

IL-35 ameliorates psoriasis by suppressing the accumulation of iNOS-expressing myeloid-derived suppressor cells

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Abstract

Background and Purpose: Psoriasis is an immune-associated disease, however, the pathogenesis of psoriasis remains unclear. We investigated the mechanism of IL-35 in psoriasis treatment. **Experimental Approach:** PASI and Baker scores were used to evaluate the Imiquimod-induced psoriasis mouse model. Flow cytometry is used to detect changes in immune cells and to select target cells. The changes of inflammatory factors were detected by ELISA. Adoptive transfused was used to demonstrate the effect of the corresponding cells. **Key Results:** IL-35 expression in patients with psoriasis was significantly increased. The number of myeloid-derived suppressor cells (MDSCs) in patients was also significantly increased. Similar results were obtained in mice with imiquimod (IMQ)-induced psoriasis. IL-35 had potent immunosuppressive effects on psoriasis model mouse, leading to a decrease in the total number of MDSCs and its subtypes in the spleen and psoriatic skin lesions. The level of inducible nitric oxide synthase (iNOS) secreted by MDSCs also decreased significantly; however, there was no difference in the level of IL-10 in MDSCs. Adoptive transfer of MDSCs from IMQ-challenged mice weakened the effect of IL-35. When MDSCs were isolated from iNOS knockout mice that were established with IMQ and transfected into IMQ-induced WT mice, there was no significant difference in psoriasis area and severity index scores between IL-35 + iNOS-/-MDSC and IL-35 treatment groups. **Conclusions and Implications:** IL-35 plays an important immunosuppressive role in psoriasis by inhibiting the counts of MDSCs expressing iNOS. This study may serve as a new therapeutic strategy for patients with chronic psoriasis or other cutaneous inflammatory diseases.

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Data Availability Statement

Data will be made available on request.

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Conflict of Interest Statement

The authors declare no conflicts of interest.

Ethics Approval Statement

All experimental protocols used in this study were approved and implemented in accordance with the guidelines of the review board of Jining Medical University.

Patient Consent Statement

The study has been conducted in accordance with The Code of Ethics of the World Medical Association. Informed consent was obtained from recruited patients for experimentation.

Author Contributions Statement

Conceptualisation: HBX, JFZ, and DMS. Data curation: ZYY, HZ, JF, and LW. Formal analysis: YSZ, DLC, and CL. Funding acquisition: JFZ, HBX, and DMS. Investigation: JFZ, FLY, CXL, and GJD. Methodology: JFZ, YSZ, HS, and ZCN. Software: MSZ and CYW. Project administration: HBX and DMS. Visualisation and Writing: JFZ and JD.

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decreased significantly; however, there was no difference in the level of IL-10 in MDSCs. Adoptive transfer of MDSCs from IMQ-challenged mice weakened the effect of IL-35. When MDSCs were isolated from *iNOS* knockout mice that were established with IMQ and transfected into IMQ-induced WT mice, there was no significant difference in psoriasis area and severity index scores between IL-35 + *iNOS*^{-/-}-MDSC and IL-35 treatment groups.

Conclusions and Implications : IL-35 plays an important immunosuppressive role in psoriasis by inhibiting the counts of MDSCs expressing iNOS. This study may serve as a new therapeutic strategy for patients with chronic psoriasis or other cutaneous inflammatory diseases.

Keywords: interleukin-35; psoriasis; myeloid-derived suppressor cell; imiquimod-induced psoriasis mouse model; inducible nitric oxide synthase

Bullet Point Summary

What's already known?

- There is conflicting evidence over the expression of IL-35 in patients with psoriasis;
- Data from registries to date suggest that IL-35 has an immunosuppressive function in psoriasis model;
- The immune function of MDSC is completely different in diseases of different backgrounds

What does this study add?

- The expression of IL-35 and the number of MDSCs in patients with psoriasis were significantly increased.
- IL-35 inhibits the development of psoriasis by inhibiting MDSCs recruitment.
- IL-35 inhibited the expression of *iNOS* in MDSCs in psoriasis model.

Clinical significance

IL-35 may be used as a biological agent in the treatment of psoriasis.

The pathogenesis of psoriasis can be inhibited by targeting pro-inflammatory MDSCs.

1. Introduction

Psoriasis is a chronic inflammatory immune-mediated skin disease caused by vascular hyperplasia, abnormal keratinocyte proliferation, and inflammatory cell infiltration into the epidermis and dermis. These extensive inflammatory cell infiltrates include T-lymphocytes, mast cells, macrophages, myeloid-derived suppressor cells (MDSCs), and neutrophils (Lebwohl, 2018). Studies on the pathogenesis of psoriasis have greatly increased our understanding of skin immunology and facilitated the introduction of innovative and efficient therapies. However, at present, psoriasis pathogenesis is incompletely understood.

MDSCs are immature heterogeneous myeloid-derived progenitor cells and can be divided into two cell subtypes, mononuclear MDSCs (M-MDSCs) and polymorphonuclear MDSCs (PMN-MDSCs), also known as granulocytic MDSCs (G-MDSCs) (Bronte et al., 2016; Tcyganov, Mastio, Chen & Gabrilovich, 2018). MDSCs have been demonstrated to modulate immune responses in inflammatory bowel disease (IBD) (Haile et al., 2008; Wang, Ding, Deng, Zheng & Wang, 2020), transplantation (Dugast et al., 2008; Pengam et al., 2019), many types of cancer (Diaz-Montero, Salem, Nishimura, Garrett-Mayer, Cole & Montero, 2009; Hoechst et al., 2008; Youn, Nagaraj, Collazo & Gabrilovich, 2008), and infections (De Santo et al., 2008; Li et al., 2020). Recent studies have revealed the expansion of MDSC populations, which produce cytokines including IL-23, IL-1 β , and C-C motif chemokine ligand 4 (CCL4), in patients with psoriasis (Cao et al., 2016; Chen et al., 2020; Oka et al., 2017; Soler & McCormick, 2011). M-MDSCs express high levels of inducible nitric oxide synthase (iNOS), which is a mediator of the suppressive function of M-MDSCs in cancer (Mundy-Bosse et al., 2011). MDSCs function as immune disruptors in patients with systemic lupus erythematosus (SLE) in an iNOS-dependent manner. The number of M-MDSCs and the expression of iNOS decreased after treatment, suggesting that these cells can be used as an indicator of treatment effectiveness (Wang et al., 2019).

IL-35 is a novel inhibitory cytokine, consisting of Epstein-Barr virus-induced gene 3 (EBI3) and IL-12 chain (p35) subunits that belong to the IL-12 cytokine family (Collison et al., 2007). IL-35 has two major effects including the inhibition of T-cell proliferation in various disease models (Kochetkova, Golden, Holderness, Callis & Pascual, 2010; Wirtz, Billmeier, McHedlidze, Blumberg & Neurath, 2011) and inhibition of the development and differentiation of Th17 cells (Collison et al., 2010; Collison, Pillai, Chaturvedi & Vignali, 2009). IL-35 gene therapy significantly alleviated psoriasis-like symptoms in psoriasis mouse models (Zhang et al., 2016). However, the contribution of IL-35 recombinant protein to the pathogenesis of psoriasis is not fully understood. Therefore, in this study, we aimed to investigate the role of the IL-35 recombinant protein in the pathogenesis of psoriasis.

2. Materials and methods

2.1 Imiquimod-induced psoriasis mouse model and therapy

BALB/c mice (8 weeks old) were administered a daily dose of 62.5 mg IMQ cream (Mingxin Pharmaceutical Co., Ltd., Chengdu, China) on the back skin of shaved mice for 7 d. For the therapeutic potential of IL-35, we administered IL-35 (5 µg) one day before the establishment of the IMQ-induced psoriasis mouse model, while three additional injections were administered every other day during the establishment of the model. At 24 h after the last injection, animals were anaesthetised with 50 µL of chloral hydrate (10 %; intraperitoneal injection) for macroscopic photography. Animals were then euthanised, and samples of serum and psoriatic lesion tissues were collected. The spleen and skin were used for flow cytometry and histological tests.

2.2 Psoriasis severity evaluation

To evaluate the severity of inflammation in the ears and neck skin of mice, we used an objective scoring system based on the clinical Psoriasis area and severity index (PASI). Erythema, scaling, and thickening were scored independently on a scale of 0 to 4: 0, none; 1, slight; 2, moderate; 3, marked; or 4, very marked (van der Fits et al., 2009).

2.3 Hematoxylin and eosin (H&E) staining and microscopy

Mouse-ear tissue and dorsal skin were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and then stained with H&E stain. Images were captured using an Olympus BX600 microscope (Olympus Corporation, Tokyo, Japan) and assessed using the Baker Scoring system.

2.4 ELISA

The expression of CXCL8 and IL-6 in the supernatant of the cell culture and other inflammatory factors, including IL-1, IL-6, IL-17A, and IL-23 in the serum and skin tissues of mice were detected by ELISA. ELISA plates were washed with PBS containing Tween 20 (0.05%) and blocked using assay diluents for 1 h at 37 °C. After washing, samples were serially diluted using assay diluents and added to the plates and then incubated for 2 h followed by the addition of diluted detection antibody at 37 °C. The plates were washed again; avidin-horseradish peroxidase (HRP) was added to the plates and incubated for 30 min. Next, the substrate solution tetramethylbenzidine (TMB) was added and incubated for 15 min; then, the reaction was stopped by adding 100 µL of acid stop solution. Absorbance was read at 450 nm within 20 min using an ELISA reader (Bio-Rad Laboratories, Hercules, CA).

2.5 Isolation of human PBMCs

The work described has been conducted in accordance with The Code of Ethics of the World Medical Association. Informed consent was obtained from all recruited subjects. Both healthy control participants and patients with psoriasis were subjected to peripheral blood drawing (6 mL/person), and human PBMCs were isolated by density-gradient centrifugation using Ficoll-Paque (GE Healthcare Bio-science AB Uppsala, Sweden). All plasma samples were stored at -80 °C for later analysis. The PBMCs at a concentration of 1×10^6 /mL were maintained in RPMI 1640 (Thermo Fisher Scientific) culture medium supplemented with 10 % FBS; Thermo Fisher Scientific).

2.6 Flow cytometry

PBS supplemented with 2 % heat-inactivated FBS was used as the staining buffer for flow cytometry. PBMCs from healthy controls and patients with psoriasis were resuspended in the staining buffer for the extracellular staining of CD4, CD19, CD14, CD15, CD11b, and HLA-DR, as well as the subsequent intracellular staining of EBI3 and p35. Directly labelled isotype-matched rat anti-mouse antibodies (BioLegend) served as controls. For intracellular staining, cells were first stimulated with 50 ng/mL phorbol myristate acetate (Sigma-Aldrich, St. Louis, MO, USA), 1.0 μ g/mL ionomycin (Sigma-Aldrich) for 2 h, and then cultured for another 4 h in the presence of monensin (2 μ L, BioLegend, San Diego, CA, USA). After extracellular staining, the cells were fixed and permeabilised using the Fixation/Permeabilization Solution Kit (BD Biosciences, St. Louis, MO, USA), according to the manufacturer's protocol, and then EBI3 and p35 staining were performed.

The spleens of treated mice were collected, homogenised, and suspended as single cells in red blood cell lysis buffer. Skin tissues from the treated mice were collected, homogenised, and suspended as single cells in collagenase IV solution. Cells were extracellularly stained with CD11b, Gr-1, Ly6G, and Ly6C for 30 min at 4 °C in the dark. Directly labelled isotype-matched rat anti-mouse antibodies (BioLegend) were served as controls. After washing twice with PBS, the cells were resuspended in 200 mL of PBS and analysed via flow cytometry. For intracellular staining, the cells were stained extracellularly and then fixed and permeabilised using Perm/Fix solution (BD Biosciences, Franklin Lakes, NJ) for 30 min at 4 °C. Finally, the cells were intracellularly stained with anti-iNOS and anti-IL-10.

The following antibodies were used: fluorescein isothiocyanate (FITC) anti-human CD4, Brilliant Violet (BV)421 anti-human CD19, BV421 anti-human CD11b, phycoerythrin (PE) anti-human CD15 (BD Biosciences, San Diego, CA, USA), FITC anti-human HLA-DR (BD Biosciences), allophycocyanin (APC) anti-human EBI3, PE anti-human p35, FITC anti-mouse CD11b, APC anti-mouse Gr-1, PE anti-mouse Ly6G, APC anti-mouse Ly6C, PE anti-mouse iNOS, and PE anti-mouse IL-10 (all unspecified antibodies were from BioLegend).

2.7 Preparation of bone marrow-derived MDSCs in vitro

Bone marrow cells were isolated from mice by flushing their femurs and tibiae, centrifuging the cell-containing elution (1800 rpm, 5 min), and resuspending them in complete RPMI 1640 (Thermo Fisher Scientific) medium supplemented with IL-6 (40 ng/mL, Peprotech, Suzhou, Jiangsu, China) and GM-CSF (40 ng/mL, Peprotech). The cells were maintained at 37 degC in a 5 % CO₂ humidified atmosphere for 4 d. To investigate the effect of IL-35 on the expansion of MDSCs, IL-35, IL-6, and GM-CSF were added to the medium at the same time.

2.8 Immunofluorescence staining

We performed immunofluorescence staining for IL-35 and M-MDSCs in the skin of patients with psoriasis and healthy controls and MDSCs and iNOS⁺MDSCs in murine skin lesions. Mice were treated with IL-35; the back-skin tissue of mice was obtained the day after the last treatment, frozen, and then stained. For staining, the primary antibodies (rat anti-human p35 and rabbit anti-mouse EBI3⁺ for human IL-35, rat anti-human CD14 and rabbit anti-human HLA-DR for human M-MDSCs, rat anti-mouse CD11b and rabbit anti-mouse Gr-1 for mouse MDSCs, rabbit anti-mouse Gr-1 and rat anti-mouse iNOS for mouse iNOS⁺MDSCs) were incubated with the samples at 4 °C overnight and secondary antibodies including goat anti-rabbit IgG-FITC, goat anti-rat IgG-TR or anti-rabbit IgG-TR, goat anti-rat IgG-FITC respectively for 1 h (all first antibodies were from Abcam, and second antibodies were from zsbio, Beijing, China). Tissue sections were examined under a fluorescence microscope (Olympus Optical, Tokyo, Japan), and images were captured at x400 magnification.

2.9 Adoptive transfer of MDSCs

For adoptive transfer, 2x10⁶CD11b⁺Gr1⁺ cells isolated from the spleen of the IMQ-induced mice (wild type/WT and iNOS gene knockout/ iNOS^{-/-}) via flow cytometry were washed twice and resuspended in

200 μ L of PBS and injected into mice via the tail vein as shown in Figure 6A. Briefly, MDSCs were injected the day before the application of IMQ and injected again on the third day after the application of IMQ.

2.10 Statistical analysis

The Prism version 6 software (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Data were expressed as the mean $\pm$ standard error of the mean (SEM) or standard deviation (SD). All P values were calculated using the Student's *t*-test or one-way analysis of variance (ANOVA). Statistical significance was set at $P < 0.05$.

3. Results

3.1 Expression of IL-35 was significantly increased in patients with psoriasis

To investigate the involvement of IL-35 in skin diseases, we first examined IL35 expression in the serum of healthy controls and patients with psoriasis. We recruited patients ($n = 53$) and age/sex-matched healthy donors ($n = 20$); the demographics are summarised in Table 1. Using ELISA, we found that the levels of IL-35 were significantly elevated in the serum from patients with psoriasis compared with those in healthy controls (Fig. 1A, $p = 0.0089$). Moreover, we observed that as the symptoms of psoriasis worsened (increased PASI score), the expression of IL-35 was increased. More specifically, the expression of IL-35 in specimens with a PASI score greater than or equal to 3 points was significantly higher than that in specimens with a PASI score less than 3 points (Fig. 1A). We also found increased populations of CD4⁺EBI3⁺p35⁺ and CD19⁺EBI3⁺p35⁺ cells in the peripheral blood of patients with psoriasis via flow cytometry (Fig. 1B-D). We demonstrated that IL-35 secreted by both T and B lymphocytes was significantly increased ($p < 0.01$ and $p < 0.05$, respectively). Moreover, immunofluorescence staining results showed that the expression of IL-35 in the skin tissue of patients with psoriasis increased significantly (Fig. 1E).

3.2 M-MDSCs were significantly expanded in patients with psoriasis

We examined whether the number of M-MDSCs is altered in patients with psoriasis. Using flow cytometry, we confirmed that the number of human M-MDSCs (CD11b⁺CD14⁺HLA-DR⁻) was remarkably increased in the peripheral blood of patients with psoriasis (Fig. 2A-B, $p = 0.0065$). Next, immunofluorescence staining of CD14⁺HLA-DR⁻ M-MDSCs in the inflamed skin of patients with psoriasis revealed that the infiltration of M-MDSC cells in psoriatic skin tissue increased significantly compared to that in healthy controls ($p < 0.01$).

3.3 IL-35 protein therapy relieved the symptoms of psoriasis models

We stimulated HaCaT cells using M5 together with IL-35 to observe the effect of IL-35 on the expression of CXCL8 and IL-6. We found consistent lower levels of CXCL8 and IL-6 in the IL-35-treated group than in the control group (Supplementary Fig. 1A, B). The results suggest that IL-35 suppresses the expression of CXCL8 and IL-6 in stimulated HaCaT cells.

We explored whether IL-35 could exhibit a therapeutic effect in K14-VEGF-A-Tg mice. Therefore, we injected K14-VEGF-A-Tg mice (10 weeks old) with IL-35 (5 μ g) recombinant protein, as shown in Supplementary Figure 2A. We found that mice in the control group showed increased ear thickening; however, mice in the IL-35-treated group were healthy (Supplementary Fig. 2B). Pathological features, such as erythema, scaling, and thickness based on PASI scores are shown in Supplementary Figure 2C. Concomitantly, we obtained the cumulative scores of these two groups (Supplementary Fig. 2C). In addition, we observed that the IL-35-treated group had lower cumulative scores than the control group. H&E staining of lesioned tissues revealed the severity of psoriatic disease in the control (PBS) group (Supplementary Fig. 2D, left panel). In contrast, we observed the reversal in ear thickness and reduction in leukocyte infiltration in the dermis and epidermis in the IL-35-treated group (Supplementary Fig. 2D, right panel). Moreover, we demonstrated that the IL-35-treated group exhibited a lower disease score based on the Baker scoring system compared to the control group (Supplementary Fig. 2E).

The IMQ-induced psoriasis mouse model is the most common model of psoriasis (Shih et al., 2020). Experimental procedures and the acquisition of mouse back skin images are shown in Supplementary Figures 3A and 3B. Based on the PASI scoring evaluation of erythema, scaling, and thickness, we examined the cumulative scores for different groups in the IMQ-induced psoriasis mouse model (Supplementary Fig. 3C). In particular, we noticed that the IMQ model group exhibited higher cumulative scores than the control group. We then used flow cytometry to detect any changes in the population of MDSCs in the spleen and skin tissues of mice in the IMQ-induced model; we found that the number of MDSCs in the spleen and skin tissues of mice was significantly higher than that in the control group (Supplementary Fig. 3D, $p < 0.001$ and $p < 0.01$, respectively). The results were consistent with those obtained using clinical samples.

We determined whether IL-35 exerts a therapeutic effect on the IMQ-induced psoriasis mouse model. The schedule of the delivery of IL-35 for the therapy of mice with IMQ-induced psoriasis is shown in Figure 3A. After treatment, we found that the control group had serious inflammation and skin flaking, which worsened by the end of the experiment (Fig. 3B). Based on the PASI scoring evaluation of erythema, scaling, and thickness (Fig. 3C), we calculated the cumulative scores of different groups (Fig. 3C). IL-35 group showed lower cumulative scores relative to the control group. In addition, H&E-stained skin sections showed less epidermal thickening in the IL-35 group compared with the control group (Fig. 3D). The Baker system-based disease score in the IL-35 treated group was significantly lower than that in the control group (Fig. 3E, $p < 0.001$).

3.4 IL-35 regulates inflammatory cytokine production in systemic and local immune microenvironment in IMQ-induced mice .

Inflammatory cytokines play important roles in the progression of psoriasis (Liang, Xu, Peng, Pan & Ye, 2014). ELISA was used to measure the levels of cytokines in serum and dorsal skin. Tissue cytokines from supernatants of extracted tissue protein were assayed and are presented in terms of picograms cytokine per milligram tissue (pg/mg). IL-17A (Fig. 4A), IL-23 (Fig. 4B), IL-1 β (Fig. 4C) and IL-6 (Fig. 4D) were detected in serum and skin tissue. Data are representative of three independent experiments. These results showed that, in addition to no significant difference in serum IL-6, other cytokines were significantly different in serum and skin tissue. Columns represent mean; bars represent SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ns, no significance.

3.5 Treatment with IL-35

reduced the number of recruited MDSCs

The above results showed that IL-35 had a clear effect in treating psoriasis and MDSCs were significantly expanded in patients with psoriasis and the mouse model. To test whether IL-35 ameliorates psoriasis by restricting the accumulation of MDSCs, we used flow cytometry to analyse the number of MDSCs in mice with IMQ-induced psoriasis with or without treatment of IL-35. The results showed that compared with that in the control group, administration of IL-35 reduced the infiltration of MDSCs in the spleen and skin tissues of mice (Fig. 5A and B). Then, we investigated whether the populations of G-MDSCs and M-MDSCs were altered in response to this therapy stress. We found that both G-MDSC and M-MDSC populations were significantly reduced in both the spleen and skin tissues (Fig. 5C-E). Finally, immunofluorescence staining of CD11b⁺Gr-1⁺ MDSCs in the inflamed skin showed that the MDSC accumulation was inhibited by IL-35 treatment (Fig. 5F). These results demonstrated that the immunosuppressive effect of IL-35 on the IMQ-induced psoriasis model was achieved by reducing the infiltration and proportion of MDSCs, and it can adjust the proportion of MDSC subtypes. Next, we examined whether IL-35 directly affects the differentiation and recruitment of MDSCs in vitro using fluorescence-activated cell sorting (FACS). IL-35 had little effect on the differentiation of MDSCs (Supplementary Fig. 3A, B IL-35 also did not significantly affect the numbers of G-MDSCs and M-MDSCs cells (Supplement Fig. 3C-E).

3.6 Adoptive transfer of MDSCs weakened the effect of IL-35 in mice with IMQ-induced psoriasis

To further explore whether the therapeutic effect of IL-35 was related to MDSCs, MDSCs from IMQ-induced mice were adoptively transferred following the experimental schedule (Fig. 6A). We found that the adoptive transfer of MDSCs derived from IMQ-induced mice aggravated disease progression. Moreover, when these MDSCs were transferred to the IL-35-treated group, the psoriasis symptoms were aggravated in these mice compared with those in mice treated with IL-35 alone (Fig. 6B). We evaluated pathological features in mice, such as erythema, scaling, and thickness, in different groups using the PASI scoring system (Fig. 6C). We observed that compared with the control (PBS) group, the PBS + MDSC group exhibited a higher PASI score. Similarly, the score of the IL-35 + MDSC group was significantly higher than that of the group treated with IL-35 alone. Furthermore, we calculated the cumulative scores from different groups and found that the IL-35-treated group exhibited the lowest cumulative scores than the other groups (Fig. 6C).

H&E-stained skin sections from these groups exhibited increased epidermal thickening in PBS + MDSC group relative to that in the control group. We also found that the skin epidermis in IL-35 + MDSC group was significantly thicker than that in the IL-35-treated group (Fig. 6D). In addition, the Baker system-based score of the IL-35-treated group was the lowest of these four groups (Fig. 6E).

3.7 IL-35 inhibited the expression of iNOS in MDSCs in mice with IMQ-induced psoriasis

The above results indicated that IL-35 inhibits the recruitment of MDSCs. Next, we investigate whether IL-35 affects the expression of inflammatory mediators in MDSCs. We used flow cytometry to analyse the expression of iNOS and IL-10, which were secreted by MDSCs in mice with IMQ-induced psoriasis with or without treatment of IL-35. The results showed that compared with the control group, the IL-35-treated group exhibited reduced expression of iNOS in the spleen and skin tissues (Fig. 7A and B). However, there was no difference in the level of IL-10 (Fig. 7C and D). Finally, immunofluorescence staining of iNOS⁺Gr-1⁺ MDSCs in the inflamed skin showed that the iNOS⁺Gr-1⁺ MDSC accumulation was inhibited by IL-35 (Fig. 7E).

3.8 Adoptive transfer of iNOS^{-/-} MDSCs had no effect on the function of IL-35 in mice with IMQ-induced psoriasis

To explore whether iNOS⁺ MDSCs play an important role in the pathogenesis of psoriasis, MDSCs from WT or iNOS^{-/-} IMQ-induced mice were adoptively transferred following the experimental schedule in Fig. 6A. The adoptive transfer of WT-MDSCs derived from mice with IMQ-psoriasis mice aggravated disease progression in the PBS group (PBS + WT-MDSCs). However, there was no exacerbation of symptoms when MDSCs from IMQ-induced iNOS^{-/-} mice were transferred to the PBS group (PBS + iNOS^{-/-}-MDSCs) (Fig. 8A). Moreover, when WT-MDSCs were transferred to the IL-35-treated group, the psoriasis symptoms were aggravated in these mice compared with those treated with IL-35 alone; however, there was no significant difference in conditions between the IL-35 + iNOS^{-/-}-MDSC and IL-35 treatment groups (Fig. 8A).

Subsequently, we evaluated the pathological features in mice, such as erythema, scaling, and thickness in different groups using the PASI scoring system (Fig. 8B). Compared with the PBS group, the PBS + WT-MDSC group exhibited a higher PASI score. Similarly, the score of the IL-35 + WT-MDSC group was significantly higher than that of the group treated with IL-35 alone. However, the score of the PBS + iNOS^{-/-}-MDSC group was similar to that of the PBS group. Furthermore, there was no significant difference in scores between IL-35 + iNOS^{-/-} - MDSC and IL-35 treatment groups. Finally, we calculated the cumulative scores from different groups and found that the score of PBS + iNOS^{-/-}-MDSC group was similar to that of the PBS group, and the IL-35-treated group exhibited the same cumulative score as IL-35 + iNOS^{-/-}- MDSC group (Fig. 8B). H&E-stained skin sections from these groups showed increased epidermal thickening, serious hyperkeratosis, and incomplete keratosis in the PBS + WT-MDSC group relative to that of the control group. PBS + iNOS^{-/-} - MDSC group exhibited reduced hyperkeratosis and incomplete keratosis compared to PBS + WT-MDSC group but almost the same morphology as that of PBS group. We also found that the thickness of the skin epidermis in the IL-35 + WT-MDSC group was significantly thicker than that in the IL-35-treated group; however, IL-35 + iNOS^{-/-}-MDSC group exhibited almost the same morphology as that of the IL-35 group (Fig. 8C). In addition, the Baker system-based score of the IL-35-treated group was

the lowest of these six groups (Fig. 8D), and these Baker scores were consistent with the phenotype of every group.

4. Discussion

Psoriasis, a common chronic inflammatory skin disease, is primarily mediated by the pathological crosstalk between immune cells and epidermal keratinocytes (Lowe, Suarez-Farinas & Krueger, 2014), which include infiltrating T-cells, macrophages, dendritic cells (DCs), MDSCs, and neutrophils (Chang et al., 1995; Liang, Sarkar, Tsoi & Gudjonsson, 2017; Soler et al., 2016). The IL-23/IL-17A Th17 axis has a crucial role in the development of psoriasis (Wu et al., 2018; Zhu et al., 2017).

MDSCs were originally identified by the CD11b⁺Gr1⁺ phenotype in tumour-bearing mice (Serafini, Borrello & Bronte, 2006). However, as the Gr1 gene homolog is lacking in humans, the surface markers of human MDSCs are different from those of mice; for instance, CD14⁺HLA-DR^{-/low} (Filipazzi et al., 2007). Cao et al. reported an increase in the number of M-MDSCs in patients with psoriasis and further studied the abnormal effects on their regulated function (Cao et al., 2016). Other studies have revealed a significant increase in the number of MDSCs in psoriasis; however, these cells also lack sufficient immunosuppressive functions (Ilkovitch & Ferris, 2016; Soler et al., 2016; Turrentine et al., 2014). Peng et al. reported that MDSCs play a proinflammatory role in IMQ-induced psoriasis-like skin inflammation by regulating the infiltration of CD4⁺ T-cells. The depletion of MDSCs by gemcitabine significantly suppressed the IMQ-mediated psoriatic phenotype (Chen et al., 2020), suggesting that targeting MDSCs might serve as a novel strategy for the treatment of psoriasis. Consistent with other studies, we also found an increased number of peripheral blood CD11b⁺CD14⁺HLA-DR^{-/low} MDSCs in patients with psoriasis compared with that in healthy controls. Concomitantly, we also found a significant increase in the number of MDSCs in the spleen and skin lesion of mice in the IMQ-induced psoriasis model.

IL-35 has been reported to play a critical role in several immune-associated diseases, such as autoimmune diseases, viral and bacterial infections, and tumours. Wirtz et al. reported that enteritis symptoms in an IBD mouse model were significantly alleviated following the vector-mediated overexpression of IL-35 (Wirtz, Billmeier, McHedlidze, Blumberg & Neurath, 2011). However, few studies have explored the role of IL-35 in the pathogenesis of psoriasis. Li et al. revealed that the serum levels of IL-35 were higher in patients with psoriatic arthritis than in patients with psoriasis and healthy controls (Li et al., 2017). Cardoso et al. did not find any differences between the levels of IL-35 in the serum of Brazilian patients with psoriasis and healthy controls (Cardoso et al., 2016). Wei et al. found that the expression of IL-35 in the peripheral blood of patients with psoriasis with vulgaris was lower than that in the control group (Chen, Du, Han & Wei, 2021). Similarly, Deng et al. revealed that IL-35 concentrations in plasma were lower in patients with psoriasis than in healthy individuals (Li et al., 2018). However, Placek et al. observed that the levels of IL-35 were higher in the serum of patients with psoriasis but without any statistically significant relationship with PASI scores (Owczarczyk-Saczonek, Czerwinska, Orylska & Placek, 2019). These studies primarily detected the expression of IL-35 in patients with psoriasis; however, there are few studies on its specific role. In this study, we found a significant increase in the serum levels of IL-35 in patients with psoriasis. In addition, the number of EB13⁺p35⁺ cells was significantly increased in the peripheral blood of psoriatic skin (Fig. 1). As an anti-inflammatory cytokine, IL-35 expression was significantly increased in patients with psoriasis, possibly to combat the severe inflammatory response of psoriasis.

We previously revealed that the overexpression of the IL-35 gene significantly inhibited the expression of proinflammatory factors in an *in-vitro* model of psoriasis and ameliorated the disease indexes of psoriasis in mouse models (Zhang et al., 2016). Similarly, we also found that the administration of IL-35 recombinant protein improved the severity of psoriasis in mice; however, the specific mechanism of IL-35 recombinant protein was not explored further (Wang et al., 2018). Our team previously found that IL-35 gene therapy can exert immunosuppressive functions by inhibiting the recruitment of macrophages in psoriatic mice and regulating the ratio between M1 and M2 to significantly improve the pathogenesis in the psoriatic mouse model. In addition, we found that IL-35 expression can significantly inhibit the proportion of CD11b⁺ myeloid cells (data not shown), suggesting that IL-35 may regulate myeloid cells. Therefore, in this study,

we focused on MDSCs.

In this study, a significant increase in the number of MDSCs was observed in the IMQ-induced psoriasis model (Supplemental Fig. 3). In contrast, the number of MDSCs was significantly decreased in mice with IMQ-induced psoriasis after treatment with IL-35 recombinant protein. Furthermore, we found that G-MDSC and M-MDSC counts were reduced in both the spleen and skin tissues in the IL-35-treated group compared with that in the control group (Fig. 5). Despite the significant reduction in the populations of MDSCs in IL-35-treated mice with IMQ-induced psoriasis, IL-35 did not play a direct role in the differentiation of MDSCs, which indicated that MDSC differentiation is potentially due to the regulation of the immune response in mice.

As mentioned, MDSCs are known to play deleterious roles in the progression of cancer and infectious diseases; however, their role in autoimmune diseases appears to be more complex (Veglia, Perego & Gabrilovich, 2018). Herein, to further study whether the role of MDSCs is to promote or inhibit inflammation, we conducted experiments of adoptive transfer of MDSCs from IMQ-induced mice during treatment with IL-35. We found that the adoptive transfer of MDSCs weakened the effect of the treatment of IMQ-induced psoriasis with IL-35 (Fig. 6). The results further support the study by Cao et al. (Cao et al., 2016). iNOS and IL-10 levels were significantly elevated in tumour-induced MDSCs, which indicates their immunosuppressive function in cancer (Hart, Byrne, Molloy, Usherwood & Berwin, 2011; Redd et al., 2017). However, there are few studies on the role of IL-10 and iNOS secreted by MDSCs in autoimmune diseases. Herein, we found that the population of MDSCs secreting iNOS, but not IL-10, were significantly increased in the IMQ-induced mouse psoriasis model. Furthermore, the adoptive transfer of MDSCs from IMQ-induced mice weakened the anti-inflammatory effects of the treatment with IL-35 in IMQ-induced psoriasis; however, this weakened effect was reversed by MDSCs from IMQ-induced iNOS^{-/-} mice. We consider that the elevated numbers of MDSCs do not necessarily play an immunosuppressive function in the IMQ-induced psoriasis mouse model. In addition, it is plausible that the immunosuppressive functions of MDSCs are impaired by secreting iNOS, leading to the promotion of immune reactions.

In summary, the administration of IL-35 attenuated the psoriasis-like skin inflammation in psoriasis mice. Mechanistic studies have revealed that IL-35 reduces the severity of psoriasis in mice by inhibiting pro-inflammatory cytokines in the skin microenvironment, suppressing the recruitment of MDSCs and the population of MDSC-secreting iNOS. This role of IL-35 has not been reported thus far. We hypothesized that IL-35 may inhibit the recruitment of MDSCs and the ability of MDSCs to secrete iNOS by affecting inflammatory factors in the skin microenvironment, corresponding mechanism diagram as illustrated in Figure 9. Hence, we conclude that supplemental treatment with IL-35 would be helpful in the management of psoriasis, and this study highlights a new therapeutic strategy for other cutaneous inflammatory diseases.

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Figure legends

Fig. 1 Expression of IL-35 increased in psoriatic serum and peripheral blood specimens . (A) Levels of expression of IL-35 in the serum of patients with psoriasis (n = 49). Psoriasis Area and Severity Index (PASI) < 3 (n = 19), PASI [?] 3 (n = 19), or control (n = 20) were measured by ELISA. Obtained values from individual controls and patients are plotted as dots. (B) The number of CD4⁺EBI3⁺p35⁺ and CD19⁺EBI3⁺p35⁺ cells in the peripheral blood of patients with psoriasis was detected by flow cytometry. (C-D) Flow cytometry proportional analysis results in B. (E) Immunofluorescence staining of IL-35 (p35⁺EBI3⁺) in patients (n = 7) and control (n = 5) skin lesions. Red represents anti-p35 Ab, green represents anti-EBI3 Ab, yellow represents p35 and EBI3 merged, and blue represents 4',6-diamidino-2-phenylindole (DAPI). Original magnification 400×. *p < 0.05, **p < 0.01, ***p < 0.001.

Fig. 2 Expansion of monocytic myeloid-derived progenitor cells(MDSCs) in the blood of patients with psoriasis. (A) Peripheral blood mononuclear cells (PBMCs) freshly isolated from blood samples of healthy donors (control) or patients with psoriasis (psoriasis) were analysed for the expression of HLA-DR and CD14, with CD11b⁺CD14⁺HLA-DR^{no/low} cells being gated (shown by a small window) (B) Mononuclear MDSCs (M-MDSCs) among PBMCs are summarised in scatter graphs, (C) Immunofluorescence staining of M-MDSCs (CD14⁺HLA-DR⁺) in patients (n = 7) and control (n = 5) skin lesions. Red represents anti-CD14 Ab, green represents anti-HLA-DR Ab, yellow represents CD14 and HLA-DR merged, and blue represents 4',6-diamidino-2-phenylindole (DAPI). Original magnification 400x. **p < 0.01.

Fig. 3 Therapy with IL-35 recombinant protein weakens the inflammatory process in the imiquimod (IMQ)-induced psoriasis mouse model . (A) Schedule of the delivery of IL-35 for the treatment of mice with IMQ-induced psoriasis. (B) Phenotype of murine skin with IMQ-induced psoriasis after therapy (n = 6). (C) Individual psoriasis area and severity index (PASI) scores of erythema, scaling, and thickness as well as cumulative PASI scores. (D) Hematoxylin and eosin (H&E)-stained skin sections from IMQ-treated mice, 200x and 400x (the 400x image is the enlarged image in the box in the 200x image). (E) Pathological scores of skin sections using the Baker scoring system. Columns represent the mean, and bars represent the standard deviation (SD). ***p < 0.001. This experiment was repeated three times.

Fig. 4 IL-35 inhibited secretion of inflammatory cytokines in serum and local lesions of imiquimod (IMQ)-induced mice. Several proinflammatory cytokines were detected by ELISA in serum and skin tissue after IL-35 treatment. Tissue cytokines from supernatants of extracted tissue protein were assayed and are presented in terms of picograms cytokine per milligram tissue (pg/mg). (A) IL-17A, (B) IL-23p40, (C) IL-1 β , and (D) IL-6. Data are representative of three independent experiments. Columns represent mean, and bars represent SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ns, no significance.

Fig. 5 IL-35 suppresses the infiltration of myeloid-derived progenitor cells (MDSCs) in mice with imiquimod (IMQ)-induced psoriasis. (A) Representative fluorescence-activated cell sorting (FACS) plots of MDSCs (CD11b⁺Gr-1⁺) in the spleen and skin tissues (gated on CD45 events) at 24 h after the last delivery of IL-35. (B) Statistics of flow cytometry results in A. (C) Representative FACS plots of granulocytic MDSCs (G-MDSCs) (CD11b⁺Ly6G⁺Ly6C^{low}) and mononuclear MDSCs (M-MDSCs) (CD11b⁺Ly6G⁺Ly6C^{high}) in the spleen and skin tissues (gated on CD45⁺ events). (D-E) Quantification analyses of flow cytometry results in c. (F) Immunofluorescence staining of infiltrated CD11b⁺Gr-1⁺ MDSCs in murine skin lesions. Green represents anti-CD11b Ab, red represents anti-Gr-1 Ab, yellow represents CD11b and Gr-1 merged, and blue represents 4',6-diamidino-2-phenylindole (DAPI). Original magnification 400 $\times$. $n = 6$, Columns represent the mean, and bars represent the standard deviation (SD). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ns, no significance.

Fig. 6 Adoptive transfer of myeloid-derived progenitor cells(MDSCs) weakens the effect of treatment of imiquimod (IMQ)-induced psoriasis with IL-35. MDSCs from mice with IMQ-induced psoriasis were adoptively transferred, following the experimental schedule (A). (B) The representative phenotype of the murine skin with IMQ-induced psoriasis after the adoptive transfer of MDSCs. (C) Individual psoriasis area and severity index (PASI) scores of erythema, scaling, and thickness as well as cumulative PASI scores. (D) Hematoxylin and eosin (H&E) stain was used to stain the skin sections from all four groups, 200 $\times$ and 400 $\times$ (the 400 $\times$ image is the enlarged image in the box in the 200 $\times$ image). (E) Scores of each group are based on the Baker scoring system. $n = 6$. Columns represent the mean, and bars represent the standard deviation (SD). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ns, no significance.

Fig. 7 IL-35 inhibited the expression of inducible nitric oxide synthase (iNOS) in myeloid-derived progenitor cells (MDSCs). IL-35 protein was used to treat imiquimod (IMQ)-induced psoriasis mouse model, and the expression of iNOS and IL-10 in MDSCs was detected. Fluorescence-activated cell sorting (FACS) analysis of iNOS and IL-10 expression in the spleen were shown in A and C (gated on CD11b⁺Gr-1⁺ events), and the results of their expression analysis in the skin were shown in (B) and (D) (gated on CD45⁺CD11b⁺Gr-1⁺ events). E, Immunofluorescence staining of infiltrated iNOS⁺Gr-1⁺ MDSCs in skin lesions. Green represents anti-iNOS Ab, red represents anti-Gr-1 Ab, yellow represents iNOS and Gr-1 merged, and blue represents 4',6-diamidino-2-phenylindole (DAPI). Original magnification 400 $\times$. $n = 5$. Columns represent the mean, and bars represent standard deviation (SD). * $p < 0.05$, ** $p < 0.01$. ns, no significance.

Fig. 8 Adoptive transfer of inducible nitric oxide synthase (iNOS)^{-/-} myeloid-derived progenitor cells(MDSCs) have no effect on the function of IL-35 in mice with imiquimod (IMQ)-induced psoriasis. MDSCs from WT and iNOS^{-/-} mice with IMQ-induced psoriasis were adoptively transferred. (A) The representative phenotype of the murine skin with IMQ-induced psoriasis after the adoptive transfer of WT-MDSCs and iNOS^{-/-}-MDSCs. (B) Individual psoriasis area and severity index (PASI) scores of erythema, scaling, and thickness as well as cumulative PASI scores. (C) Hematoxylin and eosin (H&E) stain was used to stain the skin sections from all six groups, 200 $\times$ and 400 $\times$ (the 400 $\times$ image is the enlarged image in the box in the 200 $\times$ image). (D) Scores of each group are based on the Baker scoring system. $n = 5$. Columns represent the mean, and bars represent standard deviation (SD). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ns, no significance.

Fig. 9 Schematic summary of possible mechanisms of IL-35 amelioration of psoriasis. This mechanism is associated with IL-35 can inhibit inflammatory factors in the skin microenvironment of psoriasis, and these inflammatory factors can induce the differentiation and recruitment of MDSCs, so that IL-35

can indirectly inhibit the recruitment and secretion of *iNOS* of MDSCs.

Supplementary Data

1. Materials and methods

1.1 Construction of an in-vitro psoriatic model

All experimental protocols used in this study were approved and implemented in accordance with the guidelines of the review board of Jining Medical University. Human keratinocyte cell line (HaCaT) cells were maintained in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10 % foetal bovine serum (FBS, Thermo Fisher Scientific), 100 µg/mL streptomycin (Thermo Fisher Scientific), and 100 U/mL penicillin (Thermo Fisher Scientific). Inflammation was induced in HaCaT cells following their stimulation with M5 (mixture of five proinflammatory cytokines including TNF-α, IL-17A, IL-22, IL-1α, and oncostatin-M at 10 ng/mL each; ProSpec, East Brunswick, NJ), thereby mimicking various characteristics of psoriasis (Guilloteau et al., 2010). Briefly, HaCaT cells were seeded in 6-well plates and cultured for 12 h. After 12 h of culture, cells were treated with different concentrations of IL-35 (0, 50, 100, or 200 ng/mL) and stimulated with M5, and the secretion of IL-6 and CXCL8 was determined. To test the levels of secreted IL-6 and CXCL8, culture supernatants were collected at 24, 48, and 72 h post-stimulation. The HaCaT cell line was provided by Professor Jiong Li from the State Key Laboratory of Biotherapy and Cancer Center (Chengdu, China).

1.2 K14-VEGF-A-Tg mouse psoriasis model and therapy

All animal experiments were performed in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. K14-VEGF-A-Tg homozygous mice overexpressing VEGF in the epidermis were used for the experiments, and these mice spontaneously developed a chronic inflammatory skin disease with many features similar to those observed in human psoriasis (Xia, Li, Hylton, Detmar, Yancopoulos & Rudge, 2003). These mice were provided by Professor Jiong Li from the State Key Laboratory of Biotherapy and Cancer Center (Chengdu, China). K14-VEGF-A-Tg homozygous mice (10 weeks old, psoriasis-like symptoms began to appear) were randomly assigned to two groups (n = 5 per group). Each mouse was subjected to a total of 10 intravenous (i.v.) injections of either phosphate-buffered saline (PBS) (control) or 5 µg IL-35 recombinant protein (10705-H02H, Sino Biological, Beijing, China) every other day. At 24 h after the last injection, the animals were anaesthetised using 50 µL of chloral hydrate (10 %; intraperitoneal injection, Tianjin Regent Chemicals Co., LTD, Tianjin, China) for macroscopic photography. The animals were then euthanised, and psoriatic lesion tissues were collected. All procedures were performed at the Jining Medical University.

1.3 Preparation of bone marrow-derived MDSCs in vitro

Bone marrow cells were isolated from mice by flushing their femurs and tibiae, centrifuging the cell-containing elution (1800 rpm, 5 min), and resuspending them in complete Roswell Park Memorial Institute (RPMI) 1640 (Thermo Fisher Scientific) medium supplemented with IL-6 (40 ng/mL, Peprotech, Suzhou, Jiangsu, China) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (40 ng/mL, Peprotech). Cells were maintained at 37 °C in a 5 % CO₂ humidified atmosphere for 4 d. To investigate the effect of IL-35 on the expansion of MDSCs, IL-35 (Sino Biological, Beijing, China), IL-6, and GM-CSF were added to the medium at the same time.

2. Figure Legend

Supplementary Fig. 1 IL-35 recombinant protein affects the production of IL-6 and CXCL8 in M5-stimulated HaCaT cells. HaCaT cells were seeded in 6-well plates and cultured for 12 h. After 12 h of culture, different concentrations of IL-35 (0, 50, 100, or 200 ng/mL) were added to the cells, which were further stimulated with M5 (10 ng/mL). The secretion of CXCL8 and IL-6 was detected by ELISA. (A) Expression of CXCL8 and (B) IL-6. *p < 0.05, **p < 0.01, ***p < 0.001. This experiment was repeated three times.

Supplementary Fig. 2. IL-35 recombinant protein alleviated pathological characteristics of psoriatic lesions in K14-VEGF-A-Tg mice. (A) Schedule of the therapeutic delivery of IL-35. (B) The phenotype of the mouse ear after therapy ($n = 5$). (C) Individual psoriasis area and severity index (PASI) scores of erythema, scaling, and thickness as well as cumulative PASI scores. (D) Hematoxylin and eosin (H&E) staining of the murine ear skin (original magnification $\times 200$ and $400\times$, the $400\times$ image is the enlarged image in the box in the $200\times$ image). (E) Pathological score of ear sections using the Baker scoring system. Columns represent the mean, and bars represent the standard deviation (SD). *** $p < 0.001$. This experiment stands for an independent experiment.

Supplementary Fig. 3 Populations of myeloid-derived progenitor cells (MDSCs) were significantly increased in mice with imiquimod (IMQ)-induced psoriasis. (A) Experimental procedures and establishment of IMQ-induced psoriasis in mice. (B) Images of murine back-skin specimens. (C) Psoriasis area and severity index (PASI) score evaluation of erythema, scaling, and thickness, and cumulative scores for different groups in the mouse model of IMQ-induced psoriasis. (D) Detection of MDSCs in the spleen and skin tissues via flow cytometry and subsequent quantification analyses. ** $p < 0.01$, *** $p < 0.001$.

Supplementary Fig. 4 Effect of IL-35 on the expansion of myeloid-derived progenitor cells (MDSCs) induced by granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-6 in vitro. (A-E) Bone marrow cells pre-treated with different concentrations of IL-35 (100, 200, and 400 ng/mL) or vehicle and were cultured with murine GM-CSF (40 ng/mL) and IL-6 (40 ng/mL). After four days, the numbers of $CD11b^+Gr-1^+$ MDSCs (A, B), $CD11b^+Ly6G^+Ly6C^{low}$ granulocytic MDSCs (G-MDSCs) (C, D), and $CD11b^+Ly6G^+Ly6C^{high}$ M-MDSCs (C, E) were analysed via fluorescence-activated cell sorting (FACS). Data are representative of three biological replicates, each using three technical replicates. ns, no significance.

















