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Indirubin ameliorates imiquimod-induced psoriasis-like skin lesions in mice by inhibiting inflammatory responses mediated by IL-17A-producing γδ T cells

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ABSTRACT

Objectives: Indirubin (IR) is a bisindole compound extracted from the leaves of Chinese herb *Indigo Naturalis*. *Indigo Naturalis* has been widely used in traditional Chinese medicine to treat inflammatory and autoimmune diseases. Psoriasis is a chronic immune-mediated inflammatory skin disease in which γδ T cells play an important role. This study aims to determine the immunoregulatory effects and the underlying mechanisms of Indirubin in psoriasis-related inflammatory responses.

Methods: BALB/c mice with imiquimod (IMQ)-induced psoriasis-like dermatitis were treated with saline (Model), 1 mg/kg methotrexate (MTX) that serves as a positive control, or 12.5, 25 and 50 mg/kg Indirubin(IR) intragastrically. Keratinocytes proliferation, inflammatory cells infiltration, the expression of inflammatory cytokines and Jak/Stat pathway-related proteins in the skin lesion were examined. The abundance of γδ T cells in lymph nodes and spleen was determined by flow cytometry. The IL-17 expression and secretion, and the activation of Jak3/Stat3 pathways in *in vitro* cultured γδ T cell were tested.

Results: Indirubin ameliorated keratinocyte proliferation, reduced the infiltration of $CD3^+$ T cells, IL-17 A-producing γδ T cells, and $CD11b^+$ neutrophils, inhibited the mRNA expression of *Il1*, *Il6*, *Il23*, *Il17a* and *Il22*, and the protein expression of Jak/Stat pathway-related molecules in the skin lesion. Indirubin also reduced the abundance of γδ T cell and $CCR6^+$ γδ T cells (the major IL-17 A producer) in spleen and lymph nodes. In cultured γδ T cells, Indirubin inhibited the mRNA expression of *Il17a* and *Ifng*, and the secretion of IL-17 A, while suppressed the activation of Jak3/Stat3 pathways.

Conclusion: Indirubin alleviates IMQ-induced psoriasis-like dermatitis mainly through reducing the inflammatory responses mediated by IL-17 A-producing γδ T cells involving Jak3/Stat3 activation. Our results highlighted the novel mechanisms by which Indirubin ameliorates psoriasis-related inflammatory responses, supporting its therapeutic potential.

1. Introduction

Psoriasis is a chronic immune-mediated inflammatory skin disease characterized by scaly erythematous plaques that may cover large body areas, abnormal keratinocyte proliferation, dermal angiogenesis, and immune cell infiltration (Boehncke and Schön, 2015). The reported prevalence of psoriasis ranges between 0.09%–11.43% worldwide, representing a significant public heath challenge (World health organization, 2016). It has been widely recognized that IL-23/IL-17 cytokine axis plays a central role in the development and progression of psoriasis, while IL-23/IL-17 inhibitors have demonstrated therapeutic benefits (Toussirot, 2012). IL-17 is the hallmark cytokine of T helper 17 cells (Th 17), a well-known mediator in the immunopathogenesis of psoriasis (Fujiyama et al., 2016; Zhao et al., 2016). However, recent studies have highlighted the novel role of IL-17-producing γδ T cells in psoriasis (Hartwig et al., 2015; Cai et al., 2011; Shibata et al., 2015; Ramirez-Valle et al., 2015), and the involvement of Jak/Stat signaling pathways downstream of IL-17 (Alves de Medeiros et al., 2016; Samadi et al., 2017).

Indirubin, a bisindole compound (Fig. 1), is the active ingredient extracted from the leaves of *Indigo naturalis*. *Indigo naturalis* has been commonly used in traditional Chinese medicine to treat autoimmune

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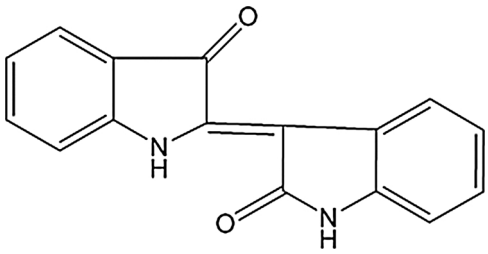

Fig. 1. The chemical structure of Indirubin. Chemical name: 2H-Indol-2-one, 3-(1,3-dihydro-3-oxo-2H-indol-2-ylidene)-1,3-dihydro- (9CI).

diseases and inflammatory diseases (McDermott et al., 2016; Zhang et al., 2015). Clinical studies have supported the application of indigo naturalis ointment as an effective treatment for psoriasis (Lin et al., 2018), but the underlying mechanisms remain incompletely understood. Several studies have focused on the effects of Indirubin on keratinocytes. Indirubin inhibited the proliferation of *in vitro* cultured epidermal keratinocytes by suppressing EGFR activation (Hsieh et al., 2012). The extracts of *indigo naturalis* also upregulated the mRNA and protein expressions of claudin-1 and improved the function of tight junction in primary human keratinocytes (Lin et al., 2013). However, the effects of Indirubin on immune cell-mediated inflammation in psoriasis have not been thoroughly characterized. In light of the emerging role of immune cells, in particular γδ T cells in the pathogenesis of psoriasis, and the broad ethnomedicinal application of *Indigo naturalis* in inflammatory diseases, this study aims to determine the role of Indirubin, the major active ingredient of *Indigo naturalis,* in regulating γδ T cells-mediated inflammatory response in psoriasis both *in vivo* and *in vitro*.

Using an imiquimod (IMQ)-induced psoriasis-like mouse model and *in vitro* cultured γδ T cells, we will determine the effects of Indirubin on: (1) the morphological features, inflammatory cells infiltration, inflammatory cytokines expression, and Jak/Stat pathway activation in the skin lesion; (2) the abundance of γδ T cells in the lymph nodes and spleen; (3) The expression and secretion of IL-17, and the activation of Jak3/Stat3 pathway in cultured γδ T cells. This study will provide novel insights into the mechanisms of Indirubin in ameliorating psoriasis through regulating γδ T cell-mediated inflammatory responses.

2. Materials and methods

2.1. Animals

BALB/c mice (male, 18 to 20 g, 8-week-old) were obtained from National Institutes for Food and Drug Control (certification NO.SCXK Jing 2014-0013), and housed under specific pathogen-free conditions, with access to food and water. All experiments adhered to the principles of the Declaration of Helsinki and were approved by the Beijing Institute of Traditional Chinese Medicine.

2.2. Establishment of psoriasis-like mouse models

Mice were divided into six groups and ten animals in each group were included. Mice in all the groups except those in the control group received a daily topical dose of 42 mg of commercially available imiquimod (IMQ, 5%, Mingxinlidi Laboratory, China) on those with shaved back for 7 consecutive days to establish a model of IMQ-induced psoriasis. While mice in the control group and model group were given normal saline by intragastric administration. Indirubin (IR, National Institutes for Food and Drug Control, China), was first dissolved in dimethyl sulfoxide (DMSO), then diluted with normal saline to achieve a final DMSO concentration of < 0.1% by intragastric administration. In the control group and model group, DMSO was used at amounts similar to those of the treatment groups. The Indirubin-high group received a dose of 50 mg/kg/day for 7 days, the Indirubin-medium group received a dose of 25 mg/kg/day for 7 days, the Indirubin-low group received a dose of 12.5 mg/kg/day for 7 days, the methotrexate (MTX) group received methotrexate dissolved in normal saline at 1 mg/kg/day for 7 days, a drug used for psoriasis treatment (West et al., 2016; Combe et al., 2016). The severity of inflammation of the back skin was monitored and graded using a modified human scoring system Psoriasis Area Severity Index (PASI) (van der Fits et al., 2009). Erythema, scaling, and thickening were scored independently on a scale from 0 to 4(0, none; 1, slight; 2, moderate; 3, marked; 4, very marked). The score for each group was averaged, and trend lines were generated to observe the changes in the mouse skin lesions. The cumulative score was used as a total score by erythema plus scaling plus thickening (scale 0–12).

2.3. Isolation of γδ T cells

Spleen samples from C57BL/6 mice [18–20 g, Beijing HFK Bioscience (China), with certification NO. SCXK Jing 2014-0004] were minced on a 200-mm mesh gauze to obtain single-cell suspensions. Total splenic T cells were purified by negative selection with the mice native γδ T Cell Isolation Kit (MiltenyiBiotec, Germany) and 'The Big Easy' Magnet (EasySep, USA) following the manufacturer's instructions. γδ T cells were seeded in 6 or 12 well plates containing anti-CD28 (2 μg/ml, BD Pharmingen, USA) antibodies at 1 or 5 × 10^6 cells/well in RPMI 1640 medium (Hyclone, Carlsbad, CA) containing 10% inactivated fetal bovine serum (Gibco, Grand Island), IL-1β (10 ng/ml, Peprotech, USA) and IL-23 (100 ng/ml, eBioscience, USA) for 48 h.

γδ T Cell cells were divided into different groups: (1) Neutral group, with cells only cultured in wells coated anti-CD28; (2) Model group, where cells were cultured in wells coated anti-CD28 with RPMI-1640 medium containing IL-1β and IL-23; (3) IR group, where cells were cultured in wells coated anti-CD28 with RPMI-1640 medium containing IL-1β, IL-23 and IR with indicated concentration.

2.4. Histology and immunohistochemistry and immunofluorescence staining

Tissue staining was performed on 5 μm paraffin sections of mouse psoriasis skin specimens. Partial sections were stained with H&E for pathological observation by light microscopy. Epidermal thickness was accurately measured by the ImagePro Plus software (Leeds Precision Instruments, Minneapolis, MN, USA). Partial sections were stained with proliferating cell nuclear antigen (PCNA, 1:500, Abcam, USA), anti-Rabbit CD3 (1:100, Abcam, USA), γδ TCR (1:50, Abcam, USA), anti-IL-17 antibody (1:500, Abcam, USA) and CD11b antibodies (1:200, Abcam, USA). DAB (Zhongshan Golden Bridge Biotechnology, China) was used for color development. Slides were observed with Zeiss Axio Imager : M2 (Germany) and semi-quantification of the staining was performed independently by two researchers using IPP6.0. Each index was presented as integrated optical density (IOD).

2.5. Flow cytometry analysis

Inguinal lymph nodes samples and spleen samples from each group were minced through a 70-mm mesh to obtain single cell suspensions. Cells were stained with anti-CD196-APC and administered anti-γδ TCR-FITC antibodies (BD Pharmingen, USA). The relevant isotype control mAbs were also used. Samples were acquired on a flow cytometer (FACScanto; BD Biosciences, USA) and analyzed using Cell Quest software (BD Biosciences).

2.6. IL-17 A ELISA

The quantitative detection of IL-17 A in the culture medium were determined using enzyme-linked immunosorbent assay (ELISA) kits (BMS6001, eBioscience, USB) with standard curves made from purified recombinant IL-17 A at various dilutions.