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IL-33 contributes to disease severity in Psoriasis-like models of mouse

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ABSTRACT

Immune cells infiltrating the psoriatic skin secrete high amounts of pro-inflammatory cytokines IL-17, TNF-α, IL-21 and IL-36 resulting in chronic inflammation. However, the exact cellular and molecular mechanisms have not been fully understood. We report here elevation of IL-33 expression in psoriatic lesions. Studies in imiquimod (IMQ)-induced mice with psoriatic inflammation confirmed a critical role for IL-33 in driving the disease. IL-33 reduces the $CD4^+$ and $CD8^+$ cells, inhibits autophagy in IMQ-treated mouse skin, and promoted tyrosyl phosphorylation of STAT3. Thus, IL-33 appears to be a major risk factor for severity of psoriasis-like skin inflammation. Our findings may open new perspectives for understanding the mechanisms and developing a therapeutic strategy for psoriasis.

1. Introduction

Psoriasis, an inflammatory skin disease, is characterized by hyperproliferation and abnormal differentiation of keratinocytes and infiltration of immune cells [1]. It effects approximately 2–3% of the global population [2]. The pathogenesis of psoriasis is not fully unclear but existing evidence indicates that immune cells infiltrating the psoriatic skin produce a large variety of proinflammatory cytokine such as Interleukin (IL)-17A, IL-21, IL-22 that stimulate keratinocyte proliferation [3].

The cytokine of interleukin-33 was identified as a member of the IL-1 subfamily approximately ten years ago [4]. IL-33 is considered as a new “alarmin” released from cell injury [5]. It was first described as a cytokine that mediates a Th2-type immune response [6]. Interleukin-33 was constitutively expressed on cells of various human tissues, in which the nucleus of endothelial cells and epithelial cells (including keratinocytes) are theirs main source [7]. Interleukin-33 expression has also been reported in psoriatic skin lesions, which is considered as a Th1/Th17-mediated immunological skin disease [8].

Although IL-17A and TNFα are effective therapeutic targets in psoriatic patients [9]. However, little is known about the role of IL-33 cytokines in psoriasis. The level of IL-33 is noteworthy growthed in affected skin of patients with psoriasis, when compared to those of healthy skin, and in patients with other inflammatory skin disease [10]. Suttle et al. [11] have also found that IL-33 is released from keratinocytes after slightly damaging of the skin in psoriasis. Additionally, recent research have demonstrated that serum IL-33 levels are elevated in patients with psoriasis than in healthy skin, indicating that IL-33 may represent as a new maker for psoriasis [12]. Despite IL-33 participate in psoriasis, little is known about IL-33 signaling and its transcriptional responses. Therefore, it is of interest to determine the importance of IL-33 expression in psoriatic lesions. In this study, we investigated the role and possible mechanisms of IL-33 upregulation using IMQ-induced mouse skin model.

2. Materials and methods

2.1. Patients

Skin samples were obtained from 30 psoriatic patients and 15 normal persons with a 4-mm punch biopsy. Sample acquisitions, including skin biopsies, were approved by the Ethics Committee of the First Affiliated Hospital of Xinxiang Medical University and handled in accordance with the declaration of Helsinki Principles. Informed consent of the subjects was obtained for all of patients.

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2.2. Cell culture

HaCaT cell was obtained from the institute of Chinese Academy of Medical Sciences & Peking Union Medical College. They were cultured in DMEM supplemented with 100 U/ml streptomycin and 10% heat-inactivated fetal bovine serum (Gibco, Gaithersburg, MD, USA).

2.3. Mice

Female BALB/c mice, aged 6–8 weeks, were purchased from Xinxiang Hualan Experimental Animal Center. Mice were maintained at 22 ± 2 °C under pathogen-free conditions according to the recommendations of the Care and Use of Laboratory Animals Guide of the United States National Institute of Health.

2.4. Cell viability assay

HaCaT cells (2×10^3 cells/well) were plated into 96-well flat-bottom dish (Corning-Costar, Corning, NY, USA) and cultured for 24 h. Then they were treated with different concentrations of IL-33. After 24 h, 10 μl of Cell Counting Kit-8 (Beyotime Institute of Biotechnology, Shanghai, China) was added to each well and the cells well incubated for another 2 h. Cell growth was quantified using a multi-well microtiter plate reader (Thermo Fisher Scientific). Each experiment contained triplicates/treatment and was repeated three times. Pooled data from all experiments were plotted and subjected to statistical analyses for determining the significance of the differences.

2.5. Detection of protein expression

In order to analyze the expression level of related proteins, skin tissues were lysed using RIPA Lysis Buffer (Beyotime Institute of Biotechnology, Shanghai, China) and the expression of various proteins was detected by Western blotting using specific antibodies for LC3 (2775), STAT3 (4904S), p-STAT3 (9131S) (Cell Signaling Technology, Massachusetts, America), β-actin(A5441) and IL-17(3605) antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.6. Flow cytometry assay

The ratios of CD3, CD4 and CD8 cells in spleen were analyzed using flow cytometry.

Briefly, 7 days after treatment with IL-33 or PBS, 3 randomly selected mice from each group were sacrificed. The spleens were isolated and homogenized in DMEM, and centrifuged at 2000 rpm for 5 min at 4 °C. The cell pellets were collected and the red blood cells were lysed using Red Blood Cell Lysis Buffer (Solarbio, Beijing, China) Splenocytes were then incubated with the following antibodies: CD3-FITC(17A2), CD4-APC(Gk1.5) and CD8-PE(SK1) (BioLegend, San Diego, CA, USA) at 4 °C for 30 min. Finally, the cells were washed and subjected to flow cytometry.

2.7. Mouse model of psoriasis

Six to eight weeks old mice (BALB/c background) were topically applied with IMQ cream (5%) (Aldara; 3 M Pharmaceuticals, St. Paul, MN) on the shaved back for 6 consecutive days daily, and then Recombinant Murine IL-33(PeproTech, USA, 1209434) injection experiment, mice (n = 10 for each group) were each injected daily intraperitoneally with 100 ng IL-33 or PBS (control) in a total volume of 100 μl. The clinical scores were assessed according to PASI. The thickness of the skin lesions on the back of the mice was measured using a digital Vernier caliper. On day 7, skin samples were collected for histological and Western blotting analyses. Serum samples were prepared from peripheral venous blood.

2.8. Detection of cytokines

Mouse serum was collected at 6 days after the development of psoriasis. The samples were centrifuged at 6000 rpm for 10 min at 4 °C. The levels of IL-33 and ST2 were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's (CUSIO) recommendations. The detection ranges for IL-33 and ST2 are 31.25–2000 (pg/ml) and 6.25–400 (pg/ml), respectively.

2.9. RNA isolation and quantitative real-time PCR

Total RNA was isolated using RNAiso Plus (TAKARA Beijing China), cDNA synthesis was performed 5 × PrimeScript RT Master Mix (Takara, Beijing China). Relative gene expression was quantified by real-time PCR using TB Green Premix Ex Taq II (Takara, Beijing China) and self-designed primers (IL-33: 5′-CGGATCCACTTCACTTTTAACAC AGTC-3′, and 5′-GAGATCTTTAGATTTTCGAGAGCTTA-3′; β-actin: 5′-GAAATAGTGCGTGACATCAAAG-3′, and 5′-TGTAGTTTCATGGATGC CACAG-3′; ST2 5′-TTACCCAGCCAGGATGTTTC-3′ and 5′-CTAGGGGC TTGGCTTCTCTT-3′). PCR conditions were as follows: initial denaturation 15 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 45 s. Relative mRNA levels were calculated by normalization to the reference genes IL-33 or ST2 using the $2^{-\Delta\Delta}$CT method.

2.10. Immunocytochemistry analyses

HaCaT cells were seeded at 1×10^6/ml in 24-well plates with cell slides, after IL-33 treatment for 24 h. The culture medium was discarded, washed once with PBS, fixed in 1 ml of 4% paraformaldehyde for 20 min, treated with Triton- X100 permeabilizing solution was for 8 min, washed with PBS containing 1% BSA three times, incubated with goat serum blocking solution for 30 min, and then primary antibody (1:100) specific for Ki-67(Bioworld technology, Nanjing, China) was added dropwise and incubated overnight at 4 °C. After washing three times, the corresponding fluorescent secondary antibody was added dropwise, incubated at room temperature for 30 min in dark, DAPI stained for 8 min, washed three times, anti-fluorescence quenched and stored in a humid container. Three replicate wells were used for each condition.

2.11. Immunohistochemistry (IHC) and immunofluorescence staining

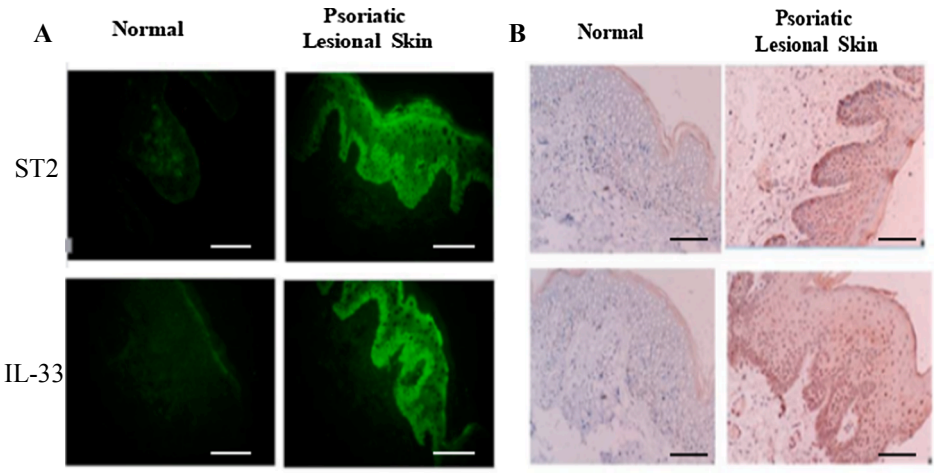
Formalin-fixed, paraffin-embedded tissue sections (~5 mm in thickness) mounted on glass slides were used for various staining procedures. For immunofluorescence and immunohistochemistry, psoriasis patient skin paraffin sections were stained with anti-IL-33(SAB3500439) and anti-ST2(PRS3363) (Sigma, America) Fluorescence images were captured with Nikon microscope, and analyzed using the ImageJ software. The thickness of epidermis or dermis was calculated based on the total area versus the length of epidermis or dermis.

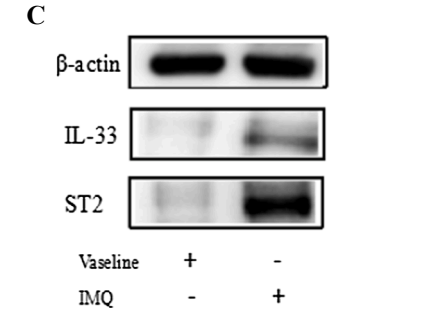
2.12. Histopathological analyses

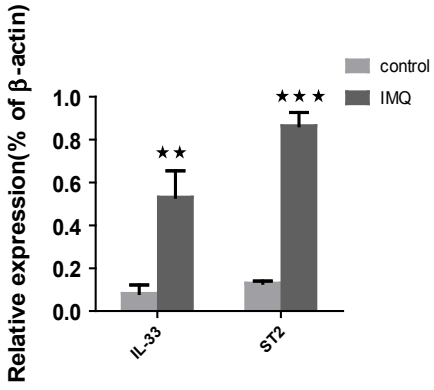
The mouse skin samples of the back were taken, fixed, dehydrated, and embedded in paraffin. The slices were sliced to a thickness of 3 μm, stained with hematoxylin-eosin (HE), and sealed with neutral gum. The epidermal changes were observed under light microscope.

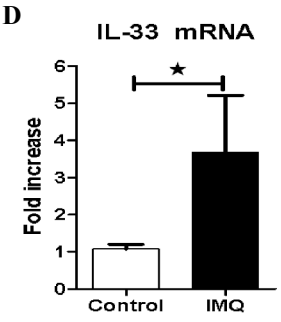
2.13. Statistical analyses

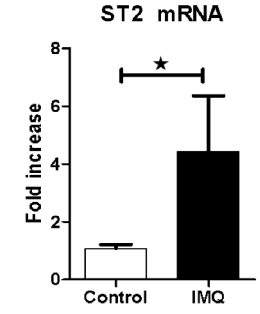
The data were analyzed by Graph Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA), and the statistical significance of the differences were analyzed by unpaired Student's *t* test or one-way ANOVA. A p value < 0.05 was considered significant.

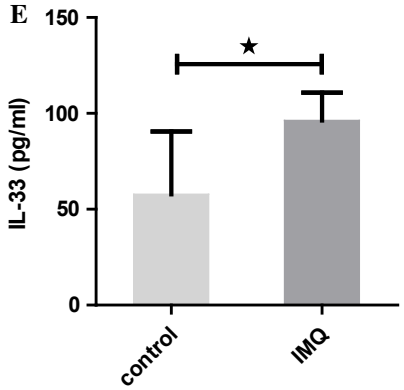












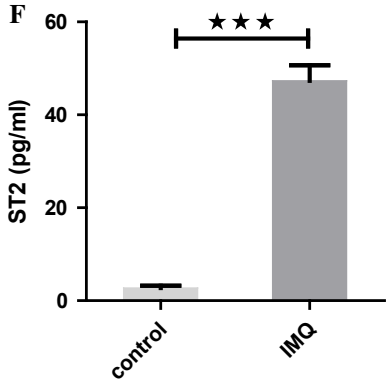


Fig. 1. IL-33 and ST2 expression is increased in psoriatic lesions. (A and B) Immunofluorescence and Immunohistochemical staining of IL-33, ST2 in the skin paraffin sections obtained from healthy control and psoriasis patients. (C and D) IL-33 and ST2 mRNA and protein expression in the skin from control mice and IMQ-treated mice. (E and F) The cytokines of IL-33 and ST2 in serum were detected using ELISA kits. All the assays were repeated three times with consistent results. p values were determined by unpaired t test. Data are given as means ± SEM. All data are representative of three independent experiments. P values were determined by an unpaired t test or two-way. $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$.

3. Results

3.1. IL-33/ST2 expression in psoriasis vulgaris lesion was elevated in human and imiquimod (IMQ)-induced psoriasis-like mouse mode

We first examined the expression of IL-33 and ST2 in skin biopsies from thirty psoriasis vulgaris patients using immunohistochemistry and immunofluorescence. The expression of IL-33 and ST2 were strongly increased in psoriatic lesions compared to the normal skin controls (Fig. 1A and B). To further confirm this observation, we also determined the expression levels of IL-33 and ST2 mRNA and protein in IMQ-induced "psoriasis-like" mouse model (after 6 days of IMQ-treatment). The mRNA and protein levels of IL-33 and ST2 were significantly increased in IMQ-treated skins (Fig. 1C and D). Consistent with these, higher levels of IL-33 and ST2 in serum were detected by ELISA (Fig. 1E and F).

3.2. IL-33 aggravates Imiquimod-induced psoriasis-like lesions

To assess the role of IL-33 in psoriasis, we injected recombinant mouse IL-33 (rIL-33) into the intraperitoneal of Imiquimod-induced psoriasis-like mouse every day. First of all, the effects of IL-33 on psoriasis-like mice was observed from histological and morphological (Fig. 2A). Consistent with the morphological results, HE staining showed a thicker epidermis in the IMQ group compared with the normal control group, with extensor extension of the epidermis accompanied by parakeratosis and micro abscess. Compared to the IMQ group, the degree of skin thickening in IMQ combine with IL-33 group was increased. On 0, 2, 4, and 7 days, PASI scores were determined using the erythema and scale indicators (Fig. 2B). Compared with the normal control group, the erythema and scales of the back skin of the IMQ alone treated mouse began to appear on the 2nd and 3rd day respectively, and the symptoms were most obvious on the 5th and 6th day of the experiment. Compared to the IMQ group, erythema and scales of IL-33 with IMQ group appeared earlier, and the symptoms were significantly aggravated. At the same time (Fig. 2C), the skin thickness in the IMQ group (0.53 ± 0.05 mm) was statistically significant ($p < 0.01$) when compared to the normal group (0.17 ± 0.05 mm) . The increase in skin thickness in IL-33 combine with IMQ group was (0.74 ± 0.05 mm) statistically significant compared to the model group ($p < 0.01$). However, when compared to normal group, IMQ group and IMQ combine with IL-33 group, the IL-33 alone group has no psoriasis-like lesions and was statistically different. Thus, IL-33 aggravates imiquimod-induced psoriasis-like lesions.

3.3. IL-33 reduces the numbers of $CD4^+$ and $CD8^+$ T cells in spleen in IMQ-treated mice

To further determine the effects of IL-33 in psoriasis, $CD4^+$ T cells and $CD8^+$ T cells in the spleen were analyzed by Flow cytometry. The mice injected with IL-33 alone are not significantly different from those in the untreated control group. Nevertheless, compared to control group, the percentages of $CD4^+$ cells and $CD8^+$ T cells were greatly reduced in the IMQ-treated group, and more obvious reduces in IL-33 combine with IMQ treated mice (Fig. 3).

3.4. IL-33 inhibited autophagy-related proteins and promoted STAT3 phosphorylation

Recently autophagy-related proteins have been reported to participate in the nascence and development of psoriasis [13]. Therefore, we investigated the expression of certain classical autophagy-related proteins in psoriatic lesions. As shown in Fig. 4, the expression of LC3 and Beclin1 were elevated in the IMQ group, whereas the protein level of LC3 and Beclin1 were decreased in IL-33 combine with IMQ group and IMQ group compared to the control group. STAT3 is known to control cell cycle and proliferation in many cell types, including keratinocytes, and has been shown to be involved in psoriasis-like skin inflammation [14]. To determine the effects of IL-33 on STAT3, we examined the phosphorylation of STAT3 by western blotting in psoriatic-skin tissue. IL-33 can induced the phosphorylation of STAT3 compare to IMQ group. Therefore, we guess that IL-33 can aggravate the inflammatory response of psoriasis by inducing phosphorylation of STAT3. IL-17 has been proved to be the pivotal cytokine which drives psoriasis directly. Hence, the expression of IL-17 was detected in the skin lesion in IMQ-induced psoriasis-like mice. There was no difference between the expression levels of IL-17 between the IL-33 combine with IMQ group and IMQ group.

3.5. IL-33 promoted HaCaT cell proliferation

We next assessed the direct impact of IL-33 on the proliferation of human keratinocyte line HaCaT. Cells were treated with various doses of recombinant human IL-33 for 24 h and cell growth was measured by MTT. At 25 ng/ml, IL-33 significantly promoted the HaCaT cell proliferation (Fig. 5A). There was no significant effect at lower doses. Consistent with this, 25 ng/ml IL-33 also increased the expression of Ki67 (Fig. 5B), a proliferation marker, which further suggests an essential role of IL-33 in regulating the proliferation of keratinocytes in the pathogenesis of psoriasis.

3.6. IL-33 induced autophagy and promoted STAT3 phosphorylation in HaCaT cells

To determine if IL-33 regulated STAT3, we examined STAT3 tyrosyl phosphorylation in IL-33 stimulated HaCaT by Western blot. IL-33 stimulated the phosphorylation of STAT3 in a dose-dependent manner (Fig. 6A). We also investigated the effect of IL-33 on autophagy-related proteins using Western blot analyses. Both Beclin1 and LC3 levels are significantly increased by IL-33 compared to the control (Fig. 6C).

4. Discussion

Interleukin-33, a new cytokine, plays a double role in inflammatory diseases [15]. In patients with psoriasis and in an IMQ-induced psoriasis-like mouse model, IL-33 expression was predominantly enriched at the epidermal layer of lesional skin, suggesting that IL-33 could serve as a major rose in psoriasis. One study reported that recombinant IL-33 treated mice develop a marked epithelial hyperplasia in the pulmonary and GI tracts, concomitant with eosinophilic infiltration [16]. IL-33 injected into the ear, can induce an increase in the thickness of the ear and an increase in inflammatory response by causing the accumulation of neutrophils [17]. Therefore, we directly studied the effects of IL-33 on HaCaT cells and mouse psoriasis-like models. Our data demonstrated that IL-33 promotes the proliferation of HaCaT cells. Disease severity was evaluated every day using an objective scoring system based on the Psoriasis Area and Severity Index (PASI). We found that the symptoms of psoriasis-like disease in the IL-33-treated group were aggravated, and it associated with the typical pathological changes of psoriasis, and increased proliferation of keratinocytes. We also examined the changes of $CD4^+$ T and $CD8^+$ T cells in the spleen. The results obtained showed that the proportion of cells reduced in IL-33 combined with IMQ group. Therefore, we believed that IL-33 could aggravate psoriasis-like symptoms in mice by inhibiting the immune response, which results in a decrease in the proportion of immune cells in mice. But the exact mechanism requires further research. Mitsui et al. [12] have confirmed that IL-33 was as a general indicator of increased psoriatic inflammation, including psoriasis vulgaris (PV), psoriatic arthritis (PsA) and

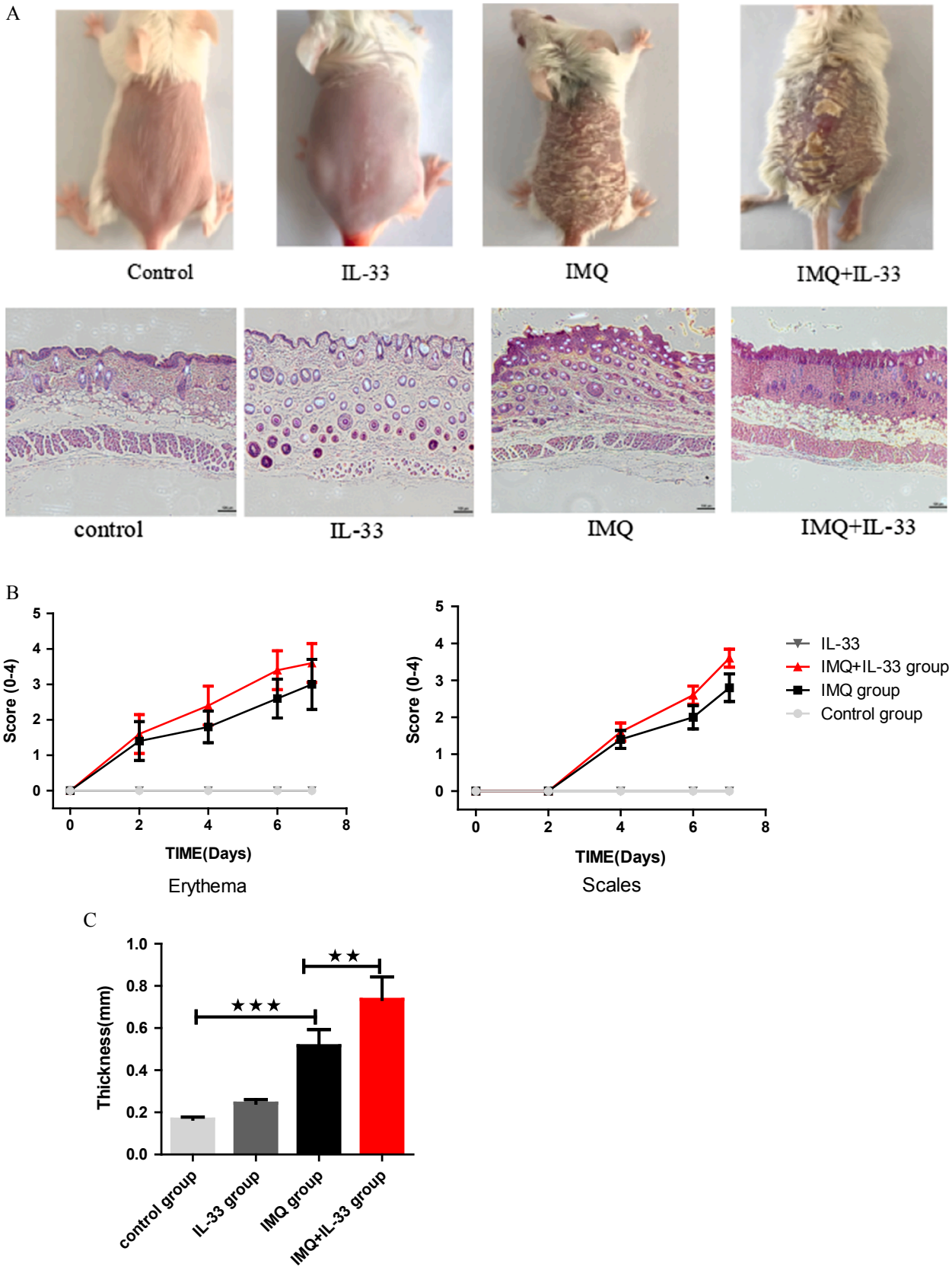


Fig. 2. IL-33 aggravated IMQ-induced psoriasis-like mouse lesions. The back skin of the mouse on three groups after being treated for seven days. The effect of IL-33 on morphology and histology of psoriatic lesion in mice(×100) (B and C) PASI scores were evaluated using the erythema and scale indicators in the 0, 2, 4, and 7 day. (D) The back skin thickness of the mouse on every group. All data represents at least three independent experiments. *p* values were determined by an unpaired *t* test or two-way. $^{***}p < 0.001$, $^{**}p < 0.01$.

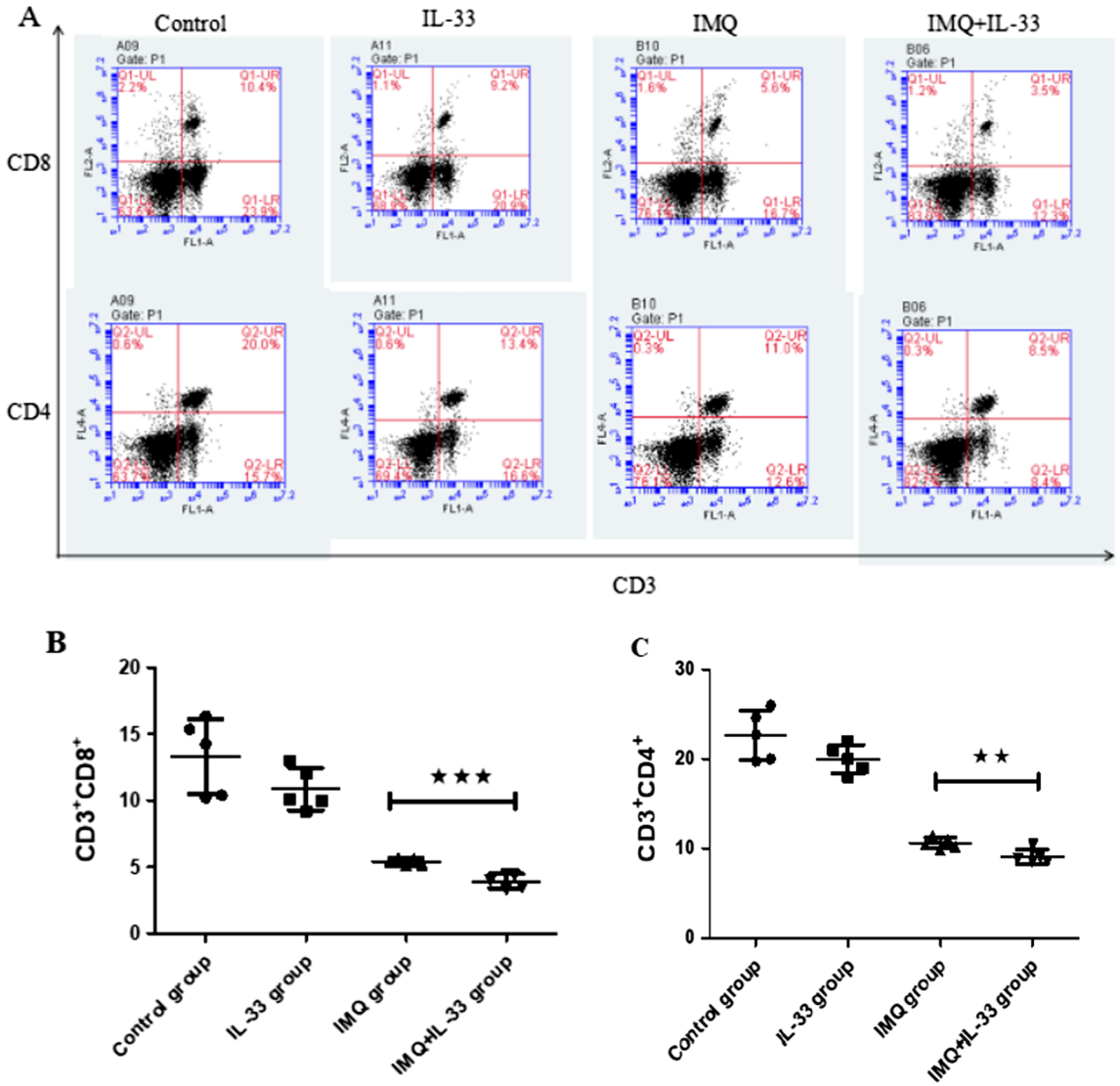


Fig. 3. IL-33 reduced the percentages of splenic $CD4^+$ and $CD8^+$ T cells IMQ-induced psoriatic mice. The ratios of $CD4^+$ and $CD8^+$ T cells in spleen. (B and C) Quantification of cellular population. All data represents at least three independent experiments. *P* values were determined by an unpaired *t* test or two-way. $^{***}p < 0.001, ^{**}p < 0.01$.

pustular psoriasis (PP). In short, these results indicate that serum IL-33 levels generally reflect increased inflammation in psoriatic patients [18]. This is consistent with that IL-33 was a general indicator of increased psoriatic inflammation. Interestingly, the study by Athari et al. found that imiquimod-induced psoriasis develop independently of IL-33 [19]. In this article, IL-33-deficient mice were used to explain that there was no different for the development in psoriasis compared with their wild-type mice. It is well known that psoriasis is a multi-factorial skin disease with a complex pathogenesis. Despite IL-33 may not be an inducer of psoriasis, some studies has proved that IL-33 was detected in lesions and serum of patients with psoriasis [12]. Our results also show the strong expression of IL-33 in psoriatic lesions and IMQ-induced psoriatic lesions. This cannot exclude that the increased expression of IL-33 may aggravate the symptoms of psoriasis. On the other hand, the mouse model used by Athari was male C57BL/6 mice, 9–11 weeks old, received a daily topical application of 62.5 mg IMQ cream on the shaved back skin for 10 consecutive days. The mouse model used in our experiment was female BALB /c mice, aged 6–8 weeks, received a daily topical application of 62.5 mg IMQ cream on the shaved back skin for 6 consecutive days. Because in the IMQ-induced mouse model, the symptoms of psoriatic lesions usually occur on the second day to the seventh day, and there is a recovery of symptoms in mice by themselves after the eighth day. We considered that the different models and strains of animals may show significant differences in the results.

Autophagy is an important physiological process that plays major roles in several pathological processes [20,21]. When autophagy occurs, LC3-I is processed and modified with phosphatidyl ethanolamine (LC3II) which is inserted into the autophagosomal membrane [22]. Beclin-1 is a protein that interacts with BCL-2 or PI3k III and plays a pivotal role in controlling autophagy and cell death [23,24]. Studies have also indicated that the expression of Beclin1, LC3 and ATG5-ATG12 complexes in the lesions skin of psoriasis is significantly decreased or even absent, but in the non-lesional skin of psoriasis and normal skin were increased [25]. Until now, little is known about the interactions of IL-33 and autophagy. A recent study showed that IL-33 down-regulates the autophagy and the inflammatory response to

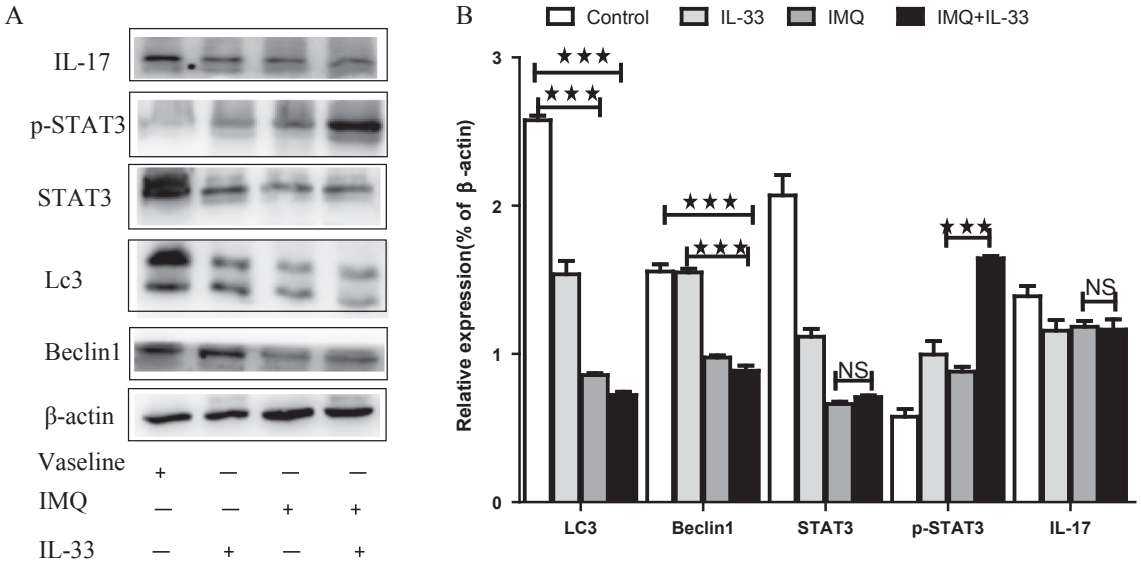


Fig. 4. IL-33 influences the expression of autophagy-related protein and STAT3 phosphorylation in IMQ-induced psoriatic mice skin lesion tissues. The western blotting results of each protein in every group. (B and C) Quantification of protein expression. All data represents at least three independent experiments. *P* values were determined by an unpaired *t* test or two-way. $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$.

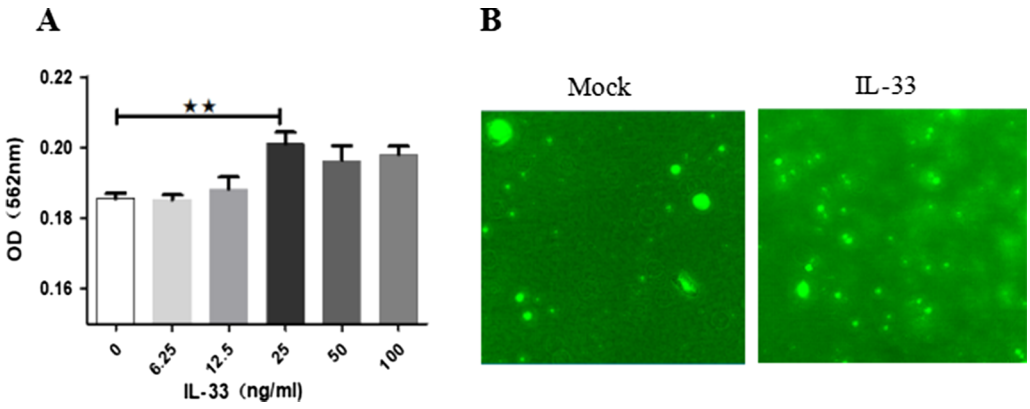


Fig. 5. IL-33 promotes HaCaT cell proliferation. IL-33 dose-dependently (ng/ml) increased the cell numbers of HaCaT cell in vitro cultures for 24 h. The viable cells were determined by Microplate reader. (B) Immunofluorescence staining of Ki67 stain in HaCaT cell stimulated by IL-33 for 24 h in cell cultures. All data are representative of three independent experiments. *P* values were determined by an unpaired *t* test or two-way ANOVA. $^{**}p < 0.01$.

protect mice from collagenase-induced intracerebral hemorrhage [26]. Similarly, IL-33 suppressed autophagy and apoptosis in neurons of neonatal rats to protect against the damaging effects of recurrent seizure [27]. Moreover, administration of IL-33 inhibited autophagy in a mouse model of traumatic brain injury [28]. In conclusion, inhibition of autophagy leads to worsening of skin inflammatory disease [29]. In our study, a decline was observed in the levels of LC3 and Beclin1 in the IL-33 with IMQ treatment group, which leads to a more serious inflammatory reaction in psoriasis-like mice. Hence, the results indicate that inhibition of autophagy aggravates the inflammatory response. However, in vitro, the expression of autophagy-related protein in IL-33 treatment group was not attenuated, which was inconsistent with the results in vivo. The reason may be that the cell line used in vitro experiments is influenced in a single factor, but in vivo animal experiments are affected by a variety of complex factors.

Previous studies have shown that STAT3 is critical for the proliferation of skin keratinocytes and development of psoriasis [30]. Studies have also indicated that IL-33 reduces the expression of filaggrin by promoting ERK and STAT3 phosphorylation in human keratinocytes, therefore alleviating atopic dermatitis [31]. In our study, the phosphorylated STAT3 expression was higher in IL-33 combined with IMQ induced psoriatic lesions than others group. Up to now, there are no reports on the relationship between IL-33 and STAT3 in psoriasis in the literature. This could be an indirect effect of IL-33 as IL-33 receptor does not depend on the classic JAK-STAT pathways for cellular signaling. We hypothesize that the exacerbation of psoriasis-like symptoms in mice treated with IL-33 may be dependent on activation of the STAT3 signaling pathway by some other unknown factor, leading to a synergy that actives downstream effects such as autophagy. Our study showed that IL-33 can inhibit the autophagy-associated proteins by promoting the phosphorylation of STAT3, which aggravate the symptoms of psoriasis-like mouse mode.

It is well established that IL-17 immune pathway plays a key role in the development of psoriasis. Increased IL-17 pathway mediated the development of psoriasis. We also detect the expression of IL-17 in IMQ-induced psoriasis-like lesion tissues. Interestingly, our results suggested that the aggravation of psoriasis-like symptoms by IL-33 are independent of IL-17. However, the detailed mechanisms need further be elucidated.

In conclusion, we demonstrated that recombinant IL-33 promoted the proliferation of keratinocytes and aggravated the symptoms of psoriasis, Our findings may open new perspectives for understanding the mechanisms and developing a therapeutic strategy by targeting the IL-33 for psoriasis.

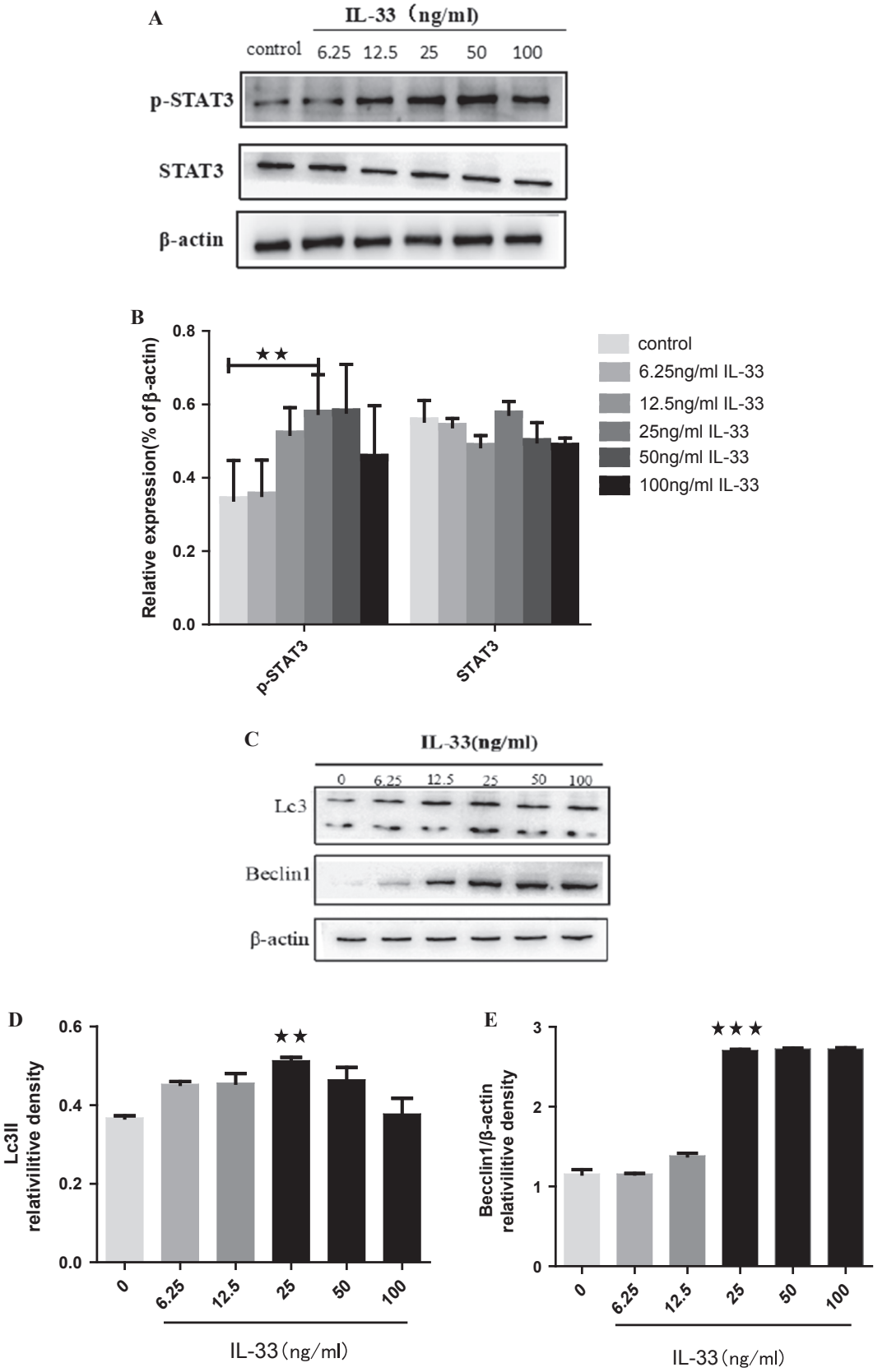


Fig. 6. IL-33 induced autophagy-related protein expression and promoted STAT3 phosphorylation in HaCaT cells. STAT3 phosphorylation were detected by western blotting in HaCaT in vitro after IL-33 in different concentration treatment for 24 h. (B) Quantification of protein expression. (C) LC3B and Beclin1 were detected by western blotting in HaCaT in vitro after IL-33 in different concentration treatment for 24 h. (D and E) Quantification of protein expression. All data are representative of three independent experiments. *P* values were determined by an unpaired *t* test or two-way ANOVA.; $^{**}p < 0.01$ and $^{***}p < 0.001$.

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Competing interests

The authors declare that they have no competing interests.

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