Assessment of para-inflammation in a wound healing model

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Abstract. A thorough understanding of the inflammatory process has substantial biological and clinical relevance. Para-inflammation has been described as an adaptive response of the immune system to low levels of tissue stress. However, the role of para-inflammation in wound repair requires further investigation. In the present study, the expression levels of several para-inflammation genes were assessed in a murine cutaneous wound healing model. The results revealed that the expression levels of the para-inflammation genes were significantly altered. Among the genes that were examined, the expression levels of solute carrier family 7 member 11 (*Slc7a11*) paralleled those of the M2 macrophage-associated genes. Further investigation indicated that the *Slc7a11* gene and its encoded protein cystine/glutamate transporter exhibited increased expression levels in IL-4-induced M2 macrophages. Notably, the inhibition of para-inflammation by sulindac prolonged wound healing process. The present study indicated that para-inflammation exhibited a protective effect in wound healing and provided new insight for host tissue repair.

Introduction

The contribution of inflammation to wound healing has attracted considerable attention as a research topic. Wound healing is a dynamic process and is usually divided into 3 main phases: Inflammation; tissue formation; and tissue remodeling. These 3 phases are overlapping. Inflammation is an adaptive response to tissue stress (1,2). Acute inflammation is important for wound healing. The infiltrating immune cells aid the degradation of debris and decrease the number of invading pathogens. Recruited monocytes can release growth factors to initiate the formation of granulation tissue (3). However, chronic inflammation can trigger delayed healing responses. In certain cases, this process can lead to tissue dysfunction and even carcinogenesis (4). Contrasting behaviors have been observed in several specific inflammatory factors; for example, interleukin (IL)-17A can promote enterocyte proliferation and maintain epithelial barrier integrity in the intestine (5). By contrast, it has also exhibited an inflammatory role in tumorigenesis (6,7). The exact role of inflammation in wound healing is yet to be identified. The mechanisms involved may be used to promote identification of potential therapeutic targets in tissue repair and regeneration.

The state between basal homeostatic conditions and inflammation has been described as para-inflammation (1), which is an adaptive response of the immune system to low levels of tissue stress (8). The physiological role of para-inflammation is to maintain homeostasis and restore tissue functionality (1). This chronic low-grade inflammation has also been demonstrated to participate in several pathological conditions. In patients with age-associated macular degeneration, the by-products of oxidative stress, such as C-reactive protein and complement, can trigger the pathophysiological para-inflammatory process (9). This dysregulated para-inflammatory response contributes to macular damage (8). Notably, para-inflammation serves a more complex role in tumor development (10), and can repress or promote tumorigenesis, depending on the activity of the tumor protein p53 (11). However, the association between para-inflammation and wound healing remains unclear. A more thorough understanding of para-inflammation has considerable biological and clinical relevance. It could be hypothesized that a disruption of para-inflammation may affect cutaneous wound repair.

Based on the aforementioned data, the present study aimed to investigate the association between para-inflammation and tissue repair using a murine cutaneous wound healing model. The results demonstrated that the expression levels of genes associated with para-inflammation were significantly altered during the wound healing process. The analysis performed to identify the para-inflammation-associated genes in wound tissues revealed elevated expression levels of solute carrier family 7 member 11 (*Slc7a11*) in IL-4-induced alternative-activated macrophages. The inhibition of para-inflammation with sulindac inhibited the wound healing process. The present study provided novel insights into the mechanism of wound healing and tissue repair.

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Materials and methods

Reagents. HyClone Dulbecco's Modified Eagle Medium (DMEM) was obtained from GE Healthcare. Fetal bovine serum (FBS) was obtained from Gibco; Thermo Fisher Scientific, Inc. Murine IL-4 and interferon-γ (IFNγ) were purchased from PeproTech, Inc. Sulindac was obtained from Xiya Chemical Co., Ltd. The cystine/glutamate transporter (xCT) monoclonal antibody was purchased from Abcam (cat. no. ab175186). GAPDH rabbit antibody was purchased from Cell Signaling Technology (cat. no. 2118). The horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was purchased from OriGene Technologies, Inc. (cat. no. ZB 2301).

Cell culture. Bone marrow-derived macrophages (BMDMs) were cultured as previously described (12), with certain modifications. All animal procedures were approved by the Sichuan University Institutional Animal Care and Use Committee. Briefly, the bone marrow was flushed from the femurs and tibias of 3 C57BL/6 female mice (8-10 weeks old). The red blood cells were lysed and the mononuclear cells were maintained in DMEM supplemented with 10% FBS and 100 ng/ml macrophage colony-stimulating factor for differentiation. Following 7 days of culture, non-adherent cells were removed, and adherent cells were stimulated by treatment with 20 ng/ml murine IL-4 or IFNγ for an additional 24 h. Cells were harvested for analysis.

Wound healing model. A total of 22 mice were purchased from Vital River Laboratories, Co., Ltd. (Beijing, China) and maintained under specific-pathogen-free conditions between 21 and 27˚C, humidity of between 40 and 60%, a light/dark cycle of 12 h and *ad libitum* access to food and water. The process of murine wound healing model preparation was performed as previously described (13,14), with certain modifications. Briefly, 19 female C57BL/6 mice (8-10 week-old) were used to establish an *in vivo* wound healing model. Prior to the production of the wound, the fur on the back of the mice was shaved following anesthetization. The tissue area was sterilized, the dorsal skin was stretched at the midline and the tissue was penetrated generating two full-thickness wounds of 6 mm in diameter on each side of the midline. For sulindac treatment, the mice were treated intraperitoneally (i.p.) with 20 mg/kg sulindac for 8 days consecutively (n=5). The control group (n=5) received a vehicle solution i.p., which was 5% DMSO, 30% PEG400 and 65% normal saline. Wound-bearing mice were held carefully during treatment and examination to avoid secondary trauma. Wound-bearing mice were also kept in separate cages to avoid secondary trauma. During the wound healing process, mice were observed for the presence of specific endpoints, including abnormal bleeding, discharge or severe infection in the wounds. Images of each wound were captured using a digital camera (Sonic; Sony Corporation) at the indicated time points. The degree of wound closure was determined using images as processed using Adobe Photoshop CS6 (Adobe Systems, Inc.). The wound area (%) was calculated as follows: (S0-St)/S0x100, where S0 was the original wound area on day 0 and St was the wound area on the indicated day.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The wounded tissues were collected at the indicated times (n=3/group at each time point). Normal skin tissues were used as controls. Total RNA was extracted from whole tissue or cultured macrophages using an RNAsimple total RNA extraction kit (Tiangen Biotech Co., Ltd.). Total RNA was reverse transcribed into cDNA using reverse transcriptase (Takara Bio, Inc.,) according to the manufacturer's protocol. qPCR was performed using SYBR® Premix Ex Taq II (Takara Bio, Inc.,) with specific primer sets. The PCR assay was performed on a CFX 96 Real-time PCR thermal cycler (Bio-Rad Laboratories, Inc.). The sequences of the primers used were as follows: Insulin like growth factor binding protein 4 (*Igfbp4*) forward, 5'-GGAGCTGTCGGAAATCGAAG-3'; *Igfbp4* reverse, 5'-TTGAAGCTGTTGTTGGGATG-3'; lactoperoxidase (*Lop*) forward, 5'-TGACCTTGCTCCAGACTGC-3'; *Lop* reverse, 5'-TTGACCCAGACCTTGACCTC-3'; prostaglandin E synthase (*Ptges*) forward, 5'-AGCACACTGCTGGTCATCAA-3'; *Ptges* reverse, 5'-TCCACATCTGGGTCACTCCT-3'; *Slc7a11* forward, 5'-TCTGGTCTGCCTGTGGAGTA-3'; *Slc7a11* reverse, 5'-CAAAGGACCAAAGACCTCCA-3'; SRY-box transcription factor 17 (*Sox17*) forward, 5'-TGAAATATGGCCCACTCACA-3'; *Sox17* reverse, 5'-CTGTCTTCCCTGTCTTGGTTG-3'; SRY-box transcription factor 4 (*Sox4*) forward, 5'-AATTGCACCAACTCCTCAGC-3'; *Sox4* reverse, 5'-TCGATTGCAGTTCACGAGAG-3'; TNF receptor superfamily member 8 (*Tnfrsf8*) forward, 5'-GAGACTCGGGAAGCCAAGAT-3'; *Tnfrsf8* reverse, 5'-GGTGGTCTTGAGTGGTCGAT-3'; toll like receptor (*Tlr*) 1 forward, 5'-GGACCTACCCTTGCAAACAA-3'; *Tlr1* reverse, 5'-TATCAGGACCCTCAGCTTGG-3'; *Tlr2* forward, 5'-GAGCATCCGAATTGCATCA-3'; *Tlr2* reverse, 5'-ACAGCGTTTGCTGAAGAGGA-3'; tumor necrosis factor receptor superfamily member (*TNFRSF*) 11b forward, 5'-ATGAACAAGTGGCTGTGCTG-3'; *TNFRSF11B* reverse, 5'-TCACACAGGAGCTGATGACC-3'; *TNFRSF19* forward, 5'-CGCTGCCATTCTCTTCCTAC-3'; *TNFRSF19* reverse, 5'-TCGATCCTTGAATTCCTGCT-3'; interleukin 1 receptor antagonist (*Il1rn*) forward, 5'-TTGTGCCAAGTCTGGAGATG-3'; *Il1rn* reverse, 5'-TTCTCAGAGCGGATGAAGGT-3'; 18S rRNA forward, 5'-CGCCGCTAGAGGTGAAATTCT-3'; 18S rRNA and reverse, 5'-CGAACCTCCGACTTTCGTTCT-3'.

Western blot analysis. BMDMs were lysed with RIPA buffer (Beyotime Institute of Biotechnology) and centrifuged for 10 min at 4˚C, at 12,000 x g to obtain the corresponding lysates. Protein was quantified using a bicinchoninic protein assay kit (Beyotime Institute of Biotechnology). The cellular lysates (40 μg protein) were resolved using a 10% SDS-polyacrylamide gel and transferred onto a PVDF membrane, which was blocked with 5% skim milk at room temperature for 1 h. The membrane was incubated with an antibody against xCT (1:1,000) at 4˚C overnight and subsequently with HRP-conjugated secondary antibody (1:10,000) at 37˚C for 1 h. The blots were visualized using the ECL System (Thermo Fisher Scientific, Inc.). Relative intensity of the indicated blot bands was quantified using ImageJ software (version 1.8.0-112; National Institutes of Health).

Statistical analysis. All data are expressed as the mean ± standard deviation. Statistical analysis was conducted with

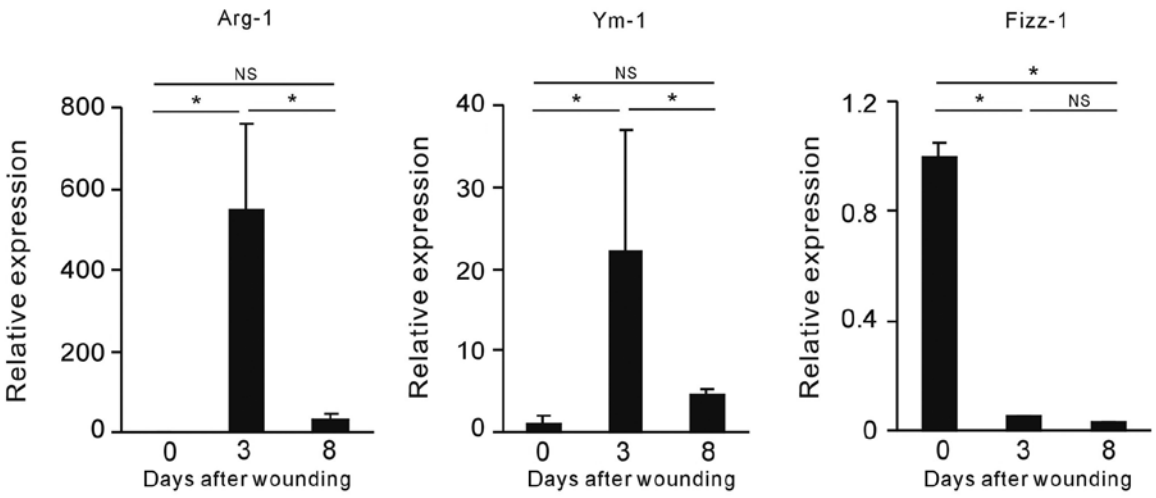

Figure 1. Expression levels of M2 macrophage-associated genes are significantly altered in the cutaneous wound healing process. Punctures were created on the back of C57BL/6 mice. Wound tissues were collected on days 3 and 8. Reverse transcription-quantitative polymerase chain reaction was performed to detect the expression levels of M2 macrophage-associated markers, including Arg-1, Ym-1 and Fizz-1. *P<0.05. Arg-1, arginase-1; Fizz-1, found in inflammatory zone 1; NS, no significant difference.

SPSS 13.0 software (SPSS, Inc.). Statistical comparisons between two groups were assessed using a Student's t-test. Statistical comparisons among three groups were analyzed using the one-way analysis of variance, followed by a Least Significant Difference post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of M2 macrophage-associated genes in the wound healing process. M2 macrophages have been demonstrated to participate in tissue repair (15). In addition, tissue-resident macrophages act as sentinels during homeostasis, in order to identify and respond to intrinsic and extrinsic stimuli (10). However, the changes in the expression pattern of M2-associated genes in wound healing are unclear. The present study firstly aimed to evaluate the expression pattern of these genes in murine cutaneous wound tissues. The skin of the mice was punctured and the wound samples were collected on days 3 and 8 following wound creation. Normal skin tissues were used as controls. The expression levels of well-known markers of M2 macrophages, including arginase-1 (*Arg-1*), Ym-1, encoded by chitinase-like protein 3, and found in inflammatory zone (Fizz)-1, encoded by resistin-like alpha (16,17) were assessed by RT-qPCR. The highest expression levels of Arg-1 and Ym-1 were observed on day 3, and were subsequently decreased on day 8 (Fig. 1). No differences in Fizz-1 expression in the wound tissues were observed between days 3 and 8 (Fig. 1). Taken collectively, the data demonstrated that the expression patterns of the main M2-associated genes were altered in the wound healing process.

Changes in expression of para-inflammation-associated genes involved in the wound healing process. Para-inflammation was identified in a mouse model of colorectal cancer (11). The expression levels of the para-inflammation-associated genes were investigated in wound tissues. The expression levels of these genes varied according to the different time intervals (Fig. 2). Notably, some of these genes, including *Ptges*, *Slc7a11*, *Il1rn*, *Tlr1* and *Tlr2* exhibited the highest expression levels on day 3. The expression levels of the genes of interest were decreased on day 8 and were similar to those of the M2-associated markers Arg-1 and Ym-1 (Fig. 2). Notably, *Slc7a11* exhibited the greatest variation during wound healing (Fig. 2). Taken collectively, the data suggested that the expression levels of the genes associated with para-inflammation were significantly altered in the wound healing process.

Slc7a11 is highly expressed in M2 macrophages. As the expression levels of specific para-inflammation genes were similar to those of the M2-associated genes, subsequent experiments focused on the expression M2 macrophage-associated genes. IFNγ and IL-4 were used to polarize bone marrow-derived macrophages (BMDMs) into M1 and M2 type macrophages, respectively. The expression levels of *Ptges*, *Slc7a11*, *Il-1rn*, *Tlr1* and *Tlr2* were analyzed. Notably, only *Slc7a11* was increased in the IL-4-induced M2 macrophages (Fig. 3A and B). Glutamate transporter xCT is encoded by *Slc7a11* (18). To confirm this observation, the protein levels of xCT were assessed by western blot analysis. Following IL-4 stimulation, the levels of xCT in BMDMs were increased. This results was consistent with that noted for *Slc7a11* mRNA levels (Fig. 3C). Taken collectively, the data suggested that IL-4 induced *Slc7a11* expression in M2 macrophages.

Suppression of para-inflammation by sulindac inhibits the wound healing process. M2 macrophages have been demonstrated to participate in the wound healing process (19). The physiological role of para-inflammation is to reset the homeostatic threshold of the tissue and restore tissue functionality (1). The present study explored whether para-inflammation affected the tissue repair process in a cutaneous wound healing model. Initially, the wounds were established in experimental mice and sulindac was subsequently administered i.p., resulting in inhibition of para-inflammation (11). The data demonstrated that the wound areas in sulindac-treated groups

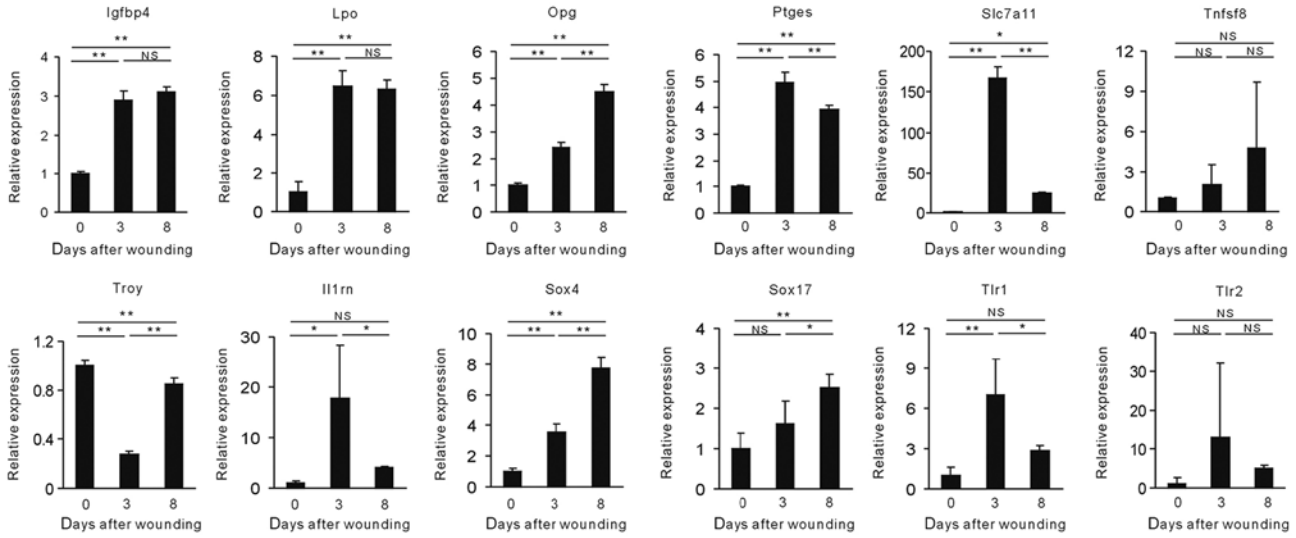

Figure 2. Expression levels of the para-inflammation genes are significantly altered during the cutaneous wound-repair process. The expression levels of the para-inflammation genes were assessed by reverse transcription-quantitative polymerase chain reaction in the wound healing tissues on days 0, 3 and 8, respectively. *P<0.05 and **P<0.01; NS, no significant difference.

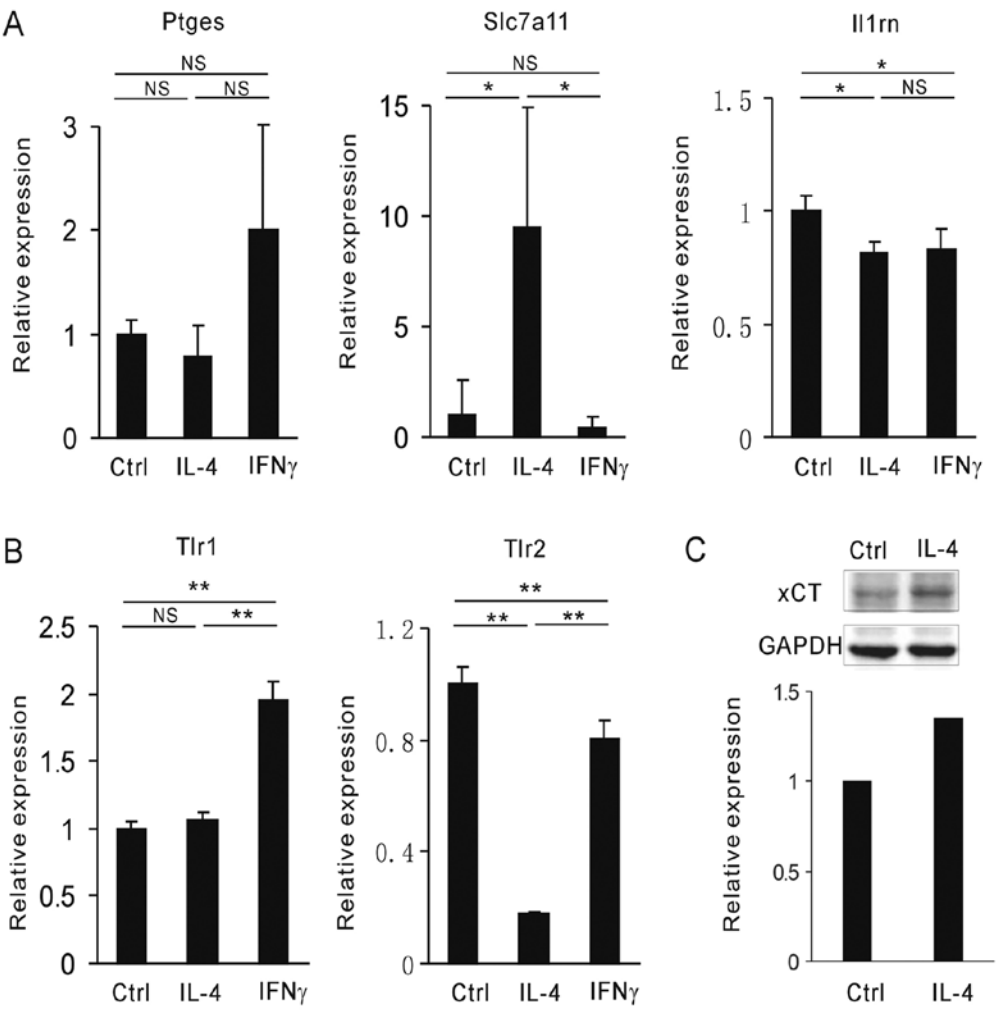

Figure 3. Levels of *Slc7a11* are elevated in the IL-4-induced M2 macrophages. (A and B) BMDMs were converted into M1 or M2 macrophages by IFNγ or IL-4 treatment, respectively. Reverse transcription-quantitative polymerase chain reaction analysis of the indicated para-inflammation genes examined in M1 and M2 macrophages. (C) The levels of xCT in BMDMs were assessed by western blot analysis. The levels of S*lc7a11* mRNA and xCT protein were elevated in IL-4-induced M2 macrophages. *P<0.05 and **P<0.01. *Slc7a11*, solute carrier family 7 member 11; IL, interleukin; IFNγ, interferon γ; BMDMs, bone marrow-derived macrophages; xCT, cystine/glutamate transporter; Ctrl, control; NS, no significant difference.

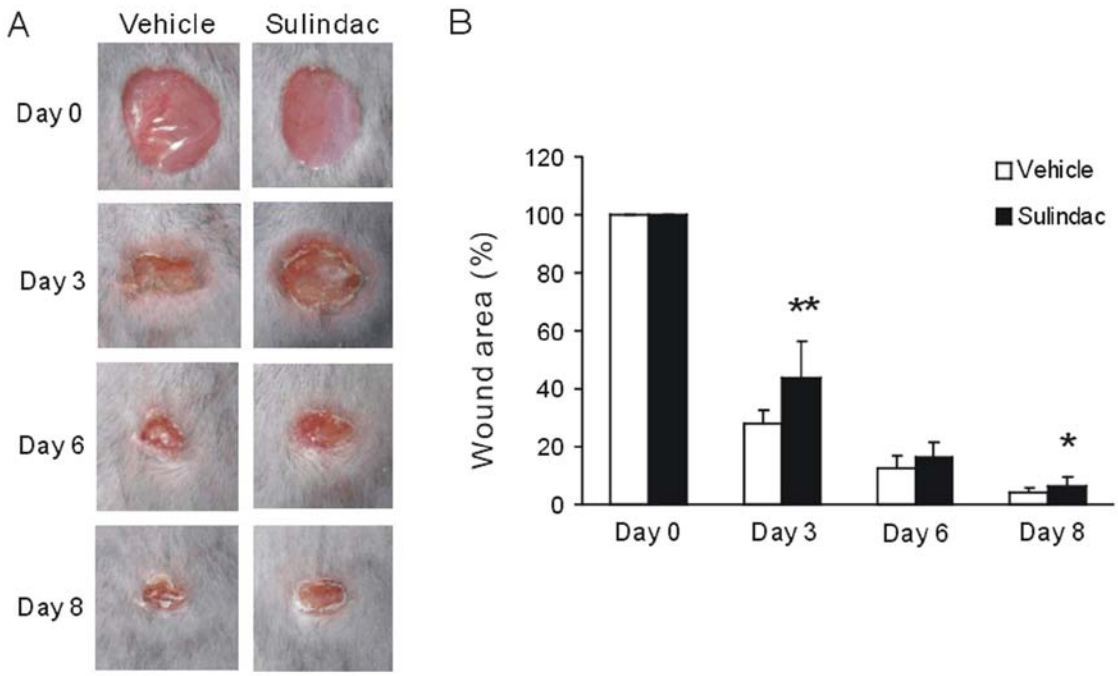

Figure 4. Suppression of para-inflammation by sulindac prolongs wound healing. The experiments included a wound healing mouse model. The animals were treated daily with 20 mg/kg sulindac and the wound healing rate was recorded. (A) Representative images of the skin lesions and consequent wound healing at the indicated time periods. (B) Percentage of wound area closed at each time point compared with the vehicle group (n=5). Sulindac inhibited wound closure. *P<0.05 and **P<0.01.

were larger compared with those in the control group; the most notable difference was observed on day 3 (Fig. 4A and B). Taken collectively, the data suggested that the regulation of para-inflammation may inhibit the wound healing process.

Discussion

The present study demonstrated that the expression levels of genes associated with para-inflammation were significantly altered during the wound healing process. Among those genes, the expression profile of *Slc7a11* in the wound tissues were similar to those associated with M2 macrophages, demonstrating an important role in tissue repair (15). In addition, the data indicated that inhibition of para-inflammation gene expression by sulindac prolonged the wound closure process.

Previous studies have indicated that the inflammation following tissue damage is a part of the protective response of the immune system (20). Accumulating evidence suggests that M2 macrophages exhibit significant roles in inflammation. Besides, pro-inflammatory M1 macrophages have also been demonstrated to facilitate tissue repair. Lipopolysaccharide (LPS) is a well-known factor used to initiate pro-inflammatory responses in macrophages (21). However, LPS has been demonstrated to activate microglia, which perform neuroprotection against experimental brain injury (22). Similarly, zymosan-activated macrophages were confirmed to induce pro-regenerative and neurotoxic functions (23). Based on these observations, inflammation is considered to exert pleiotropic roles in the tissue repair and regeneration process. Para-inflammation is a state between basal homeostatic conditions and a classic inflammatory response (1). In a murine cutaneous wound healing model, several para-inflammation genes were expressed in the wounded tissues. Following administration of sulindac, a potent para-inflammation inhibitor, the wound closure rate was decreased. The results of the present study suggested that para-inflammation exerted a protective role in the wound healing process. The therapeutic strategies that induce the para-inflammation process may be promising for tissue repair and regeneration.

In the present study, *Slc7a11* was expressed in M2 macrophages. Initially, *Slc7a11* expression levels were similar to those of Arg-1 and Ym-1, all of which were highest on day 3 and were subsequently decreased on day 8 following wounding. This result was confirmed using BMDMs. In concordance with these data, IL-4-induced M2 macrophages exhibited increased levels of *Slc7a11* mRNA expression, as demonstrated by RT-qPCR analysis, and increased levels of xCT protein, as indicated by western blot analysis. xCT is a member of a family of amino acid transporters and is a key player in glutamate/cysteine/glutathione homeostasis (18). The results were in concordance with the results from previous studies conducted in microglia: Although IL-4 suppressed the induction of xCT expression in the presence of β-amyloid, it increased the expression of the xCT protein in microglia in the absence of β-amyloid (24).

It is important to note that the present study did not fully explore the mechanism of para-inflammation in wound healing promotion. Firstly, the function of *Slc7a11* on M2 macrophages was not fully clarified. Certain amino acids were demonstrated to be essential for the development of M2 macrophages: Glutamine provided UDP-GlcNac required for the N-linked glycolysis of macrophages. In the absence

of glutamine, the expression levels of M2-associated genes were decreased in IL-4-induced M2 macrophages (25). As xCT is an important transporter of glutamate, the function of xCT in the development of macrophages has to be thoroughly explored. Notably, the expression levels of xCT were also enhanced in macrophages stimulated by LPS or by an electrophilic agent (26). High levels of reactive oxygen species (ROS) induced tissue damage. To prevent the damage caused by ROS, macrophages utilize a cytosolic redox-buffering system that consists primary of glutathione (GSH) (27), which can scavenge intracellular ROS and nitric oxide (NO (28). The glutamate/cystine transporter system is important for transporting cystine into cells and exporting glutamate. Cystine is then rapidly reduced into cysteine, which is the rate-limiting precursor of GSH (29). A recent study demonstrated that intracellular cysteine supplied by xCT contributed to NO production and the decrease of oxidative stress in macrophages. The ROS levels in xCT-deficient macrophages were increased compared with those of the wild-type cells (30). xCT deficiency in xCTmu/mu mice causes sustained inflammation due to the impaired survival of activated macrophages at the inflammatory site (31). Certain anti-inflammatory reagents, such as dimethylheptyl-cannabidiol, can upregulate *Slc7a11*/xCT expression (32). Further studies are required to clarify the role of xCT on macrophage polarization and function, in particular during wound healing. In addition, whether there is an association between eicosanoid levels and *Slc7a11* expression remains unknown. Aspirin is a well-known cyclooxygenase (COX) inhibitor that can inhibit xCT mRNA and protein levels in a dose-dependent manner (33). The exact interaction between COX inhibitors and the expression levels of xCT requires further clarification in future studies.

In summary, the present study demonstrated that para-inflammation served a protective role in the wound healing process. The results improve the current understanding of the contribution of para-inflammation to tissue repair and its significant potential clinical applications.

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Availability of data and materials

The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

Authors' contributions

TY and GBS designed the experiments. TY and GPW wrote the manuscript. GPW and TY performed the experiments. TY, GPW and GBS analyzed the data. XSJ and ZXC collaborated to design experiments and analyze the data. All authors read and approved the final version of this manuscript.

Ethics approval and consent to participate

All animal procedures were approved by the Sichuan University Institutional Animal Care and Use Committee (Chengdu, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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