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*J Immunol* 2010; 184:3964-3977; Prepublished online 22 February 2010;
doi: 10.4049/jimmunol.0903356
http://www.jimmunol.org/content/184/7/3964

Supplementary Material
http://www.jimmunol.org/content/suppl/2010/02/22/jimmunol.0903356.DC1

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Differential Roles of Macrophages in Diverse Phases of Skin Repair

Tina Lucas,* Ari Waisman, † Rajeev Ranjan,* Jürgen Roes, ‡ Thomas Krieg,*⁄§ Werner Müller, † Axel Roers, ‡ and Sabine A. Eming*  

Influx of macrophages plays a crucial role in tissue repair. However, the precise function of macrophages during the healing response has remained a subject of debate due to their functional dichotomy as effectors of both tissue injury and repair. We tested the hypothesis that macrophages recruited during the diverse phases of skin repair after mechanical injury exert specific functions to restore tissue integrity. For this purpose, we developed a mouse model that allows conditional depletion of macrophages during the sequential stages of the repair response. Depletion of macrophages restricted to the early stage of the repair response (inflammatory phase) significantly reduced the formation of vascularized granulation tissue, impaired epithelialization, and resulted in minimized scar formation. In contrast, depletion of macrophages restricted to the consecutive mid-stage of the repair response (phase of tissue formation) resulted in severe hemorrhage in the wound tissue. Under these conditions, transition into the subsequent phase of tissue maturation and wound closure did not occur. Finally, macrophage depletion restricted to the late stage of repair (phase of tissue maturation) did not significantly impact the outcome of the repair response. These results demonstrate that macrophages exert distinct functions during the diverse phases of skin repair, which are crucial to control the natural sequence of repair events. The Journal of Immunology, 2010, 184: 3964–3977.
site is timely restricted to the early stage of the wound healing response, macrophages persist through all stages of the repair response. Their number increases during the phase of inflammation, peaks during the phase of tissue formation, and gradually declines during the maturation phase (9).

Experiments in the 1970s established the concept that under sterile conditions, the influx of macrophages is essential for efficient healing of incisional skin wounds, whereas the influx of neutrophils might not be crucial (10, 11). This dogma has been challenged by recent reports, thereby arguing against an essential role of inflammatory cells in wound repair: early fetal wounds heal with minimal scarring, which is associated with little inflammation (12). Furthermore, wounds in the neonatal PU.1 null mouse, which lacks macrophages and neutrophils (but also B cells, mast cells, eosinophils), heal without scar and, surprisingly, with a similar time course as wild-type siblings (13). However, the need of macrophages for physiological repair in adults is supported in recent wound healing studies in various murine knockout models in which impaired wound healing is associated with an attenuated number of macrophages at the wound site (14–18). Although all of these studies emphasize that leukocytes significantly affect the quality of the healing response, knowledge of these models is limited, because they either do not target pathways mediated exclusively by macrophages or they address a neonate repair response, which is known to differ from healing in the adult organism (19). Furthermore, the objective of most of the earlier studies was the impact of the global inflammatory response on the outcome of the overall healing response.

This study tested the hypothesis that macrophages present at the wound site during the different stages of skin repair exert specific functions. In a mouse model that allows the conditional depletion of macrophages during distinct phases of the repair response in skin, we demonstrate in this study differential roles of macrophages in diverse phases of skin repair and report on macrophage-dependent and -independent repair mechanisms that are crucial to restore tissue homeostasis postinjury.

Materials and Methods

Animals

To generate mice in which macrophages can be depleted in a temporally and -independent repair mechanisms that are crucial to restore tissue homeostasis postinjury.

Flow cytometric analysis

Cells were isolated from the peritoneal cavity by irritation with ice-cold PBS (150 mM NaCl, 5.4 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 10 mM HEPES, 10 mM glucose, pH 7.2) and stained with FITC-conjugated anti-F4/80 (AbD Serotec), PE-conjugated anti–Gr-1 (Miltenyi Biotec, Bergisch Gladbach, Germany), alpholycoycin-conjugated-anti CD19 (Miltenyi Biotec), PE-conjugated anti-CD11b (Mac-1; Miltenyi Biotec), and allo-

Wound phase-restricted depletion of macrophages

To characterize macrophage function during distinct phases of repair, we inflicted full-thickness excisional skin wounds on the back of LysMCre/iDTR mice (hetero- or homozygous for the LysMCre/transgenic mouse, which lacks macrophages and neutrophils (but also B cells, mast cells, eosinophils), heal without scar and, surprisingly, with a similar time course as wild-type siblings (13). However, the need of macrophages for physiological repair in adults is supported in recent wound healing studies in various murine knockout models in which impaired wound healing is associated with an attenuated number of macrophages at the wound site (14–18). Although all of these studies emphasize that leukocytes significantly affect the quality of the healing response, knowledge of these models is limited, because they either do not target pathways mediated exclusively by macrophages or they address a neonate repair response, which is known to differ from healing in the adult organism (19). Furthermore, the objective of most of the earlier studies was the impact of the global inflammatory response on the outcome of the overall healing response.

This study tested the hypothesis that macrophages present at the wound site during the different stages of skin repair exert specific functions. In a mouse model that allows the conditional depletion of macrophages during distinct phases of the repair response in skin, we demonstrate in this study differential roles of macrophages in diverse phases of skin repair and report on macrophage-dependent and -independent repair mechanisms that are crucial to restore tissue homeostasis postinjury.

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phycocyanin-conjugated anti-CD115 (eBioscience, San Diego, CA). Cells were incubated for 30 min at 4°C and washed three times thereafter in PBS (0.5% BSA and 2 mM EDTA). The cells were analyzed on an FACS Calibur using CellQuest Pro Software (BD Biosciences, Heidelberg, Germany).

Wound phase-restricted depletion of macrophages

To characterize macrophage function during distinct phases of repair, we inflicted full-thickness excisional skin wounds on the back of LysMCre/iDTR or LysMCre mice (referred to as control mice). To achieve wound phase-restricted depletion of macrophages, LysMCre/iDTR and control mice were injected with DT according to three regimens. For macrophage depletion during the inflammatory phase, mice were injected with DT 2 and 1 d prior to wounding as well as 2 and 4 d postsurgery (regimen A). The phase of tissue formation mice were injected with DT 3, 4, 6, and 8 d postinjury (regimen B), and the phase of maturation mice were injected with DT 8, 9, 11, and 13 d postinjury (regimen C). At indicated time points, mice were sacrificed, and the wound tissue was excised for analysis.

Wounding and preparation of wound tissues

Mice were anesthetized by i.p. injection of Ketanest/Rompun (Ketanest S, Park Davis, Karlruhe, Germany; Rompun 2%, Bayer, Leverkusen, Germany). The back was shaved, and two 6-mm diameter full-thickness wounds were generated using a standard biopsy punch (Stiefel, Offenbach, Germany). For histological analysis, wounds were excised at indicated time points. Wounds were bisected in caudocranial direction, and the tissue was either fixed overnight in 4% paraformaldehyde or embedded in OCT compound (Tissue Tek, Miles, Elkhardt, IN). Morphometric analysis was performed on serial sections from the central portion of the wound.

Immunohistochemistry

For immunohistochemical stainings, 5-μm cryosections were fixed either in 4% paraformaldehyde or in acetone and blocked with 10% normal goat serum or with 10% FCS in PBS to reduce nonspecific Ab binding. Sections were incubated overnight at 4°C with the primary Ab diluted in DakoCytomation Ab Diluent (Carlsberg, Denmark). As a secondary Ab, Alexa-conjugated (Invitrogen, Karlsruhe, Germany) or peroxidase-conjugated (Southern Biotechnology, Birmingham, AL) secondary Abs for 1 h at room temperature, followed by counterstaining with DAPI, propidium iodide (Invitrogen), or hematoxylin. As primary Abs, we used rat mAbs against F4/80 (Dianova BMA, Augst, Switzerland), CD31 (Pecam-1, BD Pharmingen, Heidelberg, Germany), Ki-67 (DakoCytomation), and –G-1 (BD Pharmingen) polyclonal rabbit Abs against found in inflammatory zone 1 (Fiz1; Peprotech, Paris, France), vascular endothelial growth factor-A (VEGF-A; A-20, Santa Cruz Biotechnology, Santa Cruz, CA), and fibrinogen/fibrin (DakoCytomation); polyclonal goat Abs against VEGF-A (R&D Systems, Wiesbaden, Germany) and TGF-81 (R&D Systems; a rabbit mAb against cleaved caspase-3 (Ab3175) Cell Signaling Technology, Boston, MA) and a mouse mAb against smooth muscle actin (α-SMA) coupled to Cy3 (Sigma-Aldrich). Specificity of primary Abs was demonstrated by replacing them with irrelevant isotype-matched Abs.

Morphometric analysis

The macroscopic wound area was quantified by processing of photographs taken at various time points and was calculated as the percentage of the wound area immediately postsurgery. Immunofluorescence microscopy was quantified as indicated at indicated magnifications (Leica DM4000B, Leica Microsystems, Wetzlar, Germany; Diskus 4.50 Software, Diskus, Königswinter, Germany). The width of the gap between the epithelial tips was measured. The distance between the edges of the panniculus carnosus was determined as a measure of wound contraction. Numbers of macrophages, neutrophils, and cells positive for activated caspase-3, VEGF-A, or TGF-81 were determined by counting cells in two representative rectangles of 200 × 160 μm² in the granulation tissue of wound sections (minimum of three wounds on three mice per time point for each group). The number of mast cells was determined by counting all cells in the entire area of the scar tissue (four wounds on four mice). Ki-67-positive cells were determined by counting positively stained cells in two representative rectangles of 200 × 160 μm² within the
FIGURE 1. Schematic representation of DT-mediated macrophage depletion in distinct stages of the repair response. To achieve wound phase-restricted depletion of macrophages, LysMCre/iDTR and LysMCre mice were injected with DT (i.v. or i.p.) according to three regimens: DT injection regimen A (i), macrophage depletion during the inflammatory phase, DT injections 2 and 1 d prior to wounding as well as at day 2 and 4 postwounding; DT injection regimen B (ii), macrophage depletion during the tissue formation phase, DT injections at day 3, 4, 6, and 8 post-injury; DT injection regimen C (iii), macrophage depletion during the maturation phase, DT injections at days 8, 9, 11, and 13 post-injury. At indicated time points, mice were sacrificed, and the wound tissue was excised for analysis.

First, we investigated how macrophage depletion can be controlled by the dose, kinetics, and route of DT application (i.v. versus i.p.) as well as the DTR gene dose (homozygous versus homozygous). As revealed by flow cytometry for macrophages (F4/80 and CD11b), a single i.p. injection of DT (25 ng/g body weight) in LysMCre/iDTR (homozygous) mutants resulted in complete depletion of peritoneal macrophages 24 h later that persisted for ~3 d (Fig. 2A). Interestingly, although the LysM gene is expressed in polymorphonuclear leukocytes, DT injection did not result in depletion of peritoneal neutrophils (Gr-1) as revealed by flow cytometry (data not shown). Numbers of peritoneal B cells (CD19) as identified by flow cytometry were similar in LysMCre and LysMCre/iDTR (homozygous) mice, demonstrating the specificity of macrophage depletion following DT injection. Depletion of tissue-resident macrophages in skin, liver, and spleen was not achieved by a single i.p. injection of DT in LysMCre/iDTR (homozygous) mice, as revealed by immunohistochimical staining for F4/80 (data not shown). Also, repetitive i.v. DT injections did not result in efficient depletion of resident macrophages in these tissues. Therefore, we investigated whether increasing the DTR gene dose and i.v. DT application affects the efficacy of tissue-resident macrophage depletion. In fact, repetitive i.v. injections of DT (25 ng/g body weight) at 2 consecutive d in LysMCre/iDTR (homozygous) mice resulted in efficient depletion of both peritoneal and tissue-resident macrophages in skin, spleen, and liver 1 d after DT injection (Fig. 2A, 2B). Furthermore, the same regimen of DT application resulted 24 h later in efficient depletion of monocytes (CD115, CD11b) and partial depletion of neutrophils (Gr-1, CD115) in the circulation (Fig. 2C). To investigate whether myeloid cells can be efficiently depleted under inflammatory conditions, LysMCre/iDTR (homozygous) and LysMCre mice received two i.v. injections of DT at consecutive days, which 24 h later was followed by a single i.p. injection with thioglycollate. Thereafter, mice received for 3 consecutive d DT injections i.p., and at day 4 post thioglycollate application, peritoneal lavage cells were analyzed. As revealed by flow cytometry for macrophages (F4/80, CD11b) and neutrophils (Gr-1, CD115), only macrophages were effectively ablated, not neutrophils (Fig. 2D). Whether increased neutrophil number in LysMCre mice is simply the result of reduced phagocytosis by macrophages or results from the lack of different macrophage-mediated control mechanisms is currently unknown. For the subsequent wound
healing studies, we used LysMCrieDTR (homozygous) and LysMCri
Macrophage function during the early stage of repair

Macrophages recruited during the inflammatory phase of repair induce granulation tissue formation, which results in scar formation. As revealed by the macroscopic analysis of wound closure, depletion of macrophages during the inflammatory phase (DT injection regimen A [Fig. 1A]) resulted in significant delay of the early repair response compared with control mice. Whereas at day 5 postinjury the wound area was reduced to 50% of the original wound size in control mice, in macrophage-depleted wounds of LysMCrieDTR (homozygous) mice, the wound size was reduced by only 25%. However, at later time points, when DT injections were discontinued, wounds in LysMCrieDTR mice demonstrated rapid wound closure comparable to control wounds (Fig. 3A, 3B). These macroscopic findings were confirmed by histological assessment. For this purpose, LysMCrieDTR and control mice were sacrificed on days 5, 10, and 14 postinjury, and the wound tissue (5–12 wounds on three to six mice per time point for each group) was excised and analyzed. To simplify the orientation in the wound histology, we included a schematic presentation outlining the histological hallmarks of the wound tissue (Fig. 3C).

At day 5 postinjury, a significantly shorter distance between the tips of the epithelial tongues was measured for the control wounds compared with macrophage-depleted wounds, as demonstrated by H&E-stained paraffin sections, representing the longitudinal diameter of the wound (Fig. 3E). Furthermore, as revealed by expression of the cell proliferation marker Ki67, the epidermal margins in control wounds represented a hyperproliferative epidermal tongue, whereas the epidermal wound edge in macrophage-depleted wounds was short and showed few proliferating keratinocytes (Fig. 3D, Supplemental Fig. 1). To analyze dermal repair, we determined the amount of granulation tissue formation in wound tissue of macrophage-depleted and control mice. Differences in the quantity of granulation tissue were analyzed in H&E-stained sections and were shown to be significantly reduced at all time points in macrophage-depleted wounds compared with controls.

FIGURE 2. Selective depletion of macrophages in LysMCrieDTR mice. A. Analysis of macrophage depletion in peritoneallavage: single i.p. injection of DT (25 ng/g body weight) in LysMCrieDTR (heterozygous) or LysMCrieDTR (homozygous) mice resulted in efficient depletion of peritoneal macrophages (F4/80, CD11b) 24 h after DT injection that lasted for 3 d; specificity of macrophage depletion was identified by a normal B cell number (CD19). DT injection in LysMCri (control) mice had no effect on macrophage number. B. Tissue-resident macrophages (stained by F4/80) in skin, spleen, and liver were efficiently depleted in LysMCrieDTR (homozygous) mice 24 h following i.v. DT injections (25 ng/g body weight) on 2 consecutive d; tissue-resident macrophages were not affected by DT injection in LysMCri mice. C. Depletion of monocytes (CD11b, CD115) and neutrophils (Gr-1, CD115) in the circulation 24 h after two i.v. DT injections on consecutive days. D. Effective depletion of macrophages (F4/80, CD11b) but not neutrophils (Gr-1, CD115) in LysMCrieDTR (homozygous) mice after thioglycollate-induced peritonitis. d, dermis; e, epidermis; rp, red pulp; sc, s.c. fat layer; wp, white pulp.
FIGURE 3. Macrophage depletion during the early stage of repair attenuates epithelialization, granulation tissue formation, and wound contraction. 

A, Macroscopic appearance of wounds in LysMCre/iDTR and LysMCre (control) mice after DT injection following regimen A; whereas wounds of control mice had already lost their scab, macrophage-depleted wounds in LysMCre/iDTR mice still carry a firmly adherent scab 7 d postwounding. B, At the time points indicated, the wound area was determined using image analysis and expressed as percentage of the wound area immediately postinjury (n = 12 wounds on six mice for each time point and genotype). C, Scheme of a wound section several days following injury: keratinocytes at the wound margin proliferate and migrate underneath the scab and above the granulation tissue and form the hyperproliferative epithelium. D, H&E staining of wounds in LysMCre and LysMCre/iDTR mice at indicated time points postinjury. Original magnification day 5 and 10, ×100; day 14, ×50. Whereas in LysMCre mice, the day 5 wound is filled with a vascularized granulation tissue, in LysMCre/iDTR mice, only scarce granulation tissue has formed (black hatched line outlines granulation tissue; white dotted line outlines hyperproliferative epithelial tongue). In day 10 wounds of LysMCre and LysMCre/iDTR mice, the granulation tissue is covered by a complete epithelium; however, the epithelium is detached. Day 14 wounds of LysMCre and LysMCre/iDTR are closed, and scar tissue is minimal in LysMCre/iDTR mice (hatched line outlines scar tissue). Morphometric analysis of wound tissue at different time points postinjury: distance between the epithelial tips (E); distance between the edges of the panniculus carnosus (F); amount of granulation tissue (days 5 and 10 postinjury) or scar tissue (day 14 postinjury) (G). Each dot represents one wound (day 5, two wounds on one mouse; days 10 and 14 one wound per mouse); horizontal bar represents the mean. H, Sirius red staining and examination with polarized light revealed increased scar formation in control wounds when compared with LysMCre/iDTR wounds. Original magnification ×100. Arrows point to the tips of epithelial tongue, white arrowheads indicate wound edges, and black arrowheads indicate edges of panniculus carnosus. d, dermis; e, epidermis; g, granulation tissue; he, hypertrophic epidermal wound edge; pc, panniculus carnosus; sm, s.c. muscle layer; st, scar tissue.
(Fig. 3G). In fact, whereas at day 5 postinjury control wounds showed a highly vascularized, cellular, and proliferative granulation tissue, in macrophage-depleted wounds, granulation tissue was scarcely vascularized and showed few proliferating cells (Fig. 3D, Supplemental Fig. 1). Wound contraction was significantly reduced in LysMCre/iDTR mice when compared with controls (Fig. 3F).

At day 10 postinjury, wounds in LysMCre/iDTR and control mice were still covered by eschar, and histological analysis revealed that in both, beneath the eschar, a complete neoepigithelium had formed (Fig. 3D). However, when compared with control mice, in LysMCre/iDTR mice, the epithelium appeared fragile, was thinner, and partially detached from the dermis, indicating immature anchorage and basement membrane formation. Furthermore, although in these wounds the quantity of granulation tissue increased when compared with day 5 postinjury, it was significantly reduced compared with control wounds (Fig. 3G). Wound contraction remained reduced in LysMCre/iDTR mice when compared with controls (Fig. 3F).

At day 14 postinjury, both wounds in LysMCre/iDTR and control mice had lost their eschar and were similar in macroscopic appearance (Fig. 3A). In contrast, major differences between wounds in LysMCre/iDTR and control mice became apparent regarding the extent of scar tissue formation. As revealed by H&E (Fig. 3H), fine collagen bundles characteristic for scar tissue were almost absent in wounds of LysMCre/iDTR mice. Morphometric quantification of scar tissue revealed a significant reduction in LysMCre/iDTR mice when compared with controls (Fig. 3G). Morphological analysis of the epidermis overlaying the scar tissue revealed a slightly hyperproliferative closed epithelium that was similar in mutant and control wounds (Fig. 3D).

**Macrophage depletion in wounds of LysMCre/iDTR mice receiving DT injections following regimen A**

To analyze whether the changes in the tissue repair response between LysMCre/iDTR and control mice corresponded with macrophage depletion, wound tissue was stained for the macrophage marker F4/80. In control wounds 5, 10, and 14 d postinjury, F4/80-positive cells were present throughout the entire layer of the granulation tissue (Fig. 4). Quantitative evaluation revealed that the number of macrophages peaked at day 5 postinjury and subsequently declined to approximately one-third until 14 d postinjury (Fig. 4). In contrast, in LysMCre/iDTR mice receiving DT injections following regimen A, F4/80-positive cells were absent at day 5 postinjury and present at days 10 and 14 postinjury (Fig. 4). Thus, in LysMCre/iDTR mice, the absence of macrophages during the early stage of repair corresponded to the time course of DT injections. Furthermore, our data revealed that after DT injections were discontinued, newly generated macrophages were recruited into the wound site during the consecutive phases of tissue formation and maturation. Finally and most important, our results demonstrate that macrophages recruited during the inflammatory phase impact repair mechanisms not only in the early stage of the repair response, but also in the consecutive mid- and late stages of repair. As revealed by staining for the neutrophil marker Gr-1 (Fig. 4C) and chloroacetatetase (data not shown), 5 d postinjury neutrophils were not effectively depleted at the wound site by DT injections in this model.

**Wound vascularization and contraction is controlled by macrophage influx during the inflammatory phase of repair**

To assess whether the influx of macrophages during the early stage of the repair response impacts wound angiogenesis, morphometric quantification of the expression of the endothelial cell marker CD31 within the area of granulation tissue was used as readout for neovascular processes at the wound site. Wounds in control mice revealed a strong vascular response 5 d postinjury, which decreased ~25% until day 10 postinjury (Fig. 5). In wounds of LysMCre/iDTR mice, vascular density was significantly reduced compared with control wounds 5 d postinjury and slightly increased by day 10 postinjury. Thereby, in LysMCre/iDTR and control mice, wound angiogenesis correlated positively with the presence of macrophages.
Supplemental Fig. 2). In contrast, in wounds of LysMCre/iDTR mice 10 d postinjury, the number of cells that stained positive for F4/80 and Fizz1 or Ym1 was significantly reduced (Fig. 6, Supplemental Fig. 2).

Analysis of macrophage depletion during the mid-stage of repair

Macrophages recruited during the inflammatory phase of tissue formation control vascular stability and transition of granulation tissue into scar tissue. To characterize the functional impact of macrophages present in granulation tissue, we first inflicted skin wounds on the back of control and LysMCre/iDTR mice, which was followed by DT injections 3, 4, 6, and 9 d postwounding (DT injection regimen B; Fig. 1B).

The macroscopic analysis of the early wound healing response in LysMCre/iDTR mice was similar compared with controls. However, DT-mediated macrophage depletion in LysMCre/iDTR mice during the mid-stage of repair significantly delayed the subsequent wound closure rate when compared with controls (Fig. 7). These macroscopic findings were confirmed by histological assessment. For this purpose, LysMCre/iDTR and control mice were sacrificed on days 7 and 10 postinjury, and the wound tissue (4–10 wounds on three to five mice per time point for each group) was excised.

As revealed on H&E-stained paraffin sections 7 d postinjury, all wounds in control mice and 6 out of 10 wounds in LysMCre/iDTR mice showed complete wound closure (Fig. 7D). Interestingly, whereas until day 10 postinjury granulation tissue in all wounds of control animals matured and showed regular transition into a scar tissue (Fig. 7C), all wounds in LysMCre/iDTR mice revealed a regression of granulation tissue maturation and appeared immature (Fig. 7C, 7F). Immature appearance of day 10 old macrophage-depleted granulation tissue in LysMCre/iDTR mice was reflected by severe hemorrhage, fibrin, and serum exudates, which were present in all wounds analyzed (eight wounds on four mice). Hemorrhage was assessed by the presence of extravascular erythrocytes (Fig. 7C) as well as immunohistochemical staining for fibrinogen/fibrin (Fig. 7G). Morphometric analysis of fibrinogen/fibrin staining revealed a significant increase in macrophage-depleted versus control mice. Furthermore, attenuated functional capacity of granulation tissue at days 7 and 10 postinjury in wounds of LysMCre/iDTR mice became evident by attenuated wound contraction, which did not reach statistical significance (Fig. 7E). In addition, hypertrophic epidermal wound edges at day 5 postinjury regressed into atrophic epidermal wound edges, thereby decreasing their wound closure capacity at day 10 postinjury (Fig. 7D).

As revealed by staining for F4/80, the morphological and functional alterations in wounds of LysMCre/iDTR mice at day 7
and 10 postinjury were characterized by a significant reduction of macrophages within the granulation tissue compared with controls (Fig. 8A). However, whereas in wound tissue of control mice at both time points postinjury neutrophils were minimal or completely absent, their number was increased in macrophage-depleted wounds (Fig. 8C, 8D). Of interest, whereas hemorrhage was present in all wounds of LysMCre/iDTR mice, the number of neutrophils was only increased in those wounds with incomplete epithelialization. Overall, these data demonstrate that DT-mediated macrophage depletion in LysMCre/iDTR mice during the phase of tissue maturation significantly disturbed the transition of the mid-stage into the late stage of the repair response. Neutrophils appeared resistant to DT-mediated cell depletion.

**Endothelial cell damage and apoptosis in macrophage-depleted granulation tissue**

To unravel the reason for the severe hemorrhage in macrophage-depleted granulation tissue, we analyzed vessel maturation and endothelial cell apoptosis in LysMCre/iDTR and control mice receiving DT injections following regimen B. Maturation of blood vessels in healing wounds is reflected by the presence of surrounding perivascular cells (28, 29). Double immunofluorescent labeling for CD31 and desmin (marker of pericytes) revealed that 10 d postinjury in both granulation tissue of control and LysMCre/iDTR wounds, desmin-positive cells that associated with vascular structures could easily be identified (data not shown). In contrast, defects in endothelial cells themselves were identified by cosetting for CD31 and cleaved caspase-3, an apoptosis marker (Fig. 9). Whereas in granulation tissue of control wounds endothelial cell apoptosis was a rare event, in macrophage-depleted granulation tissue, numerous apoptotic endothelial cells could be identified. These findings suggest that hemorrhage observed in macrophage-depleted granulation tissue in LysMCre/iDTR mice was caused by endothelial cell damage rather than altered vessel maturation.

To identify mechanisms that might mediate endothelial cell damage, we stained wound tissue for VEGF-A and TGF-β1; both are signature mediators of vascular homeostasis and endothelial cell survival (30, 31). As demonstrated earlier in Fig. 5, in control wounds 10 d postinjury, double staining for F4/80 and VEGF-A or TGF-β1 showed that macrophages present a significant fraction of TGF-β1– or VEGF-A–expressing cells in granulation tissue. Consistently, depletion of macrophages in LysMCre/iDTR mice during the phase of tissue formation resulted in a significant reduction of
staining for both growth factors (Fig. 9C, 9D). Our results suggest that sudden withdrawal of both growth factors due to macrophage depletion contributes to endothelial cell damage.

To substantiate the hypothesis that a reduction in myeloid cell-derived TGF-β1 contributes to the hemorrhagic phenotype in LysMCre/iDTR mice, we wounded mice in which TβRII has been depleted on myeloid cells (LysMCre/TβRII). Interestingly, wounds in LysMCre/TβRII mice at day 7 and 10 postinjury revealed a significant increase in hemorrhages revealed by numerous extravascular erythrocytes (on H&E histologies, data not shown) and fibrin exudate when compared with control littermates (Fig. 9E), which was similar to the wound phenotype in macrophage-depleted LysMCre/iDTR mice. Earlier studies have shown that TGF-β1 synthesis in macrophages is induced through binding of TGF-β1 to TβRII (32). Consistently, using real-time PCR analysis, we could show that TGF-β1 gene expression is reduced in peritoneal macrophages of LysMCre/TβRII mice following stimulation with human TGF-β1 in vitro (Fig. 9E). Furthermore, double staining for F4/80 and TGF-β1 of wound tissue in LysMCre/TβRII mice revealed reduced TGF-β1 synthesis in macrophages (data not shown). Therefore, these results suggest a role for macrophage-derived TGF-β1 on vascular cell function during wound healing in skin. Ongoing studies in our group further investigate the role of TβRII-mediated TGF-β1 synthesis in macrophages on wound angiogenesis.

Analysis of macrophage depletion during the late stage of repair

Macrophages present at the late stage of repair do not impact tissue maturation. To characterize the functional impact of macrophages present during the phase of tissue maturation and after restoration of the epidermal barrier, we first inflicted skin wounds on the back of control and LysMCre/iDTR mice, which was followed by DT injections 8, 9, 11, and 13 d postwounding (DT injection regimen C; Fig. 1C).

Depletion of macrophages during the phase of tissue maturation did not result in macroscopic alterations of the wound tissue compared with control wounds. Fourteen days postinjury, both macrophage-depleted and control wounds had lost their eschar and revealed similar scar tissue (Fig. 10A). These macroscopic findings were confirmed by histological assessment. For this purpose, LysMCre/iDTR and control mice were sacrificed on days 10 and 14 postinjury, and the wound tissue (four to seven wounds on four to seven mice per time point for each group) was excised. As revealed on H&E-stained paraffin sections 14 d postinjury, all wounds in control and LysMCre/iDTR mice showed complete wound closure by a slightly hyperproliferative neoeppidermis covering scar tissue (Fig. 10C). Sirius Red staining analyzed in polarized light revealed fine collagen bundles typical for scar tissue of both macrophage-depleted and control wounds (data not shown). Morphometric analysis revealed that the amount of scar tissue was similar in control and LysMCre/iDTR wounds (Fig. 10C). Scar tissue stained for F4/80 revealed the presence of macrophages in control mice from day 10 until day 14 postinjury and their significant reduction in DT-injected LysMCre/iDTR mice (Fig. 10D). Of interest, at all time points analyzed, neutrophils were absent in both control and macrophage-depleted wounds (Supplemental Fig. 3). Furthermore, as revealed by Giemsa staining at day 14 postinjury, the number of mast cells present in scar tissue was similar in control and LysMCre/iDTR mice (Supplemental Fig. 3).

Discussion

In this study, we show that macrophages play a crucial role during skin repair in the adult organism and that their timely restricted depletion during distinct phases of the wound healing response has profound impact on phase-specific repair mechanisms. Our results show that repair mechanisms controlled by macrophages recruited during the early stage of the repair response encompass induction of granulation tissue and myofibroblast differentiation, which ultimately control the degree of scar formation. During the mid-stage of the repair response, macrophage function is crucial for stabilization of vascular structures and transition of granulation tissue into scar tissue. In contrast, macrophages present at the late stage of the repair response do not impact tissue maturation and scar formation. Therefore, our studies provide evidence that macrophages exert different roles at diverse stages of the repair response and that they orchestrate the natural sequence of repair phases in skin, which are essential to restore solid tissue homeostasis and integrity postinjury. Overall, our study suggests a crucial and varied role for macrophages in wound healing and adds to the previous knowledge.

To assess macrophage function at distinct stages of skin repair, we developed a mouse system that allows the cell-type specific and timely restricted depletion of macrophages in skin wounds. The role of macrophages during skin repair has remained a subject of debate due to their functional dichotomy as effectors of both tissue injury and repair (33). Furthermore, in earlier studies of skin injury, macrophages have been depleted by administration of...
antimacrophage serum and/or hydrocortisone, methods that have pleiotropic effects and lead to unspecific and partial cell depletion (10, 11). To circumvent these difficulties, we used a transgenic mouse line, LysMCre/iDTR, in which minute amounts of DT can efficiently, specifically, and in a timely restricted manner deplete tissue-resident and inflammatory macrophages recruited to the site of skin injury. It was surprising that neutrophils could not be efficiently depleted in the presented model by DT, because LysM promoter activity has been reported in neutrophils (21). Inefficient neutrophil depletion might be explained by low LysM promoter activity, reduced phagocytosis by macrophages, and/or short-lived turnover of neutrophils, so that DT might fail to efficiently interfere with the high number of neutrophils that infiltrate the wound site (34, 35). These findings are consistent with those of previous studies showing that DT treatment did not significantly affect neutrophil numbers (36).

Our study provides evidence that macrophages exert different functions during the distinct phases of skin repair. Specifically, in control mice, macrophages recruited during the early stage of the repair response induce a vascularized and fibroblast-rich...
granulation tissue that promotes dermal as well as epidermal repair. Consistently, wound closure at day 5 postinjury was significantly delayed in mice in which macrophages were depleted specifically during the early stage of repair. However, macrophage influx subsequent to their depletion rescued the delayed wound closure rate during the late stage of repair. Yet, the overall amount of granulation tissue that developed under these conditions, as well as vascularization, cellularity, contractile force, and, most important, the extent of scar formation, remained reduced when compared with control wounds. These findings demonstrate that macrophages that infiltrate the wound site immediately postinjury induce a robust, highly vascularized granulation tissue associated with myofibroblast differentiation and wound contraction. Although these events ensure rapid wound closure, they result in significant scar formation. However, a healing response that lacks specifically the initial burst of macrophage influx results in minimal scarring. These results are consistent with a recent study published during the time when our manuscript was under revision (36). Based on these findings, it is intriguing to speculate that providing a pathogen-free environment and preventing macrophage influx selectively during the early stage of repair improves the quality of the wound healing response with less scar formation and without compromising the rate of wound closure. However, one should take into account that scar formation and wound contraction in mice differs significantly from scar formation in the human system. Therefore, to validate our observations, future studies in other model systems that are more adequate to study scar formation are needed.

We have also investigated whether the different repair phenotypes in control and LysMCre/iDTR mice in which macrophages were exclusively depleted during the early stage of repair might originate in different activation states of macrophages present at the wound site. The current conceptual model of tissue macrophage activation is based on the hypothesis that macrophages are plastic cells and adapt their response to microenvironmental signals. Several activation states and related functions of macrophages have been described in mice and humans (37–39). The best characterized activation states in mice encompass classical activated macrophages (also called M1), which exert proinflammatory activities, eradicate invading microorganisms, and promote type I immune responses, and alternatively activated macrophages (also called M2), which are hyporesponsive to proinflammatory stimuli and are involved in debris scavenging, angiogenesis, connective tissue remodeling, and resolution of inflammation (paradigm of M1/M2 polarization) (40). The latter is considered to exert repair and regenerative activities (27, 41). However, conclusive evidence as to whether the concept of classical/alternative macrophage activation is operative at the cutaneous wound site and which of the microenvironmental cues might direct macrophage activation is still missing. Recent studies in mice report on a critical role of alternative macrophage activation for regeneration of skeletal muscle and myocardium postinjury (42, 43).

Our studies reveal the presence of alternatively activated macrophages (as defined by expression of Fizz1 and Ym1) in control skin wounds particularly during the early stage of the repair response and to a lesser extent also during the mid-stage of repair. In contrast, expression of both markers was absent in macrophages infiltrating the wound site, which was deprived of macrophages during the early stage of repair. These findings indicate that environmental factors that induce alternative macrophage activation are primarily present during the early stage of repair. Furthermore, a healing response, which lacks macrophage recruitment during the early stage of repair in mice, is inefficient in alternative activation. In addition, our data reveal that alternative macrophage activation correlates positively with the extent of a highly vascularized and cellular granulation tissue as well as ultimately with the degree of scar formation.

In support of a functional relevance of Fizz1- and Ym1-positive (and thereby alternatively activated) macrophages for robust angiogenesis, myofibroblast differentiation, and scar formation is the
positive correlation of the staining of both markers with VEGF-A or TGF-β1. Both growth factors are considered important mediators of alternatively activated macrophage function (40). Thus, our data emphasize that in physiological repair, macrophages recruited during the early stage of the repair response are alternatively activated, resulting in the expression of VEGF and TGF-β1, which contributes to wound angiogenesis and myofibroblast differentiation. In contrast, macrophages recruited at a later stage of repair, into a wound site previously deprived of the macrophage influx during the phase of inflammation, do not undergo alternative activation and consistently reveal attenuated expression of VEGF and TGF-β1, which ultimately attenuates the degree of granulation tissue and scar formation.

Depletion of macrophages during the mid-stage of the repair response, consequent to the development of a highly vascularized and cellular granulation tissue, resulted in the abrogation or even retrogression of the physiological repair cycle. This process was evident by severe hemorrhage and fibrin exudate, suspicious for destabilization of vascular structures. Indeed, apoptosis of endothelial cells was significantly increased in macrophage-depleted wounds when compared with controls 7 and 10 d postinjury. In addition, epidermal wound edges appeared atrophic and detached from the underlying granulation tissue, indicating severe disturbances in epidermal-dermal interactions. In addition, the number of neutrophils increased in macrophage-depleted wounds that had not yet completed epithelialization. During physiological skin repair in our model, the number of neutrophils declines within the initial 5 d postinjury (35). Thus, high numbers of neutrophils present in macrophage-depleted granulation tissue at day 7 and 10 post-injury are abnormal.
Several mechanisms underlying the morphological and functional changes associated with macrophage depletion during the mid-stage of the repair response might be discussed. As we have shown, macrophages present in granulation tissue of control mice provide a source for VEGF and TGF-β1. Sudden withdrawal of these growth factors by macrophage depletion from the metabolically highly active granulation tissue might result in severe alterations in tissue homeostasis. Withdrawal of VEGF and TGF-β1, both of which are potent survival factors for endothelial cells, might explain endothelial cell apoptosis and vessel destabilization observed in macrophage-depleted wounds (24, 44). Furthermore, withdrawal of TGF-β1, a potent immunosuppressive mediator (45), might be responsible for the increased influx of neutrophils into the macrophage-depleted granulation tissue. In turn, neutrophils are rich in highly active tissue degrading proteases and reactive oxygen species, which could contribute to the severe endothelial cell damage observed (46). Although at this stage we cannot exclude neutrophil-mediated vascular cell damage, we consider it an unlikely event, because hemorrhage and endothelial cell apoptosis were also present in macrophage-depleted granulation tissue, which did not present increased neutrophils. Overall, our data clearly illustrate that macrophages present in granulation tissue are crucial for the progression of the mid-stage of the repair response into the late stage characterized by tissue maturation.

Finally, depletion of macrophages during the late stage of repair did not cause significant morphological changes, indicating a minor role of macrophages present during the phase of tissue maturation and scar formation. Indeed, as outlined above, macrophages recruited during the early stage of repair significantly control the degree of scar formation. Furthermore, absence of morphological alterations in macrophage-depleted day 14 wounds of LysMCre/iDTR mice demonstrates that DT-mediated macrophage apoptosis itself does not inevitably cause cellular changes in the wounded tissue. Therefore, in our model, the altered healing response observed in macrophage-depleted wounds during the early or mid-stage of repair is a consequence of macrophage deficiency and not a sequela of their apoptosis.

In conclusion, our study adds to previous knowledge on the function of macrophages in skin repair, but also uncovers several novel aspects. First, our findings substantiate previous work, demonstrating a crucial role of macrophages in healing skin wounds in the adult organism to achieve tissue homeostasis. Secondly, we identified repair mechanisms that are dependent or independent from macrophage function. Third, we revealed that macrophages exert distinct functions during the diverse phases of skin repair that are complementary to restore skin integrity. Finally, our findings suggest that different macrophage functions in skin repair can originate in different macrophage activation states. Overall, our findings delineate cellular and molecular mechanisms that control the kinetics of skin repair and link macrophage function as the critical force to the dynamics of wound repair. Future studies will analyze whether selective modulation of macrophage activation and function during specific stages of repair might be an effective therapeutic strategy to normalize tissue regeneration in pathological healing conditions. However, one has to take into account that skin repair in mice differs significantly from the human
system. Therefore, we suggest that additional experimental studies in mice and/or other model organisms should be paralleled by analysis of macrophage activation and function during wound repair in the human system.

Acknowledgments
We are grateful to Luca Borradori for critically reading the manuscript and Sabine Werner for support in the fibrinogen/fibrin stain. We also thank Joel Leibovich for helpful discussion and advice on macrophage biology and Michael Piekarek and Margot Junker for excellent technical assistance.

Disclosures
The authors have no financial conflicts of interest.

References
Legends of supplementary Figures:

Supplementary Figure 1: Macrophages present during the early stage of repair control cell proliferation. LysMCre/iDTR and control mice received DT injections following regimen A. Left, day-5 wounds of LysMCre and LysMCre/iDTR mice stained for Ki67; the number of proliferating cells in the hyperproliferative epidermis and granulation tissue (arrowheads indicate Ki67 positive cells, yellow) is increased in wounds of LysMCre mice compared to LysMCre/iDTR mice; right, morphometric quantification of Ki67 positive cells in the hyperproliferative epithelium; data is expressed as mean ± SD, n=3 wounds on 3 mice for each and group; (propidium iodide counterstain in red) (hatched line indicates basement membrane.

Supplementary Figure 2: Fizz1-expressing macrophages in wound tissue of LysMCre/iDTR and control mice receiving DT injections following regimen A. (A) In granulation tissue of control mice at day 5 and 10 post injury double labeling for F4/80 (red) and Fizz1 (green) revealed expression of Fizz1 by macrophages; in contrast, in day-5 wounds of LysMCre/iDTR mice F4/80 and Fizz1 expressing cells are not detectable, in day-10 wounds F4/80 positive cells are negative for Fizz1 staining.

Supplementary Figure 3: Neutrophils are absent and mast cell number is similar in LysMCre/iDTR and control mice receiving DT injections following regimen C. (A) Neutrophils (stained by Gr-1) are absent in scar tissue of control and LysMCre/iDTR wounds 14 days post injury. (B) Giemsa staining of control and LysMCre/iDTR wounds reveal similar numbers of mast cells in scar tissue 14 days post injury. e, epidermis; st, scar tissue; data is expressed as mean ± SD.
Suppl. Fig. 1

Day 5 post injury

LysMCre
LysMCre/iDTR

Ki67 Ki67

100µm 100µm

Ki67 + cells/
hyperproliferative epithelium

p = 0.003

Day 5 post injury
Suppl. Fig. 2

Day 5 post injury

Day 10 post injury

B
Suppl. Fig. 3

A

LysMCre
Gr-1
LysMCre/iDTR

Day 14 post injury

B

LysMCre
LysMCre/iDTR

Day 14 post injury

Suppl. Fig. 3

Day 14 post injury

LysMCre LysMCre/iDTR

5 Mast cells/wound
35
25
15
5

Day 14 post injury

LysMCre LysMCre/iDTR

100µm
100µm
100µm
100µm