







Original Research

# Exploring the Potential Role of Interleukin-33 in Inducing Eosinophil-Mediated Fibrosis in Hepatic Alveolar Echinococcosis

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## Abstract

**Background:** Alveolar echinococcosis (AE) is a serious zoonotic parasitic disease. This study aimed to investigate the mechanisms underlying the formation of the dense fibrotic band surrounding hepatic alveolar echinococcosis (HAE) lesions, which impedes chemotherapeutic drug penetration. Additionally, the roles of Interleukin-33 (IL-33) and eosinophils in the progression of fibrosis within this band were examined. **Methods:** IL-33/suppression of tumorigenicity 2 (ST-2) expression levels were compared between patients with HAE and healthy controls, as well as between close to lesion tissues (CLT) and distant from the lesion tissues (DLT) using enzyme-linked immunosorbent assay (ELISA), western blot, and immunohistochemistry. Immunofluorescence co-localization analysis was performed to examine IL-33/ST-2 and eosinophil distribution. Masson's trichrome staining was used to evaluate fibrosis in AE lesions. Cellular assays were carried out to assess the effects of IL-33 on eosinophil phagocytosis and migration, as well as its impact on  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) expression in hepatic stellate cells (HSCs). **Results:** ELISA findings indicated significantly elevated serum IL-33/ST-2 levels in patients with AE compared with healthy controls ( $p < 0.05$ ). Immunohistochemistry and western blot analyses demonstrated higher IL-33/ST-2 expression in CLT than in DLT ( $p < 0.05$ ), with IL-33/ST-2 and eosinophils exhibiting a highly consistent distribution within CLT. Masson's trichrome staining confirmed increased fibrosis in CLT. Cellular assays showed that IL-33 enhanced eosinophil phagocytosis and migration, while IL-33 stimulation upregulated  $\alpha$ -SMA expression on the HSC surface, with this effect being more pronounced in the presence of eosinophils. **Conclusion:** IL-33 contributed to microenvironmental fibrosis within AE lesions via eosinophil-mediated mechanisms, highlighting a potential therapeutic target to improve chemotherapy efficacy in patients with AE.

**Keywords:** alveolar echinococcosis; eosinophils; hepatic fibrosis; IL-33/ST-2 signaling pathway; lesion microenvironment

## 1. Introduction

Alveolar echinococcosis (AE) is a zoonotic parasitic disease caused by the larval stage of *Echinococcus multilocularis*, predominantly found in the Northern Hemisphere, particularly in China, Central Asia, Russia, Europe, and North America [1,2]. AE poses a significant threat to human health and has substantial implications for livestock development, primarily affecting the liver [3]. The primary management strategies for AE include imaging-based diagnosis and surgical removal of the parasitic lesion. Benzimidazole derivatives, particularly albendazole, are used as adjunctive therapy; however, their long-term administration is associated with adverse side effects [4,5].

Immunological studies indicate a complex interaction between the host immune system and the parasite, influencing disease progression [6,7]. AE research has found that there is a delicate balance between the host's self-protection and the growth of the parasite body, in which the immuno-

logical regulatory mechanism plays an important role [6]. Eosinophils, as key innate immune cells, play diverse roles in parasitic infections and are regulated by interleukin-5 (IL-5) and the receptor siglec-8 [7,8]. Interleukin-33 (IL-33) functions as both a nuclear factor and a cytokine involved in immune regulation. ST-2, as the receptor of IL-33, is widely distributed on the surface of eosinophils. The mRNA encoding *ST-2* generates three splice variants: sST-2 (soluble form), ST-2L (transmembrane form), and ST-2V (variant form), with sST-2 functioning as a decoy receptor that downregulates IL-33 activity. A previous study has indicated that polymorphisms in the *IL-33* and *ST-2* genes correlate with increased eosinophil levels in humans [9].

*In vivo* studies have demonstrated that IL-33 induces eosinophilia by activating eosinophils. As primary effector cells in allergic and inflammatory diseases, eosinophils exhibit increased superoxide production, enhanced adhesion molecule expression, and prolonged survival [10–



12]. Consequently, IL-33 plays a critical role in basophil- and eosinophil-mediated allergic diseases and inflammatory responses. Elevated serum IL-33 levels in acute schistosomiasis suggest its pro-inflammatory role during the acute phase, with levels closely correlating with eosinophil counts and disease progression [13]. Nine weeks following a single-dose praziquantel treatment, patients with schistosomiasis exhibit significantly elevated serum IL-33, along with elevated eosinophil counts, IL-13 levels, and enhanced ST-2 expression on eosinophils [14]. Studies have found that eosinophils are involved in the occurrence of allergic reactive diseases such as asthma and allergic conjunctivitis mainly through the binding of IL-33 to the ST-2 receptor on the cell membrane surface, thereby promoting the proliferation, activation and degranulation effect of eosinophils [15]. IL-33 was significantly elevated in the serum of patients with Schistosomiasis japonicum and infected mice. Moreover, studies have shown that eosinophil count and the course of schistosomiasis or other inflammatory diseases are closely related to IL-33 levels [13,16,17]. In a *Schistosoma mansoni* egg-induced mouse lung granuloma model, ST-2 deficiency resulted in reduced production of T-helper type 2 (Th2) cytokines, including interleukin-4 (IL-4) and interleukin-5 (IL-5), as well as decreased eosinophil infiltration within granulomas [18]. Study on filariasis have demonstrated that IL-33/ST-2 signaling is essential for the splenic clearance of microfilariae from peripheral blood [19]. From what has been discussed above, we know the IL-33/ST-2 signaling pathway thus exerts diverse biological effects across various infection models, tissues, and disease stages, thereby influencing disease progression and outcomes.

Hematoxylin-eosin (HE) and Masson's trichrome staining of AE lesions reveals dense inflammatory fibrous bands that, while limiting lesion expansion, also hinder drug penetration, posing challenges for chemotherapy [20–22]. In the context of fibrosis, IL-33 has been identified as a key regulator of fibrosis development. Hepatic stellate cells (HSCs), the primary collagen-producing cells in liver injury, represent the main source of IL-33 [23]. Studies have indicated that IL-33 expression is upregulated in both mouse and human hepatic fibrosis [24,25]. Research indicates that elevated IL-33 expression alone can drive extensive collagen fiber formation and extracellular matrix (ECM) deposition, whereas IL-33 gene-deficient mice exhibit significantly reduced hepatic fibrosis [26]. These findings highlight the crucial role of IL-33 in liver fibrosis.

Therefore, in order to explore how IL-33 induces peripheral fibrosis in AE lesions and verify whether IL-33 changes the fibrosis process of AE lesions by altering eosinophil behavior, we conducted the following experiments. enzyme-linked immunosorbent assay (ELISA), immunohistochemical staining, and Western blot analysis were performed to assess ST-2 and IL-33 protein expression in the tissue surrounding AE lesions. Multiple immunofluo-

rescence co-localization analysis was conducted to examine the co-expression of eosinophils with these proteins, while Masson's trichrome staining was used to quantify hepatic fibrosis within AE lesions. Additionally, *in vitro* cell experiments were conducted to investigate the relationship between IL-33, eosinophils, and fibrosis. These methodologies facilitated a detailed characterization of the ST-2/IL-33 signaling pathway in local immune responses and fibrotic progression in echinococcosis through eosinophil activation, providing a theoretical basis for potential advancements in prevention and treatment strategies.

## 2. Materials and Methods

### 2.1 Materials

The included subjects were all patients with hepatic alveolar echinococcosis diagnosed for the first time, who met the requirements of surgery and did not undergo drug chemotherapy. The tissues were mainly from the surgical specimens of AE patients, and a total of 21 tissue specimens were collected. All 21 (12 females/9 males, mean age was 32 years) AE patients were initially diagnosed by B-ultrasound and were eligible for surgery for the first time. The surgically resected tissues of patients undergoing AE surgery have a complete structure, including lesion tissues, close to lesion tissues (<3 cm from the lesion, CLT), and distant from the lesion tissues (>3 cm from the lesion, DLT). Tissue samples were analyzed by western blotting (WB), immunohistochemistry, immunofluorescence and Masson. 12 blood specimens were obtained from the patients before the above-mentioned surgeries for ELISA.

### 2.2 Isolation of Peripheral Blood Eosinophils

50 mL of healthy volunteers' venous blood was diluted with PBS 1:1, and the diluent was slowly added into the centrifuge tube containing Percoll separation solution (Solarbio, Beijing, China, density  $1.130 \pm 0.005$  g/mL) at 2:1. After centrifugation, the target cell layer was collected under the Percoll layer. Objective after destroying the red blood cells, the cells were centrifuged at 4 °C, 1500 rpm and 5 min to collect the bottom cells containing eosinophils and neutrophils. PBS was washed and counted. 50  $\mu$ L anti-CD16 immunomagnetic beads (Cat. #: 130-092-010, Miltenyi biotech, Bergisch Gladbach, Germany) were added to  $5 \times 10^7$  cells, mixed well, and incubated in a shaker at 4~8 °C for 30 minutes. After incubation, the cell antibody solution was added to the LS magnetic column (Cat. #: 130-090-544, Miltenyi biotech, Bergisch Gladbach, Germany) and the eluate was collected. At 4 °C, 1500 rpm (400 g), centrifuge for 5 min, discard the supernatant, and resuspend the cells with 1 mL RPMI-1640 (containing 10% FCS), which is the eosinophil (Eos) obtained from separation and purification.

### 2.3 Acquisition of *E. multilocularis* Antigen

The lesions of *Echinococcus multilocularis* were isolated from Mongolian gerbils infected with *Echinococcus multilocularis* preserved in our laboratory. Mongolian gerbils were sacrificed (After anesthesia with isoflurane (concentration: 1.5–2%, Continuous inhalation was maintained for 2–3 minutes to achieve adequate anesthesia prior to the procedure), euthanasia was performed by cervical dislocation (neck fracture) to reduce the suffering of experimental animals), and the metacystode tissues were isolated from the abdominal cavity, and an appropriate amount of sterile normal saline was added to wash the blood water on the cyst surface repeatedly. After being crushed by a tissue homogenizer (OSE-Y50, TIANGEN, Beijing, China), it was centrifuged at 4 °C and 12,000 rpm for 20 min. The supernatant was filtered through a sterile filter (0.22 µm, Cat. #: SLGP033RB, Merck Millipore, Darmstadt, Germany), and the protein was quantified to 20 mg/mL by BCA (Cat. #: A55860, Thermo Fisher, Waltham, MA, USA) and stored at –80 °C. *E. multilocularis* antigen (*E.m*-Ag) was prepared for intervention *in vitro*. All animal experimental procedures were also approved by the Ethics Committee of Qinghai University Affiliated Hospital (Approval No.: P-SL-2019039). All efforts were made to minimize animal suffering and reduce the number of animals used in accordance with the ARRIVE guidelines.

### 2.4 Enzyme-linked Immunosorbent Assay for Interleukin-33 and ST-2 Expression in Serum

Blood samples were obtained from 12 individuals with hepatic alveolar echinococcosis (HAE) (From patients diagnosed with HAE by B-ultrasound) and 12 healthy control participants (Healthy volunteers from the physical examination center) after obtaining informed consent. Samples were collected using anticoagulated tubes and plasma was separated by centrifugation at 2300 g for 15 minutes. The plasma samples were then stored at –80 °C. ELISA kits (Cat. #: JL19282-48T/JL18366-48T, J&L Biological, Shanghai, China) were used to measure serum levels of secreted ST-2 and IL-33, with a standard curve correlation coefficient of  $R^2 = 0.998$ .

### 2.5 Western Blot Analysis of IL-33 and ST-2 Expression in Perilesional and Distal Tissue

Tissue samples (n = 15) were obtained from individuals with HAE, including close to lesion tissues (<3 cm from the lesion, CLT) and distant from the lesion tissues (>3 cm from the lesion, DLT). Total protein was extracted and adjusted to a concentration of 3 mg/µL, with 15 µg loaded into each well. After electrophoresis, protein transfer, and blocking with 10% skimmed milk powder at room temperature for 1 hour, membranes were incubated overnight at 4 °C with primary antibodies (antibody concentration: IL-33 (1:1000, ab207737, Abcam, Cambridge, UK), ST-2 (1:2500, ab25877, Abcam, Cambridge, UK)). After equilibra-

tion to room temperature, secondary antibody (1:10,000, Cat. #: C31460100, Thermo Fisher, MA, USA) incubation was conducted at 37 °C for 90 minutes. Each step was followed by three washes with PBS containing 0.1% Tween-20. Protein bands were visualized using a Bio-Rad chemiluminescence imaging system (Bio-Rad, Hercules, CA, USA), and band intensity was analyzed using NIH Image software (version 1.53; NIH, Bethesda, MD, USA, <https://imagej.nih.gov/ij/>).

### 2.6 Immunohistochemical Analysis of IL-33 and ST-2 Expression in Perilesional Tissue and Distant Hepatic Tissue

Surgically excised lesion tissues (n = 15) were fixed in 4% formaldehyde, dehydrated, embedded in paraffin, and sectioned into 3 µm slices. The sections were baked at 60 °C for 30 to 45 minutes, followed by sequential deparaffinization and rehydration using xylene I and II (15 minutes each), absolute ethanol I and II (5 minutes each), 95% ethanol I and II (5 minutes each), 90% ethanol I and II (5 minutes each), and 80% ethanol I and II (5 minutes each). Antigen retrieval was performed using high-pressure or microwave treatment for 10 to 15 minutes in 0.5M EDTA (pH 8.0) retrieval solution. Target areas were circled using a histochemical pen, followed by sequential incubation with an endogenous peroxidase blocker and a non-specific staining blocker (serum or 5% skimmed milk) for 10 minutes at room temperature. Primary antibody (antibody concentration: IL-33 (1:800), ST-2 (1:500)) incubation occurred overnight at 4 °C, followed by secondary antibody incubation at 37 °C for 1 hour after reaching room temperature. Streptavidin-peroxidase was applied for 10 minutes. Each step was followed by three washes with PBS for 3 minutes. DAB (Cat. #: YT075, Biolab, Beijing, China) was used for color development, followed by hematoxylin counterstaining, with staining duration monitored under a microscope (TISSUEFAXS SPECTRA, TissueGnostics, Vienna, Austria). Slides were rinsed with tap water for bluing, sealed, and examined. The following antibodies were utilized: anti-ST-2 antibody (ab25877) (1:500, Abcam, Cambridge, UK) and anti-IL-33 antibody (ab207737) (1:800, Abcam, Cambridge, UK).

### 2.7 Multiple Immunofluorescence Analysis of IL-33, ST-2, and Eosinophil Co-Localization

Pathological tissue (n = 7) processing, deparaffinization, hydration, and antigen retrieval were conducted as described in the immunohistochemistry protocol. Following antigen retrieval, tissue sections were immersed in a blocking solution for 10 minutes. Primary antibody (antibody concentration: IL-33 (1:2000), ST-2 (1:50)) incubation was carried out for 1 hour at room temperature or overnight at 4 °C, followed by secondary antibody incubation for 10 minutes. Fluorescent dye was applied at room temperature for 10 minutes, followed by antigen retrieval. For multiple fluorescence staining, each staining round was verified using a

fluorescence microscope before proceeding to the next step. After all staining procedures were completed, nuclear staining was performed with DAPI working solution at 37 °C for 5 minutes. Slides were mounted with an anti-fading fluorescence quenching mounting medium and examined under a fluorescence microscope (Cytation5, BioTek, Winooski, VT, USA). Eosinophils were defined as CD66<sup>+</sup>CD16<sup>-</sup> cells. The following antibodies were used: anti-ST-2 antibody (ab25877), anti-IL-33 antibody (ab207737) (Abcam, Cambridge, UK), CD66 (Cat. #: MA5-48180, Thermo Fisher, MA, USA), and CD16 (Cat. #: MA5-36143, Thermo Fisher, MA, USA).

### 2.8 Masson's Trichrome Staining Analysis of Fibrosis in Perilesional and Distant Tissues

Masson's trichrome staining was conducted using a staining kit (Cat. #: G1340, Solarbio, Beijing, China). Tissue (n = 15) processing, including hydration, was performed as described for immunohistochemistry. Sections were stained with Weigert's iron hematoxylin for 5 to 10 minutes and differentiated with acidic ethanol for 1 to 2 seconds. Masson's blue solution was applied for blue staining, followed by ponceau S staining for 5 to 10 minutes and immersion in phosphomolybdic acid solution for 1 to 2 minutes. After aniline blue staining for 1 to 2 minutes, sections were washed with a weak acid working solution for 1 minute. The sections were then dehydrated, cleared, mounted, and analyzed.

### 2.9 Effects of IL-33 on Eosinophil Migration

Eosinophils (EOL-1) were cultured in RPMI-1640 complete medium until stabilization. After cell counting, a well-mixed cell suspension was transferred to the upper chamber of Transwell plates (Cat. #: 140620, Thermo Fisher, USA) (450  $\mu$ L per well,  $3 \times 10^5$  cells/mL), while the lower chamber contained complete culture medium with induction factors. The experiment included the following groups: blank control (PBS, 50  $\mu$ L/mL; n = 5), *E.m-Ag* (20 mg/mL; n = 5), and IL-33 (3 ng/mL; ab281811, Abcam, Cambridge, UK) treatment groups (n = 5). Cells were incubated for 24-hours in a CO<sub>2</sub> incubator (MCO-20AIC, SANYO, Osaka, Japan). Samples were centrifuged at 110 g for 5 minutes and the supernatant was discarded. An equal volume of PBS was added, followed by DAPI staining. The mixture was thoroughly mixed, and 100  $\mu$ L was examined. The number of eosinophils in the lower chamber was detected by the multifunctional cell imaging microplate detector (Cytation5, Biotek, Winooski, VT, USA), and the bright field count and DAPI count were carried out at the same time. The average value of the single chamber bright field count and DAPI count was taken as the final value. This experiment was independently repeated three times.

### 2.10 Effects of IL-33 on Eosinophil Phagocytosis

EOL-1 ( $5 \times 10^5$  cells/mL) were cultured in 10% FBS (Cat. #: A5669701, Thermo Fisher, USA) RPMI-1640

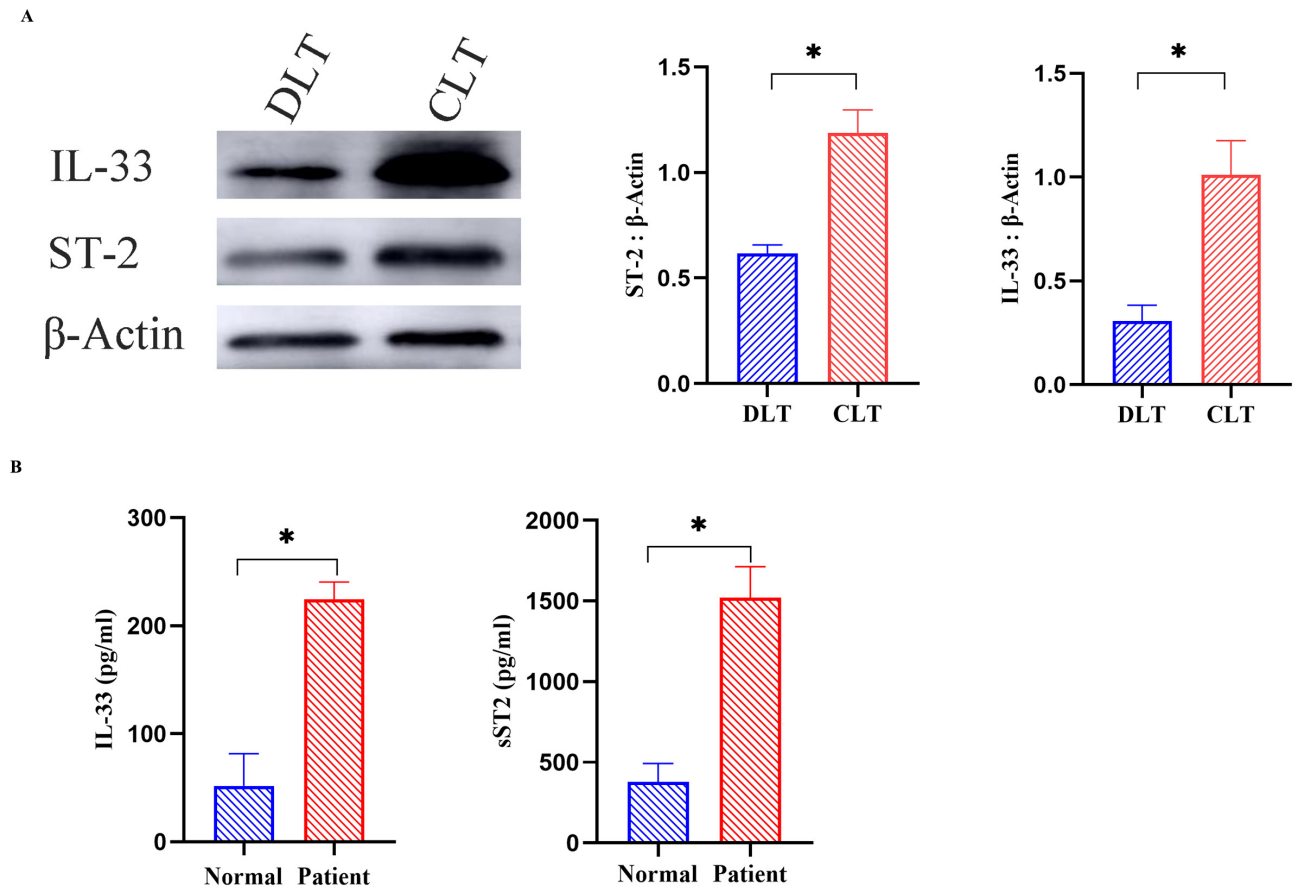
(Cat. #: PM150110A, Procell Life Science & Technology Co., Ltd., Wuhan, Hubei, China) complete medium until stabilization. After counting, the well-mixed cell suspension was evenly divided into three groups (n = 5), each containing five samples. Following a 48-hour stimulation with various factors—recombinant human IL-33 protein (active) (3 ng/mL) (ab281811, Abcam, Cambridge, UK), *E.m-Ag*, or PBS—cells were incubated with green fluorescent *E. coli* (1:500) on a shaker for 2 hours. After centrifugation and PBS washing to remove unselected *E. coli*, the isolated eosinophils were analyzed using flow cytometry (FACSCelesta, Becton, Dickinson & Co., Franklin Lakes, NJ, USA). This experiment was independently repeated three times.

### 2.11 Effects of IL-33 and ST-2 on $\alpha$ -SMA Expression in Hepatic Stellate Cells

Human hepatic stellate cells (HSCs) LX-2 (Cat. #: CL-0560, Procell Life Science & Technology Co., Ltd., Wuhan, Hubei, China) were cultured in DMEM (Cat. #: PM150210, Procell Life Science & Technology Co., Ltd., Wuhan, Hubei, China) medium until stabilization. After digestion, cells ( $5 \times 10^6$  cells/mL) were thoroughly mixed and divided into five groups (n = 5), each containing five plates. After 24 hours, the following treatments were applied: blank control (no additions), positive control (*E.m-Ag*), eosinophil intervention, IL-33 adenovirus intervention (constructed by Cyagen Biosciences Inc., Santa Clara, CA, USA), and eosinophil + IL-33 virus intervention. After 72 hours of culture, the culture medium and suspended eosinophils were removed, and the plates were washed three times with PBS. Adherent HSCs were digested and collected. Total protein was extracted, quantified, and  $\alpha$ -SMA (1:1500, Cat. #: MA1-06110, Thermo Fisher, MA, USA) expression was analyzed by Western blot. This experiment was independently repeated three times. All cell lines were validated by STR profiling and tested negative for mycoplasma.

### 2.12 Statistical Analysis

Immunohistochemistry images were analyzed using the TISSUE CONSTICS Analysis System (version 7.1; StrataQuest, Austria, <https://tissuegnostics.com>), following panoramic scanning. Multiple immunofluorescence and Masson's trichrome staining were analyzed using ImageJ (version 1.53; NIH, Bethesda, MD, USA, <https://imagej.nih.gov/ij/>). Data are expressed as mean  $\pm$  standard deviation (SD). Statistical analysis and graphing were conducted using GraphPad Prism 8.2.1 software (GraphPad, Inc., La Jolla, CA, USA, <https://www.graphpad.com>). Student's *t*-test was used for comparisons between two groups, and one-way analysis of variance (ANOVA) was applied for comparisons among multiple groups. A significance level of  $\alpha = 0.05$  was applied.



**Fig. 1. Western blot analysis of IL-33 and ST-2 expression in CLT and DLT (n = 15), along with ELISA quantification of serum IL-33 and sST-2 levels in patients with AE and healthy controls (n = 12).** (A) Western blot analysis of IL-33 and ST-2 expression in CLT and DLT (n = 15) with grayscale intensity quantified using ImageJ. (B) ELISA-based evaluation of serum IL-33 and ST-2 levels in patients with AE and healthy controls (n = 12). Data are expressed as mean ± standard deviation. \* $p < 0.05$  indicates statistical significance. CLT, close to lesion tissues; DLT, distant from the lesion tissues; ELISA, enzyme-linked immunosorbent assay; IL-33, Interleukin-33; AE, alveolar echinococcosis.

### 3. Results

#### 3.1 Western Blot Analysis of ST-2 and IL-33 Expression in Hepatic Alveolar Echinococcosis

Histopathological examination of HAE lesions indicated a distinctive inflammatory fibrous band surrounding the lesion, forming a unique microenvironment with diverse biological functions. To investigate the potential role of IL-33 and ST-2 expression in this region, their expression levels were assessed in tissue sample areas close to the lesion (CLT) (<3 cm) and distant from the lesion (DLT) (>3 cm). Western blot analysis showed significantly higher expression of ST-2 and IL-33 in CLT compared to DLT (Fig. 1A). Grayscale analysis of protein bands confirmed statistically significant differences ( $p < 0.05$ ), indicating that the IL-33/ST-2 signaling pathway may contribute to local immune regulation in hepatic echinococcosis.

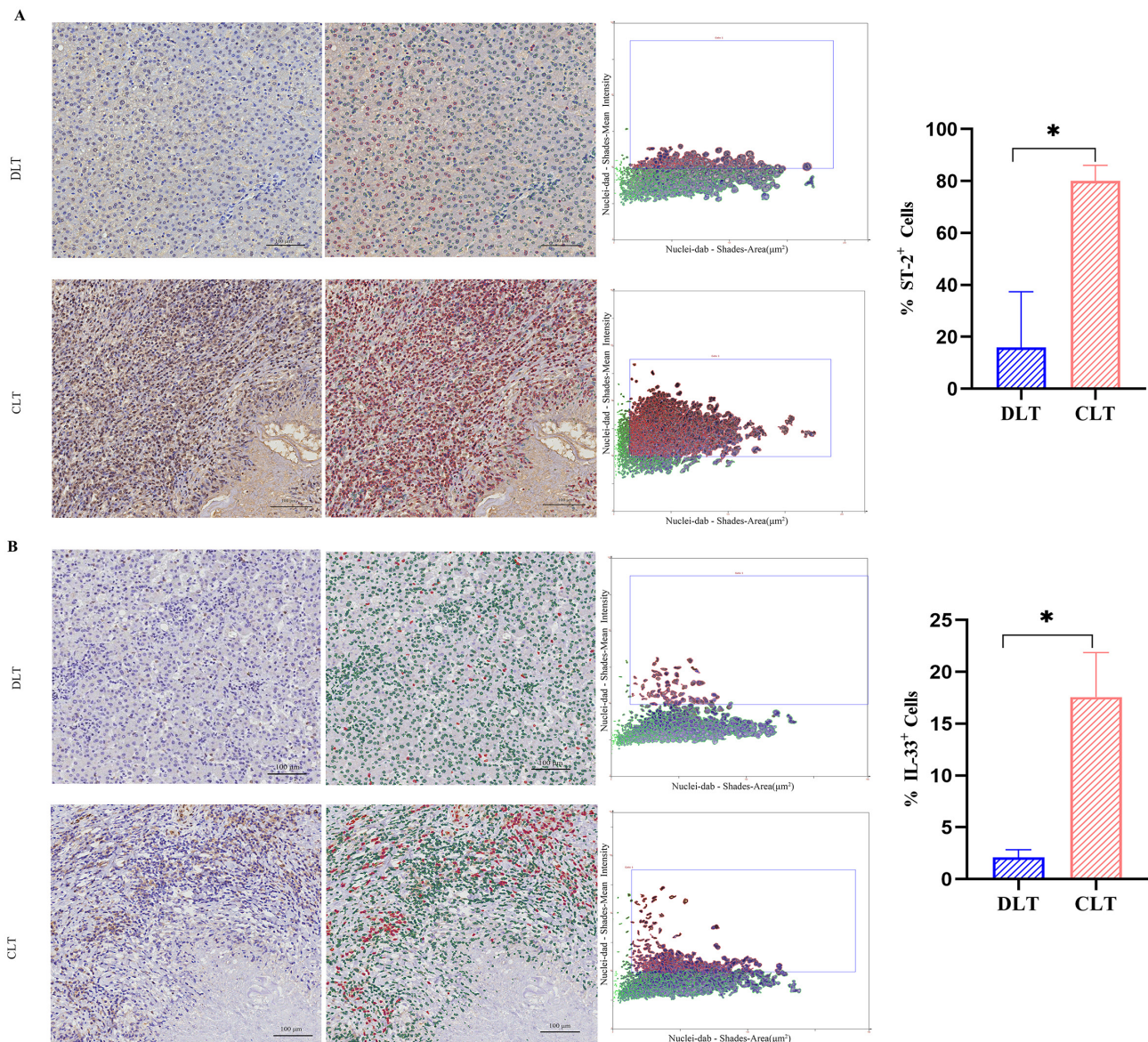
#### 3.2 ELISA Analysis of Plasma ST-2 and IL-33 Levels

IL-33 is widely recognized as an “alarm” cytokine in inflammatory diseases. To assess its systemic involvement

in HAE, plasma levels of IL-33 and sST-2 expression were measured in patients with AE and compared to healthy individuals using ELISA. As shown in Fig. 1B, plasma concentrations of sST-2 and IL-33 were significantly higher in AE patients than in healthy individuals ( $p < 0.05$ ), suggesting that the IL-33/ST-2 signaling pathway is activated in the systemic immune response to hepatic echinococcosis.

#### 3.3 Immunohistochemistry Analysis of ST-2 and IL-33 Expression in Hepatic Alveolar Echinococcosis

Western blot analysis indicated elevated IL-33 and ST-2 expression in CLT. To further investigate the spatial distribution of IL-33 and ST-2, immunohistochemical staining was performed on tissue samples from both CLT and distant from the lesion tissue (DLT) in AE patients. After panoramic scanning, analysis was conducted using the TISSUE CONSTICS Analysis System (Fig. 2). The software classified negative cells (green) and positive cells (red) in DLT and CLT for statistical analysis. Results demonstrated that the proportion of IL-33 (Fig. 2B) and ST-2 (Fig. 2A)



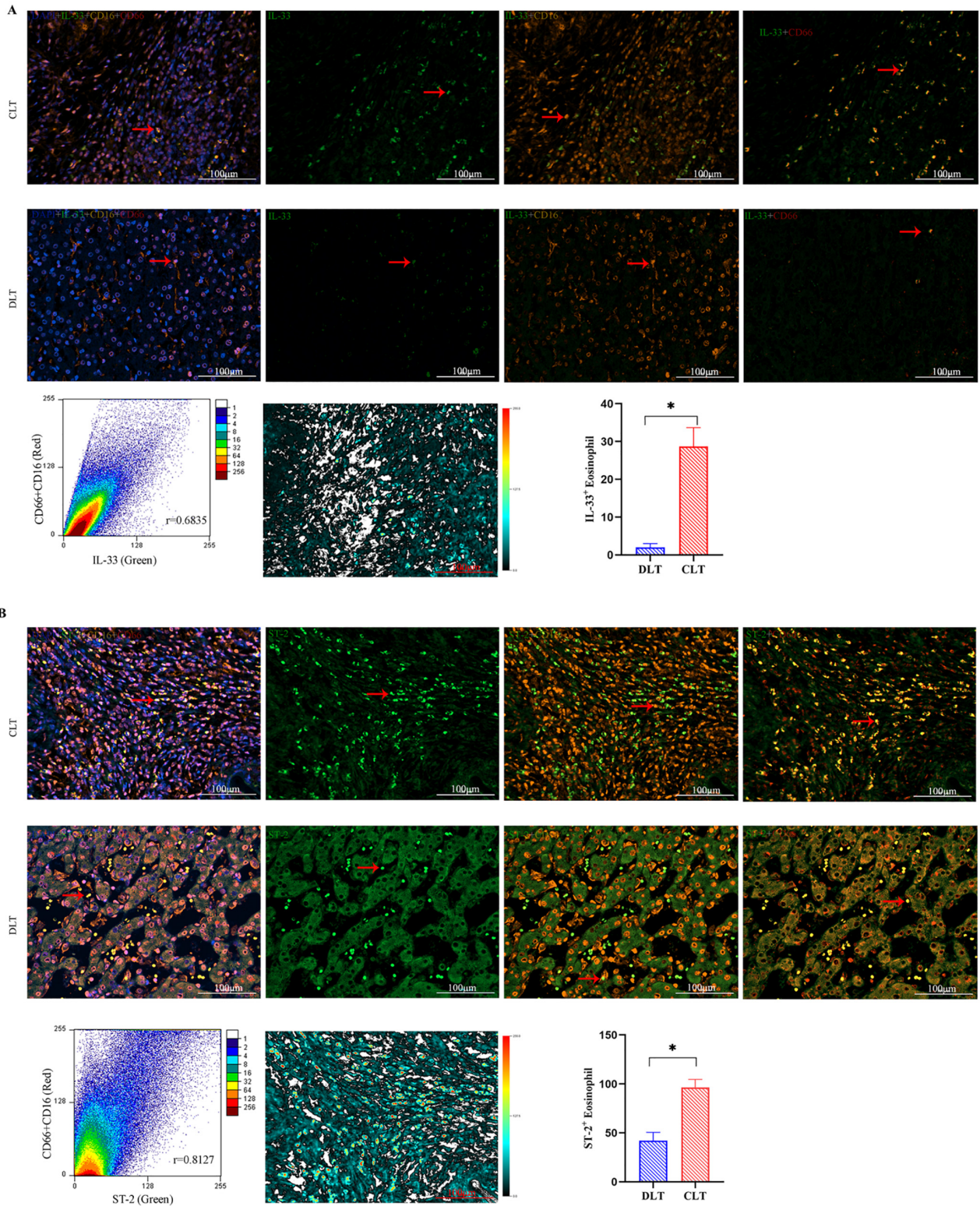
**Fig. 2. Immunohistochemical analysis of IL-33 and ST-2 expression in CLT and DLT (n = 15).** (A) Representative immunohistochemical images showing ST-2 expression in DLT and CLT. (B) Immunohistochemical images depicting IL-33 expression in DLT and CLT. Quantification was performed using the TISSUE CONSTICS Analysis System, which identified and measured negative cells (green) and positive cells (red). Scale bar: 100  $\mu\text{m}$ . Data are expressed as mean  $\pm$  standard deviation. \* $p < 0.05$  indicates statistical significance.

positive cells in CLT was significantly higher than in DLT ( $p < 0.05$ ), suggesting that IL-33 and ST-2 expression contributes to immune regulation within the AE lesion microenvironment.

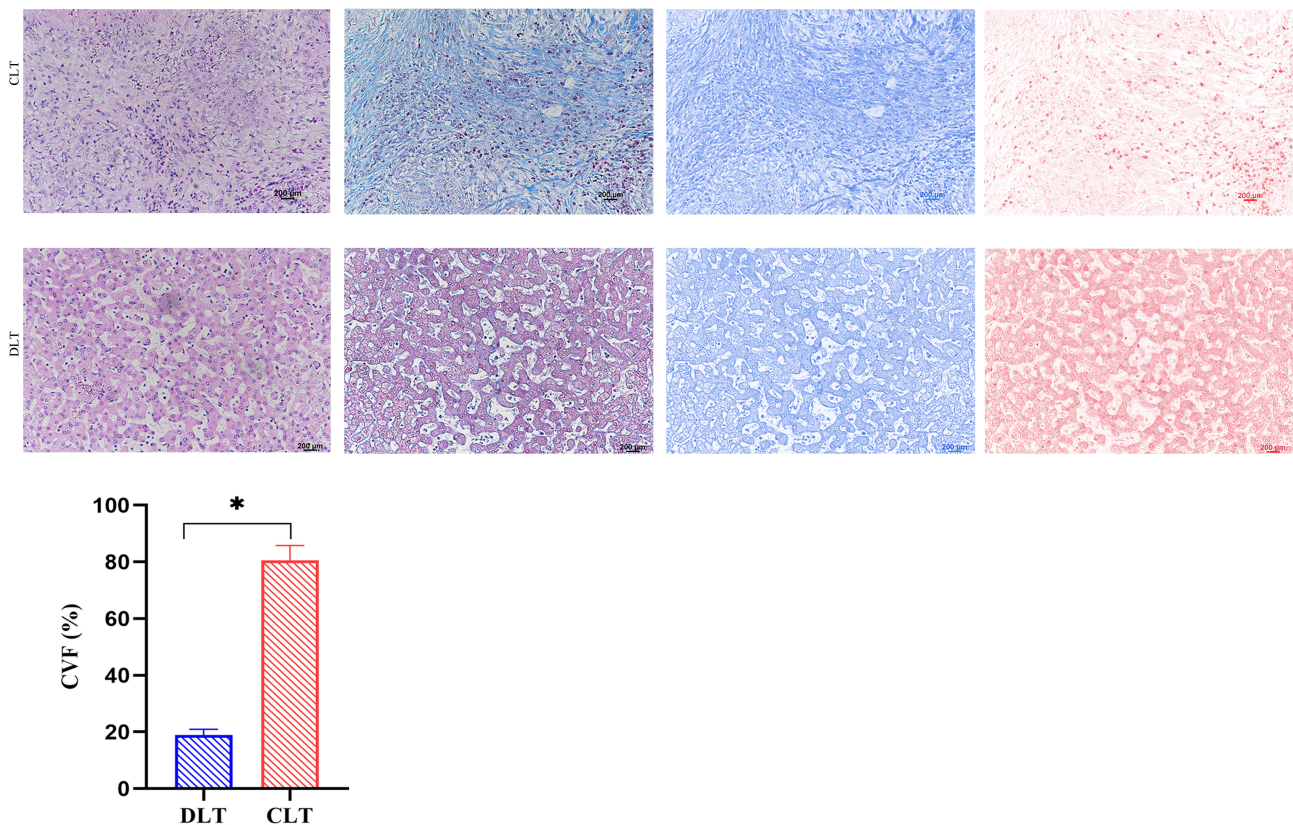
### 3.4 Co-Localization Analysis of ST-2 and Interleukin-33 With Eosinophils

Eosinophils play a crucial role in various parasitic infections, yet their role in AE remains unclear. Since ST-2, the receptor for IL-33, is widely expressed on eosinophils, this study investigated the co-localization of IL-33 and ST-2 with eosinophils within AE lesions. Immunofluorescence staining (Fig. 3) showed higher IL-33 and ST-

2 expression (green) in CLT compared to DLT, aligning with immunohistochemistry findings (Fig. 2). Furthermore, analysis of IL-33 expression and eosinophil distribution (Fig. 3A) (yellow) exhibited a strong correlation. The color distribution in the deviation plot generated by ImageJ analysis was relatively uniform (Fig. 3B), indicating a high degree of co-localization between the two groups ( $R = 0.6835$ ). This co-localized expression in CLT was significantly higher than in DLT ( $p < 0.05$ ). Similarly, ST-2 expression and eosinophil distribution exhibited an even stronger co-localization ( $R = 0.8127$ ) (Fig. 3B), with significantly greater ST-2-eosinophil co-localization in CLT compared to DLT ( $p < 0.05$ ). These findings



**Fig. 3. Immunofluorescence analysis of ST-2 and IL-33 expression in DLT and CLT and their co-localization with eosinophils (n = 7).** (A) Representative immunofluorescence images showing IL-33 and eosinophil co-expression, with yellow indicating regions of overlap. (B) Immunofluorescence images depicting ST-2 and eosinophil co-expression, with yellow indicating co-localization. Quantification of ST-2 and IL-33 expression, along with their co-expression with eosinophils, was performed using Image J. Error analysis plots illustrate the extent of ST-2 and IL-33 co-localization with eosinophils. Scale bar: 100 μm. Data are expressed as mean ± standard deviation. \* $p < 0.05$  indicates statistical significance. The target cells are indicated by the red arrows.



**Fig. 4. Hematoxylin-eosin (HE) and Masson's trichrome staining analysis of CLT and DLT (n = 15).** Representative HE and Masson's trichrome-stained images of CLT and DLT. Quantitative analysis using Image J software, which identified and extracted collagen fibers (blue) and tissue structures (red). Fibrosis levels in CLT and DLT were quantified by calculating the collagen volume fraction (CVF). Scale bar: 200  $\mu$ m. Data are expressed as mean  $\pm$  standard deviation. \* $p < 0.05$  indicates statistical significance.

suggest that IL-33 accumulation in the fibrotic infiltration area surrounding AE lesions may enhance eosinophil function through ST-2 binding. However, further *in vitro* experiments are necessary to confirm its specific effects on eosinophils and their biological functions.

### 3.5 Analysis of Fibrosis in DLT and CLT Tissues of Patients With Hepatic Alveolar Echinococcosis Using Masson's Trichrome Staining

IL-33 plays a key role in fibrosis development and was found to be highly expressed in the tissues surrounding AE lesions (CLT). Given that HAE is characterized by a dense inflammatory fibrous band, fibrosis formation in AE lesions was analyzed. HE staining demonstrated a relatively preserved structure in the DLT compared to CLT (Fig. 4). Masson's trichrome staining further demonstrated a significantly higher abundance of collagen fibers (blue) in CLT than in DLT. Quantitative analysis using ImageJ software allowed for the identification, isolation, and extraction of collagen fibers (blue) and tissue (red), from which the collagen volume fraction (CVF) was calculated. The results indicated a significantly higher CVF in CLT than in DLT ( $p < 0.05$ ), indicating that IL-33 may contribute to fibrosis formation in AE lesions.

### 3.6 Flow Cytometry Analysis of Eosinophil Phagocytosis

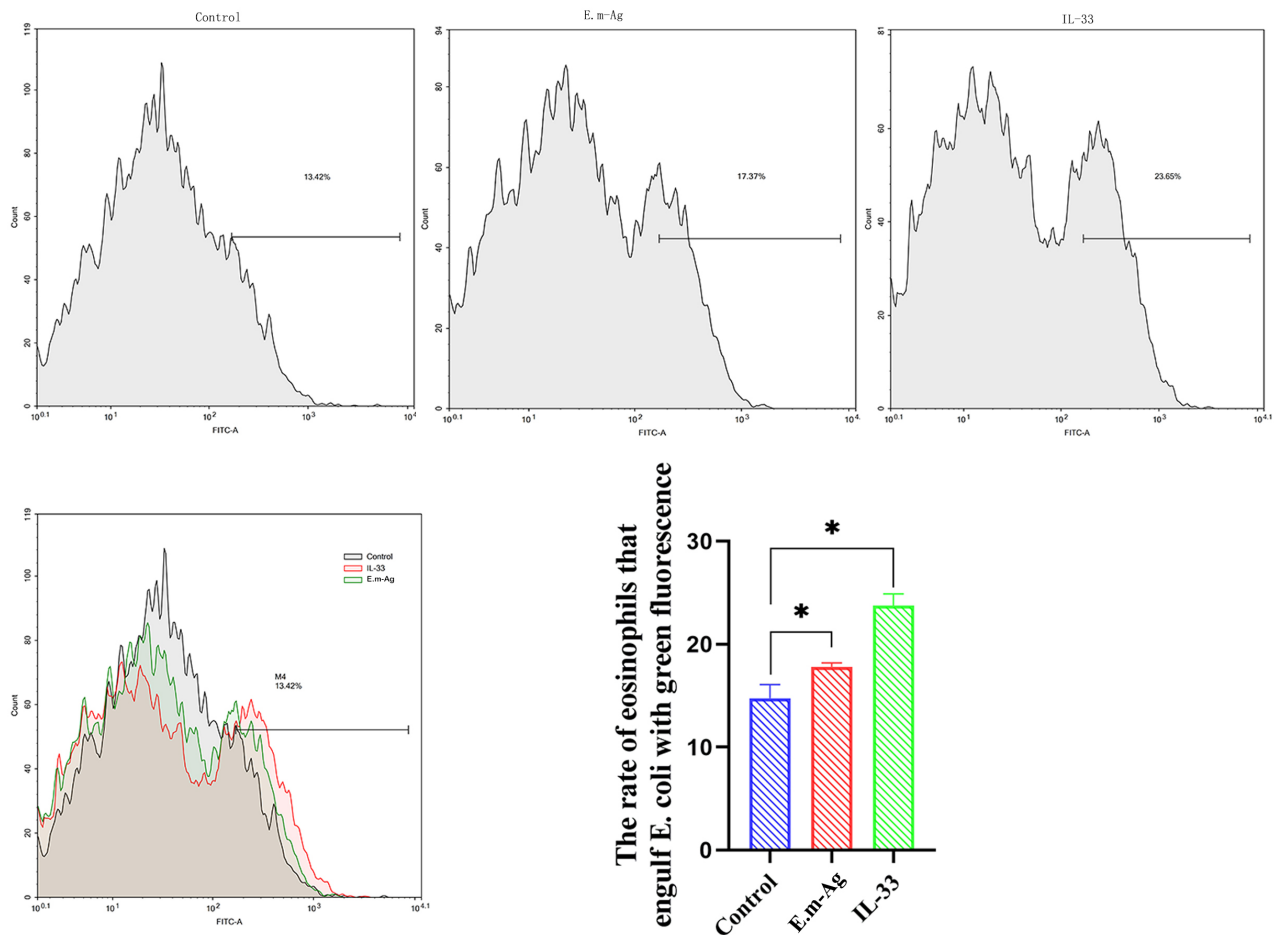
To assess the effect of IL-33 on eosinophil phagocytic function, eosinophils were divided into three groups: blank control, *E.m*-Ag, and IL-33 intervention. Flow cytometry results (Fig. 5) indicated that eosinophil phagocytosis of *E. coli* was significantly enhanced following stimulation with IL-33 and *E.m*-Ag, with IL-33 inducing a more pronounced increase in eosinophil phagocytic activity compared to the *E.m*-Ag stimulation ( $p < 0.05$ ). These findings suggest that eosinophil behavioral changes during echinococcosis infection are influenced by the disease state and IL-33 production, with IL-33 playing a dominant regulatory role.

### 3.7 Transwell Assay for Eosinophil Migration

To evaluate the effect of IL-33 on eosinophil migration, a Transwell assay was performed. The results demonstrated that IL-33 intervention significantly increased eosinophil migration through the Transwell chamber compared to both the blank control and *E.m*-Ag groups ( $p < 0.05$ ) (Fig. 6A).

### 3.8 Expression of $\alpha$ -SMA in HSC Under Different Stimuli

A dense fibrous band is a characteristic feature surrounding AE lesions, and IL-33 has been implicated in fi-



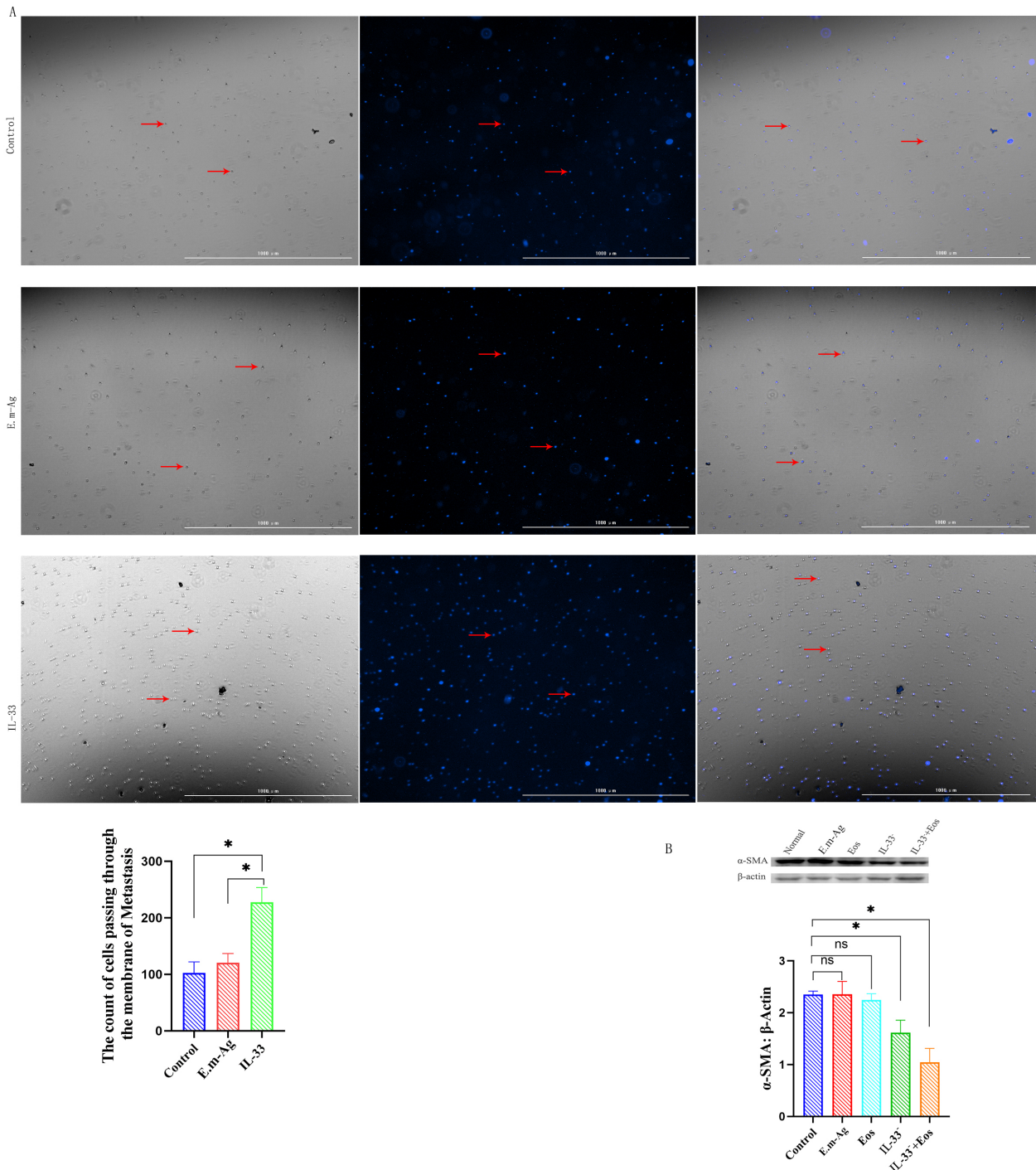
**Fig. 5. Eosinophil phagocytosis of green fluorescent *E. coli* following different treatments.** Phagocytosis assay results for the control group (PBS, 50  $\mu$ L/mL; n = 5), IL-33-treated group (3 ng/mL; n = 5), and *E.m-Ag*-treated group (20 mg/mL; n = 5). Flow cytometry to detect GFP signals in eosinophils. Data are expressed as mean  $\pm$  standard deviation, \* $p < 0.05$ .

brosis formation. To further investigate the effect of IL-33 on lesion fibrosis during AE infection, five experimental groups were established: blank control, *E.m-Ag*, eosinophil intervention, IL-33 inhibition, and eosinophil + IL-33 inhibition combined intervention. Following various interventions on HSCs, Western blot analysis of  $\alpha$ -SMA expression was performed. Results demonstrated that both eosinophil addition and IL-33 inhibition reduced  $\alpha$ -SMA expression in HSCs. Notably, the combination of eosinophil addition and IL-33 inhibition led to a more significant decrease in  $\alpha$ -SMA expression (Fig. 6B), suggesting that IL-33 influences HSC  $\alpha$ -SMA expression through eosinophil regulation.

#### 4. Discussion

Increasing evidence highlights the critical role of the IL-33/ST-2 signaling pathway in infectious diseases, with its function influenced by factors such as pathogen type, affected organ, infection stage, and immune microenvironment. In this study, IL-33 was identified as a key mediator in the immune response to hepatic alveolar echinococcosis

(AE). Findings demonstrated significantly elevated IL-33 and ST-2 levels in the peripheral blood of patients with AE compared to healthy individuals, which were somewhat different from the existing report, this might be more prominent because previous studies mainly focused on patients with AE rather than only on hepatic alveolar echinococcosis, underscoring its role in HAE progression [27]. This was further validated by Western blot and immunohistochemistry analyses, which showed increased IL-33/ST-2 expression in CLT relative to DLT. This discovery is similar to the reported study, reinforcing its involvement in local echinococcosis lesions [28]. IL-33, a member of the IL-1 family, exerts its biological effects by binding to its receptor ST-2 [29]. ST-2 is predominantly expressed on lymphocytes, including macrophages, Type 2 Innate Lymphoid Cells (ILC2), and eosinophils [24,30,31]. Given the well-established role of eosinophils in AE, their interaction with IL-33 was examined. Immunofluorescence analysis demonstrated a significant increase in eosinophils and IL-33 expression in CLT, with strong co-localization. In



**Fig. 6. Eosinophil migration and  $\alpha$ -SMA expression in HSCs following different treatments.** (A) DAPI staining, bright-field imaging, and merged DAPI + bright-field images of eosinophils across three experimental groups ( $n = 5$ ). Statistical analysis of eosinophil migration through the Transwell membrane under various stimulating conditions. (B) Western blot analysis and quantification of  $\alpha$ -SMA expression in HSCs following different stimulations ( $n = 5$ ). Scale bar = 1000  $\mu\text{m}$ . Data are expressed as mean  $\pm$  standard deviation. \* $p < 0.05$  indicates statistical significance;  $p > 0.05$  denotes no significant difference (ns). The target cells are indicated by the red arrows.

*in vitro* studies confirmed that IL-33 intervention enhanced eosinophil phagocytosis and migration, demonstrating its role in activating and promoting the function of eosinophils.

Patients with AE develop a dense fibrous band surrounding their lesions, creating a distinct immune microen-

vironment. While this fibrotic barrier limits lesion expansion, it also obstructs chemotherapeutic drug penetration, reducing antiparasitic efficacy and complicating treatment [19,31]. Hepatic fibrosis in AE arises primarily from HSC activation and an imbalance between extracellular ma-

trix (ECM) synthesis and degradation, leading to excessive ECM accumulation. Notably, IL-33 expression correlates directly with fibrosis severity [22,25,32]. To explore IL-33's role in AE-related fibrosis, *in vitro* HSC experiments demonstrated that IL-33 inhibition significantly decreased  $\alpha$ -SMA expression, confirming its involvement in fibrosis. Furthermore, co-treatment with eosinophils and IL-33 inhibition resulted in an even greater reduction in  $\alpha$ -SMA expression, suggesting that IL-33 modulates AE-related fibrosis through eosinophil regulation. Masson's trichrome staining analysis further indicated that fibrosis progression stems from IL-33-induced collagen fiber deposition, contributing to ECM accumulation [33]. Collectively, these findings, along with prior research, highlight IL-33's pivotal role in hepatic fibrosis progression, with eosinophils acting as key mediators in this process.

This study provides a comprehensive analysis demonstrating significantly elevated IL-33/ST-2 expression within the lesion microenvironment of patients with AE. *In vitro* experiments confirmed that IL-33 promotes  $\alpha$ -SMA expression in HSCs, indicating its role in fibrosis development. As a "danger signal" protein, IL-33 is secreted in response to tissue injury, infection, or necrosis, driving eosinophil proliferation, activation, and degranulation, which contribute to allergic and inflammatory diseases [15,34]. Under homeostatic conditions, IL-33 is constitutively expressed and retained in the nucleus of various barrier cells, primarily epithelial cells (e.g., in the skin, respiratory tract, and gastrointestinal mucosa), endothelial cells, and resident stromal cells such as fibroblasts and group 2 innate lymphoid cells (ILC2s). In these cells, IL-33 functions as a nuclear factor regulating gene expression rather than acting as a cytokine [35]. However, upon cellular stress, infection, or necrosis—events that characterize tissue damage in diseases like AE—IL-33 is rapidly and passively released into the extracellular milieu. This release alerts the immune system to tissue injury and initiates type 2 immune responses [36]. In addition to passive release from damaged cells, certain immune cells can actively secrete IL-33. For instance, activated macrophages and mast cells have been shown to upregulate and release IL-33 in response to specific pathogen-associated molecular patterns (PAMPs) or inflammatory signals [37]. Therefore, the elevated IL-33 levels observed in our study are likely attributable to the combined effect of passive release from damaged host tissues (e.g., liver parenchyma and infiltrating immune cells) and active secretion by local innate immune cells in response to the parasitic infection. Moreover, the observed correlation between increased eosinophils, elevated IL-33 levels, and enhanced ST-2 expression on eosinophils with fibrosis progression suggests that targeting IL-33 could modulate the AE lesion microenvironment [19]. This approach may improve therapeutic efficacy when combined with drug treatment.

Thus, the IL-33/ST-2 signaling pathway plays a pivotal role in immune regulation during AE infection, contributing to fibrosis within the immune microenvironment of AE lesions. By modulating eosinophil activity, this pathway influences both disease progression and clinical outcomes. However, at the same time, we have to admit three limitations of our research. First, the peripheral blood eosinophil count of the subjects was not available. Because the level of blood eosinophils may vary greatly among individuals and affect local inflammatory infiltration, this is a potential confounding factor. Secondly, our research mainly focused on the lesion microenvironment, while the significant reduction of peripheral eosinophils in AE infection (Wang and Gottstein, 2016 [38]) as an immune escape mechanism has not been further studied. Finally, the obtained *E.m*-Ag antigen contains a trace of host blood components, which may have a potential impact on the experimental results. Further experiments are needed in the future to determine the relationship between global immunity and local immunity, peripheral and local eosinophil responses.

## 5. Conclusion

The expression of IL-33 and ST-2 and the distribution of eosinophils in the microenvironment of hepatic alveolar echinococcosis lesions increased abnormally. *In vitro* experiments showed that IL-33 could drive eosinophils to participate in the activation of hepatic stellate cells and promote the generation and expression of collagen fibers. Therefore, it is concluded that IL-33 promotes microenvironment fibrosis in AE lesions through the eosinophil mediated mechanism, highlighting the potential therapeutic targets for improving the efficacy of chemotherapy in AE patients.

## Abbreviations

AE, Alveolar echinococcosis; CVF, Collagen volume Fraction; CLT, Close to the Lesion Tissue; DLT, Distant from the Lesion Tissue; HAE, Hepatic Alveolar Echinococcosis; EMC, Extracellular matrix; Eos, Eosinophil; IHC, Immunohistochemistry; HSC, Hepatic Stellate Cell; IL, Interleukin; ELISA, EnzymeLinked Immunosorbent Assay; WB, Western Blotting; IF, Immunofluorescence;  $\alpha$ -SMA, Alpha-smooth muscle actin; ST-2, suppression of tumorigenicity 2; ILC2, Type 2 Innate Lymphoid Cells; *E.m*-Ag, *E. multilocularis* antigen.

## Availability of Data and Materials

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

## Author Contributions

SLC completed the design of the whole subject, completed all the experimental operations, and analyzed the ex-

perimental data. BJW and YXY made substantial contributions to the study design, experiment operation and assisted in data processing and statistical analysis. XMM participated in the conception and design of the study, coordinated data collection, and contributed to data analysis and interpretation and drafted the manuscript. YGZ gave guidance in the multiple immunofluorescence experiment and assisted in data processing and statistical analysis. HNF supervised and controlled the quality of the whole experiment and designed the experimental workflow, supervised the research process. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

### Ethics Approval and Consent to Participate

All animal experimental procedures and the human sample experiments involved in this study were reviewed and approved by the Ethics Committee of Qinghai University Affiliated Hospital (Approval No.: PSL-2019039). The study was carried out in accordance with the guidelines of the Declaration of Helsinki. The samples referred to in the manuscript have obtained the informed consent of the the patients or their families/legal guardians/sample experiment. All efforts were made to minimize animal suffering and reduce the number of animals used in accordance with the ARRIVE guidelines.

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### Conflict of Interest

The authors declare no conflict of interest.

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