

IL-33 and ST2 in Atopic Dermatitis: Expression Profiles and Modulation by Triggering Factors

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In the acute phase of atopic dermatitis (AD), T-helper type 2 (Th2) cytokines characterize the inflammatory response in the skin. IL-33 is a new tissue-derived cytokine, which is mainly expressed by cells of barrier tissues, and is known to activate Th2 lymphocytes, mast cells, and eosinophils. IL-33 signals through a receptor complex consisting of IL-33-specific receptor ST2 and a co-receptor IL-1RAcP. As IL-33 is known to promote Th2-type immunity, we examined expression profiles of IL-33 and its receptor components in human AD skin, in the murine model of AD, and in various cell models. We found increased expression of IL-33 and ST2 in AD skin after allergen or staphylococcal enterotoxin B (SEB) exposure, as well as in the skin of 22-week-old filaggrin-deficient mice. In addition, skin fibroblasts, HaCaT keratinocytes, primary macrophages, and HUVEC endothelial cells efficiently produced IL-33 in response to the combined stimulation of tumor necrosis factor- α and IFN- γ , which was further enhanced by a mimetic of double-stranded RNA. Finally, the increased expression of IL-33 and ST2 caused by irritant, allergen, or SEB challenge was suppressed by topical tacrolimus treatment. These results suggest an important role for IL-33–ST2 interaction in AD and highlight the fact that bacterial and viral infections may increase the production of IL-33.

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INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease affecting about 10 million people worldwide. A majority of AD patients exhibit hyperproduction of total IgE, and most of them have allergen-specific IgE in the sera. Classical T-helper type 2 (Th2) cytokines such as IL-4, IL-5, and IL-13 are expressed in the acute eczematous lesions, whereas in chronic lesions IFN- γ -producing Th1 cells are dominant (Bieber, 2010). In addition to classical Th2 cytokines, new tissue-derived cytokines such as TSLP and IL-33 have been suggested to have a significant role in AD (Kinoshita *et al.*, 2009; Smith, 2009). In the skin lesion, signals from

barrier disruption, allergens, and microbial colonization are integrated and transmitted to diverse immune cells, which initiate and maintain the skin inflammation (Carmi-Levy *et al.*, 2011). Patients with AD are particularly susceptible to *Staphylococcus aureus* infections, partly because of impaired expression of endogenous antimicrobial peptides and defective stratum corneum barrier (Ong *et al.*, 2002; Nomura *et al.*, 2003). Besides microbial skin infections, patients with AD are susceptible to infection of certain viruses (Wollenberg *et al.*, 2003) due to disturbed skin barrier function.

IL-33 (IL-1F11) is a recently described cytokine that belongs to the IL-1 family and is linked to Th2-type immune responses. IL-33 is expressed by cells of barrier tissues (e.g., skin, gut, and lung) and is known to activate naive and Th2 lymphocytes, mast cells, and eosinophils to produce Th2-type cytokines (Schmitz *et al.*, 2005; Liew *et al.*, 2010). However, IL-33 is not produced by Th2 cells, and its signaling pathway distinguishes from classical Th2 cytokines. IL-33 signals through a membrane-bound IL-33-specific receptor ST2 (also known as IL1RL1, T1, DER4, and Fit-1) and IL-1 receptor accessory protein (IL1-RAcP), which serves as a shared co-receptor. The ST2 gene encodes at least three isoforms of ST2 proteins: ST2L, a transmembrane form, the soluble ST2, a secreted form that can serve as a decoy receptor of IL-33, and a variant ST2 (Liew *et al.*, 2010). Membrane-bound expression of ST2 is highest on mast cells (Moritz *et al.*, 1998; Allakhverdi *et al.*, 2007), Th2 cells (Xu *et al.*, 1998), and eosinophils (Cherry *et al.*, 2008), which

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Abbreviations: AD, atopic dermatitis; HDM, house dust mite; OVA, ovalbumin; poly(I:C), polyinosinic-polycytidylic acid; SEB, staphylococcal enterotoxin B; Th, T-helper; TNF- α , tumor necrosis factor- α

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are critical cells in AD. IL-33 receptor complex is activated when IL-33 binds to ST2 and IL-1RAcP, and recruits the adaptor molecule MyD88 and IL-1R-associated kinase IRAK. Activated receptor complex induces activation of signaling proteins, including transcription factor NF- κ B and the mitogen-activated protein kinase pathway (Schmitz *et al.*, 2005; Kurowska-Stolarska *et al.*, 2008).

In the present study, we explored the expression of IL-33, ST2, and IL-1RAcP in human AD skin, in the murine model of AD and in various cell models. We show increased expression of IL-33 and ST2 in AD skin after allergen or staphylococcal enterotoxin B (SEB) exposure, and efficient production of IL-33 by skin fibroblasts, HaCaT keratinocytes, primary macrophages, and HUVEC endothelial cells in response to the combined stimulation of tumor necrosis factor (TNF)- α and IFN- γ . In addition, 22-week-old filaggrin-deficient mice spontaneously express IL-33 and ST2 in the skin. Finally, the increased expression of IL-33 and ST2 caused by irritant, allergen, or SEB challenge is inhibited by topical tacrolimus treatment.

RESULTS

ST2 and IL-1RAcP are upregulated in lesional skin of patients with AD

Th2-type cytokines are produced in acute skin lesions of patients with AD, and IL-33 is known to drive the production of Th2-associated cytokines (Schmitz *et al.*, 2005). To investigate the expression of IL-33 and ST2 in AD, lesional and nonlesional skin of patients with AD and skin from healthy volunteers were investigated. We found a 2-fold increase of ST2 and a 1.8-fold increase of IL-1RAcP mRNAs, (Figure 1a) as well as a 3-fold increase of TNF- α and IL-13 mRNAs in lesional skin compared with nonlesional skin (Figure 1b). IL-33 mRNA was also increased in lesional AD skin, but the difference did not reach statistical significance. IL-33 and ST2 were expressed at the same level in healthy and nonlesional skin (Figure 1a). Immunohistochemical staining revealed increased numbers of IL-33⁺ cells in suprabasal keratinocytes and in endothelial cells of lesional skin compared with nonlesional skin (Figure 1c–f). In addition, increased numbers of ST2⁺ cells were found in infiltrating inflammatory cells in the dermis and to some extent also in the epidermis of the lesional AD skin (Figure 1g–j and Supplementary Figure S1a and b online). Isotype control-stained AD skin did not show any positive staining (Supplementary Figure S1c online).

Allergen exposure and filaggrin deficiency amplify the expression of ST2 and IL-33 in mouse AD skin and in bone marrow-derived mast cells

We investigated the expression of ST2 and IL-33 also in AD mice, which were epicutaneously sensitized with ovalbumin (OVA) allergen (modified from Wang *et al.*, 2007). Skin specimens were investigated after the first and third sensitization weeks. A 3-fold increase of ST2 expression was found after the third sensitization week compared with the ST2 expression after the first sensitization week in OVA-sensitized skin (Figure 2a). Similar to ST2 expression,

eosinophils and mast cells were increased in OVA-exposed skin along with epicutaneous sensitization (Supplementary Figure S2a online). Furthermore, mRNA levels of Th2-type cytokines, IL-4 and IL-13, were upregulated (Supplementary Figure S2c online) in accordance with ST2 expression. The number of CD3⁺ and CD4⁺ cells was already increased after 1 week of OVA exposure (Supplementary Figure S2b online), together with the enhanced expression of IL-33 in the OVA-sensitized skin (Figure 2a). Moreover, OVA-specific IgE was upregulated in the serum of OVA-sensitized mice (Supplementary Figure S3a online).

Filaggrin gene is closely linked to the increased risk to develop AD, and it is essential for skin barrier function (Palmer *et al.*, 2006; Howell *et al.*, 2007; Boguniewicz and Leung, 2011). As IL-33 is a barrier tissue-derived cytokine, we investigated IL-33 expression in filaggrin-deficient (*fl/fl*) mice, which are known to develop AD-like skin lesions with age, and start to produce IL-17, IL-23, IL-6, and IL-4 at the age of 32 weeks (Oyoshi *et al.*, 2009). We found that both IL-33 and ST2 were spontaneously upregulated in the skin of 22-week-old mice, and a 10-fold increase and a 15-fold increase of ST2 and IL-33 expressions were found, respectively, in 38-week-old *fl/fl* mice compared with 4-week-old *fl/fl* mice (Figure 2b).

Increased numbers of mast cells have been described in the lesional skin of patients with AD (Irani *et al.*, 1989; Soter, 1989). In the present study, increased numbers of mast cells were detected in the sensitized skin of AD mice (Supplementary Figure S2a online). We also examined whether cross-linking of IgE with specific allergen can stimulate bone marrow-derived mast cells to produce both IL-33 and ST2. Indeed, IgE + allergen stimulation elicited enhanced expression of ST2 mRNA and vigorous production of IL-33 mRNA in murine mast cells (Figure 2c).

House dust mite (HDM) and SEB induce IL-33 and ST2 expressions in AD skin

HDM is a common indoor allergen and an external triggering factor in AD (Hammad *et al.*, 2009). Therefore, ST2 and IL-33 mRNA levels were investigated in the skin of AD patients with histories of HDM allergy after HDM atopy patch test. We found that mRNA expression of IL-33 and ST2 was upregulated after 6 and 2 hours of HDM exposure, respectively (Figure 3a).

Furthermore, AD skin is frequently colonized with SEB-producing strains of *S. aureus* that affects the degree of disease severity (Michie and Davis, 1996; Breuer *et al.*, 2000). We investigated whether SEB can induce the expression of IL-33 and ST2 in the skin of patients with AD. IL-33 and ST2 were upregulated after SEB patch test, but it was not an immediate reaction and increased after 24 hours of exposure (Figure 3b).

We have earlier shown that dermal exposure to SEB induces a mixed Th1- and Th2-type skin inflammation in mice (Savinko *et al.*, 2005). A 4.5-fold upregulation of ST2 and a 2-fold upregulation of IL-33 were found after repeated topical SEB exposure in mouse skin compared with phosphate-buffered saline-treated skin (Figure 4a and b). Along

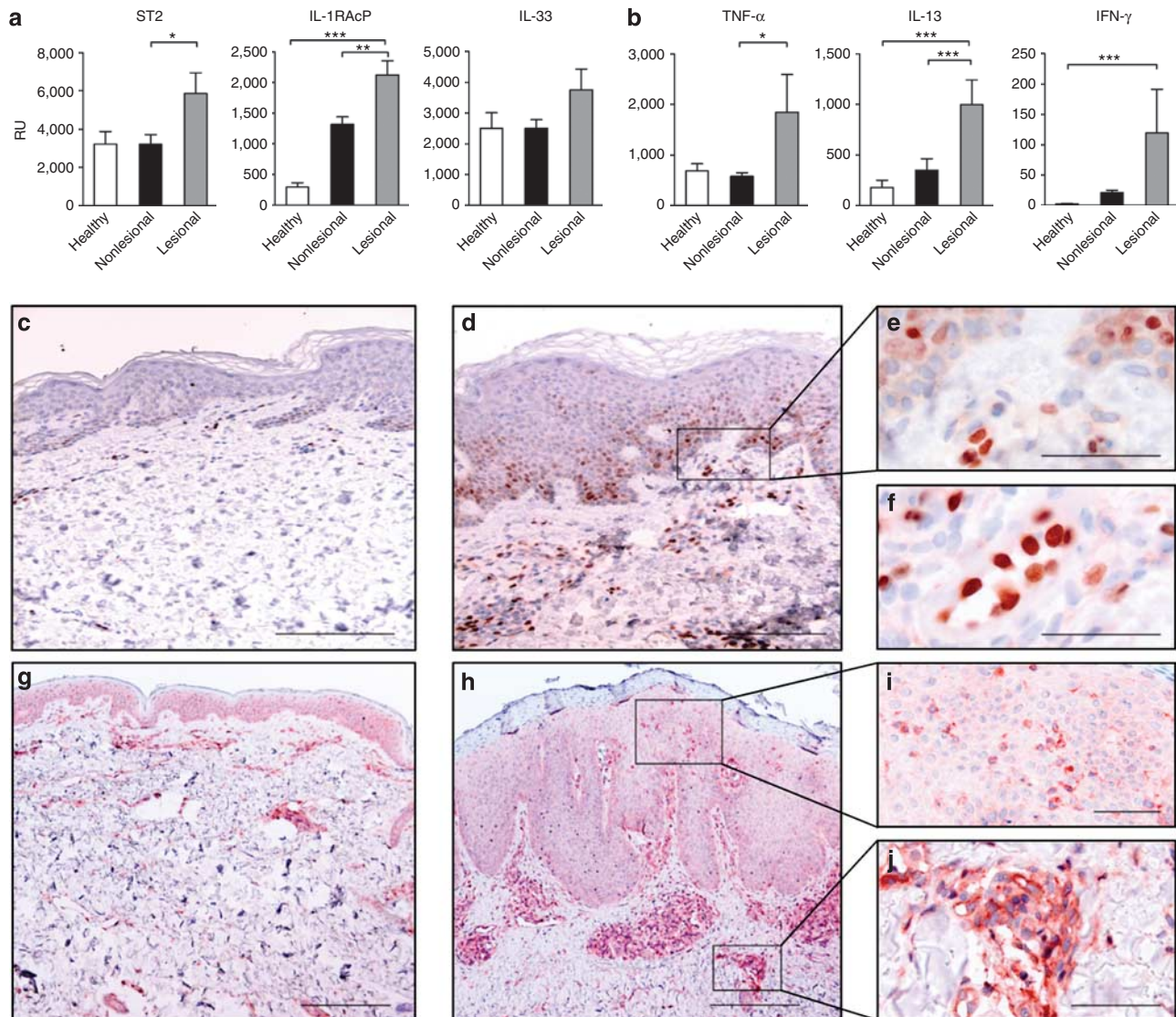


Figure 1. ST2 and IL-1RAcP are increased in lesional skin of patients with atopic dermatitis (AD). (a) mRNA levels of ST2, IL-33, and IL-1RAcP and (b) proinflammatory T-helper type (Th)2 and Th1 cytokines were analyzed by real-time reverse transcription-PCR in lesional and nonlesional skin of AD patients and in the skin of healthy volunteers. Columns and error bars represent means $\pm$ SEMs ($n = 13$ –15). Immunohistological staining of IL-33 in (c) nonlesional and (d) lesional skin of AD patients ($n = 8$). (e) Keratinocytes and (f) endothelial cells were IL-33⁺. Immunohistological staining of ST2 in (g) nonlesional and (h) lesional skin of AD patients ($n = 4$). ST2⁺ cells were found from the (i) epidermis and (j) dermal inflammatory cell infiltrates. * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$. Bar = 200 μ m in c, d, g, and h; 50 μ m in e, f, i, and j. RU, relative units; TNF, tumor necrosis factor.

with IL-33 and ST2, also TNF- α and IFN- γ (Figure 4c and d), as well as IL-4 and IL-13 (Figure 4e and f), mRNA levels were increased in SEB-exposed skin. In addition, SEB-specific IgE was similarly upregulated (Supplementary Figure S3c online).

Topical tacrolimus treatment decreases the expression of IL-33 and ST2 in mouse AD skin

Corticosteroids and calcineurin inhibitors are the most widely used drugs in AD (Akdis *et al.*, 2006). We examined whether the topical treatment with corticosteroid or calcineurin inhibitor has an effect on the mRNA expression of ST2 or IL-33 in the OVA/SEB-exposed mouse skin. A 4-fold decrease of ST2 (Figure 5a) and a 2-fold decrease of IL-33 (Figure 5b)

after tacrolimus treatment were observed, but no changes were found after the treatment with class III corticosteroid betamethasone-17-valerate compared with the OVA/SEB group (Figure 5). Notably, increased expression of IL-33 was also found from vehicle-treated skin (vehicle = acetone/phosphate-buffered saline—used as a solvent for betamethasone and tacrolimus), suggesting that mechanically (tape stripping) and chemically (acetone solvent) induced skin injury activates IL-33. Irritation-induced IL-33 expression was not affected by tacrolimus or betamethasone, whereas ST2 expression was increased in the vehicle group after betamethasone treatment. IL-33 or ST2 was not detected in the skin of untreated naive mice (Figure 5). However, tacrolimus

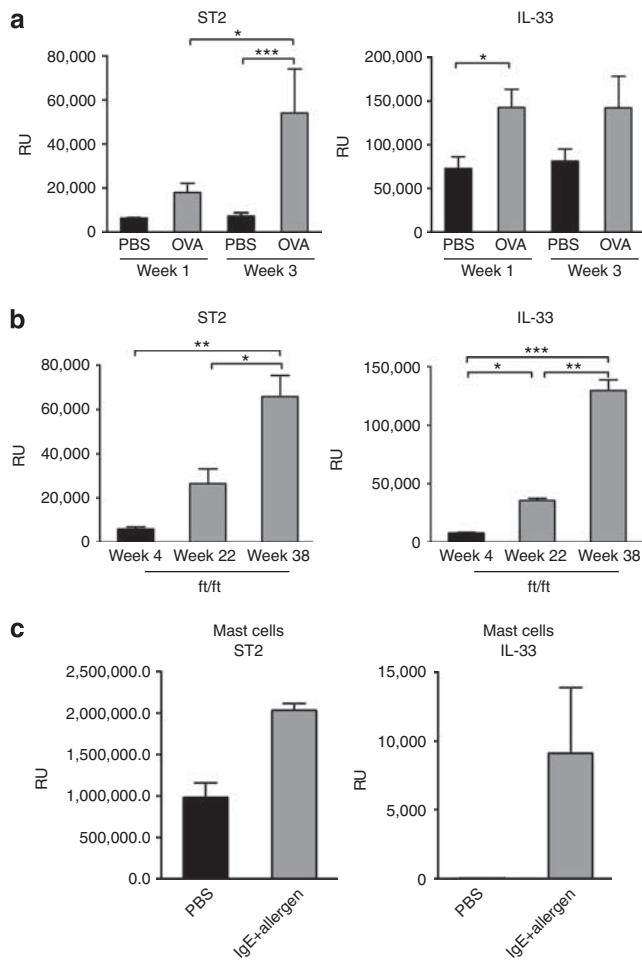


Figure 2. ST2 and IL-33 mRNA expressions in mouse atopic dermatitis (AD) skin and activated mast cells. (a) ST2 and IL-33 expressions in mouse ovalbumin (OVA)-sensitized skin after the first and third sensitization week were analyzed by real-time reverse transcription-PCR, $n = 8$ mice per group, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. (b) ST2 and IL-33 were increased in 38-week-old ft/ft mice compared with 4- or 22-week-old ft/ft mice. (c) ST2 and IL-33 expressions are shown in IgE + allergen-stimulated bone marrow-derived murine mast cells. The columns and the error bars represent means $\pm$ SEMs of three replications. PBS, phosphate-buffered saline; RU, relative units.

treatment increased OVA-specific IgE levels in mouse serum (Supplementary Figure S3b online), whereas IL-33 and ST2 mRNA levels were downregulated (Figure 5).

Human dermal fibroblasts, HaCaT keratinocytes, macrophages, and HUVEC endothelial cells produce IL-33 after stimulation with the combination of TNF- α and IFN- γ

Keratinocytes and fibroblasts are major structural cells of the skin and likely have an important role in the pathogenesis of AD. In addition, skin-infiltrating macrophages and endothelial cells are important in the inflammatory response associated with AD. We examined the expression of IL-33 in primary dermal fibroblasts, primary macrophages, HaCaT keratinocytes, and HUVEC endothelial cell lines at different cytokine environments relevant for AD. Fibroblasts, HaCaT keratinocytes, and HUVEC endothelial cells, which were

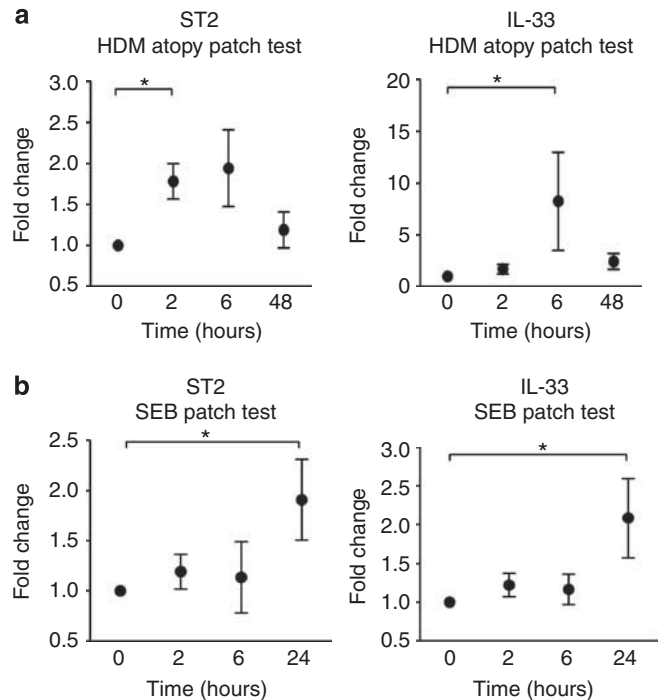


Figure 3. ST2 and IL-33 mRNAs in human atopic dermatitis skin after house dust mite (HDM) and staphylococcal enterotoxin B (SEB) exposure. mRNA expression of ST2 and IL-33 after (a) HDM atopy patch test and (b) SEB patch test was analyzed by real-time reverse transcription-PCR. Control mRNA expressions are adjusted to 1, and other expressions are shown as the fold change compared with the control. The columns and the error bars represent means $\pm$ SEMs, $n = 10$ patients per group; $*P < 0.05$.

stimulated with the combination of TNF- α and IFN- γ , produced high levels of IL-33 mRNA after 18 hours of exposure (Figure 6a, Supplementary Figure S4 online). The expression of IL-33 in TNF- α + IFN- γ -stimulated macrophages and HaCaT keratinocytes peaked already at 2 and 6 hours (Figure 6a), but the expression was lower than in primary fibroblasts. Moreover, IFN- γ -stimulated fibroblasts expressed low levels of IL-33 after 18 hours of exposure (Figure 6a). TNF- α or IL-4 alone, or the combination of TNF- α and IL-4, had no effect on IL-33 expression in fibroblasts or HaCaT keratinocytes.

Patients with AD are susceptible to infection of certain viruses. To mimic viral exposure in the skin, fibroblasts were transfected with a mimetic of double-stranded RNA (dsRNA) polyinosinic-polycytidylic acid [poly(I:C)] for 3 and 6 hours with or without cytokine stimulation (TNF- α + IFN- γ). Poly(I:C)-transfected fibroblasts did not express IL-33 (Figure 6b). However, fibroblasts that were first stimulated with TNF- α + IFN- γ for 18 hours, and subsequently transfected with poly(I:C) for 3 and 6 hours, produced enhanced levels of IL-33 compared with the levels without poly(I:C) transfection (Figure 6b). IL-33 protein expression was confirmed by western blotting using fibroblast extracts. We found full-length IL-33 protein (31 kDa) from fibroblasts, which were stimulated with the combination of TNF- α and IFN- γ for 18 hours (Figure 6c). In addition, poly(I:C) transfection

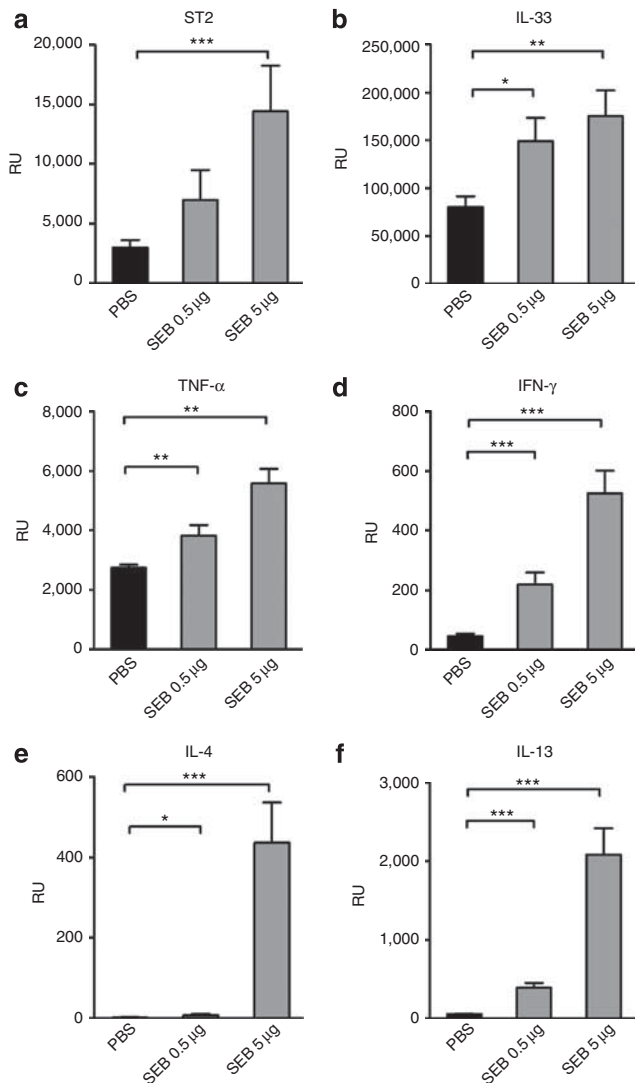


Figure 4. Staphylococcal enterotoxin B (SEB) increases the expression of ST2, IL-33, tumor necrosis factor (TNF)- α , IFN- γ , IL-4, and IL-13 in murine skin. (a) ST2 mRNA and (b) IL-33 mRNA are dose dependently increased after topical SEB exposure in murine skin. Topical SEB exposure increases the expression of (c) proinflammatory cytokine TNF- α , (d) T-helper type (Th)1 cytokine IFN- γ , and Th2 cytokines (e) IL-4 and (f) IL-13. The columns and the error bars represent means $\pm$ SEMs. * P <0.05; ** P <0.01; and *** P <0.001; n =8 mice per group. PBS, phosphate-buffered saline; RU, relative units.

enhanced cytokine-induced IL-33 expression. Poly(I:C) transfection alone had little effect on IL-33 protein expression (Figure 6c).

DISCUSSION

IL-33 is a recently described cytokine that promotes Th2-type immune responses. Recent data suggest an important role of IL-33 during the development and exacerbation of allergic asthma, but its role in allergic skin inflammation is poorly known. However, genetic polymorphism within the *ST2* gene region has been previously reported to have a strong association with AD (Shimizu *et al.*, 2005). To better understand the role of IL-33 in AD, we investigated

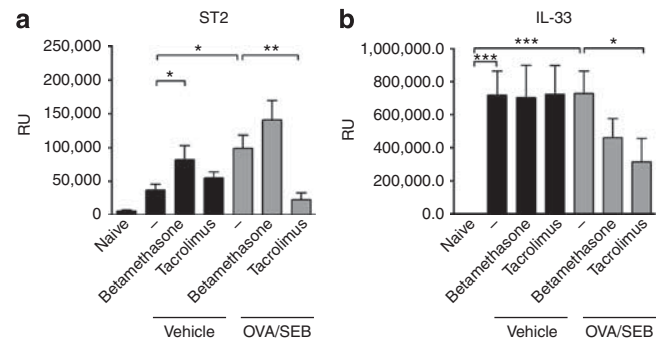


Figure 5. Tacrolimus downregulates the expression of ST2 and IL-33 in murine atopic dermatitis skin. (a) ST2 and (b) IL-33 expressions in ovalbumin (OVA)/staphylococcal enterotoxin B (SEB)-sensitized skin and control skin after topical corticosteroid (betamethasone-17-valerate) and tacrolimus treatment were analyzed. Phosphate-buffered saline with acetone was used as a vehicle. The columns and the error bars represent means $\pm$ SEMs. * P <0.05; ** P <0.01; and *** P <0.001; n =8 mice per group. RU, relative units.

expression profiles of IL-33 and its receptor components in patients with AD, in mouse models, and in various cell models.

As IL-33 is known to promote Th2-type immunity, we sought to examine expression profiles of IL-33 and its receptor components in the skin of patients with AD. Our results show elevated levels of ST2 and its co-receptor IL-1RAcP expressions in lesional AD skin. Used primers detect both the shorter and longer variant of *ST2* gene, and therefore it might be that also soluble ST2 is analyzed together with the membrane-bound ST2 receptor. Furthermore, immunohistochemical staining revealed increased numbers of IL-33⁺ cells in suprabasal keratinocytes and in endothelial cells. ST2 was found in dermal infiltrating cells and to some extent also in epidermal cells. Our results revealed that IL-33 and its receptors are increased in lesional skin of patients with AD, and thus may contribute to the pathogenesis of the disease.

To investigate the kinetics of IL-33 and ST2 during the development of the disease, we utilized the mouse model of AD. Mice were epicutaneously sensitized with OVA and investigated in the early phase of the disease, i.e., after the first sensitization week, and in the more chronic phase, i.e., after the third sensitization week. ST2 expression was progressively upregulated during the sensitization in parallel with the recruitment of the eosinophils, mast cells, and expression of Th2 cytokines. In contrast, IL-33 was already induced in OVA-sensitized skin after the first sensitization week, as well as in tape-stripped and acetone-treated vehicle skin, whereas no IL-33 was found in the skin of untreated naive mice. These results suggest that skin injury *per se* is sufficient to elicit the expression of IL-33. This is also in line with a finding by Dickel *et al.* (2010) who found upregulation of IL-33 mRNA after tape stripping in human skin. AD is clinically characterized by intense pruritus, which induces scratching of the skin. Thus, mechanical trauma to the skin, induced by the repeated scratching in AD patients, induces a strong expression of IL-33, which together with the recruitment of ST2-expressing cells (mast cells, eosinophils, and Th2

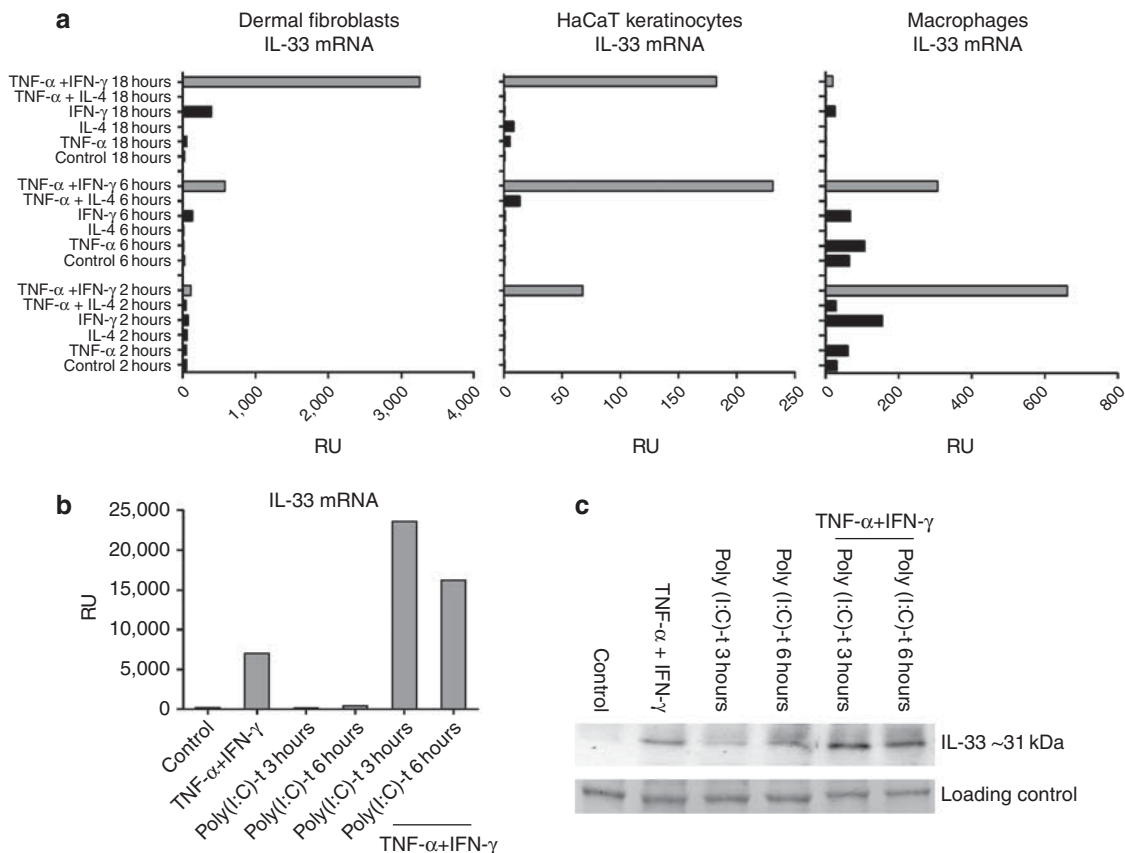


Figure 6. IL-33 protein is produced by stimulated dermal fibroblasts, HaCaT keratinocytes, and primary macrophages. (a) Primary human dermal fibroblasts, HaCaT keratinocytes, and macrophages express IL-33 mRNA after stimulation with both tumor necrosis factor (TNF)- α and IFN- γ , and (b) poly(I:C) transfection further increases IL-33 mRNA expression in fibroblasts. (c) Stimulated dermal fibroblast extracts were western blotted, and SYPRO Ruby staining of the immunoblot confirmed equal loading and transfer of proteins from cell lysate samples. Culture medium was used as a control. RU, relative units.

cells) enhances IL-33-ST2 interaction and promotes the production of Th2 cytokines.

Flaky tail (gene symbol *ft*) is a mutation in chromosome 3 within the mouse epidermal differentiation complex (Lane, 1972). *Ft/ft* mice lack filaggrin and express a truncated profilaggrin instead of the normal high-molecular-weight profilaggrin (Presland *et al.*, 2000). A previous report showed that 32-week-old *ft/ft* mice spontaneously develop eczematous lesions (Oyoshi *et al.*, 2009). We investigated ST2 and IL-33 expression in the skin of 4-, 22-, and 38-week-old *ft/ft* mice, and found that both IL-33 and ST2 were highly upregulated in 38-week-old filaggrin-deficient mice. As filaggrin-deficient mice have defects in skin barrier function, it is evident that also environmental factors can easily penetrate the skin barrier and influence the development of AD-like lesions. We suggest that in *ft/ft* mice skin structural cells respond to environmental factors and start to produce cytokines related to innate immunity.

Allergens and bacterial toxins are known triggering factors in AD. We demonstrate increased expression of IL-33 and ST2 in AD skin exposed to a common indoor allergen, HDM, or superantigen SEB. Similarly, topical exposure to SEB elicits increased expression of IL-33 and ST2 in mouse AD skin. We

also find high levels of IL-33 in mast cells after IgE-mediated activation with an allergen. This is in accordance with the recent finding by Hsu *et al.* (2010) who found IL-33 expression in mast cells after IgE/antigen stimulation. Taken together, IL-33-ST2 interaction is regulated by common triggering factors in AD, which may be critical in the development of the disease. To our knowledge, no earlier reports are available demonstrating that external triggering factors of AD, HDM, and SEB are potent inducers of IL-33 and its specific receptor in AD skin.

Current topical treatment possibilities for AD are corticosteroids and calcineurin inhibitors (Akdis *et al.*, 2006). We investigated how these treatment modalities affect the expression of IL-33 and ST2 in the murine AD skin. IL-33 and ST2 were suppressed with a calcineurin inhibitor, tacrolimus, in the OVA/SEB-sensitized murine skin. In contrast, a corticosteroid, betamethasone-17-valerate, slightly increased the expression of ST2, when it was used together with the phosphate-buffered saline/acetone vehicle. This was a betamethasone-specific phenomenon, and we did not find upregulation of ST2 after tacrolimus treatment in the vehicle group. This is in accordance with a previous study with another corticosteroid, dexamethasone, which failed to

reduce the transcription of IL-33 in stimulated primary airway smooth muscle cells (Prefontaine *et al.*, 2009). We have previously shown that both tacrolimus and beta-methasone-17-valerate downregulate Th2- and Th1-type cytokines. However, tacrolimus was more effective in downregulating IFN- γ levels in the sensitized mouse skin (Lehto *et al.*, 2010). As IFN- γ (in combination with TNF- α) is essential for upregulation of IL-33 (described below), this phenomenon can explain why tacrolimus is superior to suppress IL-33 expression in allergen/SEB-induced skin inflammation.

We identified a cytokine milieu that is sufficient to upregulate IL-33 in various cells. We show that the combination of TNF- α and IFN- γ is crucial to highly upregulate IL-33 expression in primary dermal fibroblasts, primary macrophages, and keratinocyte and endothelial cell lines. In line with our results, a synergistic increase of IL-33 was demonstrated when airway smooth muscle cells were stimulated with the combination of TNF and IFN- γ (Prefontaine *et al.*, 2009). These results may reveal a mechanism as to how topical SEB exposure increases IL-33 production. As demonstrated in the present study, topical exposure to SEB induces a strong expression of TNF- α and IFN- γ in addition to Th2 cytokines. Thus, SEB exposure may activate T cells or antigen-presenting cells to produce IFN- γ and TNF- α , resulting in enhanced expression of IL-33 in fibroblasts and keratinocytes. Moreover, it has been shown that both TNF- α and IFN- γ are increased in the whole blood of patients with chronic AD compared with patients with acute AD (Vakirlis *et al.*, 2010).

In patients with AD, genetic predisposition for skin barrier dysfunction and defects in innate and adaptive immunity can lead to higher frequency of bacterial and viral infections. *Herpes simplex* and *Molluscum contagiosum* are the most common virus infections in patients with AD (Wollenberg *et al.*, 2011). Both are DNA viruses that are known to produce dsRNA (Weber *et al.*, 2006). In the present study, we observed a weak induction of IL-33 in fibroblasts that were treated with a mimetic of dsRNA. In line with our finding, a recent study shows that human bronchial epithelial cells do not produce IL-33 after dsRNA stimulation alone (Chustz *et al.*, 2011). However, we found that fibroblasts that were stimulated with the combination of TNF- α and IFN- γ before dsRNA produced enhanced levels of IL-33, suggesting that viral infections may aggravate the production of IL-33 in certain disease conditions. Recently, Hong *et al.* (2010) found a new splice variant of IL-33, and its significance in different disease conditions should be further investigated.

In conclusion, we demonstrate upregulation of IL-33 and ST2 in the lesional AD skin by known triggering factors of AD. In addition to allergen exposure, irritants, scratching, as well as bacterial and viral infections, may increase the transcription of IL-33. We also show that topical tacrolimus treatment strongly suppresses the expression of IL-33 and ST2 in AD skin. These results recommend an important role for IL-33-ST2 interaction in the pathogenesis and disease severity of AD.

MATERIALS AND METHODS

Patients

Punch biopsies (6 mm) were taken, after obtaining informed consent, from either nonlesional or lesional skin of 15 patients with chronic AD and from the skin of 13 healthy volunteers. AD patients were identified according to the criteria defined by Hanifin and Rajka (1980). AD patients with a history of HDM allergy underwent AD patch testing as described previously (Darsow and Ring, 2000). Patients were without topical medication (glucocorticosteroids, antibiotics, tacrolimus) for 1 week and without systemic medication for 4 weeks before taking skin biopsies. Patch tests are described in Supplementary Materials and Methods online. Studies were approved by the appropriate local ethics committees and conducted according to the Declaration of Helsinki Principles.

Mice and sensitization

Female Balb/c mice (aged 6–8 weeks) were obtained from Scanbur A/S (Karlslunde, Denmark), and filaggrin-deficient mice (flaky tail ft/ft, ma/ma, double homozygous for the flaky tail, and matted mutations) were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained under pathogen-free conditions. All animal experiments were approved by the State Provincial Office of Southern Finland. Sensitization protocols are described in Supplementary Materials and Methods online.

Cell cultures

Primary human dermal fibroblasts isolated from adult skin were purchased from Gibco (Paisley, UK) and cultured in Medium 106 according to the manufacturer's instructions (Gibco). Human immortalized HaCaT keratinocytes (Rintahaka *et al.*, 2008) were cultured in DMEM (Lonza, Verviers, Belgium) containing 10% fetal bovine serum, 2 mM Ultraglutamine1 (Lonza), and antibiotics. Human primary macrophages were obtained from leukocyte-rich buffy coats from healthy blood donors (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland). Monocytes were isolated and differentiated as previously described (Pirhonen *et al.*, 1999). Bone marrow cells of C57BL/6 mice were cultured and identified as previously described (Gombert *et al.*, 2005). The cells were identified as mast cells by May-Grünwald-Giemsa staining and by flow cytometric analysis of *c-kit* (>99% of *c-kit*⁺ cells) and IgE receptor (89% of the cells positive) expression. Stimulations are described in Supplementary Materials and Methods online.

Quantitative real-time reverse transcription-PCR (Taqman) analysis

Quantitative real-time reverse transcription-PCR analyses were performed as previously described (Savinko *et al.*, 2005; Lehto *et al.*, 2010). Briefly, skin biopsies were homogenized with Ultra-Turrax T8 (IKA Labortechnik, Staufen, Germany) to TRIzol (Invitrogen Life Technologies, Karlsruhe, Germany). RNA was isolated and extracted from stimulated cells, or from skin biopsies, according to the TRIzol instructions.

cDNA synthesis and Taqman analysis are described in Supplementary Materials and Methods online.

Immunohistochemistry

Paraffin-embedded tissue sections (4 μ m) were stained with anti-human IL-33 and ST2 antibodies. Staining protocol and antibody

details are described in Supplementary Materials and Methods online.

Western blotting

IL-33 protein was analyzed by western blot analysis using whole-cell extract as previously described (Kankkunen *et al.*, 2010). Blotting conditions are described in Supplementary Materials and Methods online.

Statistics

The data were expressed as means ($\pm$ SEM), and differences between means were analyzed with the Graph Pad Prism software using a Student's *t*-test with a two-tailed test of significance. A Mann-Whitney *U*-test was used when variances were different between groups for unpaired comparisons. Differences at $P < 0.05$ were considered to be statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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Supplementary Materials and methods

Patch tests

Atopy patch tests were performed with HDM antigen containing a mix of *Dermatophagoides farinae* and *D. pteronyssinus* species (Chemotechnique Diagnostics, Malmö, Sweden). Skin specimens were obtained before and 2, 6, and 48 h after HDM-exposure. For staphylococcal enterotoxin B (SEB) patch tests, SEB (Sigma-Aldrich, St. Louis, MO) at 0.226 µg/µl in 0.9% NaCl solution was applied in Finn chambers (Epitest, Hyrylä, Finland) on healthy-appearing dorsal skin of AD patients. Skin specimens were obtained before and 2, 6, and 24 h after SEB-exposure.

Sensitization protocols

In the kinetics-study mice were epicutaneously sensitized with ovalbumin 100 µg in 100 µl PBS in each patch as earlier described (Wang *et al.*, 2007). Skin samples were investigated after first and third sensitization week. In the second mouse experiment, mice were sensitized topically with 100 µl of SEB-solution as earlier described (Savinko *et al.*, 2005). SEB was purchased from Sigma-Aldrich and dosed at 0.5 and 5 µg /patch. In the pharmacological research, mice were sensitized as earlier described (Lehto *et al.*, 2010): drugs were applied to the skin during the third sensitization week together with OVA/SEB-mixture in 75% acetone/PBS-solution. Drug concentrations were as follows: betamethasone-17-valerate (Sigma-Aldrich) 15 µg/100 µl, tacrolimus (FK-506, Sigma-Aldrich Co) 100 µg/100 µl. PBS was used as a vehicle during the first and second sensitization week and 75% acetone in PBS during the treatment week.

Cell stimulations

Cells were stimulated with TNF-α (20 ng/ml, Biosource International, Camarillo, CA, USA), IL-4 (20ng/ml, Immuno Tools), IFN-γ (500 IU/ml, Immuno Tools, Friesoythe, Germany), with the combination of TNF-α and IL-4 and with the combination of TNF-α and IFN-γ for 2, 6 and 18 hours. In further studies, human dermal fibroblasts were stimulated with the combination of TNF-α and IFN-γ for 18 hours, after which cells were transfected with a mimetic of dsRNA polyinosic-polycytidylic acid [poly(I:C)], 10 mg/ml using Lipofectamine 2000 transfection reagent (Invitrogen Life Technologies, Karlsruhe, Germany) for 3 and 6 hours according to the manufacturer's instructions.

HUVECs (ATCC CRL 1730) were cultured in RPMI 1640 and supplemented with 10 % FBS, 2mM Ultraglutamine1 (Lonza) and antibiotics and stimulated for 2 and 18 hours.

Mast cells were incubated with 10 µg of anti-DNP IgE (Sigma-Aldrich) in 2 ml of culture medium for 2 h on ice. The plates were centrifuged, supernatant discarded, and the cells resuspended in 2 ml of medium with 200 ng of DNP-human serum albumin (Sigma-Aldrich). The cells were incubated for 6 hours.

cDNA synthesis and Taqman analysis

cDNA was synthesized with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). PCR primers and probes were obtained from Applied Biosystems as predeveloped reagents. Human and mouse ST2 primers detecting transcript variant 1 and transcript variant 2 (Hs00249384_m1 and Mn00516117_m1) were used. RT-PCR was performed with 7500 Fast Real-Time PCR System (Applied Biosystems). The gene expression between different samples was normalized with endogenous 18S rRNA, and the target gene expression was calculated by the comparative CT-method according to the instructions of Applied Biosystems. Results are shown as relative units (RU).

Immunohistochemistry

Endogenous peroxidase activity was inhibited with hydrogen peroxide block (Thermo Scientific Cheshire, UK). Slides were transferred into Pretreatment Module (Thermo Scientific) in buffer at pH 9 (Thermo Scientific) to deparaffinise and perform heat induced antigen retrieval. Normal antibody diluent (Immunologic, Netherlands) was used to dilute primary antibodies, anti-human IL-33 antibody (Nessy-1, Alexis Biochemicals, San Diego, USA) and anti-human ST2 antibody (HPA007406, Sigma-Aldrich) or mouse mAb IgG1 isotype control (Cell Signaling, Danvers, MA, USA). After primary antibody, post-antibody blocking solution (Immunologic bv) was used and slides were transferred to poly-HRP-goat anti-mouse/rabbit/rat IgG. AEC (Thermo Scientific) was used as a substrate. Samples were counterstained with Mayer's hematoxylin.

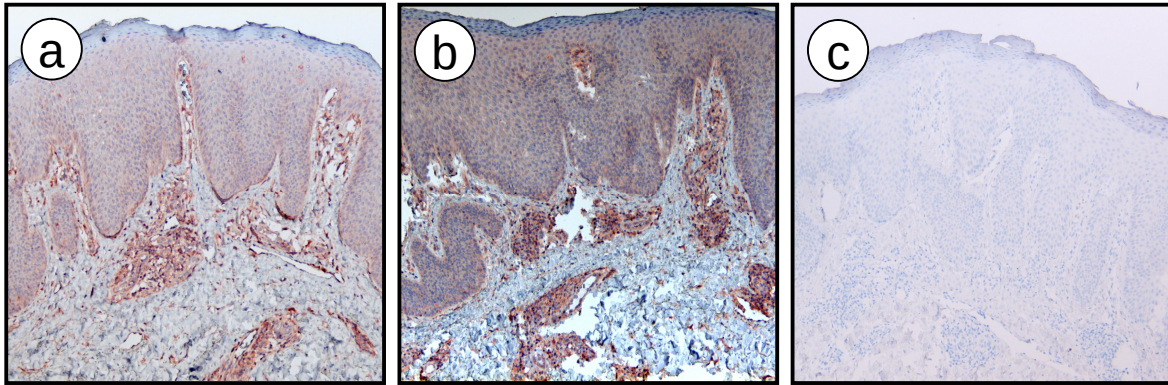
Western Blotting

20 µg of protein from whole lysed cell extracts was separated on SDS-PAGE and transferred onto Immobilon-P Transfer Membranes (Millipore). Membrane was stained with goat anti-human IL-33 (R&D) and incubated with HRP-conjugated polyclonal rabbit anti-goat immunoglobulins (Dako Cytomation). Proteins were visualized by Luminescent Image Analyzer (Image Quant LAS4000mini, GE Healthcare, Sweden).

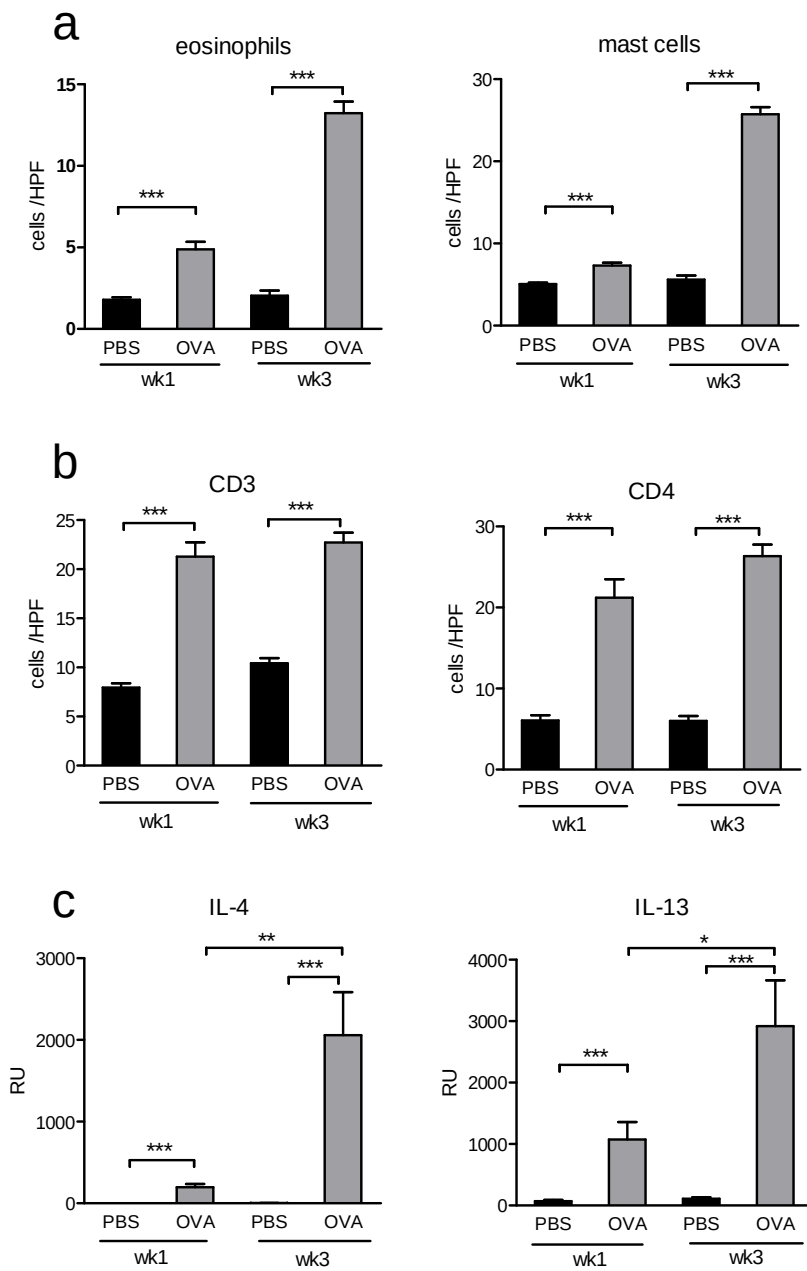
OVA- and SEB-specific IgE antibodies in the serum of sensitized mice

Serum levels of antigen specific antibodies were measured by the straight ELISA method as described previously (Savinko *et al.*, 2005).

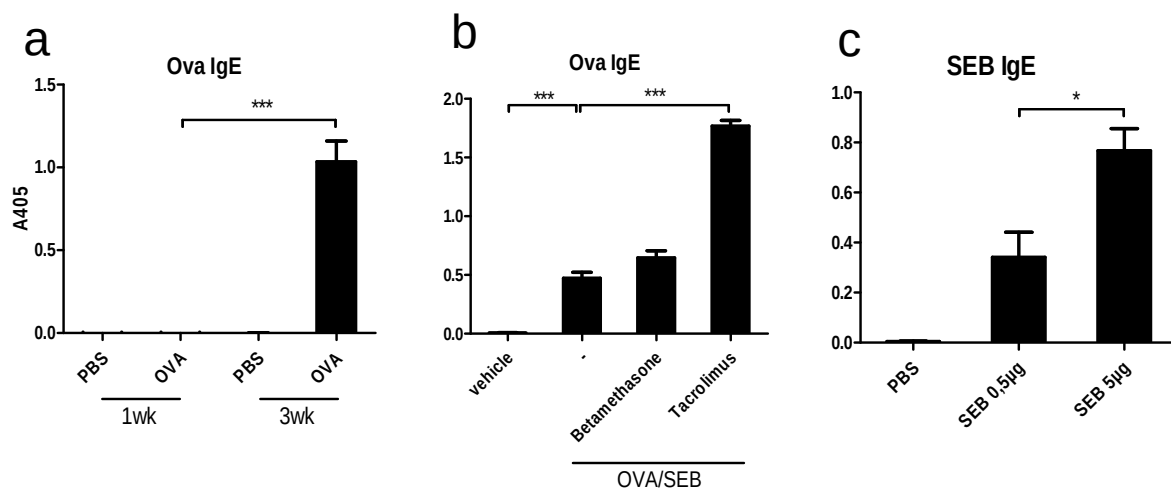
Supplementary figures



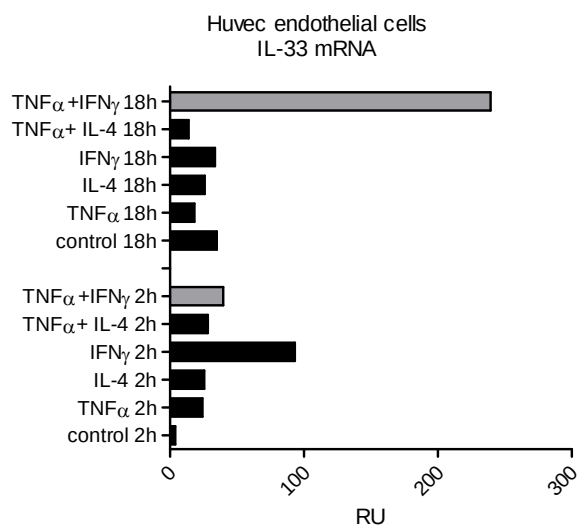
S1. (a) and (b) Immunohistochemical staining with ST2 antibody shows positive cells in the epidermis and dermis in the skin of AD patient (c) Isotype control stained AD skin shows no positive cells.



S2. Upregulation of eosinophils, mast cells, CD3, CD4 cells and Th2 type cytokines in mouse skin after epicutaneous sensitization with OVA. Increased numbers of (a) mast, eosinophils (b) CD3+ and CD4+ cells in the skin of OVA-sensitized mouse. (c) mRNA expression of Th2 type cytokines in OVA-sensitized murine skin.



S3. Ova- and SEB-specific IgE levels in mouse studies. (a) OVA-specific IgE in the serum of OVA-sensitized mice after the first and third sensitization week was analyzed with ELISA. (b) OVA-specific IgE in the serum of OVA/SEB-sensitized, OVA/SEB-sensitized and betamethasone or tacrolimus and treated skin was analyzed with ELISA. (c) SEB-specific IgE in the serum of SEB-sensitized mice was analyzed with ELISA.



S4. TNF α + IFN γ –stimulated HUVEC endothelial cells produce IL-33 mRNA.