ST2 Regulates Allergic Airway Inflammation and T-Cell Polarization in Epicutaneously Sensitized Mice

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IL-33 is an inducer of proinflammatory and T-helper type 2 (Th2) cytokines, which have an important role in atopic dermatitis (AD) and allergic asthma. ST2 is a specific receptor for IL-33 and is expressed on Th2 cells, eosinophils and mast cells. A murine model of AD was used to characterize the role of ST2 in allergen-induced skin inflammation and allergic asthma. ST2−/− and wild-type (WT) mice were epicutaneously sensitized with ovalbumin (OVA) and staphylococcal enterotoxin B, and intranasally challenged with OVA. ST2−/− mice exhibited increased production of IFNγ and increased number of $CD8^+$ T cells in the sensitized skin and in the airways compared with WT mice. The number of eosinophils was decreased, and Th2 cytokines were downregulated in the airways of epicutaneously sensitized ST2−/− mice compared with WT controls. However, dermal eosinophil numbers were as in WT, and the levels of Th2 cytokines were even elevated in the sensitized skin of ST2−/− mice. ST2−/− mice had elevated numbers of neutrophils and macrophages and increased levels of proinflammatory cytokines in the sensitized skin. The role of ST2 differs between different target tissues: ST2 is dispensable for the development of Th2 response in the sensitized skin, whereas it is a main inducer of Th2 cytokines in asthmatic airways.

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INTRODUCTION

The first clinical manifestation of atopy is generally considered atopic dermatitis (AD) that starts the atopic march. Atopic march is characterized by the progression of AD to asthma and allergic rhinitis later in life. Indeed, >50% of young children with severe AD will develop asthma and approximately 75% will develop allergic rhinitis (Kulig *et al.*, 1999). Environmental and genetic evidence suggests that a defect in epithelial barrier integrity may contribute to the onset of AD and progression of atopic march. Recent genome-wide association studies have shown that variations in genes encoding epithelial cell-derived cytokines, including IL-33 and IL-33-specific receptor ST2 have a strong association with asthma (Moffatt *et al.*, 2010; Ober and Yao, 2011) and allergic diseases (Shimizu *et al.*, 2005).

The epidermis is a primary defense and sensor to the external environment. Skin-barrier defect promotes easy entry to the pathogens, allergens, and other molecules (toxins, irritants, and pollutants) (Boguniewicz and Leung, 2011; Carmi-Levy *et al.*, 2011). In AD skin, keratinocytes respond to environmental triggers and are able to produce a unique profile of proinflammatory and pro-T-helper type 2 (Th2) cytokines, including IL-33 (Smith, 2009) and TSLP (Kinoshita *et al.*, 2009), and promote the Th2 inflammation. In the more chronic phase, Th1 type cytokines dominate over Th2 cytokines. Impaired skin barrier can also increase the susceptibility to the bacterial colonization of *Staphylococcus aureus* (Michie and Davis, 1996). Bacterial superantigens can further stimulate T-cell receptors and augment skin inflammation. Epidermal-barrier dysfunction and the increased antigen uptake through the skin can finally lead to the systemic sensitization, which may further promote respiratory allergy when the same antigen is inhaled. We have earlier demonstrated that repeated intradermal allergen exposure without external adjuvant-induced airway hyperreactivity to inhaled methacholine, local Th2-dominated lung inflammation, and systemic IgE response in mice (Lehto *et al.*, 2005).

IL-33 is a member of IL-1 family and is mainly produced by cells of barrier tissues: skin keratinocytes, fibroblasts, endothelial cells, macrophages, airway epithelial cells, and smooth muscle cells are known to secrete IL-33 (Prefontaine *et al.*, 2009, 2010). Secreted IL-33 activates surrounding ST2-expressing cells, such as Th2 cells (Xu *et al.*, 1998), eosinophils (Cherry *et al.*, 2008), basophils (Blom *et al.*, 2010), and mast cells (Allakhverdi *et al.*, 2007), which are all important cell types in allergic diseases. ST2 is selectively upregulated and expressed on IL-4, IL-13, and IL-5 producing Th2 cells, but not on IFNγ-producing Th1 cells (Xu *et al.*, 1998; Lecart *et al.*, 2002). In addition to cytokine function, IL-33 can act as a nuclear factor and can interact with the

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Abbreviations: AD, atopic dermatitis; BALF, bronchoalveolar lavage fluid; OVA, ovalbumin; SEB, staphylococcal enterotoxin B; TNFα, tumor necrosis factor-α; WT, wild-type

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transcription factor NF-κB, and therefore might also dampen the inflammatory response (Ali *et al.*, 2011). However, biological effects of nuclear IL-33 are unclear at present.

The functional role of ST2–IL-33 interaction in allergic diseases, such as AD and asthma, is poorly known. In the present study, we explored the role of ST2 in the development of allergen-induced skin inflammation and allergic asthma after epicutaneous sensitization in ST2 −/− mice. Our results demonstrate that ST2 critically regulates allergen-induced skin and airway inflammation. However, the role of ST2 differs between target tissues.

RESULTS

The number of neutrophils, macrophages, and CD8$^+$ cells is increased in the OVA/SEB-sensitized skin of ST2 −/− mice

IL-33 and ST2 are upregulated in the skin of patients with AD after external triggering factors, such as allergens, irritants, and scratching (Savinko *et al.*, 2012). We used ST2 −/− mice to investigate the role of ST2 in the murine model of AD. Histological examination revealed increased number of inflammatory cells in the sensitized skin of ST2 −/− mice (Figure 1a–f). Neutrophils were significantly increased in the skin of ST2 −/− mice, whereas eosinophils and mast cells were similarly upregulated in wild-type (WT) and ST2 −/− mice (Figure 1e). Immunohistological staining of T cells in the sensitized skin revealed increased number of CD3$^+$ (Figure 1f, Supplementary Figure S1a–d online) and CD8$^+$ cells (Figure 1f, Supplementary Figure S1e–h online), as well as F4/80$^+$ macrophages (Figure 1f, Supplementary Figure S1i–h online), in the skin of ST2 −/− mice compared with WT mice. CD4$^+$ cells were slightly upregulated in the skin of ovalbumin (OVA)/staphylococcal enterotoxin B (SEB)-treated ST2 −/− mice compared with WT, but did not reach the

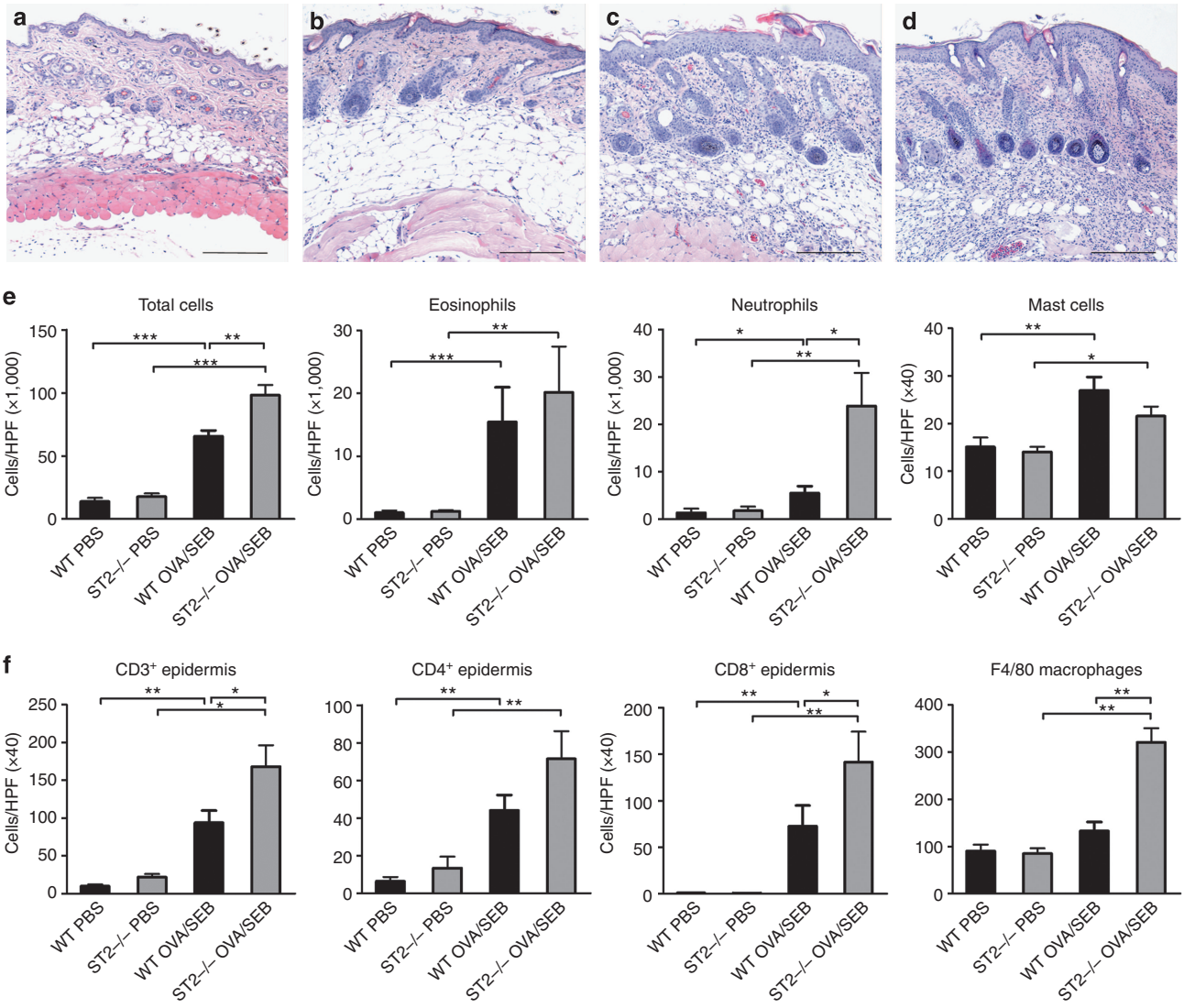

Figure 1. Total inflammatory cells and neutrophils are increased in the ovalbumin (OVA)/staphylococcal enterotoxin B (SEB)-sensitized skin of ST2 −/− mice. Representative histological figures of (**a**) phosphate-buffered saline(PBS)-sensitized wild-type (WT), (**b**) PBS-sensitized ST2 −/−, (**c**) OVA/SEB-sensitized WT, and (**d**) OVA/SEB-sensitized ST2 −/− mice. Bar = 200 μm. (**e**) Total inflammatory cells, eosinophils, neutrophils, and mast cells were counted from skin samples of WT and ST2 −/− mice after epicutaneous sensitization with PBS and OVA/SEB. (**f**) CD3$^+$, CD4$^+$, and CD8$^+$ cells were counted from the epidermis and F4/80$^+$ cells from the dermis of sensitized WT and ST2 −/− mice. HPF, high-power field. *P<0.05; **P<0.01; ***P<0.001.

statistical difference (Figure 1f). Upregulation of $CD3^+$ and $CD8^+$ cells were only seen in epidermis, whereas dermis showed no difference in the OVA/SEB-sensitized WT and ST2 −/− mice.

Proinflammatory cytokines and Th1 cytokine IFNγ are increased in the OVA/SEB-sensitized skin of ST2 −/− mice

IL-33, which binds to its specific ST2 receptor, has been reported to drive the production of Th2 cytokines (Schmitz *et al.*, 2005; Kurowska-Stolarska *et al.*, 2008). Proinflammatory, Th2, and Th1 cytokines have an important role in the pathogenesis of AD. We found a significant increase in mRNA expressions of proinflammatory cytokines IL-1β and IL-6, and Th1 cytokine IFNγ, whereas tumor necrosis factor-α (TNFα) showed no difference (Figure 2a, Supplementary Figure S2 online). Th2 cytokines (IL-4, IL-13, and IL-5) were not downregulated and were even slightly upregulated (albeit IL-13 and IL-4 not statistically significantly) in the sensitized skin of ST2 −/− mice compared with the sensitized skin of WT mice (Figure 2b, Supplementary Figure S2 online). Transcription factor for regulatory T cells, Foxp3, was increased in the sensitized skin of ST2 −/− mice as compared with WT skin, although IL-10 remained at the same level in the sensitized skin of WT and ST2 −/− mice (Supplementary Figure S2 online).

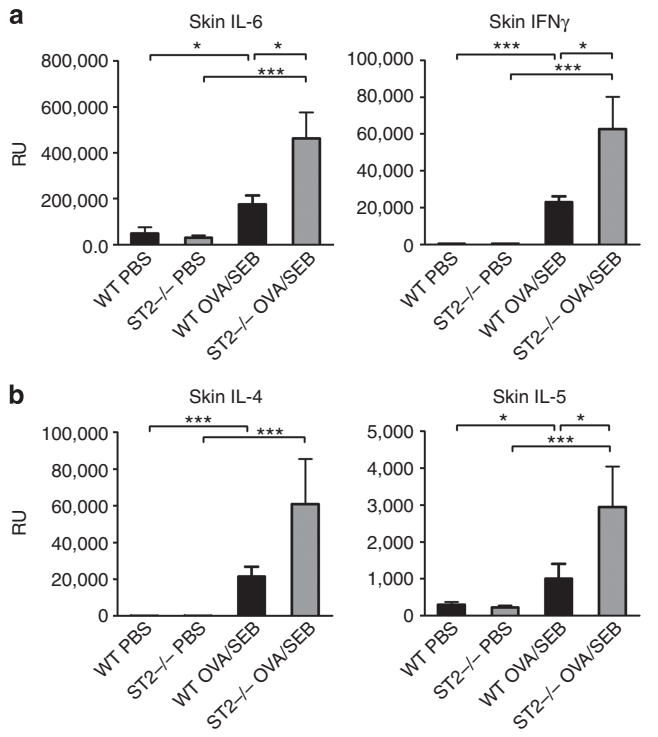

Figure 2. Increased mRNA expression of IL-6, IFNγ, and IL-5 was found in the sensitized skin of ST2 −/− mice as compared with wild-type (WT) mice. (**a**) mRNA expression of proinflammatory cytokine IL-6 and mRNA expression of Th1 cytokine IFNγ were investigated in the skin of WT and ST2 −/− mice after epicutaneous sensitization with phosphate-buffered saline (PBS) and ovalbumin (OVA)/staphylococcal enterotoxin B (SEB). (**b**) mRNA expression of T-helper type 2 (Th2) cytokines, IL-4 and IL-5, was measured from the sensitized skin. *$P<0.05$; ***$P<0.001$.

Eosinophils are downregulated and $CD8^+$ cells are upregulated in the airways of epicutaneously sensitized and intranasally challenged ST2 −/− mice

Next, we explored whether the airway inflammation is differently regulated in the epicutaneously sensitized ST2 −/− mice in comparison with WT mice. Mice were first sensitized as in AD model, and after 1 week of the last sensitization, three intranasal OVA challenges were given. Histological analysis revealed that WT and ST2 −/− mice had severe inflammation in the lung tissue after OVA challenge (Figure 3c–f). Further examination of bronchoalveolar lavage fluid (BALF) showed a remarkable downregulation of eosinophils in ST2 −/− mice compared with WT mice. Neutrophils and macrophages were similarly upregulated in the BALF of ST2 −/− and WT mice (Figure 3a). Airway hyperreactivity to inhaled methacholine was similar in OVA-sensitized WT and ST2 −/− mice (Supplementary Figure S3 online).

As T cells are known to mediate inflammatory reactions in asthmatic airways, we stained $CD3^+$, $CD4^+$, and $CD8^+$ cells in the lung tissue. Immunohistochemical staining showed increased number of $CD3^+$ and $CD8^+$ cells in the interstitial area of the lung tissue in ST2 −/− mice, whereas the number of $CD4^+$ cells remained at the same level in the lungs of ST2 −/− and WT mice (Figure 3b, Supplementary Figure S4 online).

Th2 cytokines are dramatically downregulated in the airways of epicutaneously sensitized and intranasally challenged ST2 −/− mice

As ST2 is known to promote the production of Th2 cytokines, such as IL-13 and IL-5, and is not expressed on Th1 cells (Xu *et al.*, 1998; Lecart *et al.*, 2002), we analyzed mRNA expression of Th2 and Th1 cytokines in the lung tissue of sensitized ST2 −/− and WT mice. All Th2 cytokines, including IL-4, IL-5, and IL-13, were downregulated in the lung tissue of ST2 −/− mice compared with WT mice (Figure 4a, Supplementary Figure S5 online). However, Th1 cytokine IFNγ (Figure 4a) and proinflammatory cytokine TNFα mRNA expressions (Figure 4a) were significantly upregulated in the lung tissue of ST2 −/− mice compared with WT mice. Protein concentrations of IL-5 and IL-13 were also decreased in the BALF of ST2 −/− mice compared with WT mice, whereas TNFα protein concentration increased in sensitized ST2 −/− mice in comparison with WT mice (Figure 4b) confirming mRNA findings.

To explore regulatory T cells in the lung tissue, mRNA expression of the transcription factor for regulatory T cells, Foxp3, and regulatory cytokine IL-10 were studied. Both Foxp3 and IL-10 were equally upregulated in the lung tissue of ST2 −/− mice compared with WT mice (Figure 4c, Supplementary Figure S5 online).

The number of IFNγ-producing $CD3^+$ $CD8^+$ T cells is increased in the BALF of ST2 −/− mice

To confirm the increase of $CD8^+$ T cells and the increased production of IFNγ in the lung tissue, the BALF of WT and ST2 −/− mice was analyzed by FACS. Similar to

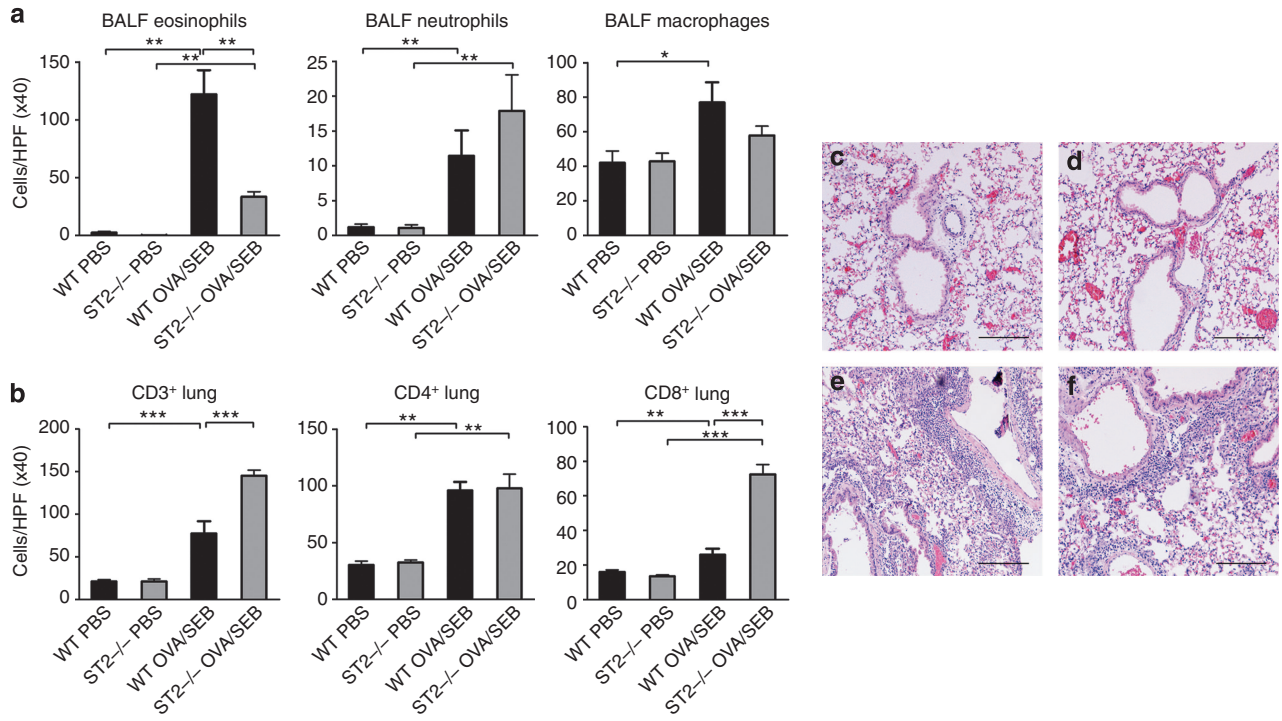

Figure 3. Eosinophils are downregulated and CD8$^+$ cells are upregulated in the airways of epicutaneously sensitized and intranasally challenged ST2 − / − mice. (**a**) Eosinophils, neutrophils, and macrophages were counted from the bronchoalveolar lavage fluid (BALF). (**b**) CD3$^+$, CD4$^+$, and CD8$^+$ cells were stained in the lung. Lung histology was investigated from (**c**) phosphate-buffered saline (PBS)-sensitized wild-type (WT), (**d**) PBS-sensitized ST2 − / −, (**e**) OVA/SEB-sensitized WT, and (**f**) OVA/SEB-sensitized ST2 − / − mice. HPF, high-power field. Bar = 200 μm (**c**–**f**). *P<0.05; **P<0.01; ***P<0.001.

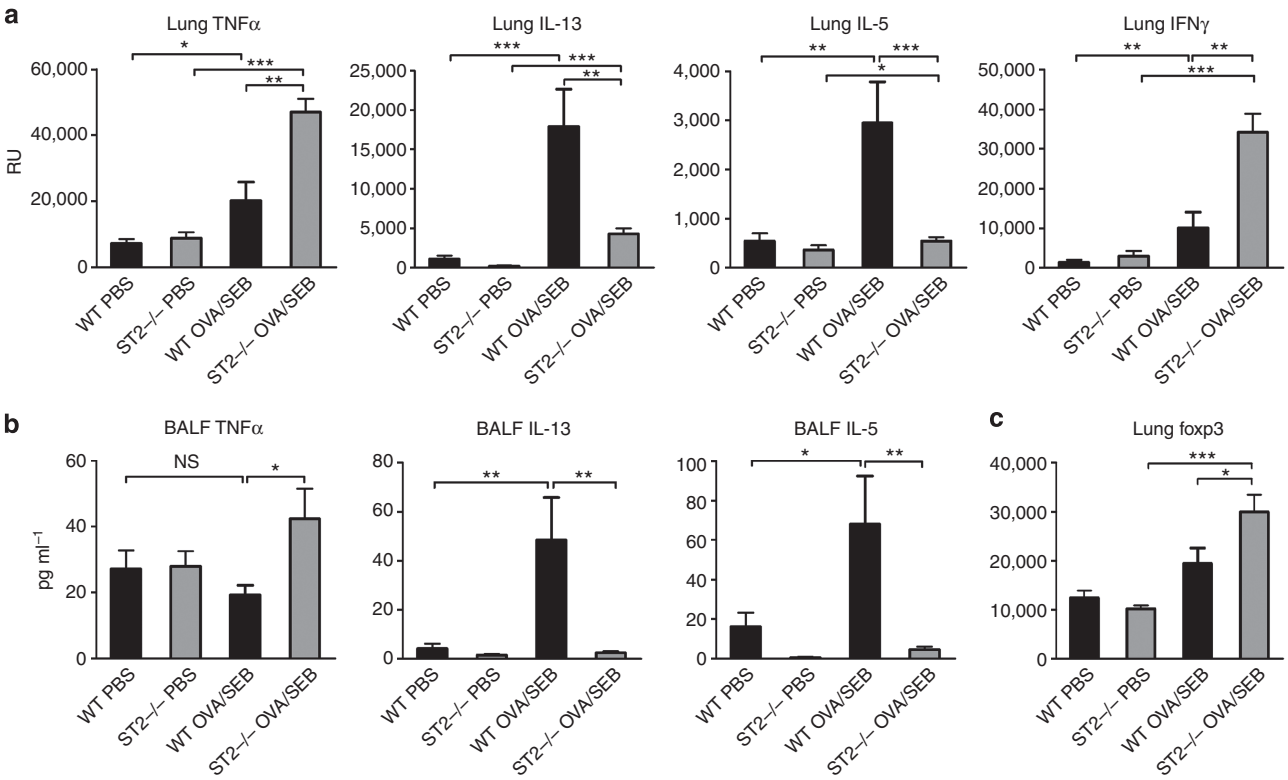

Figure 4. T-helper type 2 (Th2) cytokines are downregulated in the lung tissue and bronchoalveolar lavage fluid (BALF) in epicutaneously sensitized and intranasally challenged ST2 − / − mice. (**a**) mRNA expression of tumor necrosis factor-α (TNFα), IL-13, IL-5, and IFNγ in the lung tissue of sensitized mice and controls was studied. (**b**) Protein concentrations of TNFα, IL-13, and IL-5 were analyzed in the BALF. (**c**) Foxp3 mRNA expression in the lung tissue was studied in sensitized mice. *P<0.05; **P<0.01; ***P<0.001.

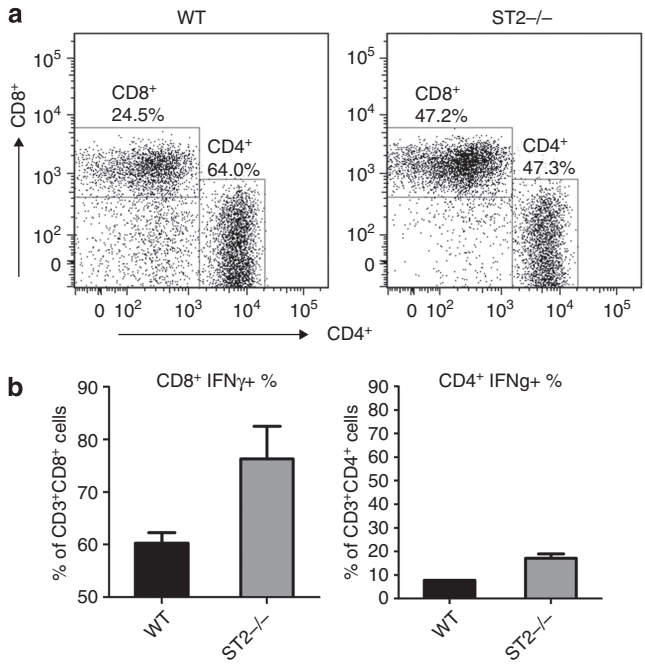

Figure 5. IFNγ-producing CD8$^+$ T cells are increased in the bronchoalveolar lavage fluid (BALF) of epicutaneously sensitized and intranasally challenged ST2 −/− mice. (**a**) Ovalbumin (OVA)/staphylococcal enterotoxin B (SEB)-sensitized and OVA-challenged ST2 −/− mice have increased number of CD8$^+$ T cells that produced (**b**) IFNγ in the BALF compared with similarly sensitized wild-type (WT) mice. Only a low production of IFNγ was detected in CD4$^+$ T cells.

immunohistochemistry data and mRNA expression data, ST2 −/− mice had more CD3$^+$CD8$^+$ T cells (Figure 5a), which produced IFNγ (Figure 5b, Supplementary Figure S6 online).

ST2 deficiency does not affect allergen-specific antibody production after epicutaneous sensitization

To investigate the systemic response, we also measured OVA-specific IgE and OVA-specific IgG2a antibodies in the serum of epicutaneously sensitized ST2 −/− and WT mice. OVA-specific IgE and IgG2a antibody concentrations were similarly increased in the serum of epicutaneously sensitized ST2 −/− and WT mice (Supplementary Figure S7a online). Moreover, OVA-specific IgE levels were slightly downregulated in ST2 −/− mice as compared with WT after epicutaneous sensitization followed by intranasal OVA challenge. However, the difference was not statistically significant (Supplementary Figure S7b online). Ova-specific IgG2a levels were at the same levels in WT and ST2 −/− mice after intranasal challenge.

DISCUSSION

Allergen exposure through the epidermis can initiate systemic allergy and predispose individuals to AD, allergic rhinitis, and asthma. The possibility that epidermal-barrier dysfunction can initiate systemic sensitization and increase the risk of asthma and other allergic diseases is supported by experimental data with the progression from AD to allergic asthma in mouse models (Spergel *et al.*, 1998; Akei *et al.*, 2006). Epithelial-derived cytokines are hypothesized to have a key role in the initiation of atopic skin inflammation. Keratinocyte and fibroblast-derived cytokine, IL-33, is known to activate dendritic cells and directly drive the polarization of naive T cells towards Th2 phenotype (Rank *et al.*, 2009). In addition, IL-33 can act on Th2 cells to increase the secretion of Th2 cytokines, such as IL-5 and IL-13 (Schmitz *et al.*, 2005; Kurowska-Stolarska *et al.*, 2008). Genetic polymorphism within the ST2 gene region is reported to have a strong association with AD (Shimizu *et al.*, 2005). However, no previous studies are available about the functional role of ST2 in AD. Therefore, in this study we examined the role of ST2 in the development of AD and asthma using ST2 −/− mice and a murine model of AD. These results demonstrate that ST2 may be more relevant for inducing Th2 responses in the lung tissue as compared with the skin.

T cells have a crucial role in mediating inflammatory reactions in patients with AD. Here we demonstrate that ST2 deficiency does not inhibit the recruitment of T cells in the sensitized skin. We further show significantly more cytotoxic CD8$^+$ cells, neutrophils, and macrophages in the sensitized skin of ST2 −/− mice compared with WT mice. Recently, it was shown that also CD8$^+$ T cells express ST2, and after TCR activation these cells produce IFNγ in response to IL-33 stimulation (Yang *et al.*, 2011). However, our results clearly show that in the absence of ST2, the number of IFNγ-producing CD8$^+$ T cells increases *in vivo*. This data also suggest that in AD skin, ST2 may inhibit in the recruitment of neutrophils and macrophages, as well as cytotoxic CD8$^+$ T cells, to the site of inflammation.

Although IL-33 is known to promote the Th2 cytokine secretion, we did not find downregulation of Th2 cytokines in the OVA/SEB-sensitized skin of ST2 −/− mice; actually, Th2 cytokines were even slightly upregulated. In addition, proinflammatory cytokines IL-1β and IL-6, as well as Th1 cytokine IFNγ, were upregulated in the sensitized skin of ST2 −/− mice. These results demonstrate that loss-of-function in ST2 gene does not downregulate Th2 response in the AD skin as would be expected. On the contrary, in the sensitized skin ST2 acts as a negative regulator of proinflammatory cytokines, which in turn may result in increased levels of both Th1 and Th2 cytokines. We did not find any differences in allergen-specific IgE or IgG2a concentrations in WT and ST2 −/− mice. This is in line with Hoshino *et al.* (1999) who found that total IgE and IgG1 were normally observed in ST2 −/− mice, which were sensitized two times with alum-emulsified OVA. Moreover, they found that ST2 −/− mice displayed almost normal Th2 responses in nematode infection.

Recent genome-wide association studies demonstrate that variations in genes encoding IL-33 and ST2 have a strong association with asthma (Moffatt *et al.*, 2010), and higher expression of IL-33 has been detected in endobronchial biopsies from human asthmatic subjects compared with healthy controls (Prefontaine *et al.*, 2009). Furthermore, a recent study by Kim *et al.* (2011) demonstrates a novel pathway for the development of glycolipid antigen-induced experimental asthma that occurs in the absence of Th2 cells

and adaptive immunity. This pathway is dependent on NKT cells, alveolar macrophages and natural helper cells, a newly described non-T, non-B, innate lymphoid cell type, and ST2 receptor. Other studies with ST2−/− mice and asthma model have been performed with OVA/alum (adjuvant) model (Hoshino *et al.*, 1999; Mangan *et al.*, 2007; Kurowska-Stolarska *et al.*, 2008). Because the results of ST2−/− mice exposed to the asthma model are controversial, we used the epicutaneous route of sensitization to mimic the atopic march. Mice were first sensitized epicutaneously with OVA/SEB and intranasally challenged with OVA. In line with previous studies (Lohning *et al.*, 1998; Coyle *et al.*, 1999; Townsend *et al.*, 2000; Kurowska-Stolarska *et al.*, 2008), our results show a drastic downregulation of eosinophils in the BALF of sensitized ST2−/− mice compared with WT mice. Consistent with the decreased number of eosinophils in the BALF, also mRNA expression of classical Th2 cytokines, that is, IL-5, IL-13, and IL-4, dropped to the phosphate-buffered saline-control level in ST2−/− mice in OVA-exposed group. Supporting the finding, it has been demonstrated that the IL-33/ST2 signaling pathway enhances the expression of CCR3, which is important in facilitating the mobilization of eosinophils from bone marrow to peripheral blood and the trafficking to the site of inflammation (Stolarski *et al.*, 2010).

However, Hoshino *et al.* (1999) demonstrated that absence of ST2 does not affect the number of eosinophils in the BALF in OVA/alum-induced asthma model. The different result might be due to the used methods and different mouse strains. Hoshino *et al.* used C57BL/6 mice and we used 129 mice. Furthermore, sensitization protocols were different and might also affect the inflammatory response; the skin sensitization is an efficient systemic sensitizer without any adjuvant, whereas the intraperitoneal model is shorter and might be therefore milder. Moreover, downregulation of Th2 cytokines in the lung tissue of ST2−/− mice or after administration of ST2 mAb has been reported in parasitic nematode infection models (Senn *et al.*, 2000; Townsend *et al.*, 2000) and in Th2 cell-mediated lung immune responses (Lohning *et al.*, 1998; Coyle *et al.*, 1999).

IL-33 can promote the production of proinflammatory cytokines via ST2 receptor (Liew *et al.*, 2010). However, we found that in the absence of ST2 receptor, the expression of TNFα was significantly upregulated in the lung tissue of OVA-challenged ST2−/− mice. Instead, IL-6 was expressed at comparable level to the WT lung tissue, suggesting that TNFα and IL-6 are differently regulated by ST2 in the lungs and skin. IFNγ expression was increased in both tissues of OVA-exposed ST2−/− mice, as was the number of $CD8^+$ cells. FACS examination of the BALF showed that IFNγ protein was mostly produced by $CD8^+$ T cells. These results suggest that ST2 might act as a negative regulator of IFNγ-producing T cells during allergic inflammation. Finally, a recent data demonstrate that suppressive $CD4^+$ $Foxp3^+$ regulatory T cells can express membrane-bound ST2 (Turnquist *et al.*, 2011). However, in the absence of ST2 receptor, the mRNA levels of Foxp3 and IL-10 increased in the lung tissue, suggesting that the regulation of inflammatory response in the lung tissue is not dependent on ST2-expressing $Foxp3^+$ regulatory T cells.

In conclusion, when mice have been epicutaneously and repeatedly exposed to OVA/SEB, ST2−/− mice exhibit increased numbers of macrophages, neutrophils, cytotoxic $CD8^+$ T cells, and IFNγ in the sensitized skin. However, in the lung tissue of sensitized ST2−/− mice, eosinophils and Th2 cytokines are significantly downregulated. These findings show that the Th2 response in the lung tissue is totally inhibited in the absence of ST2. On the contrary, in mouse AD-like skin other pathways than IL-33–ST2 interaction are critical in the development of Th2 response. However, we could not link the Th2 cytokine production in the sensitized skin or lung tissue to a specific cell type, which might help to explain obtained results.

Importantly, these results suggest that the disruption of ST2–IL-33 pathway cannot be used to target Th2 responses in the AD skin, but is very essential in inhibiting Th2 responses in allergic asthma. These results open new avenues to understand and study in more detail the basic mechanisms in AD and asthma.

MATERIALS AND METHODS

Mice and sensitization

ST2−/− mice (strain 129-Il1rl1, substrain 129/SvEv) were purchased from the European Mouse Mutant Archive (Monterotondo, Italy) and bred in our animal facilities. WT littermates (129/SvEv) were used as controls.

SEB is a well-known triggering factor of atopic skin inflammation. We have recently shown that IL-33 and ST2 are upregulated in the skin of patients with AD after exposure to SEB (Savinko *et al.*, 2012). Therefore, SEB was used together with OVA allergen to epicutaneously sensitize the mice skin to mimic natural exposure into AD skin. All animal experiments were approved by the State Provincial Office of Southern Finland. Sensitization protocol is described in Supplementary Materials and Methods online.

Skin and lung histology

Skin and lung biopsies were fixed in 10% buffered formalin and embedded in paraffin. Skin and lung sections of 4 μm were cut and stained with hematoxylin and eosin. Skin sections were stained with o-toluidine blue for mast cell counts, and lung sections were stained with periodic acid-Schiff solution and examined under light microscopy (DM 4000B; Leica, Wetzlar, Germany). Inflammatory cells were counted in 15 high-power fields.

Immunohistochemistry

Staining of $CD3^+$, $CD4^+$, and $CD8^+$ cells was made as earlier described (Savinko *et al.*, 2005; Lehto *et al.*, 2010) and described in Supplementary Materials and Methods online.

Cytokine analysis by RT-PCR

RNA isolation was made as earlier described (Savinko *et al.*, 2005) with slight modifications: Eurozol (EuroClone, Siziano, Italy) was used instead of Trizol, and RNA content was measured by NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). cDNA was synthesized from 0.5 μg of total RNA in 25 μl reaction mixture with High Capacity cDNA Reverse Transcription Kit (Applied

Biosystems, Foster City, CA). PCR primers and probes were obtained from Applied Biosystems, and quantitative real-time PCR was performed with 7500 Fast Real-Time PCR System and SDS Software v.1.4.0 (Applied Biosystems). The gene expression between different samples was normalized with endogenous 18S rRNA, and the target gene expression was calculated by the comparative C_T method according to the instructions of Applied Biosystems.

Bronchoalveolar lavage fluid

To collect BALFs, the trachea was surgically exposed, cannulated with a syringe, and flushed with 0.8 ml of phosphate-buffered saline. Cells in the lavage fluid were counted by hemacytometer, and BALF cell differentials were determined on slide preparations stained with May-Grünwald-Giemsa and counted in 15–20 high-power fields under light microscopy (Leica DM 4000B).

ELISA

Serum levels of OVA-specific antibodies were measured by the straight ELISA method (Savinko *et al.*, 2005). In brief, plates were coated with 100 μg ml^{-1} OVA for OVA-specific IgE, 2 μg ml^{-1} OVA for OVA-specific IgG2a. Bound antibodies were detected either with biotin-conjugated rat anti-mouse IgE (clone R35-118) or with biotin-conjugated rat anti-mouse IgG2a (clone R11-89) (BD Biosciences, San Jose, CA). Streptavidin horseradish peroxidase, followed by substrate, was used to detect the bound antibody levels. Optical density was measured at 405 nm.

Luminex

For analysis of TNF-α, IL-13, and IL-5 proteins in BALF supernatants, we used a Bio-Plex Pro Mouse Cytokine Assay (BioRad Laboratories, Hercules, CA) according to the manufacturer's protocol. A total of 3% BSA (Sigma-Aldrich, St Louis, MO) in phosphate-buffered saline was added at a concentration of 0.5% to samples, controls, and standards to ensure sufficient protein amounts for the assay. Assay was performed using Luminex xMAP Technology (Bio-Plex 200 System; BioRad).

FACS analysis

Flow cytometry was performed using a CantoII instrument (Becton Dickinson, Fraklin Lakes, NJ), and the data were processed with the FlowJo Software (Tree Star, Ashland, OR). BALFs from epicutaneously OVA/SEB-sensitized and intranasally OVA-challenged WT and ST2−/− mice were stimulated with phorbol myristate acetate (20 ng ml^{-1}) and ionomycin (1 μg ml^{-1}), including brefeldin A (Sigma, St Louis, MO) at 37 °C for 4 h. After stimulations, cells were washed with cold phosphate-buffered saline including 2% fetal bovine serum. Fragment crystallizable receptors were blocked with an excess of anti-mouse CD12/32 (eBioscience, San Diego, CA) and surface stained with phycoerythrin–Cy7-conjugated anti-CD3, FITC-conjugated TCR_β, PeCy5-conjugated anti-CD4, and Alexa700-conjugated anti-CD8. Cells were permeabilized with intracellular Fix and Perm staining kit (Caltag, Burlingame, CA), and stained with phycoerythrin-conjugated anti-IFNγ.

Statistics

Analysis between groups was examined with nonparametric Mann–Whitney *U*-test. Results are expressed as means (±SEM), and *P*-values <0.05 were considered to be statistically significant. Statistical analysis were performed by GraphPadPrism (GraphPad Software, La Jolla, CA)

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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