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Impaired Wound Healing with Defective Expression of Chemokines and Recruitment of Myeloid Cells in TLR3-Deficient Mice

Qing Lin,* Dan Fang,* Jiazhu Fang,* Xiangrong Ren,* Xiaoping Yang,† Feng Wen,* and Shao Bo Su*

Skin injury evokes both innate and adaptive immune responses to restore tissue integrity. TLRs play a critical role in host responses to injurious insults. Previous studies demonstrated that RNAs released from damaged tissues served as endogenous ligands for TLR3. In this study, we investigated the involvement of TLR3 in skin restoration after injury. Full excisional wounds were created on the skin of mice with TLR3 deficiency. We found that skin wound closure in TLR3−/− mice was significantly delayed compared with control littermates. Wound healing parameters, including re-epithelialization, granulation formation, and neovascularization, were decreased in TLR3−/− mice. Further studies revealed that the absence of TLR3 led to defective recruitment of neutrophils and macrophages, in association with decreased expression of the chemokines, MIP-2/CXCL2, MIP-1α/CCL3, and MCP-1/CCL2, in the wound. Moreover, in wild type mice, the mRNA level and protein content of TLR3 was significantly upregulated in wounded skins and silencing of TLR3 signal adaptor Toll/IL-1R domain-containing adapter inducing IFN-β with small interfering RNA retarded wound closure. These results indicate an essential role for TLR3 and Toll/IL-1R domain-tion, were decreased in TLR3 with control littermates. Wound healing parameters, including re-epithelialization, granulation formation, and neovascularization, were decreased in TLR3−/− mice. Further studies revealed that the absence of TLR3 led to defective recruitment of neutrophils and macrophages, in association with decreased expression of the chemokines, MIP-2/CXCL2, MIP-1α/CCL3, and MCP-1/CCL2, in the wound. Moreover, in wild type mice, the mRNA level and protein content of TLR3 was significantly upregulated in wounded skins and silencing of TLR3 signal adaptor Toll/IL-1R domain-containing adapter inducing IFN-β with small interfering RNA retarded wound closure. These results indicate an essential role for TLR3 and Toll/IL-1R domain-containing adapter inducing IFN-β in wound healing by regulating chemokine production and recruitment of myeloid cells to wound for tissue repair. The Journal of Immunology, 2011, 186: 3710–3717.

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**The State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou 510060, China; and †Cardiology Division, Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD 21287.

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Address correspondence and reprint requests to Dr. Shao Bo Su, The State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, 54 South Xianlie Road, Guangzhou 510060, China. E-mail address: shaobosu@yahoo.com.

Abbreviations used in this article: MPO, myeloperoxidase; siRNA, small interfering RNA; SMA, smooth muscle actin; TRIF, Toll/IL-1R domain-containing adapter inducing IFN-β; WT, wild type.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1003007
inducing IFN-β (TRIF) (14). TRL3 expression was demonstrated previously in healthy and psoriatic skin (15), as well as in cultured normal skin epidermal keratinocytes (16). Emerging evidence reveals that RNAs released from either damaged tissue or within endocytosed cells serve as the endogenous ligands for TRL3 to induce TNF-α and enhance TLR3 expression in keratinocytes (17, 18). TLR3-deficient mice had decreased inflammation with lower expression of IL-6 and TNF-α at the wound edge after 3 d of aseptic injury (18). Thus, TLRs appear to regulate inflammatory responses during skin wound healing.

In this study, we investigated the role of TLR3 in a mouse wound healing model. Multiple wound parameters, including inflammatory cell infiltration, the expression of chemokines, and their receptor were assessed at the different time intervals. We found that TLR3−/− mice exhibited delayed wound healing with impaired neovascularization and compromised neutrophil and macrophage recruitment associated with decreased expression of chemokines MIP-2/CXCL2, MIP-1α/CCL3, and MCP-1/CCL2 in the wounds.

Materials and Methods

Reagents and Abs

Goat anti-rabbit IgG-peroxidase Ab, goat anti-rat IgG-peroxidase Ab, and rabbit anti-goat IgG-peroxidase Ab were purchased from Sigma (St. Louis, MO). TRIZol reagent and Hoechst 33424 were from Invitrogen (Carlsbad, CA). ExScript RT reagent kit was from TaKaRa (TaKaRa Biotechnology, Dalian, China). Brilliant SYBR Green QPCR Master Mix was from Stratagene (La Jolla, CA). FITC-conjugated anti-mouse IgG/fluorescein isothiocyanate (FITC/FA) and FITC-conjugated anti-mouse CD3 were from eBioscience (San Diego, CA). Rabbit anti-mouse Y1/2 monoclonal Ab was from StemCell Technologies (Vancouver, British Columbia, Canada). Rabbit anti-mouse FIZZ1 was from PeproTech (London, U.K.). Rabbit anti-mouse keratin/cytokeratin Ab was from Millipore (Billerica, MA). Rabbit anti-mouse F4/80 and rabbit anti-goat IgG-peroxidase Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Diaminobenzidine substrate-chromogen was from Dako Cytomation (Carpinteria, CA). ELISA kits for mouse MIP-2/CXCL2, MIP-1α/CCL3, and MCP-1/CCL2 werefrom R&D Systems (Minneapolis, MN). Quick start Bradford protein assay ELISA kit was from Bio-Rad Laboratories (Hercules, CA). Most of the other reagents such as salt and buffer components were analytical grade and obtained from Sigma.

Animals

TLR3−/− mice were purchased from Taconic Farms. The mice were backcrossed ≥10 generations onto the C57BL/6 background, and were then intercrossed to obtain the knockout genotype and wild type (WT) littermates (as control). Littermates of both sexes between 8- and 12-wk-old were used in all experiments. Animals were housed individually in cages under specific pathogen-free conditions, and given water and standard laboratory chow ad libitum during the experiments. Genotyping of animals was performed by PCR of DNA obtained from tail biopsies. Primers were synthesized according to the genotyping protocol (stock number 005217) from The Jackson Laboratory (Bar Harbor, ME). Animal care and all experimental procedures were approved by the Institutional Laboratory Animal Care and Use Committee.

In vivo wound model

Full-thickness wounds were created in the dorsal skin under sterile conditions. In brief, mice were anesthetized with i.p. administration of Kessodrol (chloral hydrates). After depliation with 10% Na2S and cleaning with povidone-iodine (Betadine) and 70% ethanol, the dorsal skin was picked up at the midline and punched through two layers of skin with a sterile disposable biopsy punch (4 mm in diameter; millex), generating one wound on each side of the midline. The same procedure was repeated, generating four wounds. Each wound area was digitally photographed (at the indicated time points) at 3- to 4-dervals, and wound areas were determined on photographs using Adobe Photoshop (version 7.0; Adobe Systems) (Fig. 2). Changes in wound areas over time were expressed as the percentage of the initial wound areas.

Histopathological and immunohistochemical analyses

Wound specimens were fixed in 4% formaldehyde and then embedded with paraffin. Sections were stained with H&E for histological analysis. Immunohistochemical analyses were performed using anti-TLR3. The sections were incubated with Abs at concentrations of 0.5–5 μg/ml at 4°C overnight. After incubation with peroxidase-conjugated secondary Abs and counterstaining with Hoechst 33342, the sections were mounted and visualized in a Zeiss fluorescence microscope (Axioplan 2 imaging; Carl Zeiss MicroImaging GmbH, Germany) with a multichannel mode and Zeiss filters (set 17 for FITC; band-pass 515–565; set 20 for Cy3; band-pass 575–640; and set 02 for Hoechst 33424; LP 420). Sections were also stained with anti-CD3 Ab to evaluate neovascularization.

Leukocyte infiltration

The wound bed was defined as the area surrounded by uninjured skin and fascia, regenerated epidermis, and eschar. Frozen cryostat sections of skin wound margins and the adjacent uninjured skin were stained with anti-MPO, -MCP-1, -MIP-2, and -MIP-1α/β with commercial ELISA kits according to the manufacturer’s instructions. Total protein in the supernatants was measured using the Bradford method. The data were expressed as the quantity of target molecule (pg) per total protein (mg) for each sample.

Total RNA isolation and quantitative real-time PCR

Total RNA was isolated from uninjured and wounded skin samples using a TRIZol reagent kit, and cDNA was prepared by reverse transcription. The mRNA expression of testing molecules in skin samples was determined by real-time PCR following the manufacturer’s protocol. Each reaction contained 12.5 μl 2 × SYBR Green Master Mix, 300 nM oligonucleotide primers (Table I) synthesized by Invitrogen Biotechnology, (Shanghai, China), 10 μl of 1:10 dilution of the cDNA and water, to a total volume of 25 μl. The thermal cycling conditions included an initial denaturation at 95°C for 10 min, 40 cycles at 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min. The concentrations of coded samples were calculated by using software (version 1.7) provided with the ABI 7700 system (Perkin Elmer Applied Systems, Foster City, CA).

Small interfering RNA synthesis and treatment

The sequences of the two oligonucleotide strands of the TRIF small interfering RNA (siRNA) duplex are as follows: sense 5′-GCAGUGGUGUUGCUUGTT-3′; and antisense 5′-CAGGGGUGUUGUACAUAGT-3′. This siRNA target sequence is present in TRIF mRNA for the mouse (National Center for Biotechnology Information Reference Sequence: NM_174989.3) gene. TRIF-specific siRNA and scrambled control siRNA were synthesized by Ribobio (Guangzhou, China). Complementary sense and antisense single strands were annealed, desalted, and lyophilized to provide the final siRNA duplex. The integrity and purity of the siRNA were confirmed by HPLC and MALDI-TOF mass spectrometric methods. Lyophilized duplex was suspended in RNase-DNase–free PBS buffer before in vivo use. After wound surgery, 5 nmol specific TRIF-siRNA in 20 μl sterile PBS solution without endotoxin was locally applied to two
wounds on the right side of mice daily, whereas the other two wounds on the left side of dorsal skin received the same volume of control scrambled siRNA or PBS, respectively. Each group was composed of eight mice. Macropscopic area of the open wound was measured and analyzed at the indicated time intervals. For assessing the efficiency of TRIF silencing, the wounded skins treated with siRNA for 5 d were cut, total RNA was isolated, and quantitative real-time PCR was performed with TRIF-specific primers as shown in Table I. The mRNA expression levels in each sample were determined after normalization against the level of GAPDH mRNA.

Reproducibility and statistical analysis

All experiments were repeated at least twice. All figures showed pooled data from replicate experiments or representative experiments as indicated. Data are expressed as the mean ± SEM for the indicated number of independently performed duplicated experiments. Statistical significance between the means was analyzed by two-tailed Student t test using the SPSS 13.0 version. A p value < 0.05 was considered statistically significant.

Results

Delayed wound healing in TLR3−/− mice

It has been shown that TLR3 was required for normal inflammatory responses after injury (18). Studies also showed that activation of TLR3 inhibited angiogenesis (19, 20). These results suggest that TLR3 plays an important role in wound healing. To test the involvement of TLR3 in skin injury, we first evaluated wound changes in TLR3-deficient mice. Four full-thickness skin circular excision wounds were created symmetrically on the backs of mice (Fig. 1A). Wound areas were analyzed throughout the healing process (Fig. 2). In control littermates, the wounds underwent gradual and progressive healing, which reached complete closure by day 12 after injury (Fig. 1B). To our surprise, wound closure was markedly delayed rather than accelerated in TLR3−/− mice (Fig. 1B). On day 7 after wounding, the control wounds had already lost their eschars and appeared completely epithelialized, whereas the wounds of TLR3−/− mice showed less re-epithelialization, granulation formation (lower left and middle panels), and neovascularization (lower right panel). Boxed areas in the left panels are enlarged in the middle panels. A stratified neoe-dermis was visible on the edge of the wounds in WT mice, whereas the epithelial tissue was disorganized in TLR3−/− wounds. Representative results from three independent experiments with four animals in each group are shown. Original magnification ×40 (left panels) and ×400 (middle and right panels).

FIGURE 1. Delayed wound healing in TLR3−/− mice. A, Full-thickness skin wounds were created in WT or TLR3−/− mice. B, Representative photographs from WT and TLR3−/− mice showing the macroscopic wound closure on different days postinjury. C, Microphotographs of wounds were analyzed at indicated time intervals to determine closure in WT and TLR3−/− mice. All values represent the mean ± SEM. *p < 0.05 indicates significantly and **p < 0.01 indicates very significantly higher values observed in WT mice compared with TLR3−/− mice. D, Histopathology (left and middle panels) and immunohistochemistry (CD31 staining, right panels) of wounds of WT and TLR3−/− mice on day 5 postinjury. TLR3−/− mice showed less re-epithelialization, granulation formation (lower left and middle panels), and neovascularization (lower right panel). Boxed areas in the left panels are enlarged in the middle panels. A stratified neoe-dermis was visible on the edge of the wounds in WT mice, whereas the epithelial tissue was disorganized in TLR3−/− wounds. Representative results from three independent experiments with four animals in each group are shown. Original magnification ×40 (left panels) and ×400 (middle and right panels).

FIGURE 2. Wound measurement and analysis. On day 0, four circular excisional wounds (4 mm in diameter) were created in the dorsal skin of sex- and age-matched WT or TLR3−/− mice. Each wound site was digitally photographed at the indicated time intervals, and wound areas were determined on photographs using Adobe Photoshop software. Changes in wound areas over time were expressed as the percentage of the initial wound areas.
Decreased leukocyte infiltration in skin wounds in TLR3<sup>−/−</sup> mice

We next examined the recruitment of leukocytes, a critical procedure for wound healing, at the wound sites. Neutrophils, macrophages, and T lymphocytes were stained by immunofluorescence with anti-MPO (Fig. 3A), anti-F4/80 (Fig. 3B), and anti-CD3 Abs (Fig. 3C), respectively. There was no accumulation of neutrophils and macrophages in the normal skin (data not shown). The influx of neutrophils reached a peak on day 1 and gradually reduced until at least on day 6 in all wounds (Fig. 3D). F4/80<sup>+</sup> macrophages started to accumulate on day 1 after injury and reached maximal levels on day 6 (Fig. 3E). The kinetics of leukocyte infiltration in the wounds was similar in control and TLR3<sup>−/−</sup> animals (Fig. 3D, 3E). However, infiltration of neutrophils in TLR3<sup>−/−</sup> mouse wounds was significantly reduced on days 1 and 3 (Fig. 3D). The recruitment of macrophages in the wounds in TLR3<sup>−/−</sup> mice was significantly attenuated on days 3 and 6 compared with control littermates (Fig. 3E). The level of CD<sup>+</sup> T cell infiltration was similar in TLR3<sup>−/−</sup> and WT littermates (Fig. 3F). Next, we examined the infiltration of alternatively activated/M2 macrophages, a subpopulation of macrophages that contributes to wound healing (19, 21–25). The markers of these macrophages, FIZZ1/ RELM<sub>a</sub> and YM1, were used for staining of wound sections. We found that on 6 d after injury, there were numerous F4/80 and YM1 or FIZZ1 double-positive macrophages in both control and TLR3<sup>−/−</sup> wound tissue. However, the number of YM1<sup>+</sup> or FIZZ1<sup>+</sup> wound-healing macrophages in TLR3<sup>−/−</sup> mouse wounds was markedly reduced as compared with WT mouse wounds (Fig. 4).

Decreased chemokine expression in the skin wounds of TLR3<sup>−/−</sup> mice

The markedly decreased infiltration of neutrophils and macrophages in the wounds of TLR3<sup>−/−</sup> mice implied the possibility of chemokine expression. To test this hypothesis, we used quantitative PCRs to examine changes in mRNA expression of chemokines (Table I). All 15 chemokine genes tested were upregulated in the wounds to varying extents with different kinetics (data not shown), which is in agreement with previous reports (4, 6, 8, 10, 26–29). However, the expression of the genes for MIP-2/CXCL2, MIP-1α/CCL3, and MCP-1/CCL2 was significantly reduced in TLR3<sup>−/−</sup> mice compared with WT controls (Fig. 5A). MIP-2/CXCL2 had a reduction of 7.5-fold on the first day of skin injury in TLR3<sup>−/−</sup> mice. Thereafter, the mRNA level of MIP-1α/CCL3 and MCP-1/CCL2 was predominantly decreased in TLR3<sup>−/−</sup> mice. Consistently, the protein level of these chemokines was significantly increased in the wounds of WT mice (Fig. 5B–D). In contrast, the protein level of MIP-2/CXCL2, MIP-1α/CCL3, and MCP-1/CCL2 in TLR3<sup>−/−</sup> skin wounds was significantly decreased compared with WT mice (Fig. 5B–D). These results suggest that TLR3 activation may induce the production of chemokines, which are required for the recruitment of leukocytes in the wounds.

TLR3 expression in wound healing

Quantitative PCR showed that mRNA expression of TLR3 was significantly upregulated in wounded skins of WT mice, most strongly on day 1 (Fig. 6A). Immunohistochemical analysis showed that although TLR3 protein was detectable in both dermis and subcutaneous of normal skin, it was significantly enhanced after skin injury (Fig. 6B). Large numbers of TLR3<sup>+</sup> cells were found in the wound and surrounding areas, which remained elevated until 6 d after wounding (data not shown). Colocalization experiments revealed that TLR3<sup>+</sup> cells were resident cells (keratin<sup>+</sup> keratinocytes, α-SMA<sup>+</sup> fibroblasts, and CD31<sup>+</sup> endothelial cells) and infiltrating leukocytes (MPO<sup>+</sup> neutrophils and F4/80<sup>+</sup> macrophages) in the skin wounds (Fig. 6C). These results suggest that activation of TLR3 by endogenous ligands from injured tissue may regulate normal wound healing.

Effect of TRIF siRNA silencing on wound healing

TRIF is the critical adaptor protein for TLR3-mediated signaling (30). To clarify the role of TLR3 signaling in skin wound healing, we examined the effect of TRIF silencing. TRIF-specific or control siRNA was topically applied to wounds. Application of TRIF-specific siRNA significantly delayed the wound closure in WT mouse wounds compared with PBS treatment, whereas administration of control scrambled siRNA significantly enhanced wound repair in WT mice (Fig. 7A, 7B). Silencing of TRIF by TRIF-specific siRNA was confirmed by real-time PCR analysis, which showed that TRIF mRNA expression on day 5 was significantly reduced as compared with scrambled siRNA- or PBS-treated wounds (Fig. 7C). We also investigated the effect of scrambled siRNA on TLR3<sup>−/−</sup> wounds. The data showed that scrambled siRNA treatment failed to enhance wound healing in TLR3<sup>−/−</sup> mice throughout a period of 14 d (Fig. 7D, 7E), suggesting a role of TLR3 in mediating a better effect of scrambled siRNA than PBS on wound healing.
healing (Fig. 7B). Taken together, these results further demonstrate an important role of TLR3-TRIF axis in wound repair process after skin injury.

Discussion

Our study showed that skin wound healing in TLR3 \(^{-/-}\) mice was delayed in association with reduced accumulation of neutrophils and alternatively activated/M2 macrophages in association with reduced chemokine expression in wounds. TLR3 was originally identified as a specific receptor for viral dsRNA (31). However, recent studies demonstrated that TLR3 also recognizes endogenous RNAs and plays an important role in inflammatory responses in animal models of cecal ligation and puncture surgery and skin incision (18, 32). Reports showed that activation of TLR3 triggered by RNAs released by necrotic cells induced TNF-\(\alpha\) and TLR3 expression in keratinocytes. Decreased production of proinflammatory cytokine and leukocyte recruitment in experimental colitis (33) and acute skin injury (18) was observed on TLR3 \(^{-/-}\) mice. These data indicate that TLR3 is required for normal inflammatory response after injury. Consistent with these observations, our study showed that skin wounding upregulated TLR3 expression in WT mice. The innate response of resident and recruited cells combats invading microbes but may also critically influence the repair process by releasing a wide spectrum of mediators. However, the impact of inflammation on the process of wound healing is controversial (34). Analyses of wound healing in a number of murine models deficient in individual inflammatory mediators, receptors, or depletion of leukocytes have yielded variable results. Wound healing is accelerated in mice deficient in TNFRp55 (8), IFN-\(\gamma\) (35), or in mice with depletion of neutrophils (36), but is impaired in mice deficient in TNF-\(\alpha\) (37), MCP-1 (38) or IL-6 (39). Our findings indicate that innate inflammatory response improves the repair process in the skin, which will be helpful to further understanding of the complex and possibly tissue-dependent process of wound healing.

Table I. Sequences of primers for real-time PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>bp</th>
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<tr>
<td>TLR3</td>
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<td>5’-GCTGCTGAATTTAGTGGC-3’</td>
<td>230</td>
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<tr>
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<tr>
<td>MIP-1(\beta)</td>
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<td>5’-CCTCCCTTTGACGCTGTGTA-3’</td>
<td>132</td>
</tr>
<tr>
<td>KC</td>
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<td>5’-CGCTGCTGAATTTAGTGGC-3’</td>
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<tr>
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The importance of TLR3 signaling in skin wound healing was further confirmed by TRIF silence. TRIF is a Toll/IL-1R domain-containing adaptor protein. Its overexpression triggers the TLR3-mediated signaling pathways, resulting in the induction of cytokine and chemokines (30, 40). In this study, TRIF siRNA silencing delayed the wound repair (Fig. 7). In contrast, scrambled siRNA promoted wound closure in WT mice (Fig. 7), but not in TLR3\(^{−/−}\) mice, indicating the role of TLR3 in mediating the effect of

![FIGURE 5. Chemokine expression in skin wounds. A, Real-time PCR analysis of chemokine mRNA expression in wounds on days 1, 3, and 5 postinjury in WT and TLR3\(^{−/−}\) mice. The relative changes were normalized against the mRNA of GAPDH and calculated using the \(2^{−\Delta\Delta \text{CT}}\) method. Results were from three separate experiments performed in duplicate and expressed as mean ± SEM of fold increase over control. *\(p < 0.05\) indicates significantly greater values in WT mice in comparison with the baseline in TLR3\(^{−/−}\) mice. B–D, The protein levels of MIP-2/CXCL2 (B), MIP-1\(\alpha\)/CCL3 (C), and MCP-1/CCL2 (D) in the wounds of WT and TLR3\(^{−/−}\) mice measured by ELISA. Values represent mean ± SEM of duplicate determinations. *\(p < 0.05\) indicates significantly greater values in WT mice. Representative results from three independent experiments with four animals in each group are shown.](http://www.jimmunol.org/)

![FIGURE 6. TLR3 expression in the skin. A, Real-time PCR analysis of TLR3 expression in skin wounds. The ratio of TLR3 to GAPDH at the wound sites on 1, 3, or 5 d after wounding was analyzed. Results were from three separate experiments that were performed in duplicate and expressed as mean ± SEM of fold increase over control. *\(p < 0.05\) indicates significantly greater values compared with normal skin. B, Immunohistochemical analysis of TLR3 expression in dermis (left panels) and subcutaneous tissue (right panel) of normal skin (upper panels) or wounded skin (lower panels) on day 1 postinjury. C, Cellular source of TLR3 expression at wound site. Double-color immunofluorescence images are shown on day 3 after injury using anti-cytokeratin, anti-CD31, anti–\(\alpha\)-SMA, anti-F4/80 or anti-MPO (green), and anti-TLR3 (red) Abs. Signals were digitally merged in right panels. For controls, the sections were stained with control Abs or TLR3 Ab and counterstained with Hoechst 33342. Representative results from three independent experiments are shown. Original magnification \(\times 400\).](http://www.jimmunol.org/)
Silencing of TRIF delays skin wound healing in WT mice. Five nanomoles TRIF-siRNA in 20 μl sterile PBS were typically applied to two wounds, whereas the other wounds received the same volume of PBS or control scrambled siRNA. A. Macroscopic appearance of healing of the wound treated with testing solutions on different days after injury. Representative results from three independent experiments with six animals are shown. B. At the time points indicated, the wound area was determined using image analysis and expressed as percentage of the original wound area. Values represent mean ± SEM. *p < 0.05, in comparison with the control scrambled siRNA treatment. †p < 0.05, in comparison with the PBS treatment. Representative results from three independent experiments are shown. C. mRNA level of TRIF in TRIF-silencing wounds. Total RNA was isolated from the wounds treated daily with TRIF-specific siRNA, scrambled control siRNA, or vehicle on day 5, and quantitative real-time PCR was performed. Results were from three separate experiments that were performed in duplicate and expressed as mean ± SEM of fold induction compared with the baseline of intact skin. †p < 0.05, in comparison with the control scrambled siRNA treatment. p < 0.05, in comparison with the PBS treatment. D and E. Effect of scrambled siRNA on wound healing in TLR3−/− wounds. Wounds in TLR3−/− mice were treated with scrambled siRNA, and wound areas were determined. Representative images of wounds (D) and kinetics of wound closure (E) from three independent experiments are shown. Values represent mean ± SEM. *p < 0.05, in comparison with PBS-treated wounds.

scrambled siRNA. These results are consistent with the reports that systemic delivery of polyinosinic-polycytidylic acid, a synthetic analog of viral dsRNA, enhanced wound healing (29), and nonspecific siRNA could also trigger innate immunity via TLR3 (19). These findings indicate that TLR3 may be a potential therapeutic target for the treatment of wounds.

Chemokines are critical mediators for leukocyte recruitment. We found that MIP-2/CXCL2 expression was decreased in TLR3−/− wounds. Murine MIP-2/CXCL2 is known as a homolog of human IL-8 (CXCL8). It has been reported that IL-8 promoted keratinocyte proliferation (41), granulation tissue maturation (42), and neovascularization (4). Our findings that the expression of MIP-2/CXCL2 was increased in the mouse skin wounds suggest an important role played by MIP-2/CXCL2 in wound repair. In GM-CSF KO mice, delayed wound closure was attributed to the reduction of MIP-2/CXCL2, which caused the reduction of neutrophil infiltration and angiogenesis (43). A study using mice deficient in the src family kinases (Hck and Fgr) demonstrated that MIP-2/CXCL2 induced neutrophil-dependent angiogenesis in vivo (44). Thus, MIP-2/CXCL2 may exert its regulatory activity directly on re-epithelialization and neo-angiogenesis, or by its neutrophil chemoattractant properties in wound healing. MCP-1/CCL2 and MIP-1α/CCL3 are CC chemokines attracting monocytes/macrophages. Previous studies demonstrated that MCP-1/CCL2 and MIP-1α/CCL3 were at high levels in the tissue after wounding (6). The time course of MCP-1/CCL2 expression correlated with macrophage infiltration (6). MCP-1/CCL2 knockout animals were characterized by significantly delayed wound re-epithelialization and angiogenesis (38). Treatment of mice with a neutralizing antibody to MIP-1α/CCL3 before injury also reduced the number of macrophages at the wound site (6). Consistently, our data showed that both recruitment of macrophages and the expression of MCP-1/CCL2 and MIP-1α/CCL3 were increased in WT mice. Therefore, the decreased recruitment of macrophages in the wounds of TLR3−/− mice may be attributed to the defective expression of MIP-1α/CCL3 and MCP-1/CCL2.

Previous in vitro studies showed that TLR3 ligand polyinosinic-polycytidylic acid-enhanced chemokine MIP-1α production depended on type I IFN in keratinocytes (28). More recently, Gregorio et al. (45) showed that the infiltrating plasmacytoid dendritic cells promoted wound healing by the production of type I IFNs in vivo. Consistent with these observations, we also found that the expression of IFN-α and IFN-β in skin wounds was up-regulated in the skin wounds of both WT and TLR3−/− mice, yet with a lesser extent in TLR3−/− mice (data not shown). Further studies are required to clarify whether chemokine production is driven in vivo by IFN-α/β after TLR3 activation.

Phagocytes play an important role in wound healing (46). Neutrophils in the wounds form the first line of defense against local infections and are a source of proinflammatory cytokines to activate fibroblasts and keratinocytes (47). It was hypothesized that neutrophils may also be involved in the initiation of angiogenesis (46). Diminished neutrophil numbers in CXCR2-deficient mice are linked to impaired cutaneous healing (9). Consistent with these observations, our data showed that the infiltration of neutrophils in the wounds in TLR3−/− mice was decreased, which may have resulted from the reduction of MIP-2/CXCL2 expression. Macrophages are essential for normal wound repair. They function as phagocytes and APCs and, in particular, are an important source of factors critical for wound closure, such as vascular endothelial growth factor, platelet-derived growth factor, TGF-β, basic fibroblast growth factor, heparin binding epidermal growth factor, and TGF-α (1, 48, 49). These factors stimulate the synthesis of extracellular matrix by local fibroblasts, generate new blood vessels, promote the granulation tissue formation, and enhance re-epithelialization (1, 48). Macrophages also bind to extracellular matrix via their integrin receptors to enhance phagocytosis of microorganisms and fragments of the extracellular matrix to control the net content of collagen within the wounds (48). The synthesis of new collagen exceeding the rate of degradation will lead to fibrosis (50). Our study showed the decrease of FIZZ21 and YM1 double-positive alternatively activated/M2 macrophages in delayed closure wounds in TLR3−/− mice (51, 52), suggesting that the recruitment and differentiation of a subset of macrophages may be the key step in TLR3-mediated wound healing.

Taken together, in this study, we show that TLR3 is important for normal wound healing, based on observations in TLR3−/− mice and inhibition of TLR3 signaling in WT mice with siRNA targeting TRIF. Our findings suggest that TLR3 signaling cascade may be a potential therapeutic target for abnormal wound healing in human diseases such as diabetic foot and liver cirrhosis.