# Diabetes-impaired wound healing and altered macrophage activation: A possible pathophysiologic correlation

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## ABSTRACT

Macrophages play a critical role in wound healing and can be activated to two distinctive phenotypes in vitro: classical macrophage activation (caM) and alternative macrophage activation (aaM). This study investigated whether the impaired cutaneous repair observed in streptozotocin-induced diabetic rats was associated with altered macrophage activation. Our results show that macrophage activation phenotypes could be observed in wound healing through double immunostaining. The caM macrophages appeared in the initial stage of wound healing, followed by aaM macrophages, which predominated in normal wounds. However, through examining markers associated with activation by immunoblotting and real-time polymerase chain reaction (PCR), diabetic wounds demonstrated insufficient caM in the early stage but excessive aaM in the later proliferative phase. Moreover, the macrophage activation markers were correlated with the instructive T helper cell type 1 (Th1)/Th2 cytokines in both groups. It was indicated that changed macrophage activation might contribute to impaired healing in diabetes wounds, and that strategies for reverting this abnormal activation could be useful for enhancing the wound healing process.

## INTRODUCTION

Wound healing is an intricately regulated biological event that involves infiltrating leukocyte subtypes and resident cells. Macrophages play a central role in wound healing[1,2] and are even considered as "orchestra leaders" for normal tissue repair, due to their participation in debridement and secretion of amounts of cytokines and growth factors. Impaired wound healing is one of the most frequent adverse effects affecting patients with diabetes.[3–5] Although previous research has identified a large number of changes at the molecular and cellular levels, the actual role and possible alteration of macrophages in diabetic wound healing has not been fully elucidated.

It is becoming increasingly clear that macrophages are a remarkable population of cells whose plasticity allows them to respond efficiently to microenvironmental signals and change their activated phenotypes.[6] The classical macrophage activation (caM) and alternative macrophage activation (aaM) are defined as two extremes forms of macrophages in the activation spectrum. The aaM arises due to the induction of interferon-gamma (IFN-$\gamma$) in combination with tumor necrosis factor-alpha (TNF-$\alpha$) or stimuli that can induce TNF-$\alpha$, such as lipopolysaccharide. The caM phenotype is typically interleukin (IL)-12$^{high}$ and IL-10$^{low}$, characterized by enhanced microbicidal capacity and the secretion of high levels of proinflammatory cytokines (such as IL-1, TNF, and IL-6, etc.), toxic intermediates such as nitric oxide (NO), and reactive oxygen intermediates (ROI). However, aaM polarization is generated in response to Th2 cytokines, such as IL-4 and IL-13. The aaM cells are typically IL-10$^{high}$ and IL-12$^{low}$, characterized by a failure to produce NO and up-regulated mannose receptor expression. The aaM cells produce low-level proinflammatory cytokines, meditating anti-inflammatory responses and adaptive Th2 immunity. These two types of activated macrophages also display different features in regard to wound healing. The caM/aaM can be generalized roughly as tissue-destructive vs. tissue-reparative macrophages.[7] Generally, caM occurs during the early inflammatory phase and scavenges debris, whereas aaM populates during angiogenesis and tissue remodeling.[8,9]

Until now, the primary functions of the caM/aaM cells in the wound were elucidated in vitro. However, few studies have focused on investigating macrophage activation in skin wound healing, especially in diabetic wounds in vivo. It is well known that the activated macrophages play a critical role, especially in the transition between inflammation and the proliferative phase.[1,2] Unfortunately, the diabetic wound is often stalled in the abnormal inflammatory state, suggesting that the transition from proinflammation to resolving inflammation is hindered.[10] The classification of caM/aaM demonstrates a great difference regarding the inflammation state, and there usually exists abnormal inflammation in the chronic wound.[11] Based on this information, it is necessary to study macrophage activation in diabetic wound repair.

To gain more insight into diabetic wound healing, we aimed to confirm the existence of activated macrophage phenotypes in a rodent model with full-thickness skin wound, and then study the activation events of macrophages under normal and diabetic circumstances.

## MATERIALS AND METHODS

### Animals

Male Sprague-Dawley rats were obtained from the Laboratory of Animals Sciences, Shanghai Medical College of Fudan University. All animal protocols were approved by the Ethical Committee on Animal Experiments of the University of Fudan Animal Care Committee. All experimental procedures were in compliance with institutional guidelines for the care and use of laboratory animals and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### Induction of diabetes mellitus

Diabetes was induced by a single 62 mg/kg intravenous injection of streptozotocin (Sigma-Aldrich, St. Louis, MO). Blood glucose levels were determined using a strip-operated blood glucose monitoring system (Glucotrend 2, Roche Diagnostics, Mannheim, Germany). The occurrence of polyuria, polydipsia, polyphagia, weight loss, and elevated blood glucose (16.7 mmol/L) confirmed the diabetic state. Rats were allowed to manifest hyperglycemia for 8 weeks before surgical wounding.

### Full-thickness skin wound preparation

All rats ($n = 60$, 30 each in the normal and diabetic groups) were anesthetized with an intraperitoneal injection of sodium pentobarbital (Westang Biotechnology Inc., Shanghai, China). The dorsal regions were shaved, followed by a depilatory agent to remove the remaining hair, and the surgical area was disinfected with benzalkonium bromide. Four full-thickness wounds (11-mm diameter) were made on the back by sterile punch, equidistant from the midline.

Wound tissues and their surrounding areas were harvested from both groups at 0, 1, 3, 7, and 13 days after wounding. The tissues were fixed in 10% neutral buffered formalin for immunohistochemistry and immunofluorescence assay, or snap-frozen in liquid nitrogen and stored at −70 °C for further analysis.

### Wound area measurement

The area of each wound was measured by tracing the edges of the wound onto a transparent plastic membrane. After the membranes were scanned, the wound area was measured using ImageJ software (National Institutes of Health, Bethesda, MD), and the percentage of wound closure over time was calculated.

### Hematoxylin and eosin (H&E) staining and immunohistochemistry

Wound tissue sections were deparaffinized in xylene, rehydrated through graded series of alcohol to phosphate-buffered saline, stained with H&E, and mounted in resin. The images were captured using a Zeiss microscope and processed using SPOT imaging software (Diagnostic Instruments, Sterling Heights, MI).

After deparaffinized and antigen retrieval, sections of wounds were blocked by incubating them in 0.3% hydrogen peroxide for 20 minutes. Slides were incubated overnight at 4 °C with a mouse anti-rat CD68 monoclonal antibody (1:200, AbD Serotec, Oxford, UK). Following incubation with biotinylated secondary antibody and streptavidin-biotin complex (Ultrasensitive SP Kit, Maixin Bio, Fuzhou, China), labeling was visualized by using 3, 3'-diaminobenzidine with hematoxylin. Stained cells were manually counted on a Zeiss microscope on the wound edge at 200× magnification. The results are expressed as the total number of infiltrating CD68+ cells per high power field (HPF).

### Immunofluorescence

Deparaffinized sections were incubated with phosphate buffered saline (PBS) containing 10% horse serum to reduce nonspecific reactions. Then, the sections were further incubated with a combination of anti-CD68 and anti-inducible nitric oxide synthase (iNOS) (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-arginase 1 (Arg-1) (Abcam, Hong Kong, China) Abs at 4 °C overnight. All Abs were used at a dilution of 1:100. After incubation with Alexa 586 conjugated goat anti-mouse, Alexa 488 conjugated goat anti-rabbit, or Alexa 488 conjugated donkey anti-goat antibody (1:2500, Molecular Probes, Eugene, OR) at room temperature for 60 minutes, tissue sections were mounted with Hoechst 33342 for nuclear staining and viewed on the wound edge using a two-photon laser confocal scanning system (LSM 510, Zeiss, Thornwood, NY).

### Western blot

Protein concentrations were determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL), and protein extracts from wound tissues and uninjured skin were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8–12%) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore Inc., Bedford, MA). Subsequently, the membranes were probed with primary antibodies against β-actin (1:5000, Kangchen Bio-tech Inc., Shanghai, China), iNOS (1:200), and Arg-1 (1:2500). The membranes were then incubated with a secondary antibody for one hour at room temperature. Proteins were then visualized with a BeyoECL plus kit (Beyotime, Nantong, China).

### Quantitative real-time polymerase chain reaction (PCR)

Total RNA extraction was performed with Trizol (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Single-stranded cDNA was synthesized according to the manufacturer's instructions (Promega, Madison, WI). Quantitative real-time PCR was performed with a Hot Start FQ-PCR Premix KIT (Transhold, Shanghai, China) through an Applied Biosystems 7300 Real Time PCR system (Applied Biosystems, Foster City, CA). Sequences of interest were amplified using the primers shown in Table 1 (Invitrogen).

**Table 1.** Primers used in polymerase chain reaction

| Primer | Sense primer (5′-3′) | Antisense primer (5′-3′) |
|---|---|---|
| iNOS | CCCTAAGAGTCACAAGCATC | AGGGTGTCGTGAAAAATCTC |
| Arg-1 | AGCAGAGACCCAGAAGAATG | TTTCCTTTCAGTTCCTTCAG |
| IFN-γ | AAAAGGACGGTAACACG | AGGTGCGATTCGATGAC |
| TNF-α | GACAAGGCTGCCCCGACTAT | GGGAGACTCCTCCCAGGTACA |
| IL-4 | TCGCTTGCCTTGGTGGTC | TGTGATGTTGCTCAGCTCCTC |
| IL-13 | TGCAACAGCAGCATGGTATGGAGCG | CGATTTTGGTATCTGGGGGGCTGGA |
| GAPDH | CCCCAATGTATCCGTTGTG | CTCAGTGTAGCCCAGGATGC |

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN-γ, interferon-gamma; IL, interleukin; TGF-α, transforming growth factor α.

### Enzyme-linked immunosorbent assay (ELISA)

The protein extract was applied to ELISA. IL-10 and IL-12 protein levels were measured with commercial ELISA kits (Senxiong Biotech, Shanghai, China). All ELISA kits were used according to the instruction manuals, and the color intensity was measured at 492 nm (IL-12) or 450 nm (IL-10).

### Statistical analysis

All data were expressed as mean ± SD and were analyzed with SPSS for Windows 11.0 (SPSS, Chicago, IL). Statistical significance was evaluated by *t* test, and the level of statistical significance was set at $p < 0.05$.

## RESULTS

### Body weights and blood glucose levels confirm the diabetic state

A significant elevation in blood glucose and a decrease in body weight were observed in the diabetic rats 8 weeks after induction. Blood glucose levels in the diabetic rats used in the present study were consistently higher than 16.7 mmol/L and were dramatically higher compared with the levels in the normal rats (27.7 ± 4.2 vs. 5.2 ± 0.8 mmol/L, $p < 0.01$). The loss in body weight observed in the diabetic rats compared with the normal rats was significant (307 ± 55 g vs. 397 ± 56 g, $p < 0.01$). In addition to the two main characteristics—elevated body glucose and weight loss, clinical manifestations of the streptozotocin-induced rats also verified diabetes mellitus.

### The H&E staining, wound healing rate, and variation of total CD+68 cells indicated the repair stage of the healing process

The H&E staining showed histological features of the wound edge at each observation point in both groups (Figure 1A and B). The images recorded on day 1 and 3 demonstrated increased infiltrating cells. Granulation tissue appeared on day 7, and the images on day 13 showed collagen deposition and reepithelialization.

The excisional wound healing rates in both groups are shown in Figure 1C. The healing curves of both groups demonstrated that there was a rapid acceleration of wound healing between days 3 and 7. However, unclosed wound percentage was higher in the diabetic group when compared with the normal group, especially on day 7. At the end of the observation, the wounds of the normal rats were nearly completely closed, whereas the wounds of the diabetic rats were still healing.

Macrophage infiltration into the wound area was assessed by immunohistochemical staining for CD68. The number of CD68+ cells in both groups increased significantly on day 1 and peaked on day 3, then decreased and began to return on day 13. However, a statistically significant difference in the number of CD68+ cells was observed between the groups on days 3, 7, and 13 (Figure 1D). The quantity of CD68+ cells was higher on day 3 in the normal group, but lower at the later observation points.

In summary, these data indicate that days 1 and 3 are part of the inflammatory stage, and day 7 belongs in the proliferative stage.[12]

### The caM macrophages (CD68+/iNOS+) increased in the inflammation phase but were succeeded by aaM macrophages (CD68+/Arg-1+) in the normal wound healing process

To characterize the macrophages phenotypically during the wound healing process, we examined the normal wound sections for the expression of CD68/iNOS or CD68/Arg-1, which were recently described as reliable markers of caM and aaM.[13,14] Co-localization showed that macrophages from the wound tissue in the early inflammation phase stained positively for iNOS (Figure 2A), and that macrophages on day 7 postwounding stained positively for Arg-1 (Figure 2B). Resident macrophages from uninjured skin in the normal group did not contain iNOS or Arg-1. No labeling was seen in the control group when the primary antibodies were omitted from the second round of staining (Figure 2A and B).

To observe the appearance of the caM and aaM phases, macrophages that stained positive for iNOS or Arg-1 at 0, 1, 3, 7, and 13 days were counted. The ratio was calculated by dividing the number of iNOS- or Arg-1-positive macrophages per field by the number of macrophages in the same field. The iNOS-positive macrophages appeared mainly on days 1 and

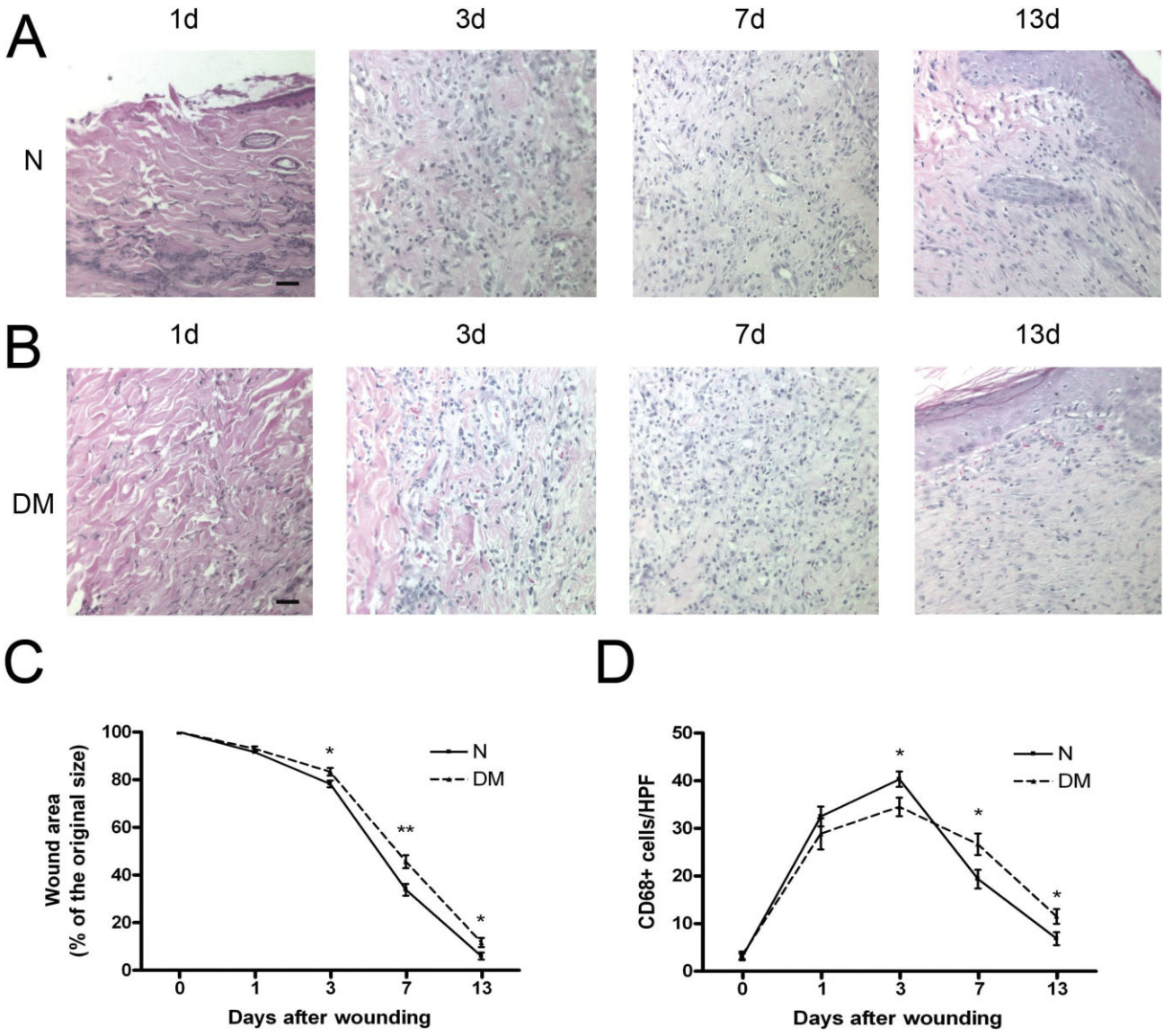


**Figure 1.** The stage of the healing process involved in the observations. (A and B) The hematoxylin and eosin (H&E) staining showed infiltrating cells, granulation tissue, and collagen deposition on wound edges at different times in both groups. Each scale bar represents 30 μm. (C) The rate of wound healing in both groups is shown; wound closure was faster in the normal group. (D) Macrophage infiltration into the wound area was assessed by CD68 positive staining. The amount of CD68 positive cells increased and decreased more slowly in the diabetic group than in the normal group ($n = 6$, $*p < 0.05$, $**p < 0.01$).

3, accounting for 10 to 15% of all macrophages. The number of iNOS-positive macrophages declined sharply after day 3 and returned to baseline on day 13. However, the ratio of Arg-1-positive macrophages increased slowly and peaked on day 7, for about one fifth of all macrophages (Figure 2C). The number of these positive cells is shown in Figure 2D. Different expressions of iNOS and Arg-1 in wound macrophages at different healing stages suggest that these two activated macrophages occurred in succession and might undertake different tasks.

### Impaired iNOS expression with late increased Arg-1 level in the diabetic wound

To evaluate the activity levels of caM and aaM, we analyzed the timing of iNOS and Arg-1 expression during the wound healing process. All three NO synthase isoforms were expressed in skin and wound healing: the calcium-dependent neuronal and endothelial NO synthases produced low levels of NO, while the iNOS generated much higher levels of NO, in a calcium-independent manner.[15] Expression of iNOS in

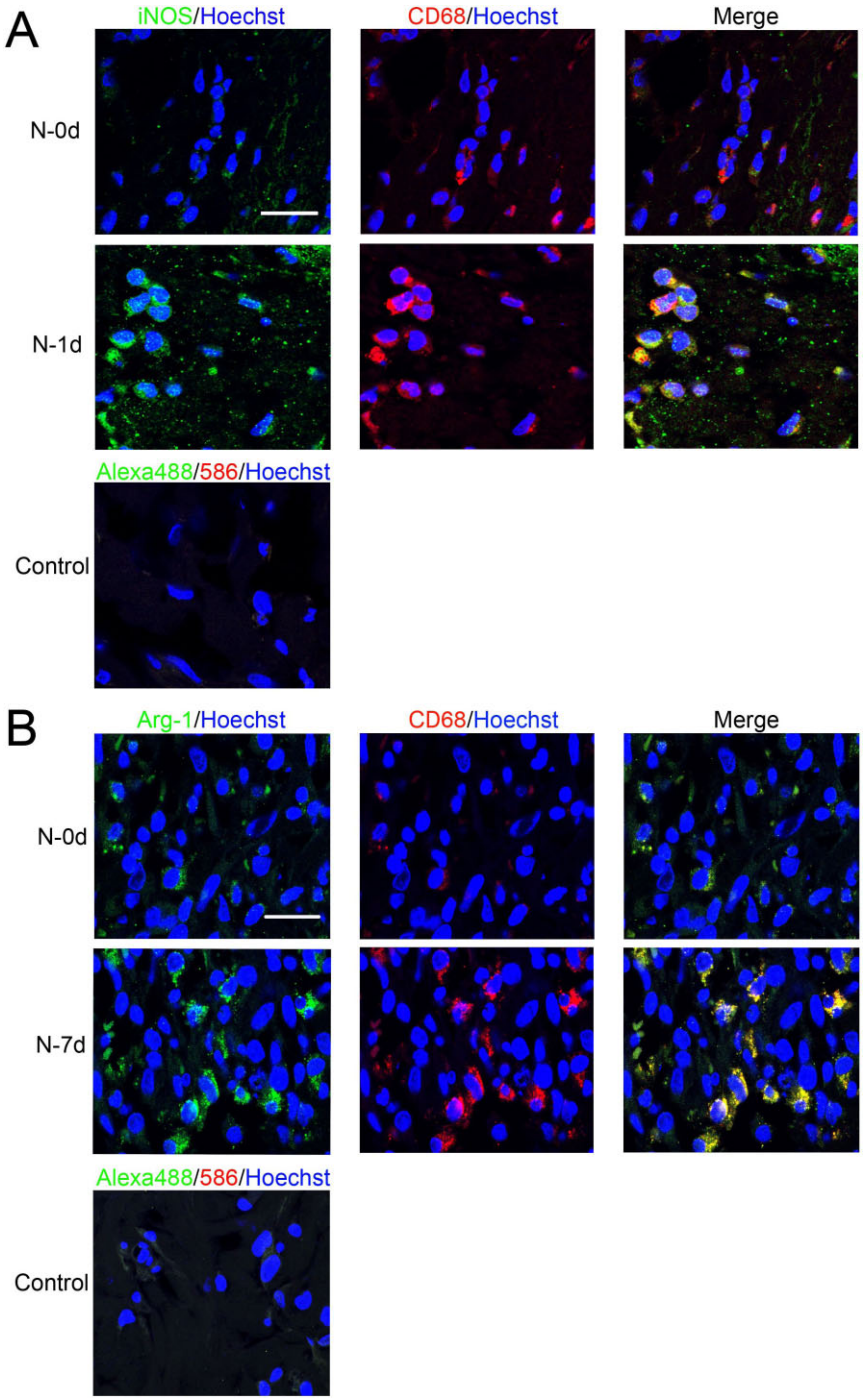


**Figure 2.** Macrophages in the normal wound (abbreviated by N) at different stages exhibit classical macrophage activation (caM) or alternative macrophage activation (aaM) states when fluorescently stained with iNOS or Arg-1. Macrophages were identified with iNOS or Arg-1 (column 1, green) and anti-CD68 (column 2, red). Marker colocalization is shown in column 3. (A) Wound macrophages on day 1 after wounding stained positive for iNOS. (B) Wound macrophages on day 7 after wounding stained positive for Arg-1. No labeling was seen in the negative control when the primary antibodies were omitted. (C) The ratios of macrophages in the normal wounds that stained positive for iNOS or Arg-1 at different times were calculated. The iNOS-positive macrophages appeared mainly on days 1 and 3 after wounding, but the ratio of Arg-1-positive macrophages peaked on day 7. Each scale bar represents 10 μm. (D) The number of CD68, iNOS, and Arg-1 positive cells is shown at each observation point ($n = 6$, $^{*}p < 0.05$, $^{**}p < 0.01$).

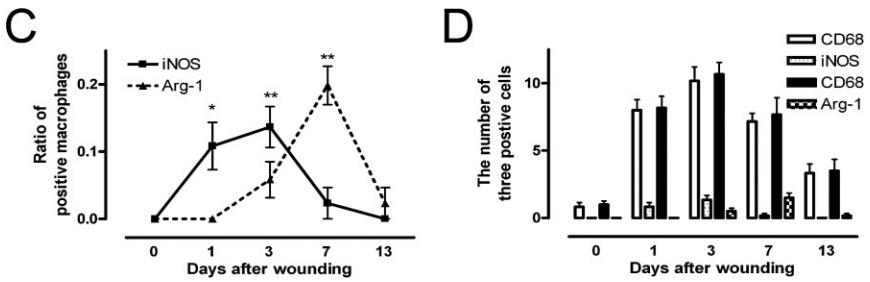


**Figure 2.** *Continued.*

healing wounds is restricted to macrophages present during the early phases of repair.[16] Accordingly, iNOS expression represents the degree of caM during wound healing.[17] Mammalian cells express two isoforms, Arg-1 and Arg-2. Arg-1 is expressed as a cytosolic enzyme, mostly in the inflammatory cells, except the liver.[18] Arg-2 is constitutively expressed, but Arg-1 expression in inflammatory cells is tightly regulated by exogenous stimuli, including IL-4 and IL-13.[8] Usually, it is assumed that Arg-1 protein is predominantly produced by macrophages following infection and wound healing.[19,20] Therefore, Arg-1 has been considered as one hallmark of aaM in tissue repair.[17,21]

The protein and mRNA levels of iNOS or Arg-1 in both groups were evaluated. Although skin lesion provoked a transient increase of iNOS in both groups, a reduction in iNOS level on days 1 and 3 was detected in the diabetic wounds, especially on day 3 (Figure 3A, B, and D). Expression of the Arg-1 protein and mRNA levels in the normal group increased slowly postwounding and peaked on day 7 to roughly sixfold and 50-fold, respectively, compared with the baseline levels. The Arg-1 protein and mRNA levels in the diabetic group were lower than those in the normal group on day 7. However, they did not decrease, as in the normal group, but increased sharply on day 13 to become higher than the Arg-1 level in the normal wounds on day 7 (Figure 3A, C, and E). These data suggest that the diabetic wounds displayed reduced iNOS expression and elevated Arg-1 levels in the later phase.

### The level of instructive cytokines of caM/aaM was correlated with iNOS/Arg-1 expression in normal and diabetic wounds

As IL-4 and IL-13, and IFN-γ and TNF-α induce aaM and caM, respectively, we examined the timing of the expression of these instructive cytokines in the normal and diabetic wounds with real-time PCR.

Overall, expression of the four instructive cytokines was higher in the normal group than in the diabetic group. This result may be related to the delayed inflammation response in the diabetic wounds. However, we should pay more attention to the relation between the instructive cytokines and the caM/aaM markers. The expression of IFN-γ and TNF-α mRNA in the normal group increased sharply after wounding, peaked on day 1, and remained at a fairly high level on day 3. On day 7, the Th1 cytokines began to decrease significantly, especially TNF-α (Figure 4A and B). The levels of IL-4 and IL-13 mRNA in the normal group also increased evidently after wounding and peaked on day 1, but they increased again on day 7 (Figure 4C and D). The fluctuations of these two cytokines were parallel to the expression of iNOS/Arg-1 at each observation point. Although all the cytokines simultaneously increased after wounding, at the beginning of the posttrauma period, the organism prefers inflammation cytokines to eliminate germs and slough rather than repair factors.

Similar correlation was also found in the diabetic wounds. Expression of the inducing cytokines of iNOS-IFN-γ and TNF-α, were lower in the diabetic wounds (Figure 4A and B). Although the expression of Th1/Th2 cytokines was generally lower on day 13 in the diabetic wounds than in the normal wounds, the level of Th1 cytokines declined more rapidly than Th2 cytokines. In other words, the expression of Th2 cytokines was higher than that of Th1 cytokines on day 13 in the diabetic group relatively (Figure 4C and D). These data indicate that the level of these instructive cytokines was correlated with the markers for caM/aaM expression in both groups.

### The expression of IL-10/IL-12 ratio in the wound did not resemble the alteration of caM/aaM in vitro

Because IL-10/IL-12 is an important indicator of macrophage activation in vitro, in these experiments, whether the IL-10/IL-12 expression ratio during wound healing reflected the transition from caM to aaM was examined.

After wounding, the normal group showed a higher IL-10/IL-12 ratio than the diabetic group overall. The ratio peaked on day 3 in the normal group and on day 1 in the diabetic group. However, neither group showed results similar to the in vitro results, in which the IL-10/IL-12 expression ratio was lower in the early stage but higher in the late stage (Figure 5A). The ratios of IL-10/IL-12 expression in both groups were elevated after wounding and decreased substantially after day 7.

## DISCUSSION

To determine the effect of diabetes mellitus on the immune state of macrophages in cutaneous wound healing, we first confirmed the existence of caM/aaM and calculated the positive rate of activated macrophage in vivo. To the authors' knowledge, the current study is the first to prove that caM and aaM are evident by double immunostaining in dorsal wound tissues of male Sprague-Dawley rats. This study demonstrated that diabetes mellitus affects the expression of macrophage activation markers such as iNOS and Arg-1 in skin

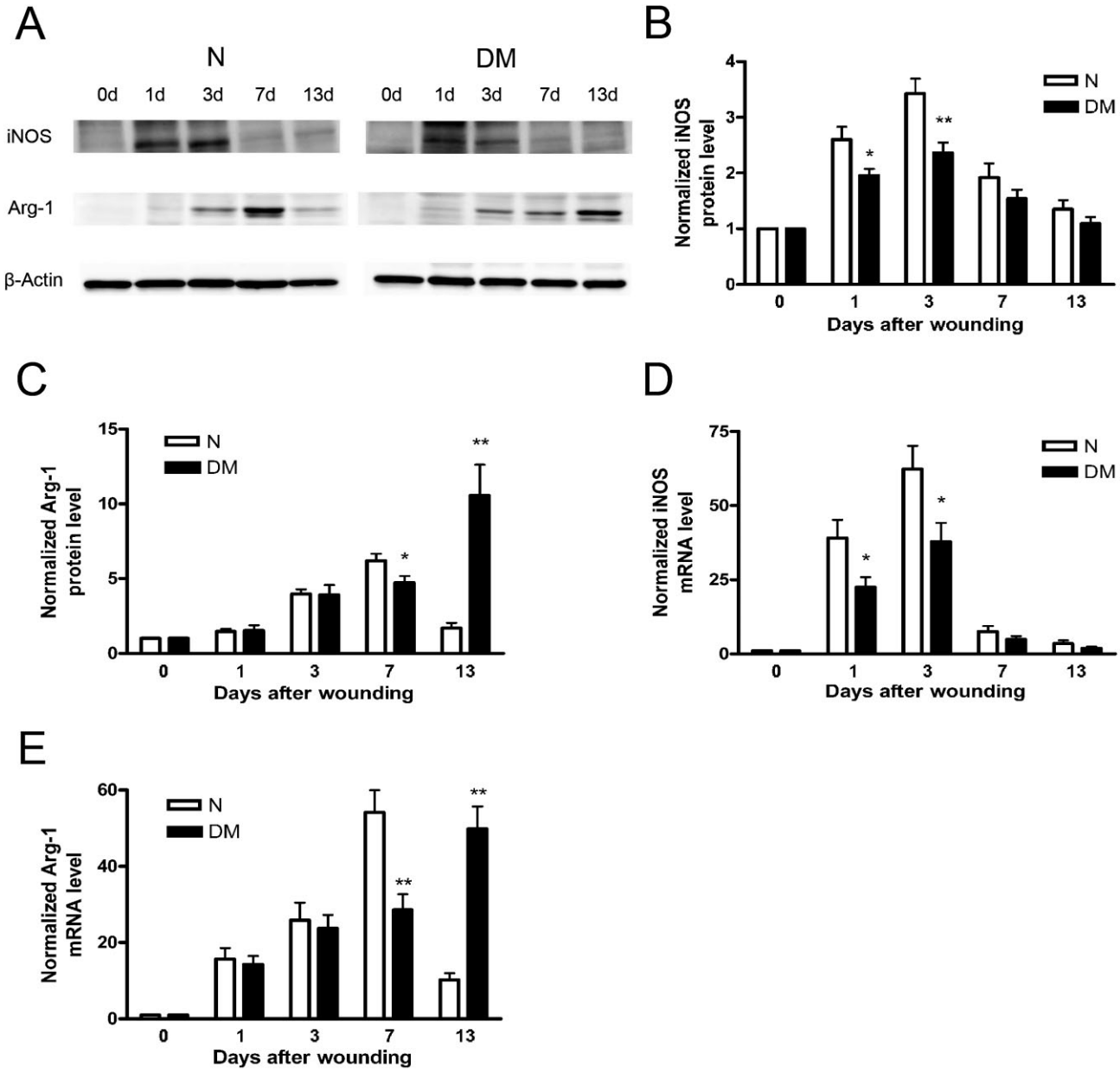


**Figure 3.** Reduced iNOS expression with late increased Arg-1 level in the diabetic wound. Western blot (A and B) and real-time polymerase chain reaction (PCR) (D) analysis showed a reduction in iNOS level on days 1 and 3 after wounding in the diabetic lesion, especially on day 3, compared with the normal group. Western blot (A and C) and real-time PCR (E) analysis showed that expression of Arg-1 in the diabetic group was lower than in the normal group on day 7. Expression of Arg-1 peaked on day 7 in the normal group, but it increased sharply on day 13 in the diabetic group ($n = 6$, $*p < 0.05$, $**p < 0.01$).

wounds. The study also demonstrated that these macrophage activation responses are mediated via instructive cytokines, which can be impacted by a hyperglycemic environment.

Increasing attention has focused on the issue of macrophage activation in vivo, especially in lung and wound tissues, and the characterization of macrophage activation has been clarified relatively well in vitro.[22–24] Recent studies on human wounds have demonstrated that in the early stage of wound repair, the expression of caM-related genes increased, but the aaM-related genes predominated later.[17,25] For the purpose of catching these two activated macrophages in rodent wound healing, we also set a time window to cover the inflammation and proliferative stages in our experiments. Consistent with the previous research on the appearance stage of activation-related genes in human wounds, we also found by immunofluorescence assay that caM macrophages appeared in the

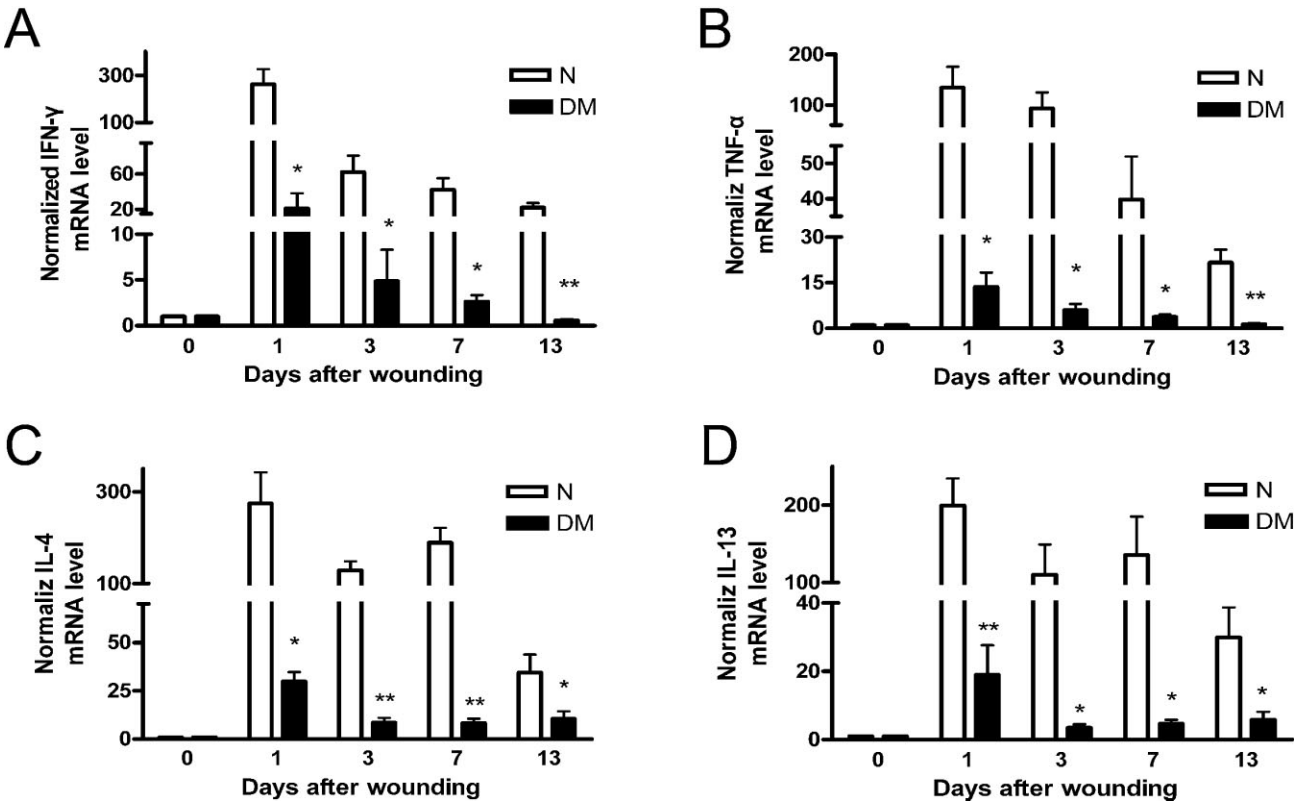


**Figure 4.** Plots of mRNA expression through real-time polymerase chain reaction (PCR) for Th1 and Th2 cytokines in normal and diabetic wounds. The expression of T helper cell type 1 (Th1)/Th2 cytokines was higher in the normal group than in the diabetic group. (A and B) The expression of interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) mRNA in the normal group peaked on day 1 after wounding and remained at a fairly high level on day 3. (C and D) The levels of interleukin (IL)-4 and IL-13 mRNA in the normal group also increased markedly after wounding and peaked on day 1, but increased again on day 7. On day 13, all the cytokines began to drop, but the level of Th1 cytokines declined more rapidly than the Th2 cytokines in the diabetic wounds ($n = 6$, $*p < 0.05$, $**p < 0.01$).

inflammation phase, but aaM macrophages increased in the proliferation stage in a normal rodent wound. Simultaneously, the measurements of caM/aaM markers by Western blot and real-time PCR also yielded similar results. This arrival sequence seems to conform to the request of wound tissue repair. After wounding, the first behaviors in a skin wound are hemostasis and inflammation, followed by cell proliferation, collagen deposition, and angiogenesis.[2] Conforming to the known functions from research in vitro, the tissue-destructive macrophages emerge first after wounding and mainly "burn something out," acting as a fire retardant. However, tissue-reparative macrophages tend to arrive slowly and act as a "builder." The complexity of the repair phase, which involves extracellular matrix formation, as well as multiple type cell infiltration, and takes more time than the inflammation stage, may explain the higher positive ratio of aaM to caM in this study. Inconsistent results were also reported in different species and wound types. The wound macrophages obtained from a PVA sponge inserted in the midline dorsal incision in mice did not express iNOS protein, and IL-4 and IL-13 were also not detected in the wound.[26] However, the sterile dermal incision often has been considered as the "simplest" wound type in the diverse field of tissue repair, due to its lower involvement of immune responses and regeneration processes.[7] In addition, dressings on the wound prevent the invasion of harmful bacteria into the wound and reduce the inflammation response.[27] Therefore, we chose open full-thickness skin defects in our studies.

It is also interesting to consider why the positive rate of activated macrophages did not exceed 20% of total macrophages activated in the wound healing. There may be two reasons accounting for these iNOS or Arg-1 negative macrophages postwounding. First, the major portion of macrophages at a wound site is recruited from capillaries rather than resident macrophages.[12] In this process, it takes time for circulating monocytes to enter the tissue space successively, transform into tissue macrophages, and display different phenotypes. In our experiments, the total number of CD68+ cells in both groups peaked on day 3 (Figure 1D), indicating that at this point there were still new monocytes being recruited from blood, which looked "naive" compared with the "furious" activated macrophages. Second, macrophages may be located in a niche that was exposed to the different microenvironment considering the complexity of wound healing in vivo. Thus,

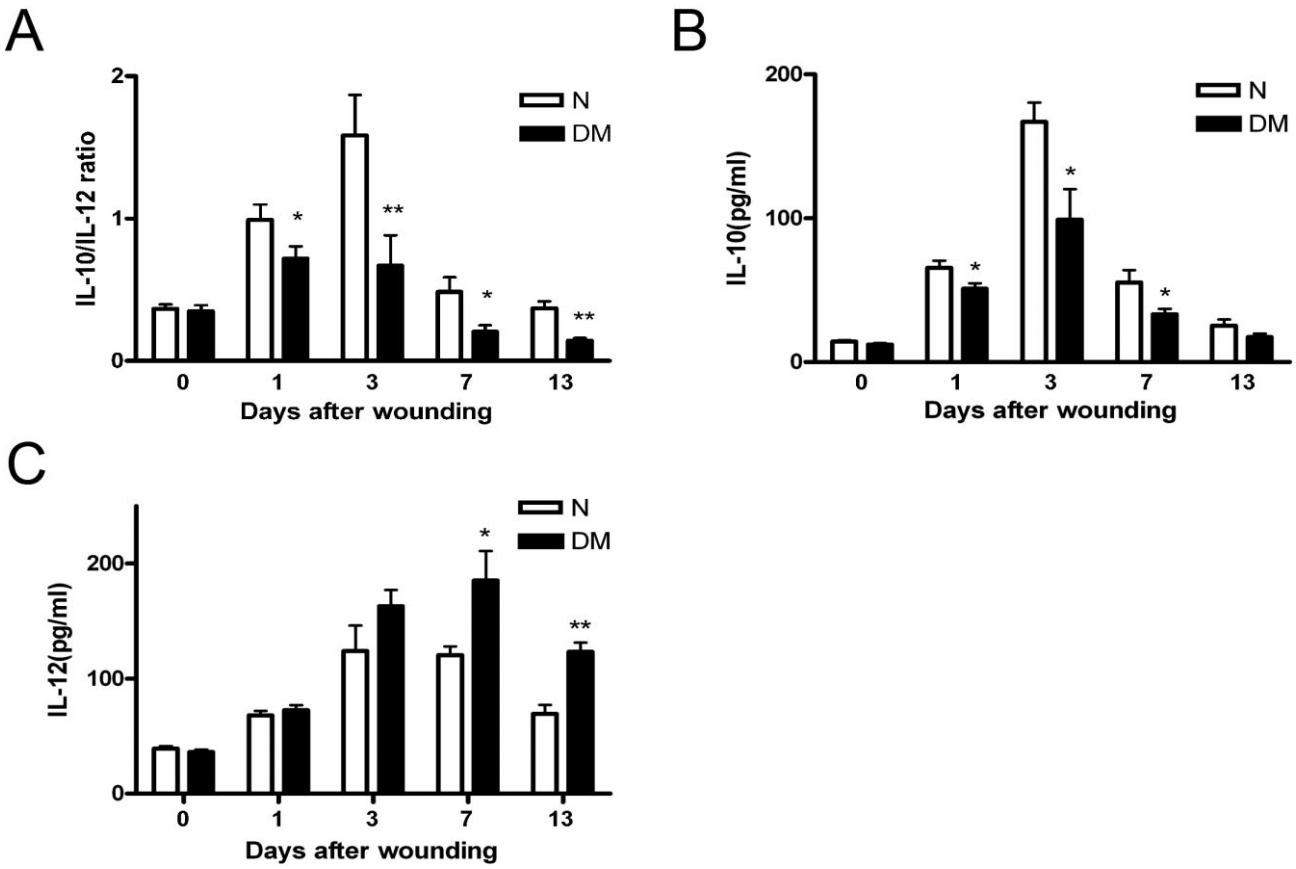


**Figure 5.** The expression of the interleukin (IL)-10/IL-12 ratio in both groups did not reflect the characteristics of classical macrophage activation (caM)/alternative macrophage activation (aaM) in vitro. Expression of the IL-10/IL-12 ratio in the wound extracts was higher in the normal group (A). Neither group showed the same results as the in vitro results, in which the IL-10/IL-12 expression ratio was lower in the early stage but higher in the late stage. (B and C) The data of IL-10 and IL-12 are also shown ($n = 6$, $*p < 0.05$ and $**p < 0.01$).

the organism may reserve a fair proportion of the total tissue macrophages, waiting for possible further requirements for wound healing. However, we have not found any clear evidence to support this conjecture.

Our current study delineated the induction of caM/aaM through Th1/Th2 cytokines in rodent skin wounds. Similar consistent results were also reported in the expression of macrophage activation-related inducing factors in mucosal wound repair of mice colons.[17] The balance of Th1/Th2 determines the outcome of a wide variety of immune responses involving infectious, allergic diseases and wound healing.[2,28,29] In addition, the defined relationship between Th1/2 cytokines and macrophage activation phenotypes in vitro was confirmed not only in normal wounds, but also in diabetic wounds.[30] However, we noticed a discrepancy in that the Arg-1 expression in the diabetic group showed a dramatic increase on day 13, but Th2 cytokines were low. As is known, iNOS/Arg-1 are reciprocally regulated by Th1/Th2 cytokines in vivo.[19] Although Th2 cytokines decreased sharply on day 13 in the diabetic group, they decreased more slowly than Th1 cytokines, which may have led to Th2 cytokines predominating. Furthermore, the reduced iNOS expression in the first 3 days prognosticated a relatively high expression of Arg-1 on subsequent days. Considering the repair ability of Arg-1 positive macrophages, the abnormally increased Arg-1 level could be a compensatory reaction to the impaired diabetic wound healing. This may also be consistent with our conjecture, previously stated, that the wound will keep parts of macrophages as a "military reserve" for possible future healing needs.

The iNOS/Arg-1 in activated macrophages regulated by Th1/Th2 cytokines is considered as an important pathway in the regulation of inflammation and tissue repair.[19,30] Our data from the normal wounds demonstrated that Th1/Th2-caM/aaM-iNOS/Arg-1 is regulated moderately and in a timely manner by the immune system. However, during the balance of the inflammation process in the streptozotocin-induced diabetic wounds, Th2-aaM-Arg-1 increased. Moreover, abnormal caM may cause problems as well. Unrestrained proinflammatory caM induced by iron and too many TNF-$\alpha$ positive macrophages, which are considered as caM cells, impairs wound healing in humans and mice.[24,31] An imbalance of caM/aaM in wound healing seems to impede skin defect restoration. It may be that one of the requirements for

successful wound healing is that macrophages must be activated in a suitable phase and extent.

However, not all the related cytokines in wounds fluctuate in the same manner as they do in vitro, such as IL-10 and IL-12. We have not found the accurate relation between IL-10/IL-12 and iNOS/Arg-1 in both groups, which seems that the trend of both cytokines may be inconsistent with the story presented that the caM and aaM predominate orderly and alter in the wound healing. Besides macrophages, the data of IL-10 and IL-12 could be affected by multiple types of infiltrating leukocyte subtypes and resident cells, such as T lymphocytes, B lymphocytes, dendritic cells, and keratinocytes, after wounding.[32,33] The ratio of IL-10/IL-12 expression in both groups demonstrated that macrophage activation appears to be more complex in vivo. Furthermore, we suppose that the IL-12, IL-10, and IL-10/IL-12 ratio maybe were not good markers to assess macrophage activation in vivo, at least they could not match the assessment quality as in vitro.

In conclusion, our findings provide new insights into the effects of diabetes mellitus on macrophage activation and should help clarify the functional importance of the macrophage state within wound healing. However, additional investigations of the mechanisms by which diabetes mellitus regulates Th1/Th2 cytokines and the utility of therapies that alter macrophage activation in the treatment of impaired wound healing are warranted.

## ACKNOWLEDGMENTS

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## REFERENCES

1. Broughton G II, Janis JE, Attinger CE. The basic science of wound healing. *Plast Reconstr Surg* 2006; 117: 12S–34S.
2. Park JE, Barbul A. Understanding the role of immune regulation in wound healing. *Am J Surg* 2004; 187: 11S–16S.
3. Margolis DJ, Allen-Taylor L, Hoffstad O, Berlin JA. Diabetic neuropathic foot ulcers and amputation. *Wound Rep Regen* 2005; 13: 230–6.
4. Fu X, Sheng Z, Cherry GW, Li Q. Epidemiological study of chronic dermal ulcers in China. *Wound Rep Regen* 1998; 6: 21–7.
5. Jiang Y, Huang S, Fu X, Liu H, Ran X, Lu S, Hu D, Li Q, Zhang H, Li Y, Wang R, Xie T, Cheng B, Wang L, Liu Y, Ye X, Han C, Chen H. Epidemiology of chronic cutaneous wounds in China. *Wound Repair Regen* 2011; 19: 181–8.
6. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* 2005; 5: 953–64.
7. Stout RD. Editorial: macrophage functional phenotypes: no alternatives in dermal wound healing? *J Leukoc Biol* 2010; 87: 19–21.
8. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol* 2003; 3: 23–35.
9. Schebesch C, Kodelja V, Muller C, Hakij N, Bisson S, Orfanos CE, Goerdt S. Alternatively activated macrophages actively inhibit proliferation of peripheral blood lymphocytes and CD4+ T cells in vitro. *Immunology* 1997; 92: 478–86.
10. Gary Sibbald R, Woo KY. The biology of chronic foot ulcers in persons with diabetes. *Diabetes Metab Res Rev* 2008; 24: S25–30.
11. Chen WY, Rogers AA. Recent insights into the causes of chronic leg ulceration in venous diseases and implications on other types of chronic wounds. *Wound Repair Regen* 2007; 15: 434–49.
12. Li J, Chen J, Kirsner R. Pathophysiology of acute wound healing. *Clin Dermatol* 2007; 25: 9–18.
13. Katakura T, Miyazaki M, Kobayashi M, Herndon DN, Suzuki F. CCL17 and IL-10 as effectors that enable alternatively activated macrophages to inhibit the generation of classically activated macrophages. *J Immunol* 2004; 172: 1407–13.
14. Misson P, van den Brule S, Barbarin V, Lison D, Huaux F. Markers of macrophage differentiation in experimental silicosis. *J Leukoc Biol* 2004; 76: 926–32.
15. Weller R. Nitric oxide: a key mediator in cutaneous physiology. *Clin Exp Dermatol* 2003; 28: 511–14.
16. Reichner JS, Meszaros AJ, Louis CA, Henry WL Jr, Mastrofrancesco B, Martin BA, Albina JE. Molecular and metabolic evidence for the restricted expression of inducible nitric oxide synthase in healing wounds. *Am J Pathol* 1999; 154: 1097–104.
17. Seno H, Miyoshi H, Brown SL, Geske MJ, Colonna M, Stappenbeck TS. Efficient colonic mucosal wound repair requires Trem2 signaling. *Proc Natl Acad Sci U S A* 2009; 106: 256–61.
18. Pesce JT, Ramalingam TR, Mentink-Kane MM, Wilson MS, El Kasmi KC, Smith AM, Thompson RW, Cheever AW, Murray PJ, Wynn TA. Arginase-1-expressing macrophages suppress Th2 cytokine-driven inflammation and fibrosis. *PLoS Pathog* 2009; 5: e1000371.
19. Hesse M, Modolell M, La Flamme AC, Schito M, Fuentes JM, Cheever AW, Pearce EJ, Wynn TA. Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism. *J Immunol* 2001; 167: 6533–44.
20. Fairweather D, Cihakova D. Alternatively activated macrophages in infection and autoimmunity. *J Autoimmun* 2009; 33: 222–30.
21. Zhang QZ, Su WR, Shi SH, Wilder-Smith P, Xiang AP, Wong A, Nguyen AL, Kwon CW, Le AD. Human gingiva-derived mesenchymal stem cells elicit polarization of m2 macrophages and enhance cutaneous wound healing. *Stem Cells* 2010; 28: 1856–68.
22. Christmann RB, Hayes E, Pendergrass S, Padilla C, Farina G, Affandi AJ, Whitfield ML, Farber HW, Lafyatis R. Interferon and alternative activation of macrophage/monocytes in systemic sclerosis-associated pulmonary arterial hypertension. *Arthritis Rheum* 2011; 63: 1718–28.
23. Sun L, Louie MC, Vannella KM, Wilke CA, LeVine AM, Moore BB, Shanley TP. New concepts of IL-10-induced lung fibrosis: fibrocyte recruitment and M2 activation in a CCL2/CCR2 axis. *Am J Physiol Lung Cell Mol Physiol* 2011; 300: L341–53.
24. Sindrilaru A, Peters T, Wieschalka S, Baican C, Baican A, Peter H, Hainzl A, Schatz S, Qi Y, Schlecht A, Weiss JM, Wlaschek M, Sunderkötter C, Scharffetter-Kochanek K. An unrestrained proinflammatory M1 macrophage population induced by iron impairs wound healing in humans and mice. *J Clin Invest* 2011; 121: 985–97.

25. Deonarine K, Panelli MC, Stashower ME, Jin P, Smith K, Slade HB, Norwood C, Wang E, Marincola FM, Stroncek DF. Gene expression profiling of cutaneous wound healing. *J Transl Med* 2007; 5: 11.
26. Daley JM, Brancato SK, Thomay AA, Reichner JS, Albina JE. The phenotype of murine wound macrophages. *J Leukoc Biol* 2010; 87: 59–67.
27. Boateng JS, Matthews KH, Stevens HN, Eccleston GM. Wound healing dressings and drug delivery systems: a review. *J Pharm Sci* 2008; 97: 2892–923.
28. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. *Nature* 1996; 383: 787–93.
29. Shi Z, Wakil AE, Rockey DC. Strain-specific differences in mouse hepatic wound healing are mediated by divergent T helper cytokine responses. *Proc Natl Acad Sci U S A* 1997; 94: 10663–8.
30. Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM. M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol* 2000; 164: 6166–73.
31. Goren I, Müller E, Schiefelbein D, Christen U, Pfeilschifter J, Mühl H, Frank S. Systemic anti-TNF alpha treatment restores diabetes-impaired skin repair in ob/ob mice by inactivation of macrophages. *J Invest Dermatol* 2007; 127: 2259–67.
32. Moore KW, O'Garra A, de Waal Malefyt R, Vieira P, Mosmann TR. Interleukin-10. *Annu Rev Immunol* 1993; 11: 165–90.
33. Watford WT, Moriguchi M, Morinobu A, O'Shea JJ. The biology of IL-12: coordinating innate and adaptive immune responses. *Cytokine Growth Factor Rev* 2003; 14: 361–8.