

The Expression and Regulation of Interleukin-33 in Human Epidermal Keratinocytes: A New Mediator of Atopic Dermatitis and Its Possible Signaling Pathway

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Interleukin (IL)-33 is a novel member of the IL-1 superfamily of cytokines that has recently become a focal point for research into the pathogenesis of atopic dermatitis (AD). However, the expression and regulation of IL-33 in human epidermal keratinocytes have not well been delineated. The aim of this study was to evaluate IL-33 and its receptor ST2L expression in skin lesions of AD patients and explored the signal transduction mechanisms leading to the IL-33 expression and of the IL-33's pharmacological action in keratinocytes from AD patients (ADKs) and those from healthy controls (NHEKs). We performed immunocytochemistry, reverse transcription-polymerase chain reaction, Western blotting, and enzyme-linked immunosorbent assay to investigate ADKs compared with NHEKs. We found that IL-33/ST2L were positively expressed in the skin lesions of AD patients and a high expression of IL-33 was induced in keratinocytes by IL-4 plus interferon [IFN]- γ or IFN- γ alone at the mRNA and protein levels. Meanwhile, IFN- γ induced IL-33 expression through extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK), epidermal growth factor receptor (EGFR), and Janus kinase/signal transducer and activator of transcription. The expression of ST2L was increased in a time- and dose-dependent manner in both types of cells incubated with IL-33, and was especially increased in ADKs compared with NHEKs. We examined the cytokine IL-6 and the chemokines CXCL-8/IL-8, CCL-20, CCL-17, CCL-5, and CCL-2 in keratinocytes, which showed increased expression in a time- and dose-dependent manner in ADKs when induced by IL-33. Furthermore, 4 signaling pathways (ERK, p38 MAPK, c-Jun N-terminal kinase, and nuclear factor- κ B) were involved in the IL-33-mediated induction in both cells. In conclusion, IL-33 is closely interlinked in AD skins and keratinocytes. IL-33 plays an important role in the pathogenesis of immune inflammatory responses in AD, which might be a possible therapeutic target in the treatment of AD.

Introduction

ATOPIC DERMATITIS (AD) is the most common chronic, relapsing, itching, and inflammatory skin disease. The prevalence of AD is estimated to be 15%–30% in children and 2%–10% in adults, while the incidence of AD has increased by 2- to 3-fold during the past 3 decades in industrialized countries (Johansson and others 2004). A predominant T-helper (Th)2 imbalance with increased immunoglobulin E (IgE) levels and eosinophilia is widely accepted in the pathogenesis of AD (Biedermann and others 2004; Fiset and others 2006; de Koning and others 2011). In skin with established chronic AD, the cytokines interferon (IFN)- γ , interleukin (IL)-12, IL-5, and granulocyte macrophage-colony-stimulating factor are dominant, which is a profile characteristic of a T helper type 1 (Th1)/0 immune environment (Grewé and others 1998).

Keratinocytes constitute a part of the innate immune defense, and also contribute to the amplification and persistence of inflammatory and immune responses in AD skin through the release of cytokines, chemokines, proteases, and bioactive lipids (Homey and others 2006). Upon stimulation by allergens, toxins, or infectious agents, keratinocytes are capable of initiating cross-talk between the adaptive and innate immune responses by activating T cells in patients with AD through the release of key molecules, and can also mediate epidermal barrier perturbation (de Koning and others 2011).

IL-33 (also known as IL-1F11), a ligand for the IL-1 receptor family member ST2L (also known as IL-1RL1), is the most recently discovered member of the IL-1 cytokine family and has recently been attributed to the epithelial “alarmin” defense system. IL-33 is released by the

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epithelial cells of various tissues and organs, including keratinocytes, endothelial cells, and immune cells. Besides, IL-33 is known to activate naive and Th2 lymphocytes, mast cells, and eosinophils to produce Th2-type cytokines, which are known to play an important role in the pathogenesis of AD (Liew and others 2010). The mitogen-activated protein kinase (MAPK) family [extracellular signal-regulated kinase (ERK), p38, and JNK] and nuclear factor (NF)- κ B signaling pathways have been implicated in many human skin diseases including AD, lichen planus, psoriasis, and skin tumors (Schmitz and others 2005; Chackerian and others 2007; Pushparaj and others 2009). And cell-specific variations in the signaling pathways induced by IL-33 have been described (Funakoshi-Tago and others 2008).

Several cytokines and chemokines have received scientific interest for their potential roles in the pathology of AD. Among them, IL-6 is released during the cutaneous response to allergen challenge in atopic individuals (Lee and others 1992). A recent clinical trial revealed that the interruption of IL-6 receptor signaling reduced the severity of AD symptoms (Navarini and others 2011). The chemokines CXCL-8/IL-8 and CCL-5 are potent chemoattractants for neutrophils and eosinophils (Kikuchi and others 2006), whereas the expression and secretion of CCL-2 are well correlated to the infiltration of monocytes, basophils, eosinophils, memory T lymphocytes, and natural killer cells (Rose and others 2003). CCL-17, a ligand for CCR4, is mainly expressed on Th2 cells. Furthermore, the serum concentration of CCL-17 is significantly higher in AD patients than in psoriasis vulgaris subjects or in normal healthy donors, and correlates well with the severity of AD (Kakinuma and others 2001). CCL-5, a chemokine secreted by epidermal keratinocytes (Park and others 2005), promotes the migration of T cells, dendritic cells, eosinophils, natural killer cells, mast cells, and basophils (Levy 2009). In the context of acute and chronic AD lesions, resident cell populations contribute to the chemoattraction of eosinophils mainly through the release of CCR3 binding molecules, including CCL-20 (Nomura and others 2003).

However, few reports have investigated the differential induction of IL-33 and its regulation of the AD-related cytokines and chemokines between ADKs and NHEKs. It was hypothesized that IL-33 plays a major role in AD and acts on activated keratinocytes to further increase the release of the cytokine and chemokines. In this study, we provide experiments to investigate IL-33 expression in skin lesions of AD patients and explored the signal transduction mechanisms leading to the IL-33 expression and of the IL-33's pharmacological action in both types of keratinocytes. And our data indicated that IL-33 takes part in ADKs and NHEKs to regulate the epidermal involvement inflammatory, and points to MAPK and NF- κ B signaling pathways as promising targets for the modulation of AD.

Materials and Methods

Patients and biopsies

All subjects provided written informed consent before participating in these studies. These studies were approved by the medical ethics committee of the General Hospital of the Air Force of the People's Liberation Army of China and were conducted according to the principles of the Declara-

tion of Helsinki. AD patients were identified according to the criteria defined by Hanifin and Rajka (1980). Skin punch biopsies (6 mm diameter) were obtained from 5 patients with AD and 5 healthy volunteers. The patients used neither topical medications for 1 week nor systemic medications for 4 weeks before taking skin biopsies.

ADKs and NHEKs isolation and stimulation

ADKs and NHEKs were cultured from foreskin or plucked hair follicles as previously described (Wang and others 2008). Cells were cultured in keratinocyte serum-free media (DermaLife® K Medium Kit; Lifeline Cell Technology, Frederick, MD), including L-glutamine (6 mM), Extract P® (0.4%, v/v), epinephrine (1 μ M), recombinant human transforming growth factor α (0.5 ng/mL), hydrocortisone hemisuccinate (100 ng/mL), recombinant human insulin (5 μ g/mL), apo-transferrin (5 μ g/mL), and calcium chloride (0.02 mM). The cells were trypsinized and passaged at 80%–90% confluency and diluted at a ratio of 1:4. Cells passaged 3–5 times were used for experiments. Before cells were stimulated with cytokines, hydrocortisone hemisuccinate, Extract P, recombinant human transforming growth factor α , recombinant human insulin, and apo-transferrin were removed for 24 h. ADKs and NHEKs were stimulated and incubated with IFN- γ , tumor necrosis factor (TNF)- α , IL-4, IL-33 (PeproTech, Rocky Hill, NJ), and combinations of those cytokines for the indicated periods of time, and were then harvested for experiments. Cells were treated with the following signaling inhibitors for 60 min before stimulation: inhibitor of NF- κ B (I κ B)- α phosphorylation inhibitor BAY11-7082 (2 μ M); ERK inhibitor U0126-EtOH (10 μ M); JNK inhibitor SP600125 (5 μ M); p38 MAPK inhibitor SB203580 (10 μ M); phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (5 μ M); epidermal growth factor receptor (EGFR) inhibitor PD153035HCl (5 μ M); and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) inhibitor ruxolitinib (5 μ M) (Selleck Chemicals, San Diego). SB203580 was dissolved in water whereas others were dissolved in dimethyl sulfoxide (DMSO). In all studies, the final concentration of DMSO was 0.1% (v/v).

Immunohistochemistry and immunocytochemistry

Tissue samples from AD patients and healthy volunteers were fixed in 10% (w/v) buffered formalin and embedded in paraffin. Staining was performed using the avidin-biotin-horseradish peroxidase method. Antigen retrieval was performed by autoclaving in a 0.01 M sodium citrate buffer (pH 6.0) for 10 min. A monoclonal antibody against human IL-33 (Nessy-1, diluted 1:100; Abcam, Cambridge, UK) and a polyclonal antibody against human ST2L (Rabbit IgG1, diluted 1:300; Millipore, Billerica, MA) were used. Color was developed using diaminobenzidine as a substrate. After stimulation, cultured cells were fixed in phosphate-buffered saline (PBS) containing 4% (w/v) paraformaldehyde for 20 min, permeabilized in PBS containing 0.2% (v/v) Triton™ X-100 for 10 min, and blocked in a serum-free blocking solution for 5 min at room temperature. Cells were then incubated with polyclonal antibody for human ST2L diluted 1:100 overnight at 4°C. After extensive washing with PBS, cells were incubated with biotinylated goat anti-rabbit IgG as the secondary antibody. Finally, nuclei were stained with hematoxylin. Slides

were examined and images were acquired using a fluorescence microscope (BZ8000; Keyence, Osaka, Japan).

RNA preparation and real-time reverse transcription-polymerase chain reaction analysis

ADKs and NHEKs were seeded in 6-well plates with 1×10^6 cells in each well. Total RNA was extracted from the cells using TRIzol[®] reagent according to the manufacturer's instructions (Life Technologies, Carlsbad, CA) after stimulation. First strand cDNA was synthesized using a Reverse Transcription System Kit (Tiangen Biotech, Beijing, China). RNA was reverse transcribed into cDNA and analyzed by real-time reverse transcription-polymerase chain reaction (RT-PCR) using an ABI PRISM[®] 7000 sequence detector (Applied Biosystems, Foster City, CA). IL-33 and ST2L mRNA levels were detected by real-time PCR in a reaction mixture of 20 μ L total volume containing 10 μ L $2 \times$ SYBR[®] TransStar Green PCR Super Mix, 0.6 μ L sense and antisense primers, and 50 ng RT product. The utilized primer sequences were as follows: IL-33 (sense) 5'-AATCAGGTGA CCGTG TTG-3' and (antisense) 5'-TGAAACACAGTTGG AGTGC-3'; ST2L (sense) 5'-CCCACATTCAATAGGA CTG-3' and (antisense) 5'-AAgAGGTGCTGTCCAGTTG-3'; and GAPDH (sense) 5'-CTCCTCCACCTTTGACG CTG-3' and (antisense) 5'-TCCTCTTGTGCTCTTGCTGG-3'. To allow comparisons to be made between treatment groups, the measured expression level of each target gene was normalized to that of GAPDH.

Western blot analysis

Cells were lysed with lysis buffer [20 mM Tris-HCl, pH 8.0, 120 mM NaCl, 1% (v/v) Triton[™] X-100, 10 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 0.05% (v/v) 2-mercaptoethanol, and 1 $\times$ protease inhibitors solution]. Then, cell debris was removed by centrifugation at 14,000 g for 15 min, and equal amounts of proteins extracted from each sample were heat denatured and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 10% (v/v) polyacrylamide gel. After electrophoresis, proteins were electrotransferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 5% (w/v) skimmed milk at room temperature for 2 h and probed with primary antibody at 4°C overnight. The primary antibodies used were IL-33 (Abcam), phosphorylated JNK1/2, total JNK1/2, phosphorylated ERK1/2, total ERK1/2, phosphorylated p38 MAPK, total p38 MAPK, β -actin (Cell Signaling Technology, Beverly, MA), anti-I κ B α , and anti-phospho-I κ B α (BD Pharmingen, San Diego, CA). After washing, membranes were incubated with the appropriate secondary antibody in 5% (w/v) skimmed milk/Tris-buffered saline containing 0.05% Tween[®] 20 for 1 h at 4°C. Proteins were detected using SignalFire[™] ECL Reagent (Cell Signaling Technology).

Enzyme-linked immunosorbent assay analysis

Supernatants from ADKs and NHEKs were harvested at 18, 24, or 72 h after stimulation. The supernatants were analyzed for IL-6, CXCL-8/IL-8, CCL-20, CCL-17, CCL-5, and CCL-2 (Boster, Wuhan, China), and the cell lysates were analyzed for IL-33 (Cloud-Clone Corp., Houston, TX)

by enzyme-linked immunosorbent assay (ELISA) following the manufacturers' respective instructions.

Statistical analysis

Data were expressed as the mean $\pm$ standard deviation (SD) of 3 independent measurements, and significance was analyzed by *t*-test and 1-way analysis of variance. Differences of $P < 0.05$ were considered significant. All analyses were performed using Statistical Package for the Social Sciences (SPSS) statistical software for Windows, Version 16.0 (SPSS, Inc., Chicago, IL).

Results

Expression of IL-33 and ST2L in AD skin

In normal control skin, positive immunostaining for IL-33 was rarely detected in keratinocytes but was observed in endothelial cells. In AD skin, a variable intensity of IL-33 immunostaining was observed among 5 cases. Cell nuclei were stained positively for IL-33 and were mostly localized in keratinocytes from the suprabasal layer to the stratum spinosum in AD lesions (Fig. 1). The nuclei of endothelial cells and infiltrating mononuclear cells also stained moderately for IL-33. We generated antibodies for both the membrane bound and soluble secreted forms of the receptor of IL-33, ST2L, for use in immunohistochemical staining. ST2L expression in AD skin was detected in the stratum spinosum and was localized to the cytoplasm, cellular membrane, and intercellular space (Fig. 1). ST2L was also stained strongly in dermal infiltrating cells, endothelial cells, and neutrophils that infiltrated the epidermis. Isotype control-stained skin did not demonstrate any positive staining.

Cytokines characteristically found in AD skin induced an increased expression of IL-33 mRNA and protein in ADKs and NHEKs

AD is currently considered to be a biphasic disease, with a Th2 immune characteristic predominating during the acute phase and a switch to Th1 in the chronic phase (Gittler and others 2012). Therefore, we tested whether the mRNA and protein expression of IL-33 could be induced by Th2 and/or Th1 cytokines present in AD skin (IL-4, IFN- γ , and TNF- α). Each cytokine was applied to ADKs and NHEKs at a concentration of 20 ng/mL. We found that IL-4 and IFN- γ either separately or in combination were inducers of IL-33 mRNA expression. TNF- α alone did not significantly regulate IL-33 mRNA expression, but showed a tendency to inhibit its up-regulation by the other tested cytokines. Comparison between ADKs and NHEKs revealed a tendency for ADKs to be more sensitive to stimulation by IFN- γ or IL-4 plus IFN- γ , as determined by their higher production of IL-33 (Fig. 2). Moreover, ADKs and NHEKs stimulated with IL-4 plus IFN- γ produced the largest amount of IL-33 mRNA expression at 6 h poststimulation (Fig. 2A). In contrast, when IFN- γ was used alone, the effect on IL-33 induction was 5.1 times stronger at 24 h than when it was used together with IL-4 in ADKs (Fig. 2B). The induction of IL-33 expression was confirmed at the protein level at 24 h post-stimulation by ELISA of cell lysates (Fig. 2C). However, neither pro-IL-33 nor mature IL-33 was detected in the cell culture media supernatants by ELISA.

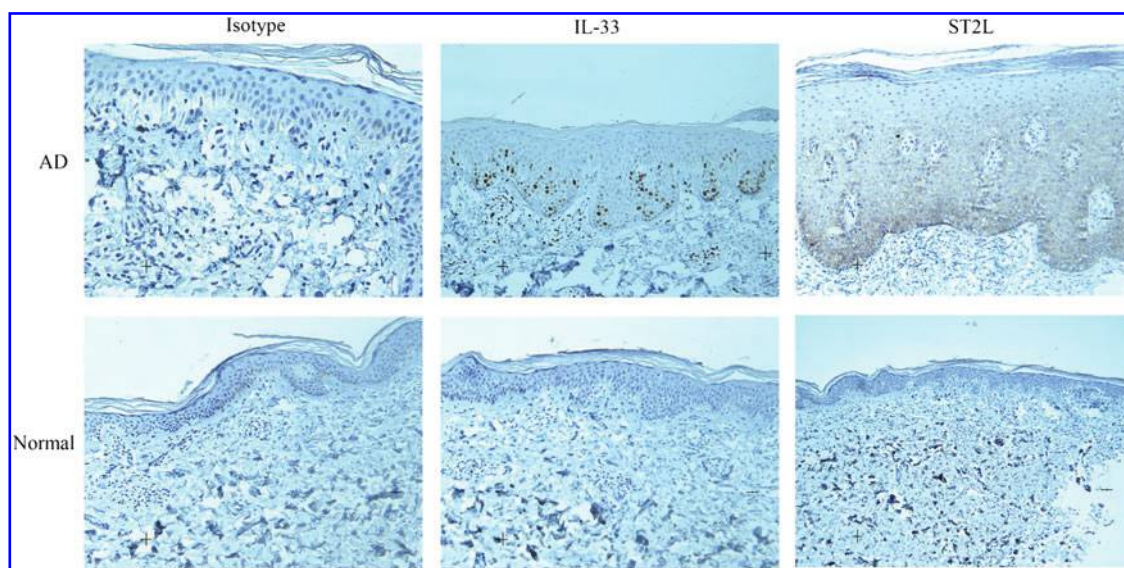


FIG. 1. IL-33 and its receptor ST2L were expressed in human AD and normal skin. Representative histology images are shown for AD patients and healthy donors ($n = 5$ per group). Magnification $100\times$; scale bar $5\mu\text{m}$. AD, atopic dermatitis; IL, interleukin.

Signaling pathways involved in IFN- γ -mediated IL-33 expression in ADKs and NHEKs

We focused on signaling pathways involved in IFN- γ -mediated IL-33 expression in ADKs and NHEKs according to the reports (Meephansan and others 2012; Seltsman and others 2013). Specific small molecule inhibitors of intracellular signaling pathways were utilized to clarify the signal transduction pathways implicated in IL-33 expression. IFN- γ -induced IL-33 mRNA expression was almost completely inhibited by SB203580, U0126-EtOH, PD153035HCl, and ruxolitinib, whereas LY294002, BAY11-7082, and SP600125 were ineffective in both ADKs and NHEKs (Fig. 3A, B). Furthermore, the Western blotting results (Fig. 3C, D) indicated that IFN- γ induced IL-33 protein expression through ERK, p38 MAPK, and EGFR-dependent pathways, and that the JAK/STAT pathway may be involved in IL-33 induction by IFN- γ (Meephansan and others 2012).

IL-33 induced ST2L mRNA and protein expression in ADKs and NHEKs

The IL-33 receptor ST2L was constitutively expressed on ADKs and NHEKs (Fig. 4A). The mRNA and protein levels of ST2L were analyzed after treatment of keratinocytes with IL-33 to determine whether IL-33 raised ST2L expression. Analysis using RT-PCR showed that both ADKs and NHEKs treated with IL-33 exhibited a significant and dose-dependent increase in the mRNA expression of ST2L compared with that in untreated cells (Fig. 4B). Those findings were further confirmed by immunostaining. The ST2L signal was enhanced on the cell membrane of ADKs and NHEKs incubated with IL-33 at the concentration of 100 ng/mL for 24 h, and it was stronger than the signal observed at 10 ng/mL of IL-33 (Fig. 4A). The effect of IL-33 on ST2L mRNA expression was also time dependent. It started at 2 h, peaked at 12 h, and returned to the

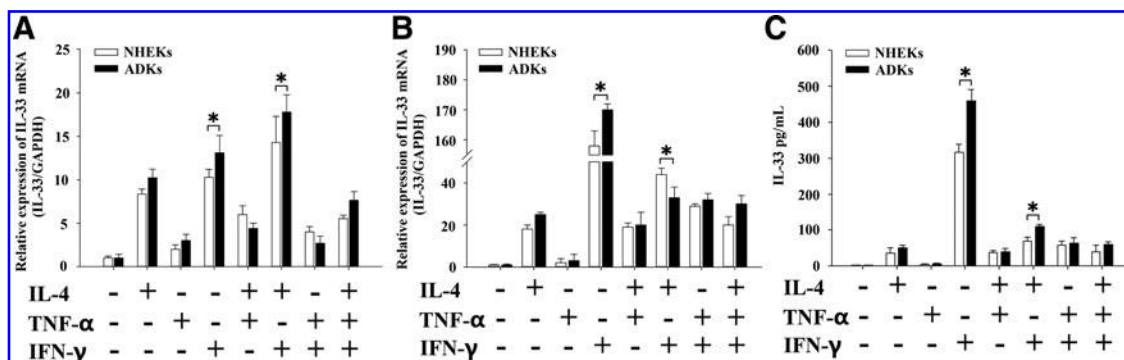


FIG. 2. Increased IL-33 mRNA and protein expression was induced by the Th2 cytokine IL-4 and the Th1 cytokines IFN- γ and TNF- α in keratinocytes from AD patients (ADKs) and healthy volunteers (NHEKs). ADKs and NHEKs were harvested at 6 h (A) and 24 h (B) after stimulation and analyzed by RT-PCR. (C) IL-33 protein in cell lysates expression was analyzed by ELISA in 3 independent experiments. All cytokines were applied at 20 ng/mL . The results are reported as the mean $\pm$ standard deviation (SD) of 3 independent experiments. $*P < 0.05$. ELISA, enzyme-linked immunosorbent assay; IFN, interferon; RT-PCR, reverse transcription-polymerase chain reaction; Th, T-helper; TNF- α , tumor necrosis factor- α .

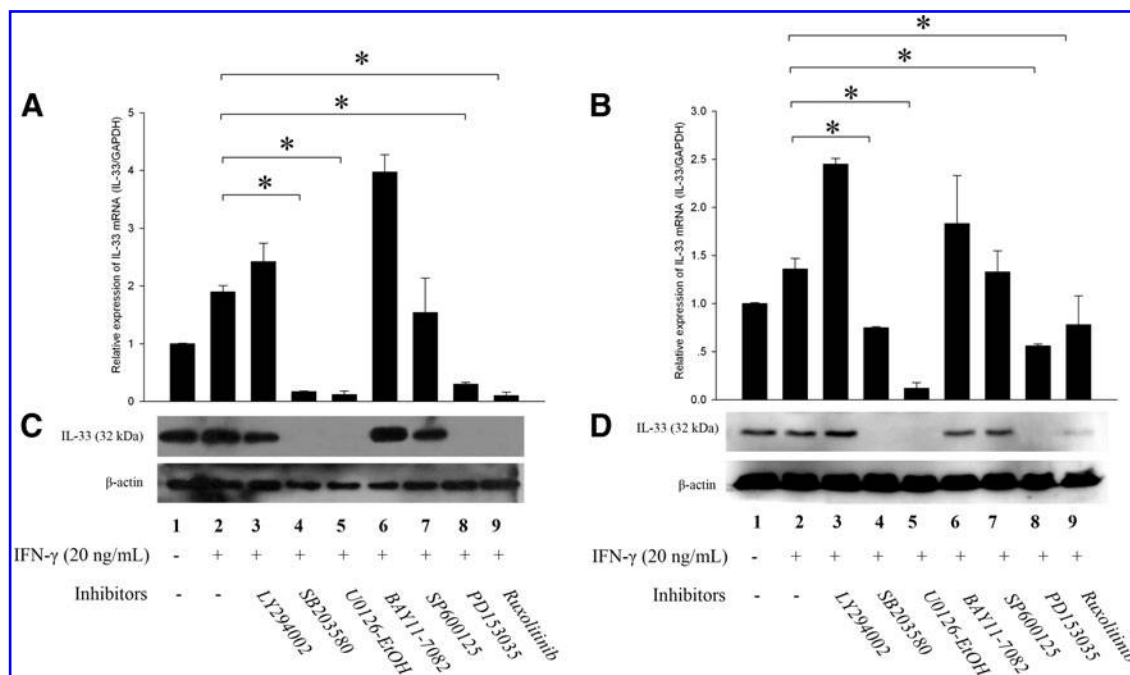


FIG. 3. Signaling pathways involved in IFN- γ -induced IL-33 expression in ADKs and NHEKs. ADKs (**A, C**) and NHEKs (**B, D**) were preincubated with inhibitors for PI3K (5 μ M LY294002), p38 MAPK (10 μ M SB203580), ERK1/2 (10 μ M U0126-EtOH), NF- κ B (2 μ M BAY11-7082), c-Jun N-terminal kinase (JNK) (5 μ M SP600125), EGFR (5 μ M PD153035HCl), and JAK/STAT (5 μ M ruxolitinib) for 1 h before IFN- γ (20 ng/mL) stimulation for 6 h for RT-PCR (**A, B**), and 24 h for Western blotting (**C, D**); loading control: β -actin. Results are expressed as the mean $\pm$ SD of 3 independent experiments. * P < 0.05. EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; JAK/STAT, Janus kinase/signal transducer and activation of transcription; MAPK, mitogen-activated protein kinase; NF, nuclear factor.

baseline level by 24 h post-IL-33 stimulation (Fig. 4C). Meanwhile, ADKs showed a significantly higher sensitivity to IL-33 stimulation than NHEKs did, especially at 12 h after stimulation with the 100 ng/mL concentration of IL-33 (all P < 0.05).

Cytokine and chemokine release induced by IL-33 in ADKs and NHEKs

Keratinocytes are a major cellular component of the skin and play a key role in establishing a local proinflammatory milieu in various cutaneous diseases (Pastore and others 2005). As shown in Fig. 5, IL-33 directly activated ADKs and NHEKs to significantly induce the release of the proinflammatory cytokine IL-6 and the chemokines CXCL-8/IL-8, CCL-20, CCL-17, CCL-5, and CCL-2 in a dose-dependent manner (0–100 ng/mL) at 18 and 72 h. Moreover, the release of IL-6, CXCL-8/IL-8, and CCL-20 occurred in a time-dependent manner; the release of these factors at 72 h was obviously higher than that at 18 h after stimulation with IL-33 at concentrations of 20–100 ng/mL (Fig. 5; * P < 0.05, ** P < 0.01). The proinflammatory factor that was most potently induced by IL-33 treatment was CCL-20, which showed a 2.1-fold increase in its concentration at 72 h compared with that at 18 h in ADKs incubated with IL-33 at 100 ng/mL (Fig. 5C). On the contrary, the release of CCL-17, CCL-5, and CCL-2 was lower at 72 h than at 18 h of incubation with IL-33 at 50 and 100 ng/mL concentration in both type of cells (Fig. 5D–F; * P < 0.05 or ** P < 0.01). However, cytokine release and chemokine release from ADKs stimulated by IL-33 at 50 and 100 ng/mL

were obviously higher than those from NHEKs stimulated in the same way (* P < 0.05).

IL-33 activates MAPK and NF- κ B signaling pathways in ADKs and NHEKs

Western blotting analysis showed that increased phosphorylation of ERK1/2, p38 MAPK, and JNK1/2 was detected upon IL-33 stimulation, and NF- κ B was also activated, as demonstrated by I κ B- α phosphorylation (Fig. 6). These 4 signaling molecules were all rapidly activated. Their phosphorylation peaked within 5 or 30 min and that of p38 MAPK returned to baseline within 60 min. There was an obvious difference in the activation kinetics of these molecules between ADKs and NHEKs. The phosphorylation signaling responses induced by IL-33 in ADKs were more rapid and stronger than those in NHEKs for p38 MAPK, JNK1/2, and I κ B- α .

The IL-33-mediated induction of cytokine and chemokine release involved the MAPK and NF- κ B signaling pathways in ADKs and NHEKs

Analysis by Western blotting showed that the MAPK and NF- κ B signaling pathways were activated by IL-33 in ADKs and NHEKs. Furthermore, various signaling molecule inhibitors were used to distinguish among these signaling pathways regarding their involvement in regulating the release of cytokines and chemokines in ADKs and NHEKs upon IL-33 stimulation. As shown in Fig. 7, the p38 MAPK inhibitor SB203580, ERK inhibitor U0126, NF- κ B inhibitor

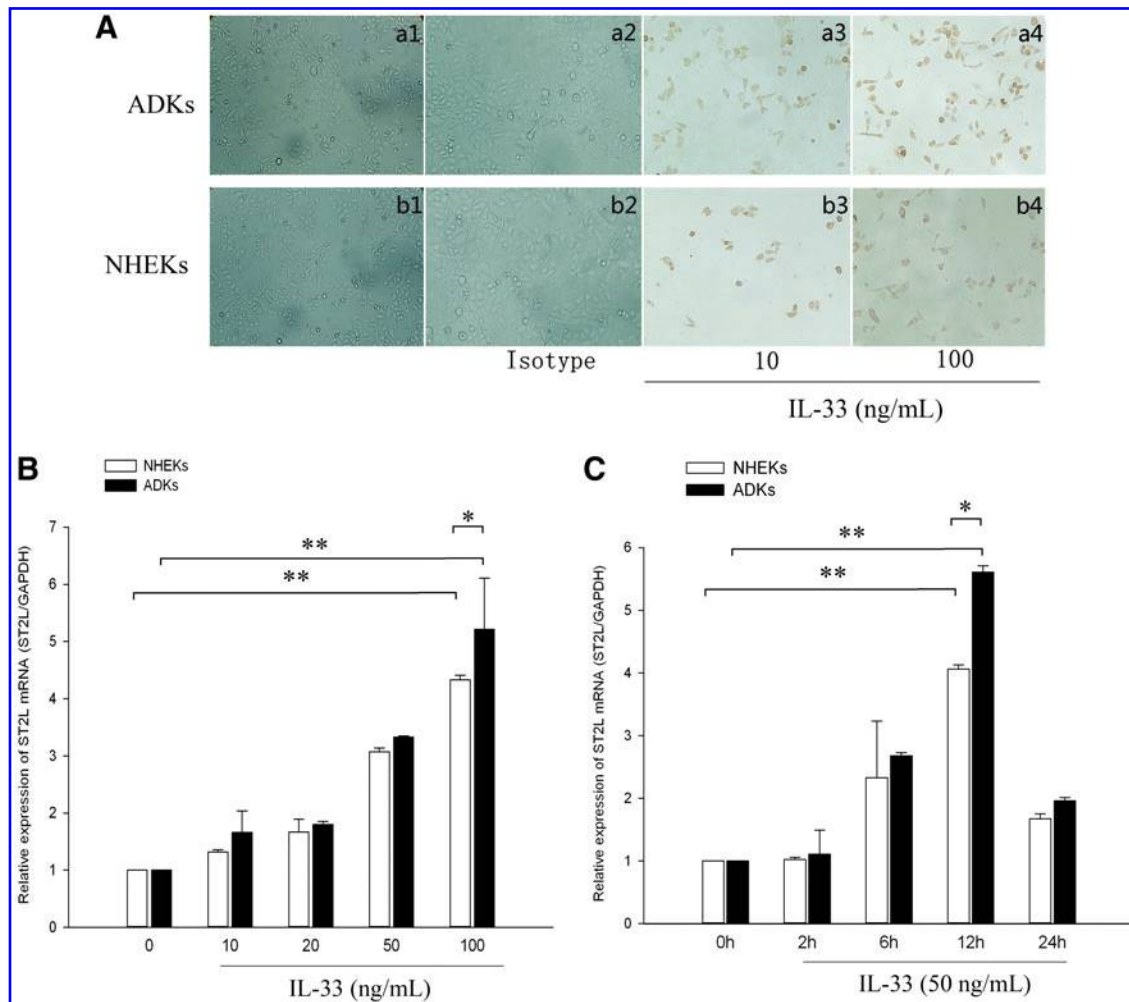


FIG. 4. ST2L mRNA and protein were expressed in ADKs and NHEKs. (A) Representative immunostaining images showing ST2L in ADKs and NHEKs incubated for 24 h; (a1, b1) show unstained cells with normal morphology, (a2, b2) show cells immunostained with isotype immunoglobulin G as a negative control, (a3, b3) and (a4, b4) were incubated with IL-33 at a concentration of either 10 or 100 ng/mL; 100 $\times$ magnification. (B) ST2L mRNA was induced by IL-33 in a concentration-dependent manner at 6 h of incubation. (C) ST2L mRNA was induced by IL-33 and its expression peaked at 12 h of incubation. * $P < 0.05$, ** $P < 0.01$.

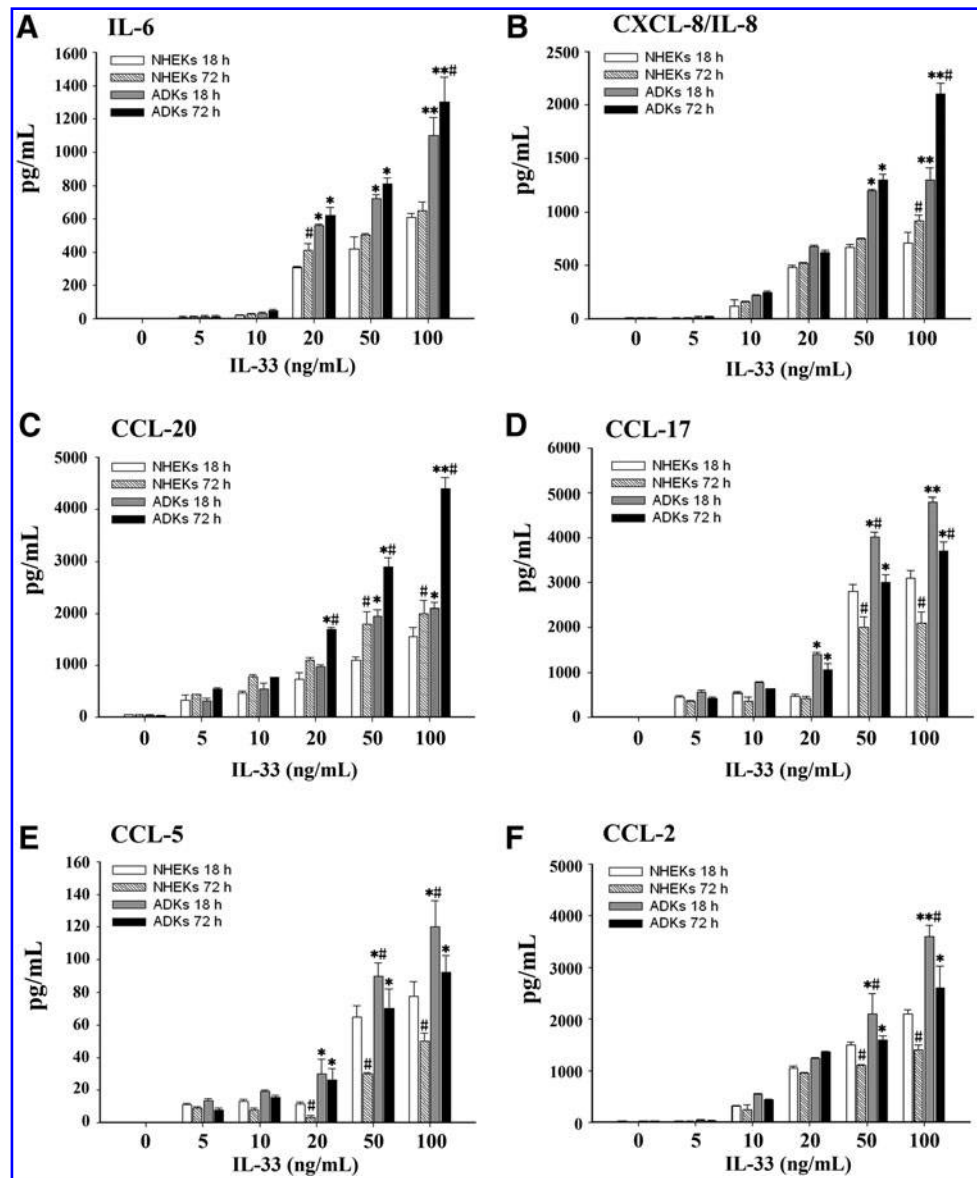
BAY11-7082, and JNK inhibitor SP600125 differentially suppressed the release of cytokines and chemokines induced by IL-33 in ADKs and NHEKs. For cytokine release and chemokine release, the IL-33-mediated induction of IL-6, CXCL-8/IL-8, CCL-20, and CCL-2 was also significantly suppressed by U0126, BAY11-7082, and SB203580 to differing degrees both in ADKs and NHEKs, whereas BAY11-7082 had little effect on CCL-17 and CCL-5 release. Meanwhile, we found that U0126, SP600125, and SB203580 significantly inhibited the induction of CCL-17 and CCL-5 by IL-33 in ADKs and NHEKs equally (Fig. 7E, F; all $P < 0.05$). On the contrary, the PI3K inhibitor LY294002 and the JAK/STAT inhibitor ruxolitinib did not significantly affect the induction of cytokine and chemokine production by IL-33 in ADKs and NHEKs. In addition, the degrees of inhibition exerted by various signaling molecule inhibitors showed no significant differences between ADKs and NHEKs ($P > 0.05$). Our measurements also suggested that ADKs produced a certain level of IL-6, CXCL-8, CCL-20, and CCL-17 in the absence of stimulation that was significantly elevated by treatment with IL-33. The solvent control treatment [0.1% (v/v)

DMSO only] did not significantly affect the release of cytokines and chemokines. Taken together, our results indicated that the 4 signaling pathways ERK, p38 MAPK, JNK, and NF- κ B were involved in the IL-33-mediated induction of cytokine release and chemokine release from both ADKs and NHEKs.

Discussion

Two topics are discussed in this article. One is the generation of IL-33 in ADKs compared with that in NHEKs, and the other is the function of IL-33 in these 2 types of keratinocytes and the signaling pathway involved. Although the active contribution of keratinocytes to the inflammation of AD has long been recognized (Ong 2014), their pathogenic role in local inflammatory regulation in AD is not well understood. Recently, there has been mounting evidence suggesting an important role of IL-33 in the pathogenesis of AD (Liew and others 2010; Cevikbas and Steinhoff 2012). IL-33 appears to drive Th2 responses in several organs, which suggests diverse roles in allergic and atopic diseases (Liew and others 2010; Balato and others 2012). AD is a common chronic

FIG. 5. Effects of IL-33 on cytokine and chemokine (IL-6, CXCL-8/IL-8, CCL-20, CCL-17, CCL-5, and CCL-2) release from ADKs and NHEKs. (A–F) ADKs and NHEKs (2×10^6 cells/well) were cultured with IL-33 (0, 5, 10, 20, 50, and 100 ng/mL) for 18 and 72 h. The expression levels of IL-6, CXCL-8/IL-8, CCL-20, CCL-17, CCL-5, and CCL-2 were determined by ELISA. Results are expressed as the mean $\pm$ SD of 3 independent experiments, * $P < 0.05$, ** $P < 0.01$ when compared between ADKs and NHEKs at the same incubation time point and treated with the same concentration of IL-33. # $P < 0.05$ when compared between the incubation times of 72 and 18 h in the same type of cells treated with the same concentration of IL-33.



inflammatory skin disease characterized by an early Th2 “immune signature”: patients suffer from relapsing eczematous and occasionally generalized (erythroderma) lesions associated with severe pruritus. Scratching reactions to pruritus typically exacerbate the inflammatory skin reactions (Hong and others 2011). Unfortunately, current AD treatments, which suppress inflammation broadly (eg, steroids and cyclosporine A), are hampered by effects on other cells and pathways that are unrelated to the disease. Investigation of the interaction between IL-33 and keratinocytes may, therefore, help to elucidate the mechanism by which the local inflammatory response is initiated and IL-33’s therapeutic potential in AD.

First of all, we sought to examine the expression of IL-33 and its receptor in the skin of AD patients using immunohistochemical staining. Our results showed that IL-33 and ST2L expression was increased in the skin lesions of AD patients. ST2L expression was found in dermal infiltrating cells and to some extent in epidermal cells. Our results revealed that IL-33 and its receptor are upregulated in skin lesions of AD patients, and thus may contribute to the pathogenesis of the disease.

It is well established that the acute phase of AD is characterized by the expression of the Th2-associated cytokines IL-4, IL-5, and IL-13, whereas the chronic phase of AD is dominated by IFN- γ expression (Werfel 2009). We showed that IL-4 and IFN- γ in combination caused the largest upregulation of *IL-33* expression in the primary phase (6 h), and its induction by IFN- γ alone was highest at the longest measurement time point (24 h). In line with our results, IFN- γ is the best characterized proinflammatory cytokine for keratinocytes, and IFN- γ -producing T-cell clones dominate psoriasis and allergic contact dermatitis lesions, but also intervene in the establishment of chronic AD lesions (Pastore and others 2005). Our results showed an increased response of ADKs to IL-4 and/or IFN- γ with more generation of IL-33 when compared with that of NHEKs. In contrast to previous reports describing the upregulation of IL-33 by TNF- α in epithelial cell cultures (Matsuyama and others 2010), there was no upregulation of IL-33 protein or mRNA expression in NHEKs. To our knowledge, these results may point to a differential regulation of the expression of IL-33 in diverse inflammatory skin diseases (Balato

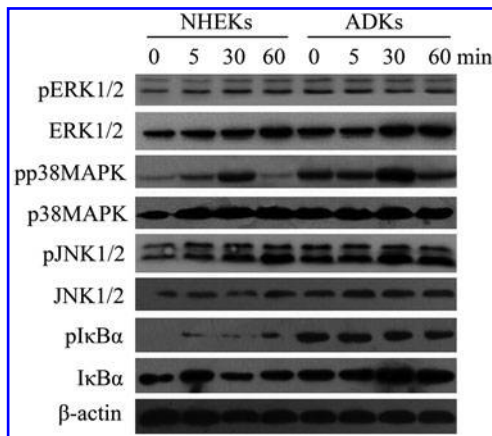


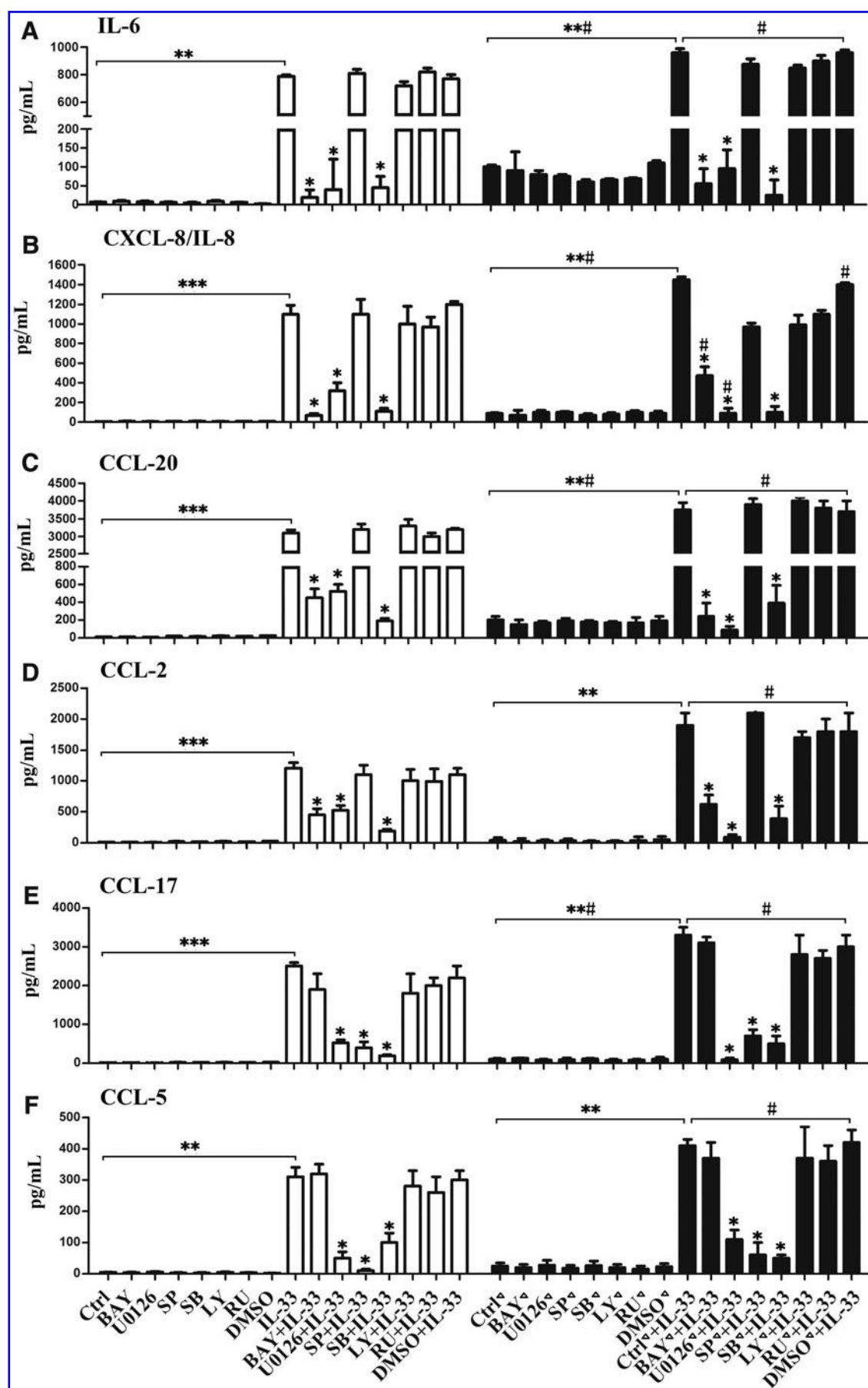
FIG. 6. The phosphorylation of ERK, p38 MAPK, JNK1/2, and NF- κ B was affected by IL-33 in ADKs and NHEKs. ADKs and NHEKs (5×10^6 cells/well) were incubated with or without IL-33 (50 ng/mL) for various lengths of time as noted. Total cellular proteins were extracted from ADKs and NHEKs for the detection of phospho-ERK, phospho-p38 MAPK, phospho-JNK1/2, and phospho-inhibitor of NF- κ B (I κ B)- α with the indicated antibodies by Western blotting.

and others 2012). IL-33 protein was not detected in the supernatants of stimulated keratinocytes by ELISA, but it was detected in cell lysates (Fig. 2C). The mechanism by which IL-33 is secreted from keratinocytes remains unclear. IL-33 has been described as an alarmin that is released after trauma or injury and necrosis (Haraldsen and others 2009; Meephansan and others 2012), so we presumed that IL-33 can be released after tissue damage or physical stress, including scratching of the skin in AD (Kakkar and others 2012). Moreover, the signaling pathways involved in the production of IL-33 by ADKs in response to IFN- γ are similar to those in NHEKs. The ADKs showed a significantly higher sensitivity to IL-33 and a higher expression of ST2L mRNA and protein than those of NHEKs. The RT-PCR results revealed that IL-33 significantly increased the mRNA expression of ST2L compared with that in untreated cells in a dose-dependent manner, and immunostaining yielded similar results (Fig. 4). Increased ST2L protein expression on the surface of keratinocytes in the skin of AD patients after atopy patch testing due to inflammation (infiltrating inflammatory cells) was reported to be more pronounced in AD than in healthy donors (Savinko and others 2012). It was presumed that a higher expression of ST2L protein on the cell membrane of keratinocytes confers a higher sensitivity to IL-33 and a greater induction of cytokine and chemokine release.

An investigation of the IL-33's pharmacological action on keratinocytes was conducted to further characterize the immunological mechanisms by which IL-33 initiates and sustains allergic inflammation. ADKs and NHEKs were directly activated by IL-33 and showed a significantly increased release of the inflammatory cytokine IL-6 and the chemokines CXCL-8/IL-8, CCL-20, CCL-17, CCL-5, and CCL-2 in a dose-dependent manner (0–100 ng/mL) at 18 and 72 h (Fig. 5). It has been reported that IL-6 can activate Th lymphocytes and induce Th2 immune responses including the synthesis of acute-phase proteins and mediate various inflammatory responses (Chattopadhyay and others 2007). The IL-33-induced release of CCL-2, CXCL-8, and CCL-5 from keratinocytes can, therefore, mediate the recruitment, infiltration, and activation of the immune effector cells in the microenvironment of inflammatory sites, thereby amplifying inflammatory responses in allergic diseases including AD. In contrast, the release of CCL-17, CCL-5, and CCL-2 was lower at 72 h than at 18 h, whereas CCL-20 was constantly higher (Fig. 5C–F). The results indicated that the amounts of chemokines produced by IL-33 stimulation changed over time. Thus, IL-33 was considered as a dual function cytokine not only because of its dual cytoplasmic and nuclear functions but also because of its dual actions on Th1- and Th2-linked inflammation. In this respect, it can be linked to other IL-1 family members such as IL-1 and IL-18, which both play a role in Th1- and Th2-associated inflammation (Garlanda and others 2013; Selmann and others 2013). In view of this, the induction of IL-6 and the chemokines CXCL-8/IL-8, CCL-20, CCL-17, CCL-5 and CCL-2 in ADKs and NHEKs upon IL-33 stimulation might play an important role in the development of local inflammatory responses in AD.

According to previous studies, IL-33 binds a heterodimeric receptor complex consisting of ST2L and IL-1R accessory protein (IL-1RAP) and induces signaling through the TIR domain of IL-1RAP. Myeloid differentiation primary-response protein88 (myD88) and IL-1R-associated kinase (IRAK) 1 and 4 are recruited to the receptor complex and induce the phosphorylation and/or activation of numerous signaling proteins, including NF- κ B, inhibitor of NF- κ B α (I κ B- α), ERK1/2, p38 MAPK, and JNK1/2 (Schmitz and others 2005; Chackerian and others 2007; Pushparaj and others 2009). In this study, the phosphorylation of ERK1/2, p38 MAPK, JNK1/2, and I κ B- α occurred more rapidly and strongly in ADKs than in NHEKs in response to stimulation with IL-33. It has been demonstrated previously that the PI3K-Akt, JAK/STAT, ERK, p38 MAPK, JNK and NF- κ B pathways can regulate cytokines and chemokines release by activated keratinocytes upon exposure to different stimuli such as leptin, Th17 cytokines, and various microbial products (Pastore and others 2005; Kim and others 2007;

FIG. 7. Effects of signaling molecule inhibitors on the release of cytokines and chemokines (IL-6, CXCL-8/IL-8, CCL-20, CCL-17, CCL-5, and CCL-2) from ADKs and NHEKs. (A–F) ADKs and NHEKs were pretreated with BAY11-7082 (2 μ M), U0126-EtOH (10 μ M), SP600125 (5 μ M), SB203580 (10 μ M), LY294002 (5 μ M), and ruxolitinib (5 μ M) for 1 h, followed by incubation with or without IL-33 (50 ng/mL) in the presence of inhibitors for a further 24 h to induce cytokine and chemokine release. Released cytokines and chemokines in the culture supernatants were determined by ELISA. DMSO (0.1%, v/v) was used as the vehicle control. * P < 0.05, ** P < 0.01, *** P < 0.001 when compared between groups denoted by horizontal lines or between groups of inhibitor treatments and IL-33 alone in the same type of cells; # P < 0.05 when compared between ADKs and NHEKs under the same treatment condition. Ctrl, control; BAY, BAY11-7082; U0126, U0126-EtOH; SP, SP600125; SB, SB203580; LY, LY294002; RU, ruxolitinib; Δ , ADKs group; DMSO, dimethyl sulfoxide.



Amano and others 2015). Therefore, it is logical that the mentioned pathways are commonly involved in the effects induced by IL-33 in ADKs and NHEKs in this study (Fig. 6).

Further elucidation of the involvement of the mentioned signaling pathways in the induction of cytokine/chemokine release in ADKs and NHEKs upon stimulation with IL-33 (Fig. 7) indicated that the release of IL-6, CXCL-8/IL-8, CCL-20 and CCL-2 was dependent on ERK, p38 MAPK and NF- κ B signaling, whereas the release of CCL-17 and CCL-5 was dependent on ERK, p38 MAPK and JNK signaling. There was no significant difference between ADKs and NHEKs in terms of the signaling molecules involved in their responses to IL-33. Regarding intracellular signal transduction, we found that MAPK and NF- κ B were selectively activated by IL-33 in both ADKs and NHEKs. In view of recent advances in the application of MAPK and NF- κ B inhibitors as potential anti-inflammatory agents in allergic diseases (O'Neill 2006), these findings may provide clues for the development of novel treatments for IL-33-mediated allergic diseases. In addition, it was presumed that the cause of the difference between ADKs and NHEKs in their sensitivity to IL-33 treatment is that the genetic predisposition for skin barrier dysfunction and defects in innate and adaptive immunity can lead to higher tropism in AD patients (de Koning and others 2011; Kezic and others 2012).

Collectively, this study conducted a meaningful investigation to explore the IL-33 expression and action in the skin and keratinocytes from the AD patients and normal human, respectively. Our data indicated that IL-33 has a potential role in AD. IL-33 and its receptor ST2L were expressed in AD skin. ADKs showed a stronger cytokine/chemokine release response to IL-33 than NHEKs, when ADKs and NHEKs were treated with cytokines and inhibitors. We demonstrated that IL-33 could stimulate ADKs and NHEKs to release proinflammatory cytokine and chemokines through signaling involved ERK, p38 MAPK, JNK and NF- κ B. Follow-up studies should be conducted to further investigate whether the mentioned signaling pathways are also involved in the activation of dendritic cells, mast cells, and CD4⁺ T cells by IL-33 in AD. Through our research, we found that IL-33 can be a possible therapeutic target for AD patients in the future clinical treatment.

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Author Disclosure Statement

No competing financial interests exist.

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