary attenuated wound contraction in MC-depleted mice (Figure 3b and c). The distance between the two edges of the panniculus carnosus is regarded as a measure of wound contraction that in rodents is a combination of contractile myofibroblasts developing