Current Medical Science
DOI https://doi.org/10.1007/s11596-019-2128-x
39(6):954-964,2019

Knockout of MicroRNA-155 Ameliorates the Th17/Th9 Immune Response and Promotes Wound Healing*

Chen-rong WANG[1†], Hong-fei ZHU[2, 4†], Yong ZHU[3, 4†]
[1]*Department of Otolaryngology, the First Hospital of Wuhan, Wuhan 430022, China*
[2]*Department of Anesthesiology, Hubei Provincial Hospital of Traditional Chinese Medicine, Wuhan 430061, China*
[3]*Department of General Surgery, Hubei Provincial Hospital,of Traditional Chinese Medicine, Wuhan 430061, China*
[4]*Hubei Province Academy of Traditional Chinese Medicine, Wuhan 430074, China*

Summary: MiRNAs are integral for maintaining immune homeostasis and self-tolerance. In this study, qPCR analyses were performed to determine which miRNAs play an important role in wound healing. Next, an experiment in a model of wound healing was performed, and histology, mRNA expression and T-cell subpopulations in wound tissue were analyzed. The accelerated experiments were performed by local injection of either rIL-17A and/or rIL-9 after wound healing. *In vitro*, the differentiation of Th17/Th9 in miR-155$^{+/+}$ or miR-155$^{-/-}$ mice was investigated, and the target genes of miR155 were analyzed. From our findings, miR-155$^{-/-}$ in mice promoted wound healing and weakened T cell-mediated inflammation, especially in IL-17/IL-9, and less severe skin fibrosis developed in the mice. rIL-17A and/or rIL-9 could exacerbate inflammatory injury and delay wound healing. We also demonstrated that miR-155$^{-/-}$ led to a defect in the differentiation of Th17/Th9 *in vitro*, and this effect of IL-17/IL-9 might be related to the expression of C-maf, which is a target gene of miR155. MiR-155 regulated IL-17/IL-9-related inflammation in wound healing and might be a potential therapeutic target to attenuate the inflammatory response in wound tissue and promote the closure of wound injuries.
Key words: wound healing; T cells; miR155; C-maf; IL-17A; IL-9

Wound healing is a complicated process that involves four key overlapping stages, including haemostasis and inflammation, proliferation, and scar formation/remodelling phases. Immune cells can impact the repair processes, and excessive inflammation delays healing and may lead to complications and chronic wounds[1]. Immune cells serve as an important source of a variety of proinflammatory mediators and cytokines that can promote inflammation, specifically leukocytic infiltration in the wound bed, especially during the time of granulation tissue formation[2, 3]. Thus, having a better understanding of the mechanisms underlying the involvement of inflammatory cells in the progression of wound healing will lead to decreased treatment costs while increasing patients' quality of life.

MicroRNAs (miRNAs) represent a novel group of RNA molecules that are approximately 22 nucleotides in length and post-transcriptionally regulate gene expression, resulting in either translational repression or mRNA degradation. Currently, more than 800 miRNAs are believed to exist in the mammalian genome and may regulate as many as one-third of all mRNAs, playing important roles in diverse physiological and pathological processes, including cellular immunity, cell proliferation and differentiation and cancer development. However, the roles of miRNAs in wound healing are still unclear because emerging studies are still addressing the relationship between miRNAs and wound healing[4–8]. MiR-155 has been one of the miRNAs particularly associated with inflammatory and immune responses[9]. Previous studies have demonstrated that knockout of miR-155 reduces the incidence of various inflammatory diseases, including rheumatoid arthritis and systemic lupus erythematosus[10–12]. In addition, the elevated expression of miR-155 in macrophages within atherosclerotic lesions has been demonstrated to be partially responsible for the negative outcome[13].

Most evidence suggests that IL-17 regulates local tissue inflammation and autoimmune diseases, primarily via the induced release of proinflammatory and neutrophil-mobilizing cytokines[14]. In psoriasis, which is a chronic inflammatory skin disease, IL-17

Chen-rong WANG, E-mail:117248994@qq.com; Hong-fei ZHU, E-mail: zhf73@126.com; Yong ZHU, E-mail: 27303356@qq.com
†The authors contributed equally to this work.
*This project was supported by the Natural Science Foundation of Hubei Province (No. 2018CFC819) and Health Commission of Hubei Province Scientific Research Project (No. WJ2019F163 and No. WJ2019F165).

has the potential to affect skin biology[15]. Recently, Rodero *et al* showed that that both IL-17A inhibition in a genetic model and IL-17A neutralizing antibodies can accelerate wound healing[16]. IL-9 is a T cell-derived factor, and it has been reported to be capable of inducing tissue inflammation in a colitis model[17]. Constitutive expression of IL-9 in lung epithelial cells resulted in an asthma-like phenotype consisting of lymphocytic and eosinophilic lung inflammation[18]. However, to the best of our knowledge, whether IL-9 is involved in wound healing has not been investigated.

The aim of this study was to elucidate the inflammatory role of miR155 in skin wound healing. Because miR-155 has emerged as a central regulator of the immune system[19], we thought that knockout of miR-155 could improve wound healing through reducing inflammatory response.

1 MATERIALS AND METHODS

1.1 Skin Wound Mouse Model

Adult male C57BL/6 (H-2b) mice were purchased from HFK Bioscience Co., Ltd. (China). B6.Cg-miR155$^{tm1.1Rsky/J}$ (miR155$^{-/-}$) mice were purchased from the Jackson Laboratory. Six- to eight-week-old male mice (18–25 g) were used for the experiments. Mice were anesthetized with 1% pentobarbital (30 mg/kg), and the hair on their backs was shaved. By punch biopsy, two full-thickness skin of 6 mm in the middle of the back was aseptically obtained. The animals were housed in a specific pathogen-free facility at Tongji Medical College (China). All animal experiments were approved by the Animal Care and Use Committee of Tongji Medical College (China).

1.2 Histology and Immunohistochemistry

Tissue from wounded areas was excised and was embedded in paraffin for haematoxylin and eosin (H&E) staining or immunohistochemistry. Wound morphology, inflammatory cell infiltrate, and vessel density were assessed in a blinded fashion, and images were captured using an OLYMPUS BX5 l microscope and were analyzed using IMAGE-PRO PLUS 6.0 software (Media Cybernetics, Inc., USA). Wound closure was evaluated by measuring total wound area and distance between follicles. Total number of neutrophils (Gr-1-positive) and T cells (CD3-positive) was determined using three randomly assigned wound images per wound. The mean number of three images was used to obtain the mean value per wound/animal.

1.3 Quantitative Real-Time PCR

Total RNA was extracted from the wound tissue using TRIzol, according to the manufacturer's instructions (Invitrogen, USA). The cDNA from the adult mRNA transcripts was amplified using PrimeScript RT Master Mix (Takara, Japan). The PCR mixture was prepared using SYBR Premix EX Taq (Takara, Japan). The results were normalized to GAPDH or U6 using the ΔΔCt method. The primers for miR155, miR142, miR196, IFNγ, IL-4, IL-17A, IL-9, Foxp3, CCL2, CXCL1, CXCL2, c-Maf, SOCS1, SOCS3, Smad2, Smad5 and GAPDH were used for quantitative real-time PCR as described in table 1.

1.4 Treatment of Wounds with Recombinant IL-17/IL-9 or CD4+ Cells

Recombinant IL-17/IL-9 (PeproTech, London, UK) was used to disturb focal cytokine expression, and the MACS CD4+ cells were used to disturb focal inflammatory cells. After the skin was wounded, recombinant IL-17/IL-9 (5 μg dissolved in 100 μL PBS) or CD4+ cells (10^6) were directly injected into the surrounding dermis of the wound at four sites on day 0, while the control wounds received equal amount of PBS. The process of wound healing was digitally photographed at an indicated time.

1.5 Murine T-cell Differentiation

The CD4+ T cells from spleens and lymph nodes were purified via magnetic selection (clone L3T4) according to the manufacturer's instructions (Miltenyi Biotec); next, they were cultured in IMDM supplemented with 15% foetal bovine serum (FBS), penicillin/streptomycin and 2-ME and stimulated with plate-bound anti-CD3 monoclonal antibody (mAb) (5 μg/mL; BD Biosciences) and soluble anti-CD28 mAb (2 μg/mL; BD Biosciences) in the presence of recombinant cytokines. Th17 cell polarization was performed in the presence of human TGF-β (2.5 ng/mL; PeproTech), IL-6 (50 ng/mL; PeproTech) and anti-IFNγ (10 μg/mL; eBioscience) and anti-IL-4 (10 μg/mL; eBioscience) antibodies. For the Th9 cells, human TGF-β (2 ng/mL; PeproTech) plus mouse IL-4 (20 ng/mL; PeproTech) were added. After 3 days, the Th17 cell cultures were expanded for 48 h, and fresh complete IMDM containing homologous recombinant cytokines and additional IL-23 (50 ng/mL; PeproTech) was added. Following 5 days and 3 days of culture, respectively, the Th17 and Th9 cells were collected for flow cytometric analysis.

1.6 Flow Cytometric Analysis

For intracellular flow cytometric staining, Th17/Th9 cells were stimulated with PMA (phorbol 12-myristate13-acetate; 50 ng/mL; Enzolife sciences), ionomycin (1 μg/mL) and BFA (Brefeldin A; 10 μg/mL; Biolegend) for 6 h at 37°C. The cells were fixed and permeabilized overnight at 4°C using Fix/Perm buffer (eBioscience) before being stained with the following fluorochrome-conjugated antibodies according to the manufacturer's protocol: FITC-anti-CD4 (Gk1.5; BD Biosciences), PE-Cy7-anti-IL-17A (eBio17B7; eBioscience). The graft-infiltrating cells were isolated as previously described. Total infiltration cell counts were undertaken via microscopy and were

Table 1 Primers for qPCR detection

Gene		5′–3′	Species
miR155	Sense	TGTGATAGGGGTTTTGGCCT	Mouse
	Anti	ACAGGTAGGAGTCAGTCAGAGG	Mouse
miR142	Sense	GTATGGATCCTCTTAGGAAGCCACAAGGAG	Mouse
	Anti	TATCAAGCTTTAAGGTGCTCACCTGTCACA	Mouse
miR196	Sense	GCCTGGCTTTCTGAACACAAC	Mouse
	Anti	TGCCATGAATCAGGTGGTTTGA	Mouse
IFNγ	Forward	AAGACTGTGATTGCGGGGTT	Mouse
	Reverse	ATCTGAGTTCAGTCAGCCGC	Mouse
IL-4	Forward	CCATATCCACGGATGCGACA	Mouse
	Reverse	AAGCACCTTGGAAGCCCTAC	Mouse
IL-17A	Forward	GCTGACCCCTAAGAAACCCC	Mouse
	Reverse	GAAGCAGTTTGGGACCCCTT	Mouse
IL-9	Forward	TGAGTTCCAGACTCCCGTCA	Mouse
	Reverse	AACAGTCCCTCCCTGTAGCA	Mouse
Foxp3	Forward	TAGAGAAGACAGACCCATGCTG	Mouse
	Reverse	CAGAGGCAGGCTGGATAACG	Mouse
CCL2	Forward	TGCCCTAAGGTCTTCAGCAC	Mouse
	Reverse	AAGGCATCACAGTCCGAGTC	Mouse
CXCL1	Forward	ACTCAAGAATGGTCGCGAGG	Mouse
	Reverse	GTGCCATCAGAGCAGTCTGT	Mouse
CXCL2	Forward	AGGGCGGTCAAAAAGTTTGC	Mouse
	Reverse	CGAGGCACATCAGGTACGAT	Mouse
c-Maf	Forward	GCAGGTAGACCACCTCAAGC	Mouse
	Reverse	GTTTTCTCGGAAGCCGTTGC	Mouse
SOCS1	Forward	CTCCTTGGGGTCTGTTGGC	Mouse
	Reverse	GCGTGCTACCATCCTACTCG	Mouse
SOCS3	Forward	GCAGTTCCAGGAATCGGGG	Mouse
	Reverse	GCAGGCGAGTGTAGAGTCAG	Mouse
Smad2	Forward	AATCATTGCAACAAGAGGCAGT	Mouse
	Reverse	ATTCCCGTCCCCATCATCCT	Mouse
Smad5	Forward	CCACGCGGGACCTGACG	Mouse
	Reverse	CGGATCCTTATCTCCCAGACACA	Mouse
GAPDH	Forward	CTCATGACCACAGTCCATGC	Mouse
	Reverse	CACATTGGGGGTAGGAACAC	Mouse

stained with FITC-anti-CD45, APC-anti-CD3, APC-Cy7-anti-CD11b and PE-Cy7-anti-Ly6G antibodies (all from eBioscience, USA). All of the samples were subsequently assessed via flow cytometry using an LSR II (BD Biosciences, San Diego, CA) and were analysed using FlowJo software (Tree Star Inc., Ashland, OR).

1.7 RNA Interference

To knock down the expression of c-Maf, CD4+T cells were purified and nucleoporated with small interfering RNA (siRNA) specific for c-Maf (Santa Cruz Biotechnology, USA). Chemically modified siRNA with FAM maker was synthesized as follows[20] (where mC, mU, mA, mT or mG indicates a methoxy nucleotide, and dG, dA, dT or dC indicates a deoxy nucleotide): siRNA specific for cMaf, 5′-mCmUGAU GAAGUUUGAAGUdGdAdAmAmAmA-3′ (sense) or 5′-dTUUCACUUCAAACUUCAUCmAmG-3′(antisense); and control siRNA, 5′-GmGmAGCGCACCAUCUU-CdTdCdAmAmTmT-3′ (sense) or 5′-dTUGAGAAGA-UGGUGCGCUmCmC-3′ (antisense). Cells were transfected using a Gene Pulser Xcell™ Electroporation Systems (BIO-RAD, USA). Briefly, CD4+ T cells were stimulated with plate-bound anti-CD3 mAb and soluble anti-CD28 mAb for 24 h. After they were stimulated, 7×10^6 CD4+ T cells were resuspended in 700 μL Nucleofector solution and were transfected with 20 nmol/L siRNA in 0.4-cm cuvettes at 960 microfarads and 400 V using a Gene Pulser Xcell™ Electroporation System. The nucleofector buffer was composed of KCl (5 mmol/L), $MgCl_2$ (15 mmol/L), HEPES (15 mmol/L), Na_2HPO_4/NaH_2PO_4 (150 mmol/L; pH7.2), and sodium succinate (50 mmol/L)[21]. Cells in the best conditions were incubated for 10 min at 0°C. After transfection, the cells were incubated for 24 h at 37°C, followed by polarization under Th9 or Th17 conditions.

1.8 Western Blotting Analysis

Cells were lysed with RIPA buffer (Thermo Scientific, USA) containing a protease inhibitor mixture (Sigma-Aldrich, USA). Western blotting analyses were performed as previously described[22]. β-actin (Santa Cruz, sc-1616r) and c-Maf (Abcam, ab76817) antibodies were used as primary antibodies. Secondary

antibodies were HRP-labelled goat anti-rabbit IgG or rabbit anti-goat IgG (Biosharp). Protein levels were normalized to β-actin. All films were scanned and analyzed using Alpha software.

1.9 Statistical Analysis

All data were analyzed using Prism software 5.0 (GraphPad Software, USA). The data were considered to be statistically significant if $P<0.05$. Differences between two individual experimental groups were determined using the unpaired Student's *t*-test.

2 RESULTS

2.1 Abundant Expression of MiR-155 in Wound Healing

In the process of wound healing, the proliferative phase is a critical stage in which inflammatory responses are related to scar formation and remodelling. To underline the role of miRNAs in wound healing, we analyzed the expression of inflammatory-related miRNAs. We found that the expression patterns of miR-142 and miR-196, known to be involved in the inflammatory response, were not significantly altered in wound tissue on day 1 and 7 compared to expression patterns in healthy skin. However, miR-155 was also reported to be closely related to the regulation of inflammatory responses, and increased miR-155 might be involved in wound healing. Compared with healthy skin, miR-155 levels in wound tissues produced 1.740-, 11.263- and 3.777-fold increases on days 1, 3 and 7 after wound injury (fig. 1). We therefore hypothesized that miR-155 is involved in the overall modulation of wound healing.

2.2 MiR-155$^{-/-}$ Mice Had Decreased Inflammatory Cells

To determine the role of miR-155 in wound healing, miR-155$^{-/-}$ mice were used to establish a mouse model of wound healing. The quantification of total wound area demonstrates a decrease in the wound area in miR-155$^{-/-}$ mice compared with wide type mice (on day 5 and 7) (fig. 2A). The animals were sacrificed, and the histological analysis showed decreased inflammatory cells on day 3 and weaker fibrosis on day 7 in miR-155$^{-/-}$ mice (fig. 2B).

We subsequently isolated the graft-infiltrating cells and performed total infiltrated cell counts via microscopy. As depicted in fig. 2C, the total number of infiltrated cells in the miR-155$^{-/-}$ mice was significantly decreased on day 3 compared with wide type groups. However, the proportion of the CD3+/Gr1+ cells was not different between the two groups. As demonstrated by CD3 and Gr1 staining of the wound tissue, the numbers of CD3+ T cells and Gr1+ neutrophils were also significantly decreased in miR-155$^{-/-}$ mice (fig. 2D and E). Taken together, these findings underscore the importance of miR-155 in the inflammatory response.

These results indicate that knockout of miR-155 is highly effective in preventing wound healing. Many researches have demonstrated that neutrophils are involved in the process of wound healing, but the role of T cells in wound healing is limited, especially in miR-155$^{-/-}$ mice.

2.3 T Cells Led to Weaker Wound Closure

Immune cells are most important in the initial stages of healing, where the priorities are haemostasis and preventing infection. Magnetic-activated cell sorting (MACS) of CD4+ splenocytes from mice was performed, and 1×10^6 T cells in solution were injected into the wound tissue. As shown in fig. 3A, the mice with CD4+ cells had weaker wound closure in the whole course of wound healing. Next, the histological analysis showed increasing numbers of Gr1+ neutrophils in mice with CD4+ cells (fig. 3B and 3C).

Taken together, these findings underscore the importance of CD4+ cells and neutrophils in wound healing.

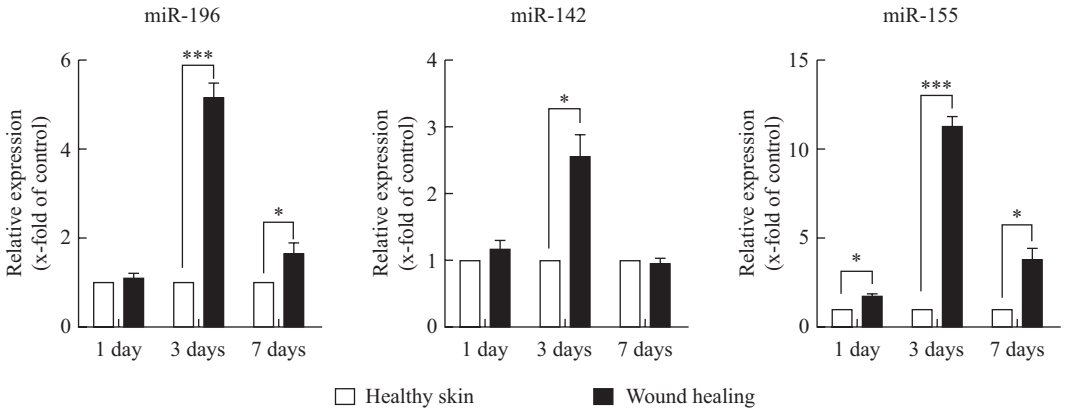

Fig. 1 MiR-155 was abundantly expressed in wound healing process
Mouse miR-155, miR-196 and miR-142 expression was quantified on day 1, 3, and 7 after wound healing. Fold changes of RNA levels by real-time PCR were normalized to the housekeeping gene, U6. Bars represent mean±SEM; *P* value was calculated by Student's *t* test between the syngeneic and allogeneic groups. $^{*}P<0.05$. $^{***}P<0.001$

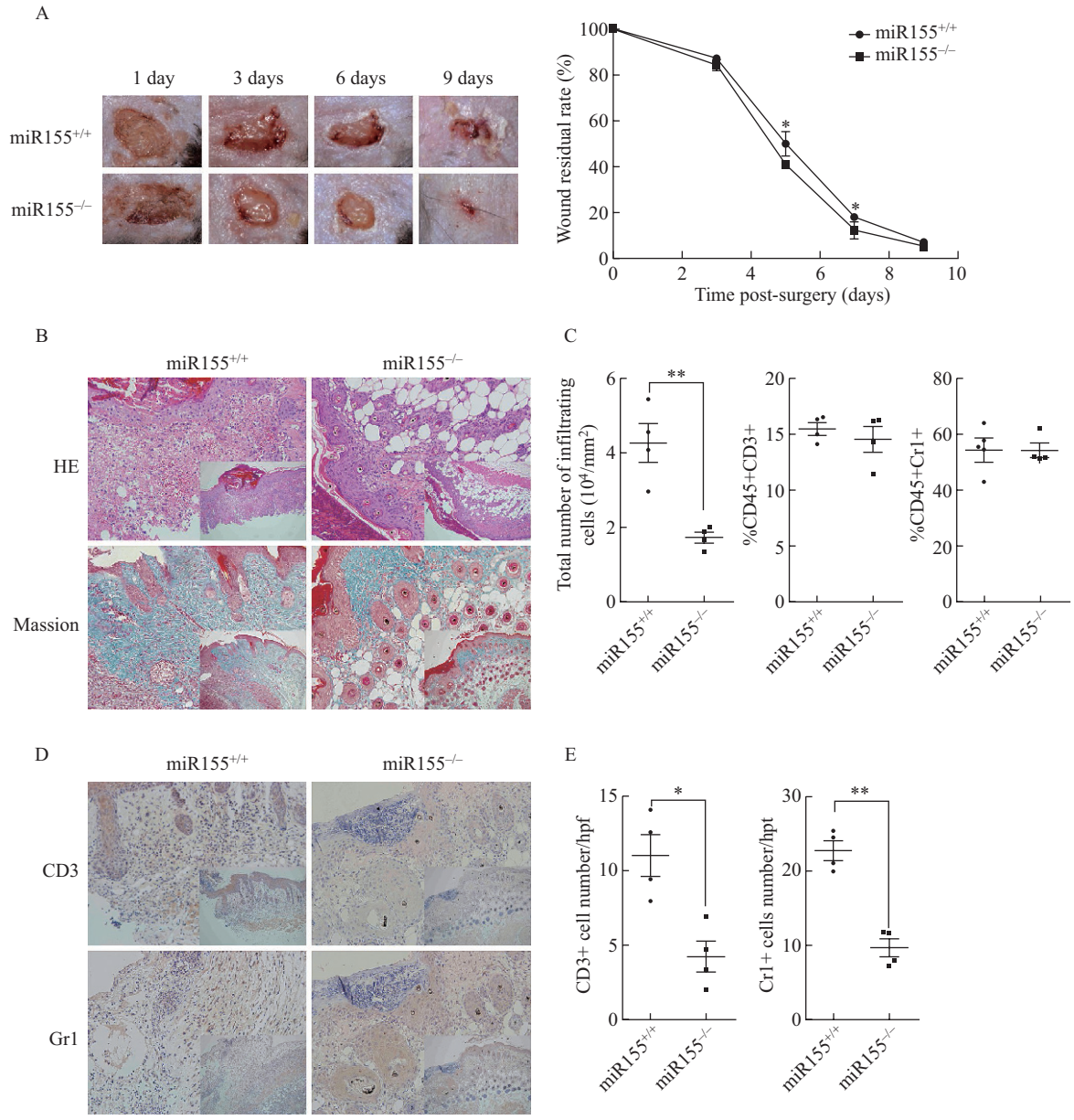

Fig. 2 Inflammatory cells in MiR-155$^{-/-}$ mice

A: Representative gross image of murine skin 1, 3, 6 and 9 day(s) after wound healing. Wound area measurement was performed by digital planimetry using Image-pro plus software. The wound residual rates were calculated as the ratio of the residual wound area at a given time point to the original wound area × 100%. B: Haematoxylin-eosin and Masson staining of wound tissue from miR155$^{-/-}$ or miR155$^{+/+}$ (C57) mice on day 3 after wound healing. C: The number of infiltrating cells and the proportion of the CD3+/Gr1+ cells in wound tissue from miR155$^{-/-}$ or miR155$^{+/+}$ (C57) mice on day 3 after wound healing. D and E: CD3 and Gr1 staining of wound tissue from miR155$^{-/-}$ or miR155$^{+/+}$ mice; the numbers of CD3+ T-cells and Gr1+ neutrophils were analyzed on day 3 after wound healing. Bars represent mean±SEM; *P* value was calculated by Student's *t* test between the miR155$^{-/-}$ or miR155$^{+/+}$ (C57) groups. hpf: hight power field; $^{*}P<0.05$, $^{**}P<0.01$

2.4 MiR-155$^{-/-}$ Mice Had a Weaker IL-17/IL-9 Response in Wound Healing

We analyzed the Th-related cytokines in the wound tissue 3 days after wound healing. PCR analyses demonstrated that the levels of IL-17A and IL-9 were decreased, whereas IFN-γ, IL-4 and Foxp3 expression was not changed in the wound tissue from miR-155$^{-/-}$ mice (fig. 4A). These results indicated that attenuated Th17/Th9 response has a key role in wound healing in miR-155$^{-/-}$ mice.

Additionally, both IL-17A and IL-9 can promote the recruitment of neutrophils and T cells into the wound tissue and delay the process of wound healing. As demonstrated by fig. 2E, CD3+ T cells and Gr1+ neutrophils were significantly decreased in miR155$^{-/-}$ mice. Consistent with the decreased IL-17A and IL-9 responses, the levels of the chemokines CCL2 and CXCL2 were significantly decreased in wound tissue

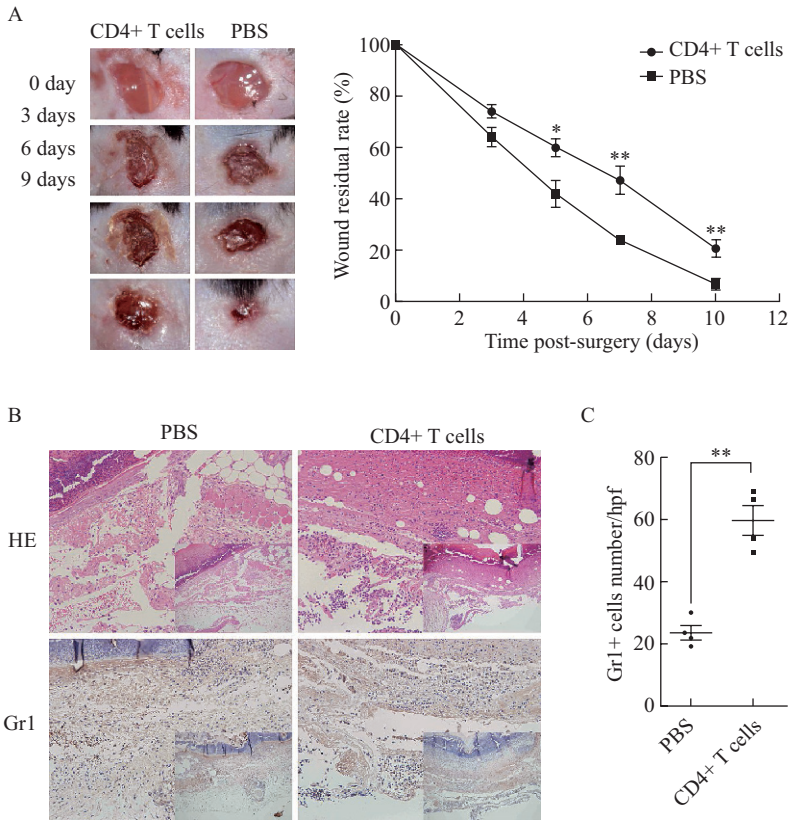

Fig. 3 T cells led to weaker wound closure

A: Representative gross image of murine skin 1, 3, 6 and 9 day(s) after PBS or T cell solution was injected into the wound tissue. The wound area measurement was also performed by digital planimetry using Image-pro plus software. B: Haematoxylin-eosin (HE) and Gr1 staining of wound tissue from PBS- or T cell solution-treated wound tissue on day 3. C: The numbers of Gr1+ neutrophils in the two groups were analyzed on day 3. Bars represent mean±SEM; *P* value was calculated by Student's *t* test between the PBS- and T cells solution-treated groups. $^{*}P<0.05$; $^{**}P<0.01$

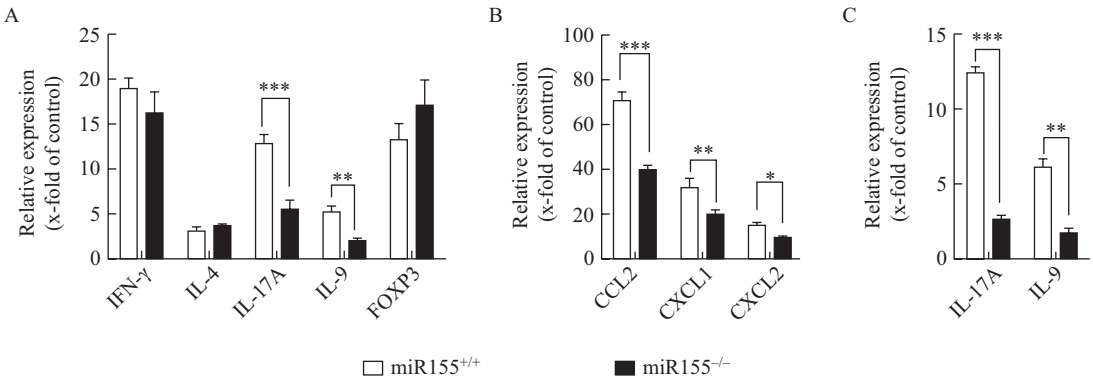

Fig. 4 MiR-155$^{-/-}$ mice had a weaker IL-17/IL-9 response in wound healing

A: Wound tissue from miR155$^{-/-}$ or miR155$^{+/+}$ mice was analyzed by quantitative PCR for expression of IFNγ, IL-4, IL-17A, IL-9 and FoxP3 relative to GAPDH on day 3 after wound healing. B: Chemokine expression was measured by quantitative PCR from miR155$^{-/-}$ or miR155$^{+/+}$ mice on day 3 after wound healing. C: CD4+ T cells from miR155$^{-/-}$ or miR155$^{+/+}$ mice were analyzed by quantitative PCR for the expression of IL-17A and IL-9 relative to GAPDH on day 3 after wound healing. Bars represent mean±SEM; *P* value was calculated by Student's *t* test between the miR155$^{-/-}$ and miR155$^{+/+}$ (C57) groups. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$

of miR-155$^{-/-}$ mice as determined by quantitative PCR (fig. 4B). Therefore, we speculated that the attenuated Th1 immunity might contribute to the Th17 response.

Next, we analyzed the Th-related cytokines in the wound tissue with miR-155$^{-/-}$ or miR-155$^{+/+}$ CD4+ cells. As expected, the expression levels of IL-17 and IL-9 were significantly decreased (fig. 4C). Those results indicated that the attenuated wound healing might be related to the miR-155$^{-/-}$ CD4+ T cells.

Therefore, the levels of IL-17A/IL-9 were playing

an important role in the pathological process of wound healing. These data indicated that miR-155 might participate in wound healing via the Th17/Th9 immune response.

2.5 Recombinant IL-17A or/and IL-9 Played an Important Role in miR155-mediated Wound Healing

The better closure of wound tissue in miR-155$^{-/-}$ mice might be related to an impaired Th17 /Th9 immune response. To assess our hypothesis, miR155$^{-/-}$ mice were subjected to wounds with rIL-17A or/and rIL-9. As shown in fig. 5A, rIL-17A or/and rIL-9 delayed the wound healing. The extent of histological tissue injury and the degree of cell infiltration were more serious in the rIL-17A or/and rIL-9 cytokine-treated miR155$^{-/-}$ group than in the PBS-treated miR155$^{-/-}$ group at 3 days following wound formation (fig. 5B and 5C). The levels of the chemokines CCL2, CXCL1 and CXCL2 were also significantly upregulated in the wound tissue of rIL-17A or/and IL-9 cytokine-treated miR155$^{-/-}$ mice (fig. 5D). These data indicated that the rIL-17A or/and rIL-9 injection increased the expression of chemokines and caused the excessive accumulation of neutrophils in the wound tissue.

These data demonstrates that IL-17A/IL-9-driven immunity plays a critical role in miR-155-related wound healing.

2.6 Mir-155 Regulated the Process of Th17/Th9-cell Differentiation Through c-Maf

We subsequently investigated the role of miR155 in the differentiation of Th17/Th9 cells *in vitro*. Consistent with the results of previous studies[23–25]; intracellular staining revealed that miR155$^{-/-}$ Th17/Th9 cells were defective in their ability to produce IL-17A/IL-9 (fig. 6A). These results indicate that miR155 may also be required for the development of Th17/Th9 cells.

Many Th-related transcripts, including c-Maf, SOCS1, STAT6 and PU.1, have been reported to be targets of miR-155. Therefore, we investigated which transcripts participate in the process of miR-155-related wound healing. As shown in fig. 6B, c-Maf was only upregulated in miR-155$^{-/-}$ mice compared with the miR-155$^{+/+}$ mice (fig. 6B).

To further analyse the role of miR-155 for Th17/Th9-cell differentiation, we detected whether c-Maf was regulated in Th17/Th9-cell differentiation. The results showed that the expression of c-Maf protein was increased in the miR-155$^{-/-}$ cells (fig. 6C). Next, we silenced the expression of c-Maf in miR-155$^{-/-}$ CD4+T cells by electroporation. The FAM-transfection efficiency was detected by fluorescence microscopy 12 h later. Those cells were differentiated in Th17/Th9 conditions. As shown in fig. 6D, the expression of IL-17A was slightly decreased, but the difference was not statistically significant. However, the expression of IL-9 significantly increased. Elyaman *et al*[26] had proven that IL-9 could promote naïve CD4+ cells into Th17 cells that synergize with TGF-β. Therefore, we concluded that the decreased production of IL-17A/IL-9 in miR-155$^{-/-}$ cells might be induced by c-Maf.

3 DISCUSSION

In this study, we highlighted the mechanism of miR155 in the process of wound healing and made several important discoveries. First, we showed that the expression of miR155 in wound tissue was obviously upregulated in a mouse model of wound healing. Second, we demonstrated that knockout of miR-155 led to an enhanced wound closure, and it was associated with a decreased number of immune cells in the wound tissue. Third, we provided a detailed analysis of the inflammatory cytokines in miR155$^{-/-}$ mice and demonstrated the importance of miR155 in IL-17A/IL-9 responses. Recombinant IL-17A and recombinant IL-9 can delay the process of wound healing. Fourth, miR-155$^{+/+}$ CD4+ T cells delayed the wound healing compared with miR-155$^{-/-}$ CD4+ T cells, and this might be related to the IL-17A/IL-9 response. Fifth, miR155 directly targeted the transcription factor c-Maf, a known positive regulator of Th2 cell differentiation, which also plays an important role in the function of Th17/Th9 cells. Additionally, miR-155 was required for T cell differentiation, especially in Th17 and Th9 cells. Taken together, these data advance our understanding of how miR-155 affects wound healing.

A major role of miRNA effectors is to regulate the expression of one-third of genes and control biological function of inflammatory cells in regulating wound healing[27–30]. For example, miR-21 promotes keratinocyte migration and boosts re-epithelialization during skin wound healing[30]. miRNA-29 is another miRNA that profoundly represses several extracellular matrix proteins which are involved in the signalling pathways and important for scarless healing[31, 32]. Here, we show data on the role of miR-155 during skin wound healing. Although it has been demonstrated that knockout of miR-155 accelerates wound closing through elevating number of macrophages and anti-fibrotic properties in wounded tissue[33, 34], we presumed that miR-155 might regulate wound healing in other ways, such as its involvement in the inflammatory reaction.

MiR-155 is known to influence the differentiation processes in the immune response[25, 35]. T cells and neutrophils are the first cell populations to arrive in the wound, and they exert bactericidal effect and clean the foreign substance on the wound. Due to the consistent pattern of immune cell infiltration in wound tissue, cells of the immune system were regarded as necessities for proper wound healing. An abundance of chemoattractants and cytokines are released by

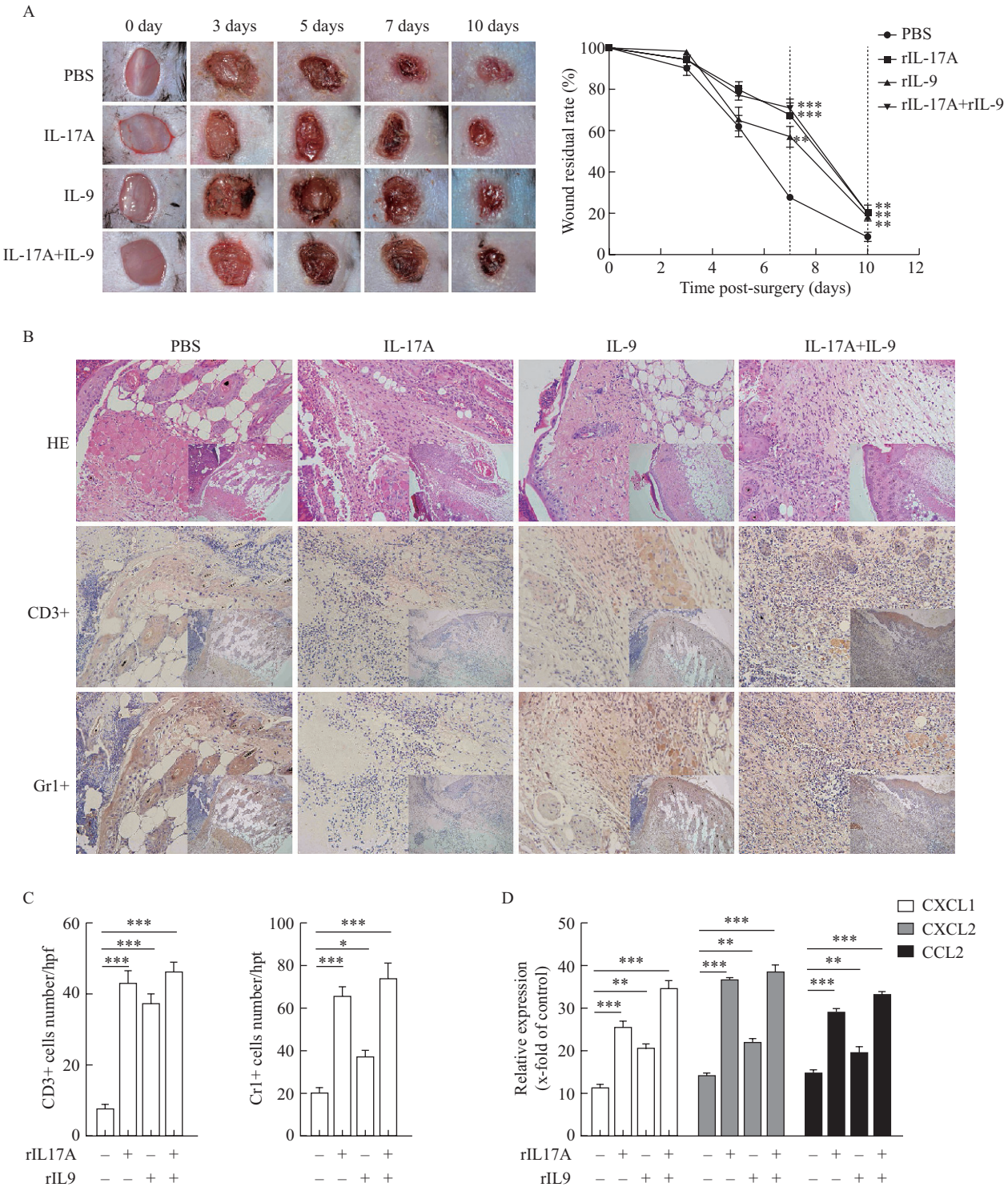

Fig. 5 Recombinant IL-17A or/and IL-9 played an important role in miR155-mediated wound healing
A: Representative gross image of murine skin 0, 3, 5, 7 and 10 day(s) after rIL-17A or/and rIL-9 solution were injected on the wound tissue. Wound area measurement was performed by digital planimetry using Image-pro plus software. B: Haematoxylin-eosin (HE), CD3 and Gr1 staining of wound tissue on day 3 following rIL-17A or/and rIL-9 cytokine treating wound tissue. C: The numbers of CD3+ T cells and Gr1+ neutrophils were analyzed among the different groups. D: Chemokine expression as measured by quantitative PCR among the different groups. Bars represent mean±SEM; *P* value was calculated by Student's *t* test between the different groups. $^{*}P<0.05$. $^{**}P<0.01$. $^{***}P<0.001$

immune cells that guide neutrophils, monocytes, and macrophages toward the wounded sites. However, at early stages of development, foetal skin has the unique ability to heal without a scar. This phenomenon of scarless, regenerative healing is highly dependent on the absence of acute inflammation and a lack of immune

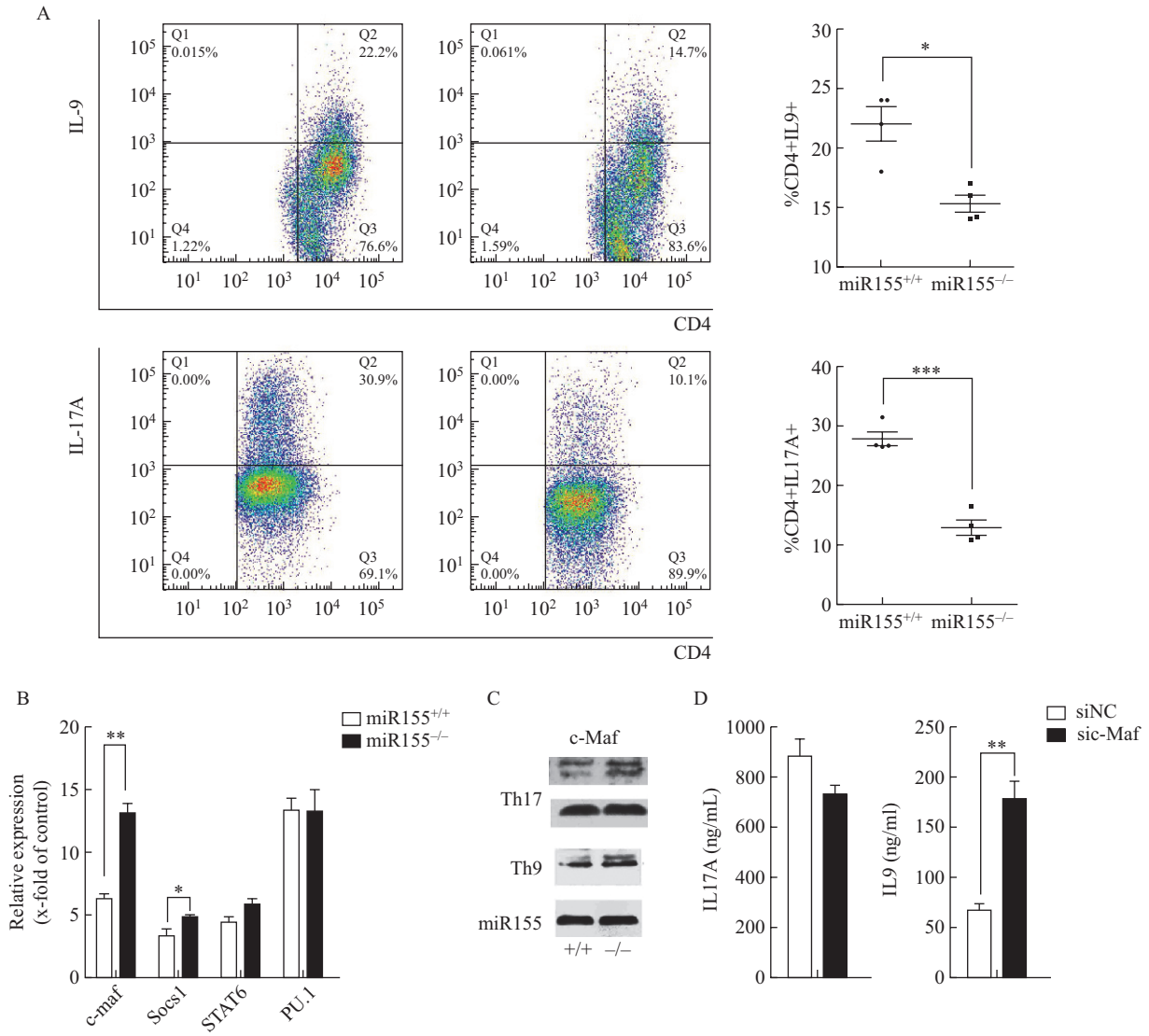

Fig. 6 Mir-155 regulates the process of Th17/Th9-cell differentiation through c-Maf

A: FACS analysis of polarized CD4+ T cells from miR155$^{-/-}$ or miR155$^{+/+}$ mice. CD4+ splenocytes were cultured under Th9/Th17-polarizing conditions. After 3 or 5 days of *in vitro* stimulation, the cells were stained for Th9/Th17-related cytokines (CD4, IL-17A, and IL-9). B: Wound tissue from miR155$^{-/-}$ or miR155$^{+/+}$ mice was analyzed by quantitative PCR for expression of c-Maf, SOCS1, STAT6, and PU.1 relative to GAPDH on day 3 after wound healing. C: The expression levels of c-Maf protein after Th9 or Th17 differentiation were analyzed by Western blotting, and β-actin served as the internal reference. D: After silencing the expression of c-Maf in miR-155$^{-/-}$ CD4+T cells by electroporation, the expression of IL-17A and IL-9 proteins in supernatants after Th9 or Th17 differentiation was analyzed by ELISA. Bars represent mean±SEM; *P* value was calculated by Student's *t* test between the different groups. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$

cell infiltration[36–38]. So, the traditional assumption that inflammatory cells are required for healing has been challenged, and by contrast, these cells in wounded tissue may actually be harmful by impeding healing and increasing scar tissue. Many studies have proven that animals with deficiency or depletion of neutrophils or macrophages exhibit accelerated healing[39, 40]. Our results show that lack of miR-155 may promote wound healing via decreasing the number of inflammatory cells.

We have demonstrated that there are attenuated IL-17A/IL-9 responses in miR155$^{-/-}$ mice during wound healing. *In vivo*, IL-17 regulates tissue inflammation. Antibodies to IL-17 inhibited chemokine expression in autoimmune disease, whereas overexpression of IL-17 in lung epithelium caused chemokine production and leukocyte infiltration[41]. Meanwhile, as a proinflammatory cytokine, it had been demonstrated that neutralizing or knocking out IL-17 promotes wound healing[42]. IL-9 was described as a Th2-related cytokine that is expressed on various cell types including T cells, B cells, eosinophils, neutrophils and airway epithelial cells. Most of these inflammatory cells are involved in the pathology of tissue inflammation,

including asthma, EAE and wound healing[43–45]. In our studies, we first proved that overexpression of IL-9 and IL-17 can delay wound healing.

c-Maf, as a target of miR-155, provides insight into the ability of miR155 regulating Th9/Th17 cell effector function. c-Maf was once a well-established positive regulator of Th2 cell development and was also shown to be involved in the development of Th9/Th17 cells. Consistent with previous studies, in this study, elevated expression of c-Maf was discovered in cultures of miR155-deficient cells in Th9/Th17 conditions. Many studies have demonstrated that c-Maf was required for IL-17 production through upregulating the expression of IL-23R[46]. The production of IL-9 also seems to be negatively correlated with the expression of c-Maf in Th9 differentiation. Transfection of c-Maf resulted in a decrease in IL-9-producing cells compared with transfection with control retrovirus[47], and c-Maf could induce the promoter activity of IL-23R[48], which negatively regulates the expression of IL-9[46, 49, 50]. Additionally, IL-10, another cytokine mainly secreted from Th9 cells, is positively correlated with c-Maf[46]. Recent studies demonstrated that miR-155 plays an important role in the pathogenesis of fibrosis by inhibiting the expression of IL-10 in skin fibrosis[51, 52]. Therefore, miR-155 may regulate the Th17/Th9 response through c-Maf.

Taken together, our findings show that miR-155 is up-regulated after skin wounding in mice and this upregulation correlates to the influx of immune cells into the wound. The wound tissue contains pro-inflammatory cytokines, especially IL-17/IL-9, which could attract more immune cells into wound tissue. In summary, miR-155 seems to be involved in the regulation of many factors influencing wound healing through c-Maf, which opens an avenue for the development of new therapeutic approaches.

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

REFERENCES

1 Wilgus TA. Immune cells in the healing skin wound: influential players at each stage of repair. Pharmacol Res, 2008,58(2):112-116

2 Roy S, Khanna S, Rink C, *et al*. Characterization of the acute temporal changes in excisional murine cutaneous wound inflammation by screening of the wound-edge transcriptome. Physiol Genomics, 2008,34(2):162-184

3 Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. Physiol Rev, 2003,83(3):835-870

4 Sehm T, Sachse C, Frenzel C, *et al*. miR-196 is an essential early-stage regulator of tail regeneration, upstream of key spinal cord patterning events. Dev Biol, 2009,334(2):468-480

5 Biswas S, Roy S, Banerjee J, *et al*. Hypoxia inducible microRNA 210 attenuates keratinocyte proliferation and impairs closure in a murine model of ischemic wounds. Proc Natl Acad Sci U S A, 2010,107(15):6976-6981

6 Wang T, Zhang L, Shi C, *et al*. TGF-beta-induced miR-21 negatively regulates the antiproliferative activity but has no effect on EMT of TGF-beta in HaCaT cells. Int J Biochem Cell Biol, 2012,44(2):366-376

7 Bertero T, Gastaldi C, Bourget-Ponzio I, *et al*. miR-483-3p controls proliferation in wounded epithelial cells. Faseb J, 2011,25(9):3092-3105

8 Yang X, Wang J, Guo SL, *et al*. miR-21 promotes keratinocyte migration and re-epithelialization during wound healing. Int J Biol Sci, 2011,7(5):685-690

9 Martinez-Nunez RT, Louafi F, Friedmann PS, *et al*. MicroRNA-155 modulates the pathogen binding ability of dendritic cells (DCs) by down-regulation of DC-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN). J Biol Chem, 2009,284(24):16334-16342

10 Divekar AA, Dubey S, Gangalum PR, *et al*. Dicer insufficiency and microRNA-155 overexpression in lupus regulatory T cells: an apparent paradox in the setting of an inflammatory milieu. J Immunol, 2011,186(2):924-930

11 Takagi T, Naito Y, Mizushima K, *et al*. Increased expression of microRNA in the inflamed colonic mucosa of patients with active ulcerative colitis. J Gastroenterol Hepatol, 2010,25(Suppl 1):S129-33

12 Stanczyk J, Pedrioli DM, Brentano F, *et al*. Altered expression of MicroRNA in synovial fibroblasts and synovial tissue in rheumatoid arthritis. Arthritis Rheum, 2008,58:1001-1009

13 Nazari-Jahantigh M, Wei Y, Noels H, *et al*. MicroRNA-155 promotes atherosclerosis by repressing Bcl6 in macrophages. J Clin Invest, 2012,122(11):4190-4202

14 Kolls JK, Linden A. Interleukin-17 family members and inflammation. Immunity, 2004,21(4):467-476

15 Nograles KE, Krueger JG. Anti-cytokine therapies for psoriasis. Exp Cell Res, 2011,317:1293-1300

16 Rodero MP, Hodgson SS, Hollier B, *et al*. Reduced Il17a expression distinguishes a Ly6c(lo)MHCII(hi) macrophage population promoting wound healing. J Invest Dermatol, 2013,133(3):783-792

17 Li H, Rostami A. IL-9: basic biology, signaling pathways in CD4+ T cells and implications for autoimmunity. J Neuroimmune Pharmacol, 2010,5(2):198-129

18 Temann UA, Laouar Y, Eynon EE, *et al*. IL9 leads to airway inflammation by inducing IL13 expression in airway epithelial cells. Int Immunol, 2007,19(1):1-10

19 O'Connell RM, Taganov KD, Boldin MP, *et al*. MicroRNA-155 is induced during the macrophage inflammatory response. Proc Natl Acad Sci U S A, 2007,104(5):1604-1609

20 Rutz S, Noubade R, Eidenschenk C, *et al*. Transcription factor c-Maf mediates the TGF-beta-dependent suppression of IL-22 production in T(H)17 cells. 2011,12(12):1238-1245

21 Chicaybam L, Sodre AL, Curzio BA, *et al*. An efficient low cost method for gene transfer to T lymphocytes. PLoS One, 2013,8(3):e60298

22 Sun Y, Ye P, Wu J, *et al*. Inhibition of intimal hyperplasia in murine aortic allografts by the oral administration of the transforming growth factor-beta receptor I kinase inhibitor SD-208. J Heart Lung Transplant, 2014,33(6):654-661
23 Divekar AA, Dubey S, Gangalum PR, *et al*. Dicer insufficiency and microRNA-155 overexpression in lupus regulatory T cells: an apparent paradox in the setting of an inflammatory milieu. J Immunol 2011,186(2):924-930
24 Kolls JK, Linden A. Interleukin-17 family members and inflammation. Immunity, 2004,21:467-476
25 Rodriguez A, Vigorito E, Clare S, *et al*. Requirement of bic/microRNA-155 for normal immune function. 2007,316(5824):608-611
26 Elyaman W, Bradshaw EM, Uyttenhove C, *et al*. IL-9 induces differentiation of TH17 cells and enhances function of FoxP3+ natural regulatory T cells. 2009,106(31):12885-12890
27 Banerjee J, Chan YC, Sen CK. MicroRNAs in skin and wound healing. Physiol Genomics, 2011,43(10):543-556
28 Sand M, Gambichler T, Sand D, *et al*. MicroRNAs and the skin: Tiny players in the body's largest organ. J Dermatol Sci, 2009,53(3):169-175
29 Bertero T, Gastaldi C, Bourget-Ponzio I, *et al*. miR-483-3p controls proliferation in wounded epithelial cells. FASEB J, 2011,25(9):3092-3105
30. Yang X, Wang J, Guo SL, *et al*. miR-21 promotes keratinocyte migration and re-epithelialization during wound healing. Int J Biol Sci, 2011,7(5):685-690
31. Li Z, Hassan MQ, Jafferji M, *et al*. Biological functions of miR-29b contribute to positive regulation of osteoblast differentiation. J Biol Chem, 2009,284(23):15676-15684
32 van Rooij E, Sutherland LB, Thatcher JE, *et al*. Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. Proc Natl Acad Sci U S A, 2008,105(35):13027-13032
33 Yang L, Liu J, Bai X, *et al*. Acute downregulation of miR-155 at wound sites leads to a reduced fibrosis through attenuating inflammatory response. Biochem Bioph Res Co, 2014,453(1):153-159
34 van Solingen C, Araldi E, Chamorro-Jorganes A, *et al*. Improved repair of dermal wounds in mice lacking microRNA-155. J Cell Mol Med, 2014,18(6):1104-1112
35 O'Connell RM, Taganov KD, Boldin MP, *et al*. MicroRNA-155 is induced during the macrophage inflammatory response. Proc Natl Acad Sci U S A, 2007,104(5):1604-1609
36 Adolph VR, DiSanto SK, Bleacher JC, *et al*. The potential role of the lymphocyte in fetal wound healing. J Pediatr Surg, 1993,28(10):1316-1320
37 Hopkinson-Woolley J, Hughes D, Gordon S, *et al*. Macrophage recruitment during limb development and wound healing in the embryonic and foetal mouse. J Cell Sci, 1994,107(Pt 5):1159-1167
38 Olutoye OO, Yager DR, Cohen IK, *et al*. Lower cytokine release by fetal porcine platelets: a possible explanation for reduced inflammation after fetal wounding. J Pediatr Surg, 1996,31(1):91-95
39 Dovi JV, He LK, DiPietro LA. Accelerated wound closure in neutrophil-depleted mice. J Leukoc Biol, 2003,73(4):448-455
40 Goren I, Muller E, Schiefelbein D, *et al*. Systemic anti-TNFalpha treatment restores diabetes-impaired skin repair in ob/ob mice by inactivation of macrophages. J Invest Dermatol, 2007,127(9):2259-2267
41 Park H, Li Z, Yang XO, *et al*. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol, 2005,6(11):1133-1141
42 Rodero MP, Hodgson SS, Hollier B, *et al*. Reduced Il17a expression distinguishes a Ly6c(lo)MHCII(hi) macrophage population promoting wound healing. J Invest Dermatol, 2013,133(3):783-792
43 Abdelilah S, Latifa K, Esra N, *et al*. Functional expression of IL-9 receptor by human neutrophils from asthmatic donors: role in IL-8 release. J Immunol, 2001,166(4):2768-2774
44 Little FF, Cruikshank WW, Center DM. Il-9 stimulates release of chemotactic factors from human bronchial epithelial cells. Am J Respir Cell Mol Biol, 2001,25(3):347-352
45 Louahed J, Zhou Y, Maloy WL, *et al*. Interleukin 9 promotes influx and local maturation of eosinophils. Blood, 2001,97(4):1035-1042
46 Bauquet AT, Jin H, Paterson AM, *et al*. The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. Nat Immunol, 2009,10(2):167-175
47 Lu LF, Lind EF, Gondek DC, *et al*. Mast cells are essential intermediaries in regulatory T-cell tolerance. Nature, 2006,442(7106):997-1002
48 Sato K, Miyoshi F, Yokota K, *et al*. Marked induction of c-Maf protein during Th17 cell differentiation and its implication in memory Th cell development. J Biol Chem, 2011,286(17):14963-14971
49 Rutz S, Noubade R, Eidenschenk C, *et al*. Transcription factor c-Maf mediates the TGF-beta-dependent suppression of IL-22 production in T(H)17 cells. Nat Immunol, 2011,12(12):1238-1245
50 Poulin LF, Richard M, Le Moine A, *et al*. Interleukin-9 promotes eosinophilic rejection of mouse heart allografts. Transplantation, 2003,76(3):572-577
51 Billeter AT, Hellmann J, Roberts H, *et al*. MicroRNA-155 potentiates the inflammatory response in hypothermia by suppressing IL-10 production. FASEB J, 2014,28(12):5322-5336
52 Quinn SR, Mangan NE, Caffrey BE, *et al*. The role of Ets2 transcription factor in the induction of microRNA-155 (miR-155) by lipopolysaccharide and its targeting by interleukin-10. J Biol Chem, 2014,289:4316-4325

(Received Jan. 20, 2019; revised Oct. 20, 2019)