

ORIGINAL ARTICLE

Anti-IL-33 Antibody Has a Therapeutic Effect in an Atopic Dermatitis Murine Model Induced by 2, 4-Dinitrochlorobenzene

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Abstract— IL-33 is a new member of the IL-1 family that plays a role in allergic disease. In this study, we evaluated the potential on the inhibition of atopic dermatitis (AD) of anti-mouse IL-33 antibody (α IL-33Ab) using 2, 4-dinitrochlorobenzene (DNCB)-induced AD mice model. We treated mice with α IL-33Ab *via* subcutaneous injection of each DNCB treatment 1 h later from day 1 to day 33 for 14 times. A control group received tacrolimus. Skin lesion and scratching behavior were compared. Ear thickness, dermatitis score, eosinophils and mast cells infiltration, and serum IgE levels were also analyzed. Correlations between serum IL-33 as well as soluble(s) ST2 and AD disease activity index in human AD were also investigated. DNCB-induced AD-like mice treated with α IL-33Ab showed improved AD-like symptoms. Eosinophils and mast cells infiltration and serum IgE levels were also significantly reduced by α IL-33Ab. Our study suggests that blockade of IL-33 has a curative effect on AD.

KEY WORDS: IL-33; α IL-33Ab; atopic dermatitis; DNCB; inhibition.

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease that is characterized by intense itching and recurrent eczematous lesions [1]. The epidermal thickness with cutaneous hypersensitivity in AD is associated with increased serum immunoglobulin E (IgE) levels and infiltration of inflammatory cells including eosinophils and mast cells [2, 3].

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As current treatment regimens of AD represent a therapeutic challenge, there is a growing interest in cytokine-targeted therapies that could improve the therapeutic armamentarium for AD [4–6]. Interleukin (IL)-33 is a newly cytokine from the IL-1 family including IL-1 α , IL-1 β , and IL-18, which may be involved in the pathogenesis of allergic inflammation [7]. IL-33 can be secreted from inflamed and damaged tissues, including epithelial or endothelial cells *via* scratching behavior, of AD [8]. IL-33 may play a crucial role in the exacerbation of allergic inflammation in allergic diseases mediated by activation of eosinophils [7] and has been shown to promote the maturation and survival of mast cells leading to the production of an array of proinflammatory cytokines, which plays important roles in type-2 innate immunity and atopic disease, including AD [9–13]. Injection of recombinant IL-33 into the mouse skin elicits production of Th2 cytokines [14] and accumulation of macrophages, eosinophils, and mast cells [15]. Furthermore, vitamin D3 analog MC903-induced AD syndrome was markedly decreased in IL-33 $-/-$ mice and IL-33 can mediate MC903-induced AD *via*

ST2-MyD88 signaling [16]. Besides, anti-IL-33 antibody treatment has been reported to inhibit allergen-induced eosinophilic airway inflammation, mucus hypersecretion, and Th2 cytokine production in mice [17].

However, it remains elusive whether anti-IL-33 antibody treatment plays a protective or deleterious role in AD. In the current study, we explored the expression of IL-33 in AD patients and evaluated the potential of IL-33 inhibition as a treatment option for AD using the 2, 4-dinitrochlorobenzene (DNCB)-induced BALB/c mice model. This study reveals that IL-33 represents a potential therapeutic target in AD disease and that inhibition of IL-33 delays AD progression.

MATERIALS AND METHODS

Subjects

The study adhered to the tenets of the Declaration of Helsinki. From March to November 2016, a total of 28 patients (18 men, 10 women; median age 7.99 ± 2.56 years old; range 0.41–38 years old) with AD were randomly selected and enrolled in the study. Diagnosis of AD was based on the criteria of Hanifin and Rajka's diagnostic criteria [18]. The severity of the disease is determined by the Atopic Dermatitis Score Index (SCORAD) [19]. The control group contains 14 healthy individuals. All subjects were at similar age and gender distributions. In addition, the patients with additional acute/chronic diseases other than allergic diseases were excluded. Peripheral blood was obtained when the eruptions in patients with AD were exacerbated. The study was approved by the local ethical committee (2016PS001K) and oral consent was obtained from all individuals.

Assay for Serum Levels of IL-33 and sST2

Serum IL-33 and sST2 levels were measured using a sandwich double monoclonal antibody enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's directions.

Animals

Female 5- or 6-week-old BALB/c mice were purchased from Beijing HFK Bioscience CO. (Beijing, China). Mice were housed in individual ventilated cages under specific pathogen-free conditions with a 12-h light-dark cycle, standard conditions of temperature ($22\text{--}25^\circ\text{C}$), and humidity (35–75%). The animals were provided with food

and tap water *ad libitum*. All animal care and experiments were performed in the Experimental Animal Center in accordance with the Guidelines for the Care and Use of Laboratory Animals at affiliated Shengjing Hospital of China Medical University, and the protocol was approved by the Ethics Committee of China Medical University (2016PS001K). All studies involving animals were reported in accordance with the ARRIVE guidelines for reporting experiments involving animals [20, 21].

Sensitization and Challenge Procedures

Mice were sensitized and repeatedly challenged at the same skin site with DNCB followed by anti-mouse IL-33 antibody (α IL-33Ab, R&D Systems:AF3626, USA) or tacrolimus as described previously [22]. Briefly, after 2 weeks of acclimation, mice were divided into four groups ($n = 6\text{--}8$ per group): (1) Vehicle, vehicle + PBS, (2) Control, DNCB + PBS, (3) α IL-33Ab, DNCB + α IL-33Ab subcutaneous (s.c.) injection ($2.5\text{ }\mu\text{g}/\text{mouse}$), and (4) Tacrolimus, DNCB + tacrolimus intraperitoneal (i.p.) injection ($0.1\text{ mg}/\text{kg}$). The dose of tacrolimus was based on that used to show antipruritic effects in other AD mouse models [23]. One day before application, the dorsal hair was completely removed (approximately 4 cm^2). On day 1, the dorsal skin was applied with $150\text{ }\mu\text{l}$ 1% DNCB dissolved in an acetone: olive oil mixture (3:1 vol/vol), and the face and surface of both ear lobes were applied with $20\text{ }\mu\text{l}$ 1% DNCB solution. On day 5, the dorsal skin as well as the face and surface of both ear lobes were applied with 150 or $20\text{ }\mu\text{l}$ 0.2% DNCB solution dissolved in an acetone: olive oil mixture (3:1 vol/vol) respectively three times a week for 4 weeks (day 5–33). One hour after each DNCB application, α IL-33Ab $2.5\text{ }\mu\text{g}/\text{mouse}$ s.c. or tacrolimus $0.1\text{ mg}/\text{kg}$ i.p. was injected. As the negative control, the same amount of acetone olive oil mixture (3:1 vol/vol) was applied to the dorsal skin, face, and surface of both ear lobes of BALB/c mice followed by PBS s.c. injection. At the end of experiments, animals were anesthetized with 2% isoflurane, the blood and the injection site were harvested for analysis. The experimental design is summarized in Fig. 1.

Skin Lesion, Dermatitis Score, and Ear Thickness

Mice were anesthetized with 2% isoflurane, and images of skin lesions were taken using a digital camera (PRAKTICA Luxmedia16-Z21C, Germany) on day 33. Mice were observed and the skin conditions were scored once a week according to the criteria described previously [24]. A total dermatitis score (maximum score 15)

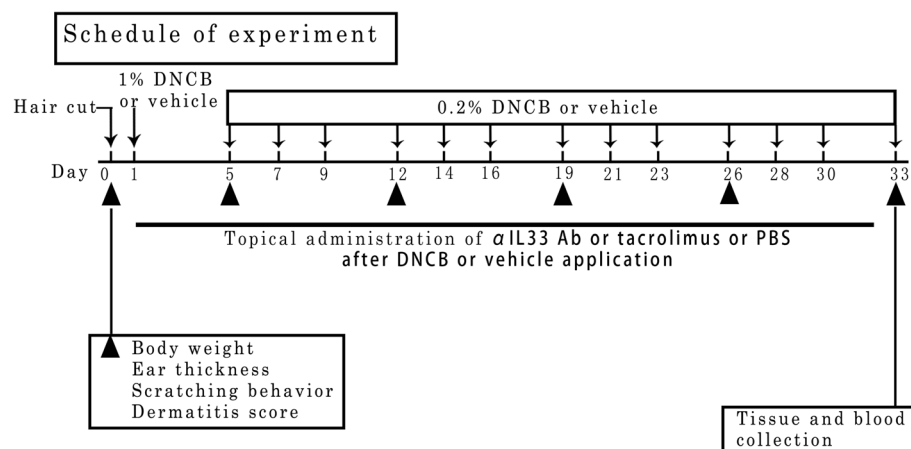


Fig. 1. Experimental design. For the induction of AD, DNCB was topically applied on BALB/c mice. One day before application, the dorsal hair was completely removed. On day 1, 1% DNCB or vehicle was applied. On day 5, 0.2% DNCB was challenged three times a week for 4 weeks (day 5–33) followed by α IL-33Ab 2.5 μ g/mouse s.c. or tacrolimus 0.1 mg/kg i.p. or PBS topically application 1 h later.

indicating clinical severity was defined as the sum of the individual scores graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe), which were given for each of five symptoms (itching, erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness). In addition, mouse ear thickness was also measured and recorded once a week with a micrometer (Mitutoyo, Kawasaki, Japan). To minimize variation, a single investigator performed all measurements [25].

Scratching Behavior

To investigate AD-like behavioral changes on mice, we measured the scratching events spent on rubbing their face, ears, and dorsal skin with their hind paws by placing each of them into a cage once a week for 10 min and observing and recording *via* video. In addition, to allocate mice to groups, we measured the scratching events without treatment. Individual mice with a high or low number of scratching events were not included into the study, and the remaining mice were distributed to groups. Thus, there were no differences between groups on the basal number of scratching events during the experiments.

Histological Analysis

To evaluate epidermal thickening and detect inflammatory cells infiltration (*i.e.*, eosinophils and mast cells), the dorsal skin lesions of each mouse were removed on day 33, fixed with 10% neutral-buffered formalin, and embedded in paraffin. Then,

4-mm-thick sections were cut and transferred onto slides. Deparaffinized skin sections were stained with hematoxylin and eosin (H&E) and toluidine blue (TB) to detect epidermal thickness and inflammatory cells, respectively. Histopathological evaluation of all skin sections occurred in a blind fashion [2]. The number of eosinophils and mast cells in five sites chosen at random was counted at $\times 400$ magnification. Tissue sections were examined using an inverted microscope and data are representative of five observations (Y-TV55, Nikon, Tokyo, Japan).

Serum IgE Measurements

Blood were collected on day 33 and stored at -80°C until use. Serum IgE levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's directions.

Statistics Analysis

Statistical analysis was performed using GraphPad Prism software (ver. 7.0 GraphPad Software, San Diego, CA, USA). The data were expressed as means $\pm$ standard error of the mean (SEM) and the data from animals and human were evaluated using one-way ANOVA with Duncan's multiple-range test and independent-sample Student's *t* test, respectively. Data are representative of three independent experiments. Differences were considered significant at $p < 0.05$.

RESULTS

Effects of α IL-33Ab on the Development of DNCB-Induced AD Mouse Skin Lesions

Firstly, to verify the toxicity of the drug, we measured body weight of BALB/c mice treated with different treatments. As seen in the Suppl. 1, we found that α IL-33Ab s.c. injection did not show any toxicity maintaining body weight. To determine the effect of IL-33 inhibition on the skin lesions, we administered α IL-33Ab following the induction of AD mouse skin lesions by using DNCB. In the AD model, severe dermatitis with edema, erosion, scarring, and hemorrhage was observed (Fig. 2a), as well as the dermatitis score gradually increased throughout the observation period (Fig. 2b). However, skin conditions significantly improved in α IL-33Ab administered groups compared to the control group (Fig. 2a, b). In addition, the inhibition effect of α IL-33Ab on skin lesions and dermatitis scores was more effective than that in the group treated with tacrolimus (Fig. 2a, b).

Effects of α IL-33Ab on Scratching Behavior and Ear Thickness in AD Mice

We next investigated the effects of α IL-33Ab on the scratching behavior in BALB/c mice. Scratching behavior of the control group was rapidly increased and a significant reduction was observed after α IL-33Ab treatment (Fig. 3a). And α IL-33Ab also significantly reduced DNCB-induced ear thickness (Fig. 3b). In addition, effects of α IL-33Ab on scratching behavior and ear thickness in AD mice were both significantly changed compared to the tacrolimus treatment group. These results suggested that α IL-33Ab has a therapeutic effect that can reduce AD symptoms in mice.

Effects of α IL-33Ab on Dorsal Skin Thickness and Eosinophil Accumulation in AD Mouse Skin Lesions

According to the previous study, we knew that multiple applications of DNCB, inducing infiltration of high levels of inflammatory cells into the skin, resulted in increased dermal thickness [26]. In

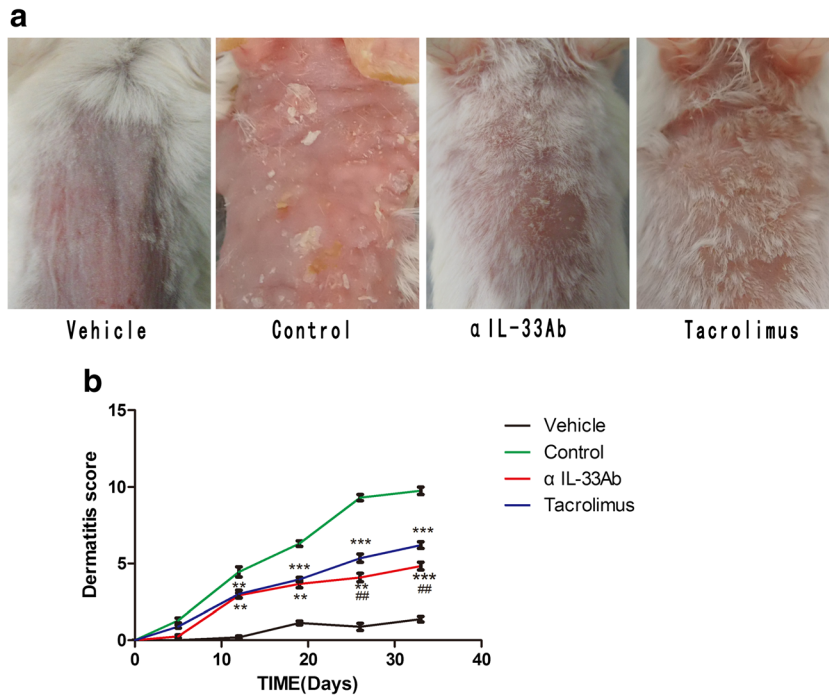


Fig. 2. Effects of α IL-33Ab on the development of DNCB-induced AD mouse skin lesions. **a** Representative effects of α IL-33Ab clinical features of DNCB-induced AD skin lesions. **b** Dermatitis scores were measured once a week for six times. Results are expressed as the mean $\pm$ SEM ($n = 6-8$). Data are representative of three independent experiments with similar results. Vehicle, vehicle + PBS treated group; control, DNCB + PBS treated group; α IL-33Ab, DNCB + α IL-33Ab 2.5 μ g/mouse s.c. treated group; tacrolimus, DNCB + tacrolimus 0.1 mg/kg i.p. treated group. ** $p < 0.01$ and *** $p < 0.001$ versus control. ## $p < 0.01$ versus tacrolimus.

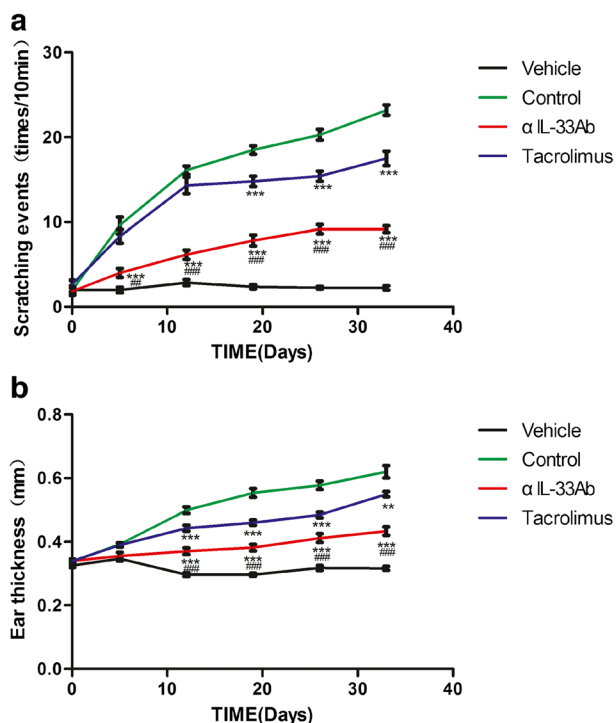


Fig. 3. Effects of α IL-33Ab on scratching behavior and ear thickness. **a** Scratching behavior was measured by placing each mouse into a cage once a week for 10 min of each time and observing and recording *via* video. **b** Ear thickness was measured once a week for six times with a dial thickness gauge. Results are expressed as the mean $\pm$ SEM ($n = 6-8$). Data are representative of three independent experiments with similar results. Vehicle, vehicle + PBS treated group; control, DNCB + PBS treated group; α IL-33Ab, DNCB + α IL-33Ab 2.5 μ g/mouse s.c. treated group; tacrolimus, DNCB + tacrolimus 0.1 mg/kg i.p. treated group. ** $p < 0.01$ and *** $p < 0.001$ versus control. ## $p < 0.01$ and ### $p < 0.001$ versus tacrolimus.

addition, it is known that eosinophils have been shown to be present in most patients with AD and correlated with the disease activity [3]. To determine whether α IL-33Ab decreases eosinophil accumulation into AD skin lesions, we preformed H&E staining on the skin after topical administration of drugs and observed the tissue under an optical microscope. We observed thickening of the epidermis and dermis and infiltration of eosinophils into both the epidermis and dermis in the control group. Whereas, α IL-33Ab significantly reduced dorsal skin thickness and suppressed eosinophil accumulation into the skin. And α IL-33Ab treatment in DNCB-induced mice showed better inhibition effect on skin thickness and eosinophils infiltration than that of the tacrolimus treatment (Fig. 4a–e).

Effects of α IL-33Ab on Mast Cell Infiltration and Serum IgE Levels in AD Mice

Hyperproduction of IgE and mast cell infiltration are major characteristics of AD. We stained sliced cross sections of skin lesions with toluidine blue for mast cells observation. Repeated cutaneous application of DNCB increased dermal mast cell number. However, this feature was significantly suppressed by α IL-33Ab compared with positive control group (Fig. 5a). Also, we investigated the effect of α IL-33Ab on total IgE production. Serum IgE levels were elevated dramatically in DNCB-induced group compared with negative control group. However, increased serum IgE levels were significantly decreased by both α IL-33Ab and tacrolimus (Fig. 5b). And α IL-33Ab treatment showed more effective than the tacrolimus one (Fig. 5a, b).

Increased Serum IL-33 and sST2 Level Was Correlated with SCORDA in AD Patients

Furthermore, we also investigated the clinical serum samples in AD patients. We found that serum IL-33 and sST2 level were markedly increased in the AD patients compared with healthy volunteers (Fig. 6a, c). Correlation analysis showed that serum IL-33 and sST2 were positively correlated with SCORDA in AD patients (Fig. 6b, d), suggesting IL-33 and sST2 as the indicators for disease activity in AD.

DISCUSSION

AD is an inflammatory disease characterized by an increase of TH2 response cells, including eosinophils, mast cells, monocytes, and macrophages. Both Th2 immune responses and increased IgE production play a pivotal role in the pathogenesis of AD [27]. Proliferation, migration, and local activation of eosinophils are common in AD. Eosinophils can secrete an array of cytokines and chemokines to attract more eosinophils to the site of inflammation. In addition, eosinophils promote a switch to chronic responses in AD [3]. In our experiment, α IL-33Ab treatment reduces eosinophil accumulation in AD mouse skin lesions (Fig. 4d, e).

Mast cells play a key role in allergic reactions *via* the production and secretion of proinflammatory mediators such as histamine, chemokines, cytokines,

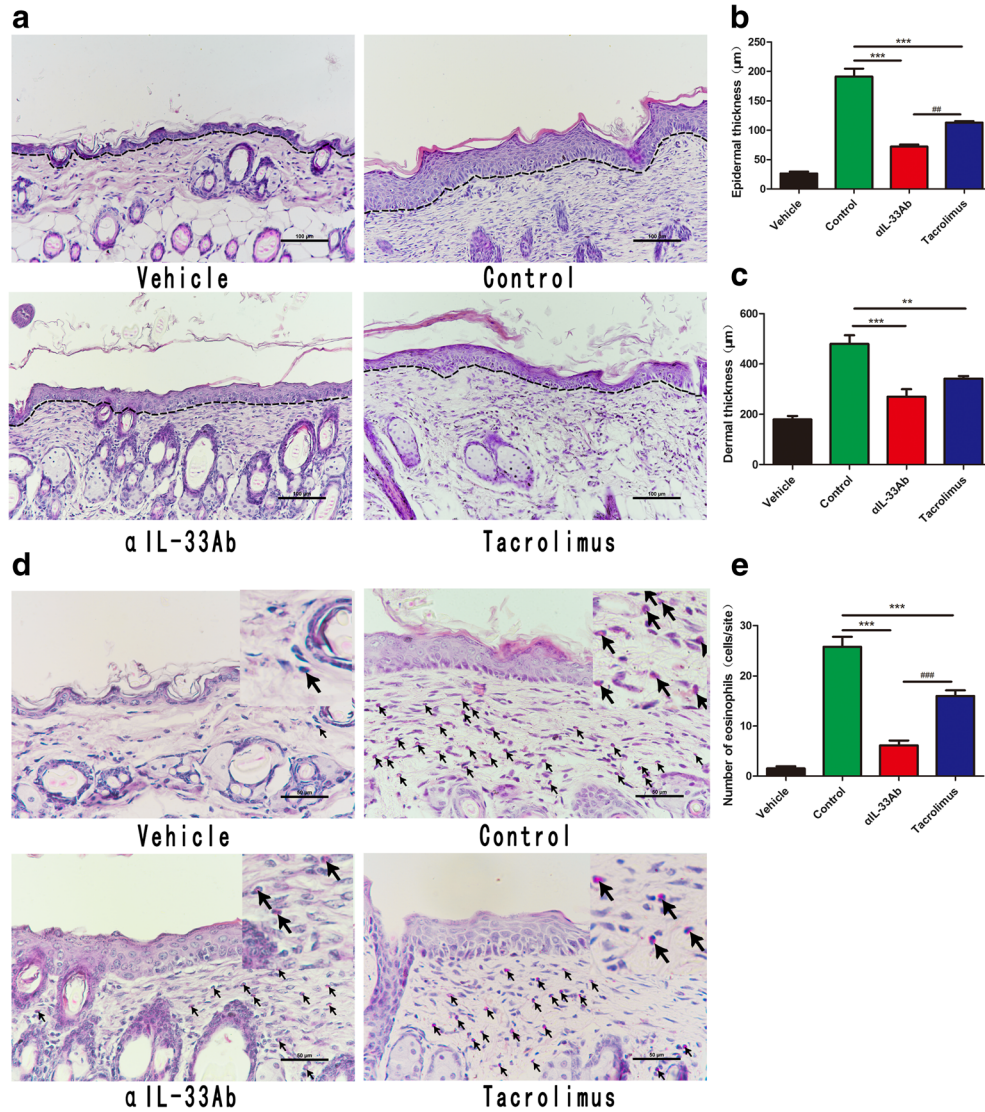


Fig. 4. Effects of α IL-33Ab on dorsal skin thickness and eosinophil accumulation in AD mouse skin lesions. **a** The epidermis findings by H&E staining of skin sections (20 $\times$, scale bar = 100 μ m). Black dotted line shows the boundary line between epidermis and dermis. **b** Determination of epidermal thickness. **c** Determination of dermal thickness. **d** Eosinophil findings by H&E staining of skin sections (40 $\times$, scale bar = 50 μ m) and their local magnifications on the right upper corner. Black arrows mark the stained eosinophils. **e** Number of eosinophils per site. Results are expressed as the mean $\pm$ SEM (n = 6–8). Data are representative of three independent experiments with similar results. Vehicle, vehicle + PBS treated group; control, DNCB + PBS treated group; α IL-33Ab, DNCB + α IL-33Ab 2.5 μ g/mouse s.c. treated group; tacrolimus, DNCB + tacrolimus 0.1 mg/kg i.p. treated group. ** p < 0.01 and *** p < 0.001 versus control. ## p < 0.01 and ### p < 0.001 versus tacrolimus.

and growth factors. On the surface of mast cells, Th2 cells produce IgE, which binds to the Fc ϵ RI on the mast cell surface [28]. Fc ϵ RI-mediated mast cell activation is triggered by antigen IgE cross-linking and leads to the degranulation and expression of proinflammatory mediators. Fc ϵ RI-activated mast cells induce IgE elevation and increase mast cells

in a majority of AD patients; therefore, mast cells are hypothesized to contribute to the pathogenesis of AD [29]. IgE plays a pivotal role in the pathogenesis of allergic inflammatory diseases, as high production of serum IgE mediates the critical clinical characteristics of AD. In particular, IgE binds with mast cells, thus inducing the inflammatory

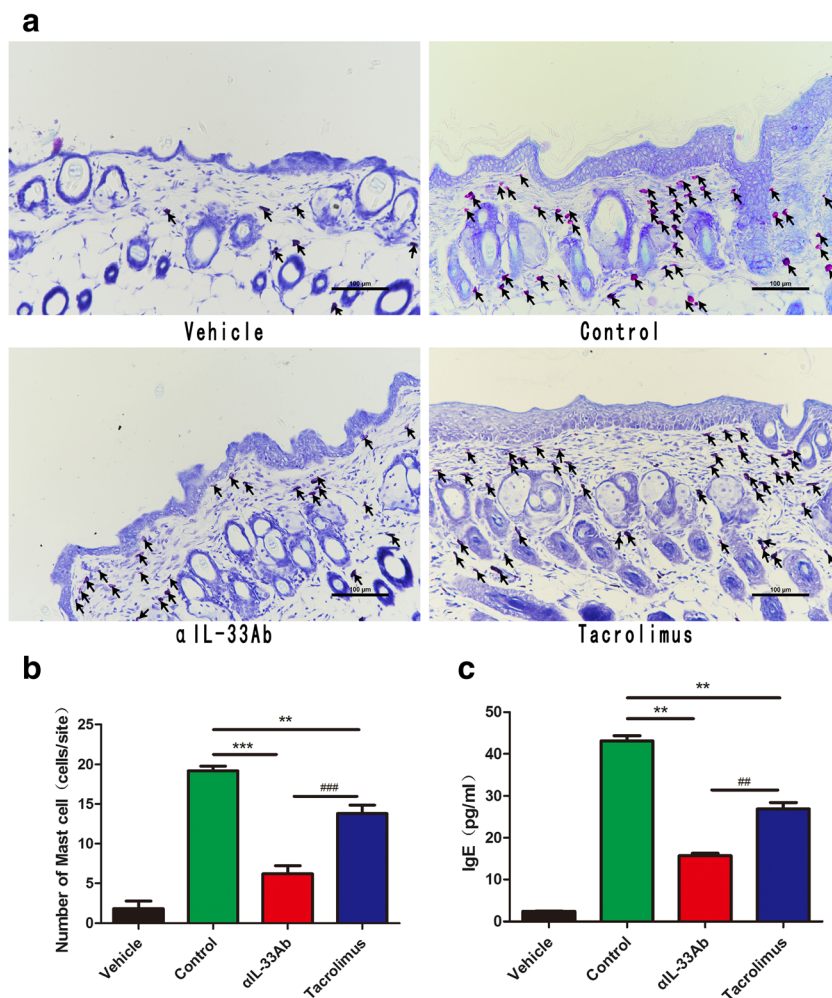


Fig. 5. Effects of α IL-33Ab on mast cell infiltration and serum IgE levels in AD mice. **a** Mast cells findings by toluidine blue staining of skin sections (40 $\times$, scale bar = 50 μ m). Black arrows mark the stained mast cells. **b** Number of mast cells per site. **c** Serum IgE level was measured by ELISA. Results are expressed as the mean $\pm$ SEM ($n = 6-8$). Data are representative of three independent experiments with similar results. Vehicle, vehicle + PBS treated group; control, DNCB + PBS treated group; α IL-33Ab, DNCB + α IL-33Ab 2.5 μ g/mouse s.c. treated group; tacrolimus, DNCB + tacrolimus 0.1 mg/kg i.p. treated group. ** $p < 0.01$ and *** $p < 0.001$ versus control. ## $p < 0.01$ and ### $p < 0.001$ versus tacrolimus.

mediators, which are associated with the severity of AD [30]. In the epidermis of AD patients, there are increased IgE-bearing Langerhans cells, which play a crucial role in presenting cutaneous allergens to Th2 cells and may promote a clinical situation, which scratching is a known prerequisite for the development of AD skin lesions [31]. Therefore, IgE is considered to be one of the most important therapeutic targets in AD. Our findings show that α IL-33Ab treatment suppresses serum IgE levels and mast cell infiltration in a DNCB-induced AD mouse model (Fig. 5a, b).

Increasing evidence has shown that IL-33 and its receptor ST2 contribute to the pathogenesis of allergic inflammation diseases. It was reported that IL-33 increased significantly in patients with AD compared those in patients with urticarial and psoriasis and in healthy control subjects, and the elevated levels of IL-33 were significantly reduced after drug treatment [32]. Studies have also indicated an important role for IL-33/ST2 interaction in AD, wherein the expression profile of IL-33 and ST2 increased significantly in AD skin after irritant, allergen, or staphylococcal enterotoxin B exposure [33]. Data also showed that *in vitro*, IL-33/ST2 axis is able to control Th2/IL-31

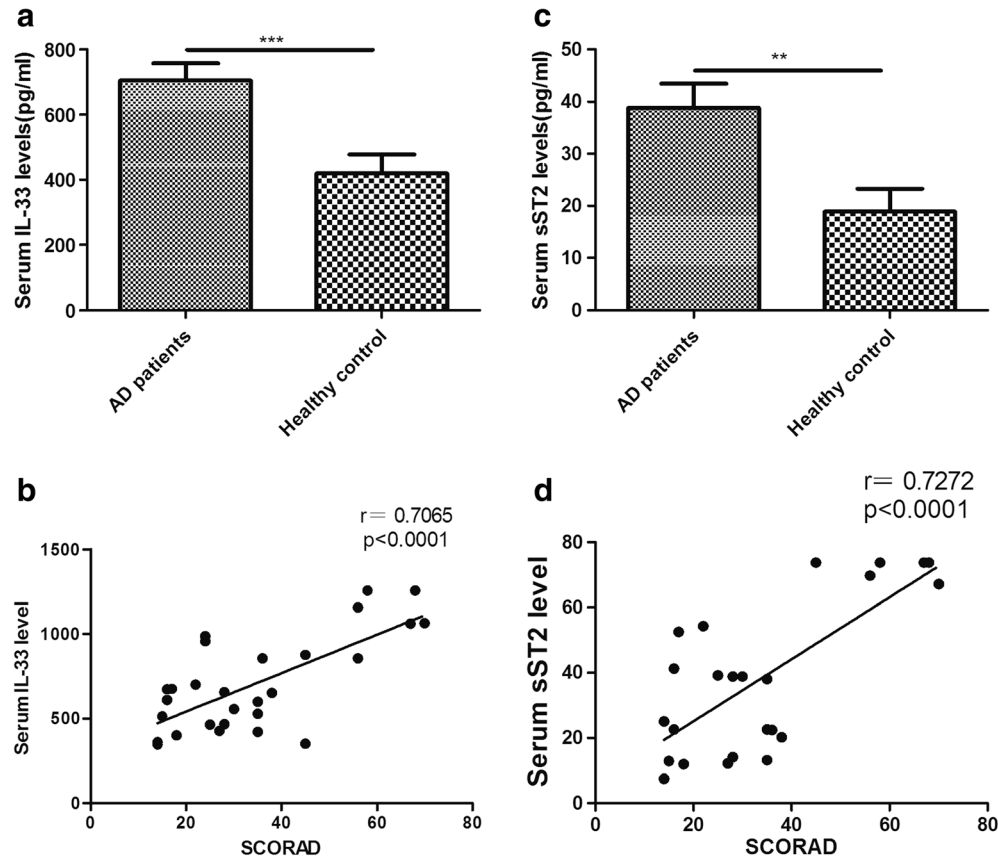


Fig. 6. Increased serum IL-33 and sST2 level were correlated with SCORAD in 28 AD patients. **a** Serum IL-33 level was markedly increased in the 28 AD patients compared with 14 healthy volunteers. **b** Correlation analysis showed that serum IL-33 was positively correlated with SCORAD in 28 AD patients ($r = 0.7065$, $p < 0.0001$). **c** Serum sST2 level was markedly increased in the 28 AD patients compared with 14 healthy volunteers. **d** Correlation analysis showed that serum sST2 was positively correlated with SCORAD in 28 AD patients ($r = 0.7272$, $p < 0.0001$, 4 individuals were excluded to avoid bias). Data are the mean $\pm$ SEM for 28 patients and 14 healthy controls. ** $p < 0.01$ and *** $p < 0.001$ versus healthy control.

and IL-17 immune response in PBMC from allergic patients [34]. And soluble(s) ST2 acts as a decoy receptor which inhibits IL-33 pathway. Elevated sST2 level has been reported in asthmatic children while compared with healthy control [35]. However, the functional role of IL-33 and sST2 in AD remains to be explored. In our present study, we observed higher levels of IL-33 and sST2 in patients with AD compared with those in the control, which was consistent with the previous study but not including higher sST2 [36]. Delivery of sST2 depresses Th2 cytokine production by the IL-33 signaling in allergic airway inflammation [37]. Our data confirm not only IL-33 but sST2 involvement in AD patients, as they were increased in serum. These results suggest, with increasing of IL-33, production of sST2 is probably inhibiting IL-33 induced inflammation. In addition, we also found serum IL-33 and

sST2 levels were associated with disease activity (SCORAD).

In this study, we presented evidence that IL-33 played a critical role in the pathological process of the AD and verified that administration of α IL-33Ab *in vivo* could significantly improve scratching behavior, ear thickness, epidermal and dermal thickness, and serum IgE levels in DNCB-induced AD mice. We observed a reduction in the infiltration of eosinophils and mast cells into the AD skin lesions following α IL-33Ab treatment. These data suggest the new IL-1 family member IL-33 is a potential therapeutic target in AD.

In conclusion, these results demonstrate that α IL-33Ab decreases AD symptoms in DNCB-induced AD mice. Our study suggests that IL-33 blockade might be a potential therapeutic option for treating AD patients.

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COMPLIANCE WITH ETHICAL STANDARDS

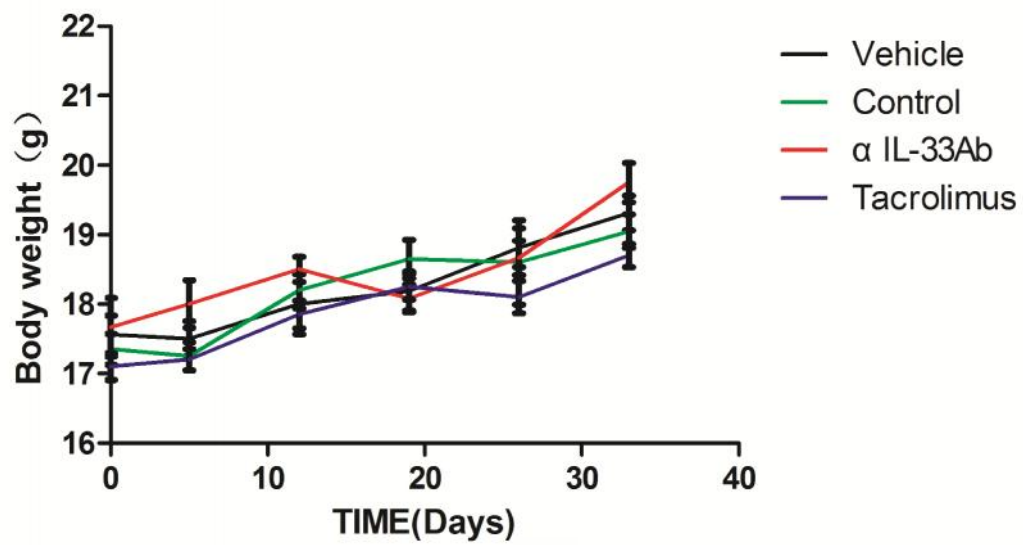
The study adhered to the tenets of the Declaration of Helsinki. The study was approved by the local ethical committee (2016PS001K) and oral consent was obtained from all individuals.

Conflict of Interest. The authors declare that they have no conflict of interest.

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Suppl. 1. Body weight. Body weight of mice were measured once a week for 6 times. Results are expressed as the mean $\pm$ SEM ($n = 6-8$). Data are representative of three independent experiments with similar results. Vehicle, vehicle + PBS treated group; control, DNCB + PBS treated group; α IL-33Ab, DNCB + α IL-33Ab 2.5 μ g/mouse s.c. treated group; tacrolimus, DNCB + tacrolimus 0.1mg/kg i.p. treated group.