and a nonspecific control siRNA were purchased from Dharmacon (Lafayette, CO, USA). The HEECs in the monolayer model were transfected with 25 nM siRNA using DharmaFECT 4 (Dharmacon). Control and negative siRNA groups were treated with the transfection reagents without siRNA, and with the transfection reagents with nonspecific control siRNA, respectively. Cells were incubated for 48 h, then the medium was changed to serum-free basal medium. After 24 h starvation, the medium was changed to new basal medium with or without IFNγ (30 ng/ml) or DCA (100 μM) for the indicated time. The culture supernatant was centrifuged to remove cellular debris, and the supernatant was stored at −80 °C for further analysis.

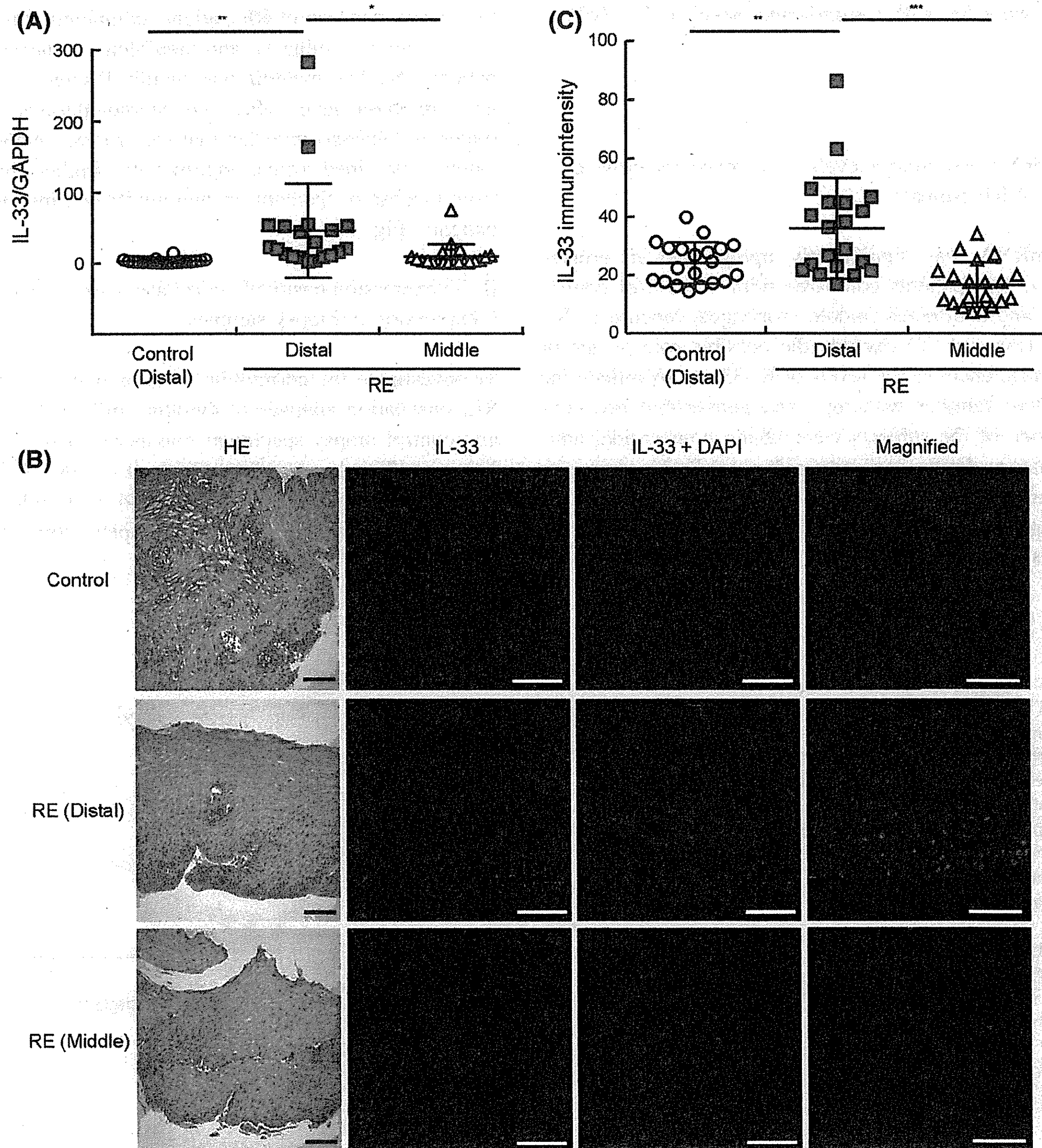

Fig. 1 IL-33 is upregulated in reflux esophagitis (*RE*). Esophageal biopsy samples were taken from the erosive part of the distal esophagus and the unaffected middle esophagus of RE patients ($n = 20$) and from the distal esophagus of control subjects ($n = 20$). **a** The IL-33 level was normalized to the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) level, and relative messenger RNA (mRNA) levels were calculated as the fold increase over the average level of the control subjects. **b** Hematoxylin–eosin (*HE*) staining and representative immunofluorescence staining of IL-33 (*red*) and 4′,6-diamidino-2-phenylindole (*DAPI*; *blue*). **c** Semiquantified IL-33 immunointensity. *One asterisk* $P < 0.05$, *two asterisks* $P < 0.01$, *three asterisks* $P < 0.001$, *white bar* and *black bar* 100 μm, *yellow bar* 50 μm

Statistical analysis

All data are presented as the mean ± standard deviation. A two-tailed Mann–Whitney *U* test or an unpaired *t* test was performed where appropriate. Nonparametric correlation analysis was performed using Spearman's rank correlation coefficient. One-way ANOVA followed by Scheffe's *F* test was performed for multiple comparisons. All tests were applied two-sided with a significance level of $P < 0.05$.

Results

IL-33 mRNA and protein levels are increased in erosive mucosa of RE patients

IL-33 mRNA was significantly upregulated in erosive mucosa of RE patients compared with mucosa of control subjects and unaffected middle esophageal mucosa of RE patients (Fig. 1a). To exclude the possible role of age or gender differences in the levels of IL-33, we examined the consecutive samples with age- and gender-matched controls. None of the subjects were taking nonsteroidal anti-inflammatory drugs, corticosteroids, antiallergic drugs, or other immunosuppressive drugs. IL-33 mRNA levels in the subgroups of RE patients according to the Los Angeles classification are shown in Fig. S1. The IL-33 mRNA level of grade B RE patients was significantly higher than that of the control subjects, but the levels of the grade A and grade B RE patients were not different.

To identify the origin of IL-33 in the esophageal mucosa, immunofluorescence staining was performed on the biopsy samples. The controls showed weak staining of IL-33 in the nucleus of esophageal epithelial cells. In contrast, strong nuclear staining of IL-33 was observed in the erosive mucosa of RE patients compared with mucosa of the control subjects and unaffected mucosa of RE patients. Nuclear staining was mainly located in the basal and suprabasal layers (Fig. 1b). Semiquantitative immunointensity analysis revealed that the erosive mucosa of RE patients provided values significantly higher than those from mucosa of the controls and unaffected mucosa of RE patients (Fig. 1c).

IL-33 expression positively correlates with IL-8 and IL-6 expression in biopsy samples

To investigate the correlation of upregulated cytokines in RE, correlation analysis of cytokine mRNA levels of RE and control biopsy specimens was performed. IL-33 level was significantly correlated with IL-8 and IL-6 levels (Fig. 2a, b). The levels of IL-8 and IL-6 mRNA were significantly increased in RE samples compared with control samples (Fig. 2c, d).

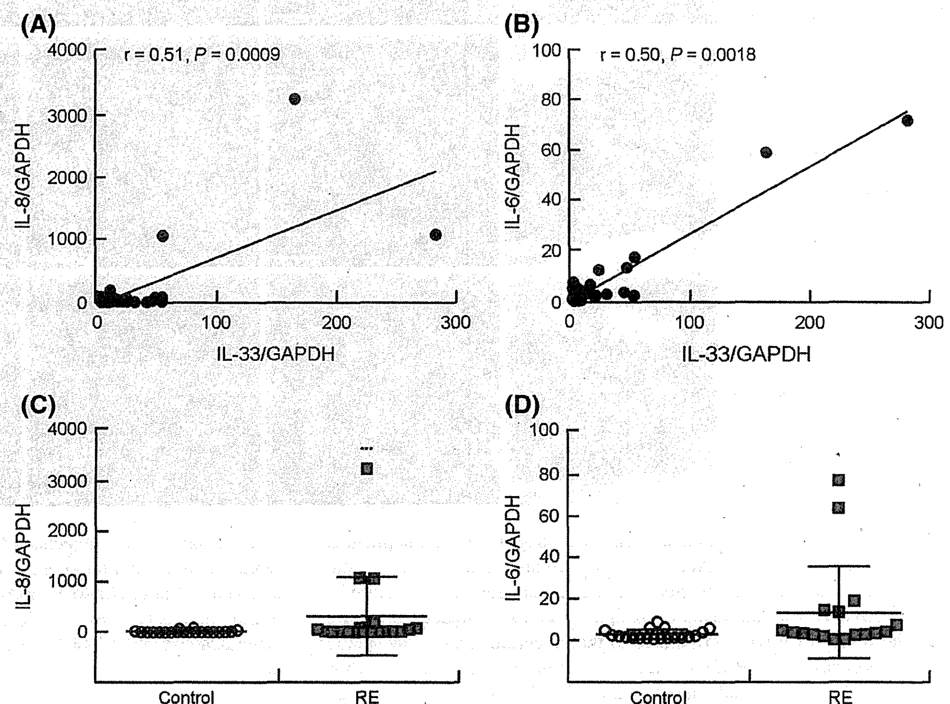

Fig. 2 IL-33 is positively correlated with IL-8 and IL-6 in biopsy samples. Esophageal biopsy samples from the erosive part of the distal esophagus of RE patients ($n = 20$) and the distal esophagus of controls ($n = 20$) were analyzed for IL-33, IL-8, and IL-6 mRNA. IL-33 was significantly correlated with IL-8 (**a**) and IL-6 (**b**). The levels of IL-8 (**c**) and IL-6 (**d**) mRNA were significantly increased in RE patients compared with the controls. *One asterisk* $P < 0.05$ versus the control group, *three asterisks* $P < 0.001$ versus the control group

IFNγ induces IL-33 mRNA and protein expression in ALI-cultured HEECs

To examine the function and the mechanisms of the upregulated IL-33 in RE, the effect of several intraluminal stimuli which may be regurgitated in RE patients, such as acid (pH 1 and 2) and bile acid [acidic DCA (400 μM, pH 6.5) and acidic CDCA (400 μM, pH 6.5)], on the production of IL-33 was examined in our esophageal stratified squamous epithelial cell model. However, 6 h of stimulation did not upregulate IL-33 mRNA (Fig. 3a). IFNγ, TNF-α, and IL-1β have been reported to induce IL-33 in other cell types, such as keratinocytes [7] and fibroblasts [17], and IFNγ, TNF-α, and IL-1β levels have also been reported to be increased in GERD patients [2, 18]. Therefore, we next investigated the effect of these cytokines on the production of IL-33 in the stratified squamous cell model. RT-qPCR revealed that IFNγ, but not TNF-α or IL-1β, significantly increased the IL-33 mRNA level in comparison with the level in untreated cells (Fig. 3a). Western blots confirmed that IFNγ-induced IL-33 was full-length IL-33, with a mass of 30 kDa. The expression level of IL-33 peaked at 6–10 h, then decreased to almost the basal level at 24 h (Fig. 3b). Immunofluorescence staining of IL-33 induced by IFNγ was mainly located in the nucleus of basal layer cells (Fig. 3c).

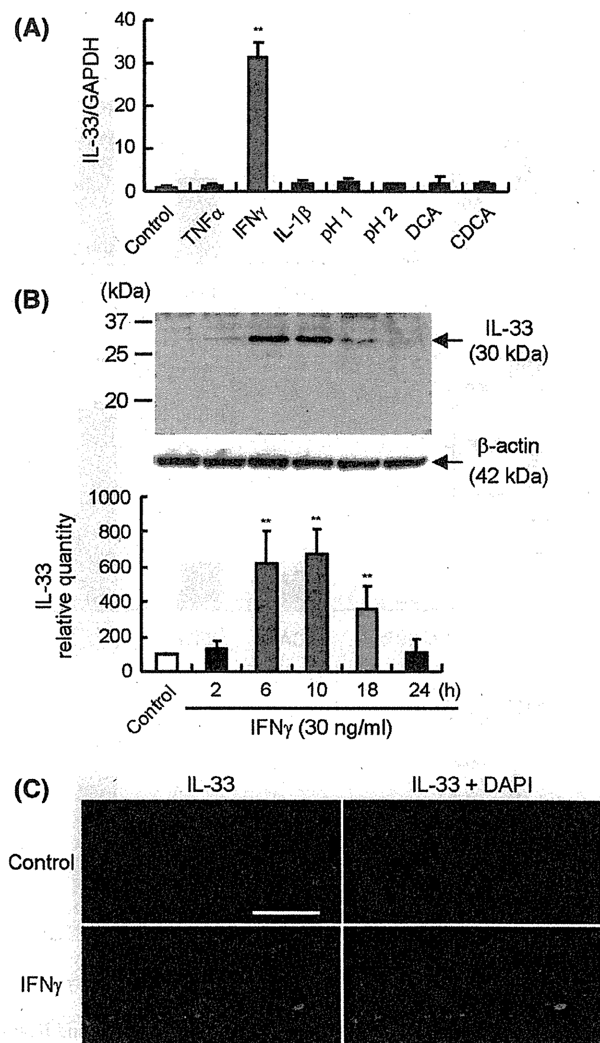

Fig. 3 Interferon-γ (*IFNγ*) induces IL-33 in the nucleus of air–liquid interface (ALI)-cultured human esophageal epithelial cells (HEECs). **a** IL-33 mRNA was analyzed by reverse transcription quantitative PCR (RT-qPCR). ALI-cultured HEECs were stimulated with IFNγ (30 ng/ml), TNF-α (20 ng/ml), and IL-1β (10 ng/ml) from the basal compartment, or acidic medium (pH 1 and 2), acidic deoxycholic acid (DCA; 400 μM, pH 6.5), and acidic chenodeoxycholic acid (CDCA; 400 μM, pH 6.5) from the apical compartment, and were harvested after 6 h. **b** Relative levels of IL-33 were assessed by western blot analysis at the indicated time point after IFNγ (30 ng/ml) stimulation. **c** Immunofluorescence staining of IL-33 (*red*) in ALI-cultured HEECs after IFNγ (30 ng/ml, 6 h) stimulation at the indicated time point; DAPI (*blue*) was used as the nuclear marker. Each value represents the mean ± standard deviation (SD) of three independent experiments. *Two asterisks* $P < 0.01$ versus the control group, *bar* 50 μm

IFNγ and DCA synergistically induce IL-33, IL-8, and IL-6 in ALI-cultured HEECs

We next investigated the combined effect of bile acid and IFNγ on the production of inflammatory cytokines. In our previous study, acidic DCA, but not neutral DCA, induced IL-8 in ALI-cultured HEECs [4]. We therefore stimulated cells with DCA (100 μM) at pH 6.5 from the apical compartment and applied IFNγ (30 ng/ml) from the basal compartment to simulate the in vivo condition. The combination of DCA and IFNγ significantly upregulated IL-33 mRNA compared with IFNγ alone after 6 h (Fig. 4a), and significantly induced more IL-8 and IL-6 than did DCA or IFNγ alone after 24 h (Fig. 4b, c). These data suggest that DCA- and IFNγ-induced IL-33 precedes the upregulation of IL-8 and IL-6.

Exogenous IL-33 does not induce IL-8 and IL-6

The transient upregulation of IFNγ-induced IL-33 impelled us to investigate whether IL-33 was released or degraded. IL-33 protein was not detected by ELISA in cell culture supernatants after IFNγ (30 ng/ml, 24 h) stimulation (data not shown). Previous studies showed that extracellular IL-33 bound plasma membrane receptor ST2, and activated NF-κB and MAPK to produce inflammatory cytokines such as IL-8 and IL-6 [8]. However, exogenous IL-33 (100 ng/ml, 24 h) induced neither IL-8 nor IL-6 in HEECs (Fig. 5a, b). We also confirmed that ST2 was expressed in HEECs at a low level, and that this expression was not affected by IFNγ stimulation (data not shown). Furthermore, to exclude the combination effects of IFNγ and possibly released small amounts of IL-33, we used sST2 to block the binding of IL-33 to ST2 and evaluated IFNγ-

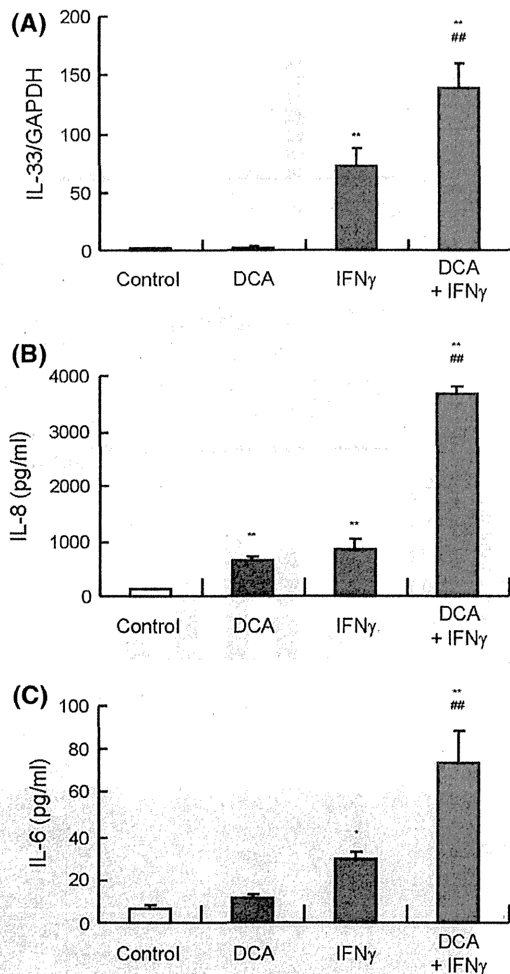

Fig. 4 IFNγ and DCA synergistically induce IL-33, IL-8, and IL-6 in ALI-cultured HEECs. Acidic DCA (pH 6.5, 100 μM) applied from the apical compartment, IFNγ (30 ng/ml) applied from the basal compartment, and a combination of the two treatments were used to stimulate ALI-cultured HEECs. **a** The IL-33 mRNA level was detected after 6 h stimulation. The production of IL-8 (**b**) and IL-6 (**c**) in the basal compartment medium by each stimulant (24 h) was detected using the Bio-Plex assay. Each value represents the mean ± SD of three independent experiments. *One asterisk* $P < 0.05$ versus the control group, *two asterisks* $P < 0.01$ versus the control group, *two pound signs* $P < 0.01$ versus the IFNγ or DCA group

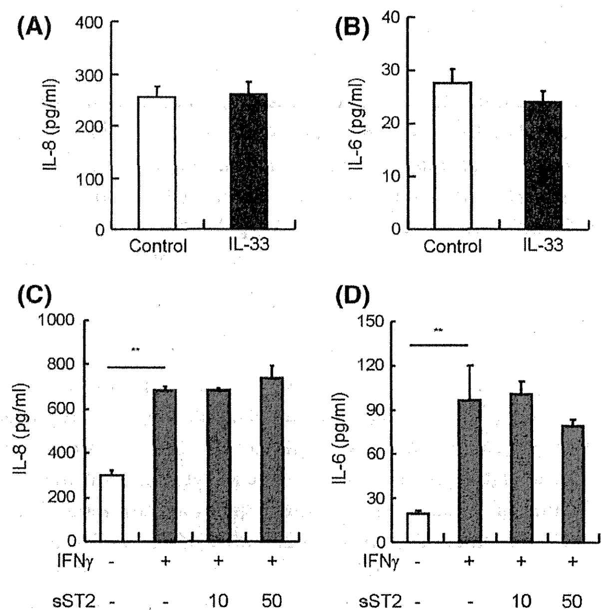

Fig. 5 Exogenous IL-33 does not induce IL-8 and IL-6. **a**, **b** ALI-cultured HEECs were stimulated with IL-33 from the basal compartment (100 ng/ml) for 24 h. The production of IL-8 (**a**) and IL-6 (**b**) was detected by enzyme-linked immunosorbent assay 24 h after IL-33 stimulation. ALI-cultured HEECs were stimulated with IFNγ (30 ng/ml) with or without soluble ST2 (*sST2*; 10 and 50 ng/ml, 24 h). The production of IL-8 (**c**) and IL-6 (**d**) was detected by enzyme-linked immunosorbent assay 24 h after the stimulation. Each value represents the mean ± SD of three independent experiments. *Two asterisks* $P < 0.01$

induced IL-8 and IL-6 production. sST2, even at high concentration (50 ng/ml), did not block IFNγ-induced IL-8 and IL-6 production (Fig. 5c, d). These data suggest that exogenous IL-33 cannot affect the production of IL-8 and IL-6.

Nuclear IL-33 affects IFNγ- and DCA-induced IL-8 and IL-6

To investigate the function of nuclear upregulated IL-33 in RE, we next suppressed its expression with siRNA in monolayer HEECs. IL-33 knockdown dampened IFNγ-induced IL-8 and IL-6 production at 24 h (Fig. 6a, b). Although bile acid did not induce IL-33, whether IL-33 influences the response of HEECs to bile acid is not known. Therefore, after IL-33 siRNA transfection, HEECs were treated with DCA (100 μM, 6 h). DCA-induced IL-8 production and IL-6 production were significantly blocked by IL-33 knockdown (Fig. 6c, d). These data suggest that nuclear IL-33 exaggerates the production of inflammatory cytokines.

Signaling pathways involved in IFNγ-induced IL-33 expression

To clarify the signal transduction pathways involved in IL-33 expression, we used inhibitors of intracellular signaling. IFNγ-induced IL-33 expression was completed inhibited by JAK inhibitor I, SB203580, and EGCG, but not by H89 in ALI-cultured HEECs (Fig. 7a). To confirm the specificity of the inhibitor and the role of STAT1, we transfected monolayer HEECs with STAT1 siRNA. IFNγ-induced IL-33 expression was completely inhibited in STAT1 knockdown cells (Fig. 7b).

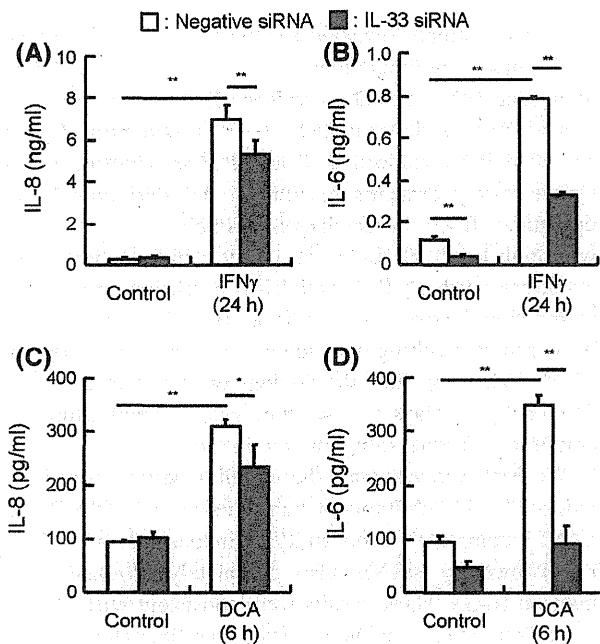

Fig. 6 IL-33 knockdown dampens IFNγ- and DCA-induced IL-8 and IL-6 production. Monolayer HEECs were transfected with IL-33 small interfering RNA (*siRNA*) and nonspecific control siRNA (negative siRNA). In the supernatant of the negative siRNA and IL-33 siRNA treated groups, the production of IL-8 (**a**) and IL-6 (**b**) 24 h after IFNγ (30 ng/ml) stimulation was detected using the Bio-Plex assay. The production of IL-8 (**c**) and IL-6 (**d**) 6 h after DCA (100 μM) stimulation was detected using the Bio-Plex assay. Each value represents the mean ± SD of three independent experiments. *One asterisk P* < 0.05, *two asterisks P* < 0.01

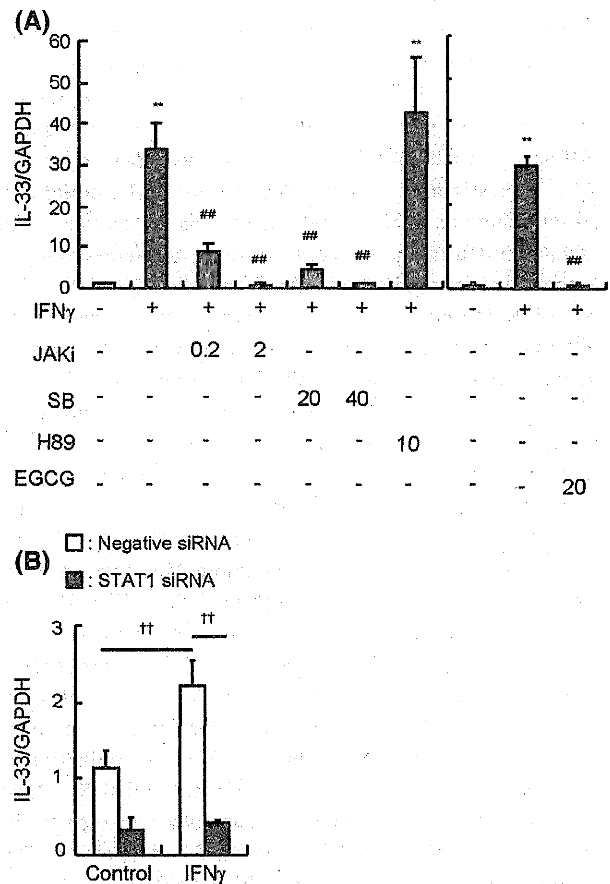

Fig. 7 Signaling pathway involved in IFNγ-induced IL-33 expression. **a** ALI-cultured HEECs were preincubated with inhibitors of Janus kinases (0.2 or 2 μM JAK inhibitor I; *JAKi*), p38 mitogen-activated protein kinase (20 or 40 μM SB203580; *SB*), protein kinase A (10 μM H89), or signal transducer and activator of transcription 1 [*STAT1*; 20 μM epigallocatechin gallate (*EGCG*)] for 1 h, and then co-incubated with IFNγ (30 ng/ml). Cells were harvested 6 h after IFNγ stimulation to evaluate IL-33 expression by RT-qPCR. **b** Monolayer HEECs were transfected with STAT1 siRNA and nonspecific control siRNA (negative siRNA) 72 h before stimulation. IL-33 expression after 6 h IFNγ (30 ng/ml) stimulation was evaluated by RT-qPCR. Each value represents the mean ± SD of three independent experiments. *Two asterisks P* < 0.01 versus the control group, *two pound signs P* < 0.01 versus the IFNγ group, *two daggers P* < 0.01

Discussion

For decades, esophageal epithelial layers were thought of as a tissue forming a barrier against caustic chemical injury [19–22]. However, the study of Souza et al. [1] revealed other functions of epithelial cells, and showed that epithelial cells can initiate inflammation by producing various inflammatory cytokines such as IL-8 and IL-1β. Here, we demonstrated that esophageal epithelial nuclear IL-33 but not exogenous IL-33 plays an important role as the upstream mediator for the production of inflammatory cytokines. IL-33 aggravates inflammation and is involved in the pathogenesis of RE.

IL-33 is a member of the IL-1 cytokine family, is mainly and constitutively expressed in epithelial and endothelial cells, and is proposed to play an important role in sensing damage caused by various infectious and inflammatory diseases [23]. Our study has shown for the first time that IL-33 is expressed in the nucleus of esophageal epithelial cells, and is upregulated in the epithelial cells of RE patients. Furthermore, immunofluorescence staining indicated that upregulated IL-33 is mainly located in the basal and suprabasal layer cells in RE patients, indicating that the basal and suprabasal layer cells may be actively involved in immune responses to injury in the pathogenesis of RE. Furthermore, we investigated the expression levels of IL-8 and IL-6 in biopsy samples: both were upregulated in erosive mucosa of RE patients, and correlated well with IL-33 expression levels.

To explore the trigger and function of IL-33 in esophageal epithelial cells, we performed in vitro experiments using an in vitro three-dimensional esophageal squamous

epithelial cell model, ALI-cultured HEECs, established by us to simulate the in vivo stratified esophageal epithelial cell layers [3, 4, 13, 24]. The character and gene expression of the cells in monolayer and stratified layers are totally different even though the cells have the same origin [15, 24]. This system is unique and useful, and intraluminal stimuli, such as acid and bile acids, can be applied to the apical compartment, whereas immune-cell-derived cytokines can be applied to the basal compartment. This pathophysiological simulation makes the experimental observations relevant. Here, intraluminal stimuli, such as acid and bile acids, did not affect the production of IL-33. However, IFNγ, but not TNF-α or IL-1β, significantly upregulated IL-33. Although IFNγ, TNF-α, and IL-1β levels are increased in RE [18, 25], and augment IL-33 expression in different cell types, IFNγ seems to be the strongest inducer of IL-33 in epithelial cells [7, 26], whereas TNF-α and IL-1β seem more effective on fibroblasts [27]. Several studies confirmed that IFNγ is overexpressed in GERD and is associated with the endoscopic and histological grading of RE [18, 28]. Consequently, IFNγ is very likely to be the cause of the elevated expression of IL-33 in RE patients. Furthermore, although bile acids alone did not induce IL-33, a combination of DCA and IFNγ induced more IL-33 than did IFNγ alone, implying that reflux contents can also synergistically enhance the effect of IFNγ on IL-33 production. The synergistic effect of DCA and IFNγ further affected the production of IL-8 and IL-6.

In RE patients, IL-8 and IL-6 levels are increased in esophageal mucosa [29, 30]. High levels of mucosal IL-8 correlated with the severity of RE [30] and predicted an increased relapse rate for RE patients [31], whereas IL-6 played a central role in the transition from the acute to the chronic phase of the inflammatory process [32]. IL-33, like other IL-1 family cytokines, can act as a dual function protein. When released from cells, IL-33 binds to its plasma membrane receptor, ST2, activating NF-κB and MAPK to produce inflammatory cytokines, such as IL-8 and IL-6 [8, 33]. Although the IL-33/ST2 axis is the most investigated IL-33 pathway [34], in our model IL-33 was not released into the cell culture medium. Exogenous IL-33 induces neither IL-8 nor IL-6, and sST2 did not block IFNγ-induced IL-8 or IL-6 production. These data indicate that the IL-33/ST2 axis is not involved in IL-8 or IL-6 production from esophageal epithelial cells.

In addition to the IL-33/ST2 axis, we considered whether IL-33 affects IL-8 or IL-6 production in an intracrine manner. The IL-1 cytokine family, IL-1α, IL-33 and IL-37, can be translocated to the nucleus by nuclear localization sequences [35], and nuclear IL-33 and IL-37 have been reported to regulate the gene expression of several cytokines and repress inflammation [7, 9, 10, 36]. However, our study interestingly revealed for the first time that nuclear IL-33 has a proinflammatory effect. IL-33 knockdown dampened IFNγ- and DCA-induced IL-8 and IL-6 production in HEECs. IFNγ-induced IL-33 production (6–10 h) preceded IFNγ-induced IL-8 and IL-6 production (24 h). On the basis of these results, immune-cell-derived IFNγ can upregulate IL-33 in esophageal epithelial cells, and cells with high levels of IL-33 can produce more inflammatory cytokines, such as IL-8 and IL-6, following intraluminal stimulation. Under these condition, more immune cells can be recruited, resulting in a vicious cycle in the pathogenesis of RE. Taken together, the findings indicate that epithelial-derived IL-33 plays an important role in maintaining and amplifying chronic inflammation in RE.

We further investigated the signaling pathways of IFNγ-induced IL-33. Inhibitors of Janus kinases, p38 MAPK, and STAT1 completely blocked IFNγ-induced IL-33. STAT1 knockdown by siRNA also completely blocked IFNγ-induced IL-33. These results were consistent with those of a previous study conducted using keratinocytes [7]. The PKA–cyclic AMP response element binding protein (CREB) pathway has also been shown to be involved in lipopolysaccharide-induced IL-33 expression in macrophages [37], and IFNγ can activate PKA and stimulate CREB [38]. However, a PKA inhibitor did not show any effect on IFNγ-induced IL-33. Further investigations are warranted to explore the nuclear-IL-33-induced transcriptional mechanism of IL-8 or IL-6.

In summary, this is the first study to show that IL-33 is upregulated in the erosive mucosa of RE, and is correlated with the expression of IL-8 and IL-6. Nuclear but not exogenous IL-33 amplified IFNγ- and DCA-induced IL-8 and IL-6 production. Esophageal epithelial-cell-derived IL-33 can aggravate inflammation in RE patients. Regulating the intracellular function of IL-33 in epithelial cells of RE patients may be an effective way to relieve inflammation.

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

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The role of basophils and proallergic cytokines, TSLP and IL-33, in cutaneously sensitized food allergy

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Abstract

Cutaneous sensitization with a food antigen before its consumption elicits the development of food allergy. Here, we report the site- and stage-dependent roles of basophils and proallergic cytokines, thymic stromal lymphopoietin (TSLP) and IL-33, in a mouse model of food allergy initially sensitized cutaneously with the food antigen. Mice were epicutaneously sensitized with the food antigen ovalbumin (OVA) followed by oral challenge with OVA. Epicutaneously sensitized mice produced OVA-specific IgE and developed IgE-dependent anaphylaxis after oral challenge. Basophil-depleted or TSLP–receptor-deficient mice did not produce OVA-specific IgE and were protected from oral challenge-induced anaphylaxis. IL-33-deficient mice produced normal levels of OVA-specific IgE. However, IL-33-deficient mice and mice treated with recombinant soluble IL-33 receptor were protected from anaphylaxis. Thus, basophils and TSLP have pivotal roles in T_h2 development in the skin during the sensitization phase of food allergy. In contrast, while IL-33 is dispensable for promoting cutaneous antigen sensitization, the cytokine is essential for inducing IgE-dependent anaphylaxis in the gut.

Keywords: anaphylaxis, gut, IgE, ovalbumin, skin

Introduction

Food allergy is a growing public health problem that compromises patient quality of life and is potentially fatal. Although the prevalence of food allergy varies study by study, it is estimated that ~5% of adults and 8% of children are affected by the disease (1). Moreover, several reports indicate an increasing trend of food allergy, especially in westernized countries (1–3).

The gastrointestinal tract digests and absorbs nutrition from ingested foods and is protected from inducing inappropriate immune responses by a mechanism termed 'oral-tolerance' (4). However, when tolerance is compromised and T_h2-type immune responses are induced to food antigens, IgE-mediated anaphylaxis, namely food allergy, can be evoked (1, 2). Currently, the management of food allergy is achieved by allergen avoidance and prompt use of self-injectable epinephrine in emergency cases (1, 2, 5). These measures significantly compromise patient quality of life and cannot completely avoid the threat of food allergy. Thus, it is essential to identify immune pathways that can be targeted to prevent food allergy development.

A growing body of evidence indicates that the route of antigen sensitization is an essential determinant for inducing either food allergy or oral tolerance (6–8). This is now termed the 'dual-allergen-exposure hypothesis' (7, 8). According to this theory, epicutaneous sensitization of a food allergen before its oral encounter elicits food allergy, while early consumption of the food antigen induces oral tolerance (7, 8). Previously, avoidance of consumption of highly allergic foods, such as peanuts, milk or eggs, in infants and their mothers was considered a preventative measure against the development of food allergy (9). However, some children developed anaphylaxis after the first consumption of a food, even if they and their mothers had avoided its consumption previously (10, 11). Thus, the efficacy of food avoidance in preventing food allergy has been questioned (12, 13) and tissues other than the gut have been considered as initiation sites for food antigen sensitization (6, 10). Du Toit *et al.* (14) compared the prevalence of peanut allergy in Jewish children in the UK (infants are forbidden to eat peanuts) and Israel (infants regularly eat peanuts)

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and demonstrated that the risk for peanut allergy was 9.8-fold higher in the UK. Furthermore, early onset of severe eczema is associated with the later development of food allergy (15). Filaggrin gene mutation, which is a risk factor for atopic dermatitis (AD) (16), is also linked to the prevalence of peanut allergy (17), even though the protein is expressed exclusively in the skin but not in the gastrointestinal tract (18), further suggesting the skin as an important site for food antigen sensitization. In addition to epidemiologic studies, animal models showed that skin-mediated antigen sensitization evoked systemic IgE responses and induced subsequent anaphylactic responses when the antigen was orally challenged (19, 20). These observations clearly demonstrate the importance of skin-mediated sensitization in the later development of food allergy. However, the cellular or molecular mechanisms underlying the process are poorly understood.

The role of basophils in the development of T_h2-type immune responses is highly controversial (21, 22). Basophils express MHC class II and costimulatory molecules and secrete large amounts of IL-4 that promote T-cell differentiation to T_h2, thus basophils might function as antigen-presenting cells (APCs) (23–25). Some groups have challenged this theory by reporting basophils did not present antigen to T cells, and that dendritic cells (DCs) had a pivotal role in initiating T_h2 responses in lung house dust mite instillation (26) or helminthic infection (27) models. More recently, other reports demonstrated the unique role of basophils in cutaneously initiated T_h2 cell differentiation (28, 29). In the skin, basophils promote T_h2 cell differentiation cooperatively with DCs as IL-4 producers (29, 30), or as APCs for hapten or peptide antigens (28). These studies also demonstrated the important role of thymic stromal lymphopoietin (TSLP) in basophil-mediated T_h2 responses in the skin (28–30). TSLP activates DCs to promote subsequent DC-T-basophil associations (29, 30). Thus, basophils might contribute to T_h2 immune responses in a context-dependent manner, and skin might be a tissue that requires basophils to induce optimal T_h2 responses. However, although previous reports showed the precise molecular mechanisms of basophil- and TSLP-mediated induction of T_h2 cell differentiation in the skin (29, 30), the importance of the pathways in initiating food antigen sensitization and following gastrointestinal responses in the context of food allergy have not been demonstrated (21).

Recent studies demonstrated the important role of IL-33 in T_h2-type immune-mediated diseases (31). Importantly, IL-33 may participate in both skin (32, 33)- and gut (34–36)-mediated T_h2-type immune responses. Furthermore, IL-33 can amplify FcεRI cross-linking-mediated mast cell degranulation (37, 38) and exacerbate anaphylaxis (38). Therefore, IL-33 could be a therapeutic target for food allergy initiated though cutaneous antigen sensitization.

In this study, we established an experimental food allergy mouse model, in which mice were epicutaneously sensitized with the food antigen ovalbumin (OVA) followed by oral challenge with OVA to induce anaphylactic responses. We demonstrate the essential roles of skin basophil-mediated initiation of T_h2 responses in the development of food allergy. Moreover, we identify the tissue- and phase-specific roles of proallergic cytokines, TSLP and IL-33, in a cutaneously sensitized food allergy model.

Methods

Mice

Wild-type (WT) BALB/c mice were purchased from Oriental yeast (Osaka, Japan). *Fcer1*$^{-/-}$ mice (BALB/c-background) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). *Kit*$^{W/W-v}$ mice were purchased from the Japan SLC (Hamamatsu, Japan). *Crlf2*$^{-/-}$ mice (BALB/c-background) were generated as previously described (39, 40). *Il33*$^{-/-}$ mice (BALB/c background) (37, 41) and *Il4*$^{eGFP/+}$ mice (BALB/c background) (23, 42) were bred at the animal facilities of Hyogo College of Medicine. Three to eight female mice, 6–10 weeks of age, were used in each study as experimental and control groups. All mice were kept under specific pathogen-free conditions and received humane care. All animal experiments were performed in accordance with guidelines of the Institutional Animal Care Committee of Hyogo College of Medicine (Hyogo, Japan).

Antibodies and reagents

PE-Cy5-anti-CD3ε (145-2C11), FITC-anti-CD45.2 (104) and allophycocyanin-anti-CD49b (DX5) antibodies were purchased from BD Biosciences (San Jose, CA, USA). Anti-FcεRI antibody (MAR-1) was from eBiosciences (San Diego, CA, USA). Anti-CD16/32 (93), PE-Cy5-anti-CD45R/B220 (RA3-6B2) and purified-anti-MCP-8 (TUG8) antibodies were from BioLegend (San Diego, CA, USA). PE-anti-IgE (23G3) antibody was from Southern Biotechnology Associates (Birmingham, AL, USA). Anti-CD200 receptor-like-3 (Ba103) and RAT IgG2b antibodies were from Hycult Biotech (Plymouth Meeting, PA, USA). Anti-DNP IgE mAb (SPE-7), OVA (grade V) and sodium dodecyl sulfate (SDS) were from Sigma-Aldrich Japan (Tokyo, Japan). Soluble ST2 (recombinant human ST2/IL-1 R4 Fc chimera) was from R&D SYSTEMS (Minneapolis, MN, USA).

Mouse model of food allergy

For epicutaneous sensitization, mice were treated with 4% SDS in sterile distilled water on their shaved back skin, and 10 min later 300 μg of OVA in 60 μl of PBS or PBS alone (control) were applied to the skin. The mice were epicutaneously sensitized with OVA three times a week, for the first 2 weeks. To induce systemic anaphylaxis, mice were intra-gastrically challenged on day 21 with 5 mg of OVA using a ball-ended mouse feeding needle. In some experiments, mice were given 1 mg of OVA intra-gastrically before epicutaneous sensitization. To block IL-33 signaling, mice were given 50 μg of soluble ST2 intra-peritoneally 24 and 2 h before oral challenge with OVA.

Measurement of systemic anaphylaxis

The changes in rectal temperature of mice were measured using a Microprobe Thermometer BAT-12 (Physitemap, NJ, USA), at 0, 5, 10, 15, 20, 30, 45 and 60 min after OVA challenge.

Vascular permeability measurements

Vascular permeability was evaluated by measuring the leakage of Evans blue dye into the skin and intestine. Evans

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blue dye (20 mg kg^{-1}) was injected in the tail vein just before challenge. At 15 min after intra-gastric challenge with OVA, mice were sacrificed and systemic circulation was perfused with saline to remove intravascular dye. The skin and intestine were dissected from the mice, blotted dry and weighed. Evans blue dye was extracted in formamide at 37°C for 24 h and the concentration was determined with a spectrophotometer at the absorbance maximum of 620 nm wavelength. The tissue content of the Evans blue dye (ng g^{-1} wet weight tissue) was calculated from a standard curve of Evans blue dye concentration.

OVA-specific IgE measurement

OVA-specific IgE levels were measured by DS Mouse IgE ELISA (OVA) (DS Pharma Biomedical, Osaka, Japan).

RT–PCR

Total RNA was isolated from tissues or $CD4^+$ T cells isolated from inguinal lymph nodes (LNs) using an RNeasy Mini Kit (*Qiagen*, Valencia, CA, USA), and cDNA was synthesized using superscript III (Invitrogen, Carlsbad, CA, USA). For quantitative PCR, DNA fragments were amplified using Premix Ex Taq (Takara Bio, Otsu, Japan) and gene-specific TaqMan probe (Applied Biosystems, Carlsbad, CA, USA). Gene-specific PCR products were measured using the Thermal Cycler Dice Real Time System II (Takara Bio). The levels of target gene expression were normalized to β-actin expression using the $2^{-\Delta\Delta Ct}$ method. The following primers and probes were purchased from Applied Biosystems: mouse *Actb* (Mm00607939_s1), *Foxp3* (Mm00475162_m1), *Il4* (Mm00445259_m1), *Il5* (Mm00439646_m1), *Il13* (Mm00434204_m1), *Il33* (Mm00505403_m1), *Mctp8* (Mm00484933_m1), *Tslp* (Mm01157588_m1).

Immunohistochemistry

Paraffin-embedded sections (4 μm thick) of the skins were deparaffinized, and heated in citrate buffer (pH 6.0) for epitope retrieval and then cooled at room temperature for 50 min before blocking. The sections were incubated in PBS containing 1.0% BSA and 0.05% Tween 20 for blocking. The sections were incubated with a primary antibody for basophils, anti-mMCP-8 mAb (TUG8) (BioLegend), at 4°C overnight, followed by incubation with a secondary antibody, biotin-conjugated goat antibody against rat IgG (Vector Laboratories, Burlingame, CA, USA) at room temperature for 30 min and staining with Alexa Fluor 594-conjugated streptavidin (Invitrogen), at room temperature for 30 min. The sections were cover-slipped with mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen). The sections were washed with PBS containing 0.05% Tween 20 before each step. Sections were examined under a Zeiss LSM 510 microscope (Carl Zeiss, Thornwood, NY, USA). Computer software, ZEN 2011 (Carl Zeiss), was used for image processing and analysis.

Hematoxylin and eosin staining of skin

Skin from the backs of mice was dissected from mice. The specimens were fixed in 4% (w/v) paraformaldehyde, embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin.

Basophil depletion

For basophil depletion, anti-FcεRI mAb (MAR-1) and anti-CD200 receptor like-3 mAb (Ba103) were used. For depletion with MAR-1 mAb, mice were intra-peritoneally injected with 5 μg of MAR-1 or isotype control (eBio299Arm) antibodies, 3 days before starting skin sensitization. Antibodies were injected twice a day for three consecutive days every week (days −3 to −1, 4–6 and 11–13). For depletion with the Ba103 mAb, mice were intravenously injected with 10 μg of Ba103 or control Rat IgG2b antibodies from 1 day before starting skin sensitization. Antibodies were injected every 4 days (days −1, 3, 7 and 11).

Flow cytometry

Single-cell suspensions were pre-incubated with anti CD16/32 mAb (93) for blocking before staining. Basophils are defined as $CD3\varepsilon^-B220^-CD49b^+IgE^+$ cells.

Statistical analyses

Data are expressed as mean ± SEM and statistical significance was analyzed by analysis of variance or unpaired Student's *t*-tests. Significance for all statistical tests is shown in figures as $P < 0.05$ (*) and $P < 0.01$ (**).

Results

Epicutaneous sensitization and oral challenge with a food antigen induces IgE-dependent anaphylaxis

WT Balb/c mice were epicutaneously sensitized with OVA or PBS at a shaven skin site topically treated with SDS, three times a week for the first 2 weeks, and then intra-gastrically challenged with OVA at day 21 (Fig. 1A). *Il4* mRNA levels in the inguinal LNs at day 20 were ~6-fold higher in epicutaneously OVA sensitized-mice compared with control mice (Fig. 1B). Mice epicutaneously sensitized and orally challenged with OVA, but not control mice, showed significantly increased OVA-specific IgE levels in their sera over time (Fig. 1C). After oral challenge with OVA, epicutaneously OVA-sensitized mice showed a prompt and significant decrease in core-body temperature (Fig. 1D). To monitor serum leakage caused by anaphylaxis, mice epicutaneously sensitized with OVA were intravenously injected with Evans blue dye just before the oral challenge. Fifteen minutes after oral challenge with OVA, but not PBS, Evans blue leaked at the sensitized skin site, as well as the upper small intestine of sensitized mice (Fig. 1E and F), similar to the symptoms of food allergy patients including skin rashes (5). To determine if the anaphylactic responses were IgE-dependent, mice deficient for the high-affinity receptor for IgE (*Fcer1*$^{-/-}$ mice) were used. Although *Fcer1*$^{-/-}$ mice produced OVA-specific IgE comparable to WT mice (Fig. 1G), they were completely protected from oral challenge-induced anaphylaxis as demonstrated by a decrease in core-body temperature (Fig. 1H) and serum leakage at skin and intestine (Fig. 1I). Thus, consistent with previous studies (19, 20), cutaneous sensitization of a food

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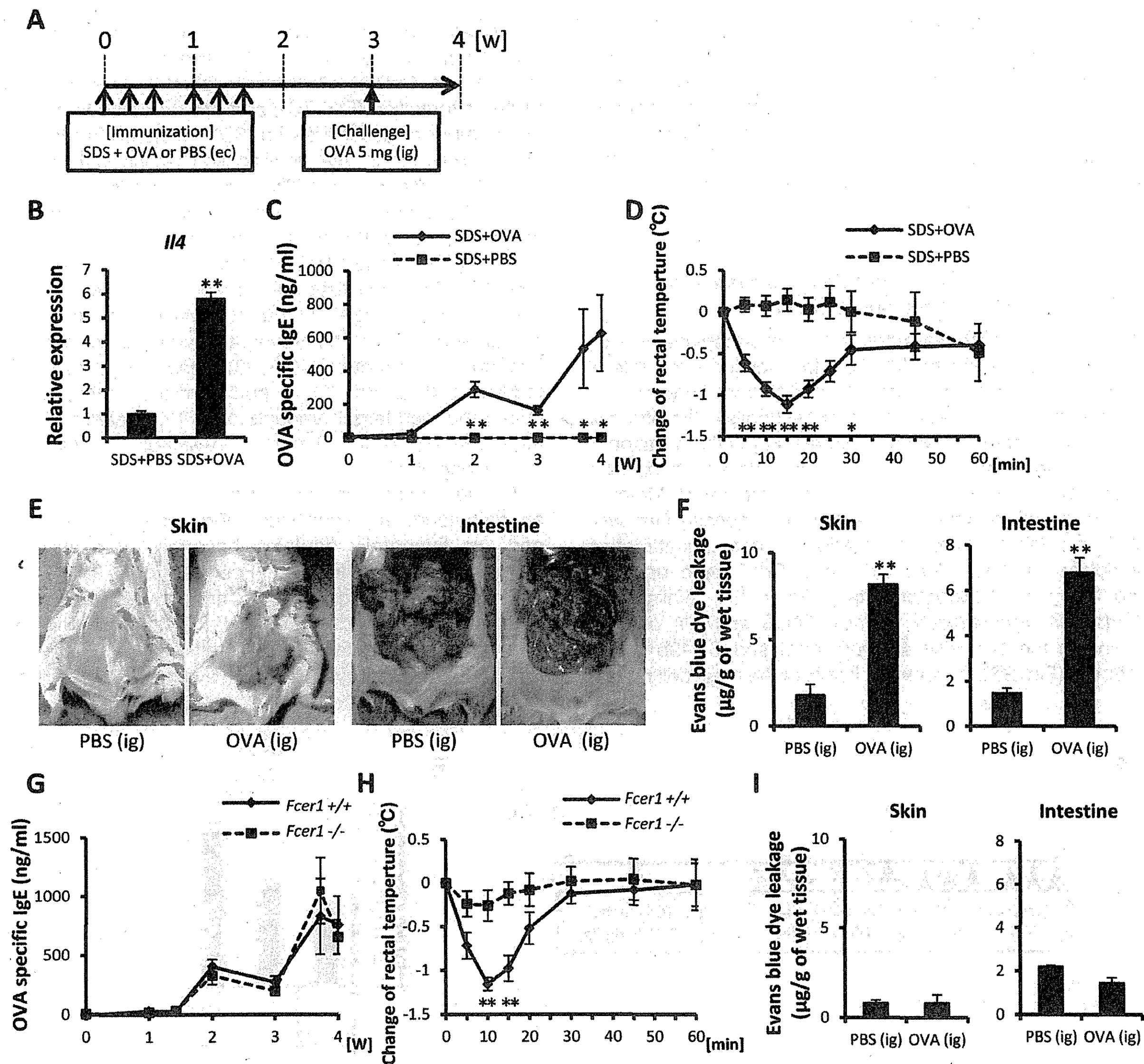

Fig. 1. Development of food allergy by epicutaneous antigen sensitization. Mice were epicutaneously sensitized and orally challenged with OVA. (A) Experimental schema. (B) Inguinal lymph nodes were obtained from mice at day 20 of epicutaneous sensitization with OVA or PBS. Total RNAs were extracted and subjected to quantitative PCR analysis for the expression of *Il4* and *Actb*. (C) Sera were collected weekly from mice epicutaneously sensitized and orally challenged with OVA or PBS. OVA-specific IgE levels in the sera were determined by ELISA. (D) The changes in rectal temperature of mice after oral challenge were measured at the indicated time points. (E and F) Serum leakage at skin and small intestine. Mice epicutaneously sensitized with OVA were injected with Evans blue dye and orally challenged with OVA or PBS. The representative images (E) and quantified graph of leaked dye (F) are indicated. (G–I) WT and *Fcer1*$^{-/-}$ mice were epicutaneously sensitized and orally challenged with OVA as in A. (G) Serum OVA-specific IgE levels. (H) Changes in rectal temperature of mice after oral challenge. (I) Serum leakage at skin and small intestine. Data represent mean ± SEM from five to seven mice of two independent experiments. $^{**}P < 0.01$, $^{*}P < 0.05$.

antigen induced systemic T_h2-type responses, and IgE-dependent anaphylaxis after oral challenge, with the symptoms closely resembling human food allergy.

Early oral exposure to food antigen prevents food allergy development

Studies have suggested that early consumption of food prevents the onset of food allergy by establishing oral tolerance before sensitization, thus the site an antigen engages first might be an important determinant for food allergy development (4, 7, 8, 14). We sought to determine whether oral introduction of OVA before epicutaneous sensitization prevented food allergy development in our model. To this end, mice were administered 1 mg of OVA intra-gastrically three times a week for 2 weeks before epicutaneous sensitization to induce OVA-specific oral tolerance (Fig. 2A). Tolerance-induced mice showed *Foxp3* mRNA up-regulation but did not show

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Il4 mRNA up-regulation in the inguinal LNs (Fig. 2B and C) or elevation of serum OVA-specific IgE levels (Fig. 2D) after epicutaneous OVA application. As a result, tolerance-induced mice were protected completely from oral challenge-induced anaphylaxis (Fig. 2E). Thus, our mouse model clearly reflects the dual-allergen-exposure hypothesis in which allergic sensitization results from cutaneous food antigen exposure before its consumption (7, 8).

Basophils play a pivotal role in cutaneous induction of T_h2 responses and food allergy development

Basophils have essential roles in the skin-mediated development of T_h2 responses (28–30). To investigate the role of basophils in an epicutaneously sensitized food allergy model, we first examined the recruitment of basophils in the affected skin and inguinal LNs. Mmcp8-expressing cells (a basophil marker) (Supplementary Figure 1, available at *International Immunology* Online) and *Mcpt8* mRNA (encoding Mmcp8) levels (Fig. 3A) increased in the skin after sensitization with OVA and peaked at day 11. In addition, basophil-attracting chemokine mRNAs, *Ccl2*, *Ccl3* and *Ccl7*, were up-regulated in the skin (Supplementary Figure 2A, available at *International Immunology* Online). FACS analysis of cells residing in the skin also showed increased CD49b$^+$FcεRI$^+$ basophils (Fig. 3B). In contrast, levels of the mast cell marker *Mcpt1* and eosinophil marker *Prg2* were not altered in the skin (Supplementary Figure 2B, available at *International Immunology* Online). Consistent with skin basophils, *Mcpt8* mRNA expression (Fig. 3C), and the percentage and absolute number of basophils (Fig. 3D) increased in the inguinal LNs at day 11 of OVA sensitization. Along with increased basophil numbers, *Il4* mRNA levels increased in the skin (Fig. 3A) and inguinal LNs (Fig. 3C), suggesting a close relationship between basophils and T_h2 development. Indeed, CD4$^+$ T cells isolated from inguinal LNs at day 11 in epicutaneously OVA-sensitized mice showed increased *Il4* mRNA levels (Supplementary Figure 3, available at *International Immunology* Online). Moreover, as basophils are considered important IL-4 producers (28–30), basophils recruited to the inguinal LNs at day 11 in epicutaneously OVA-sensitized mice produced larger amounts of IL-4 compared with naive mice (Supplementary Figure 4, available at *International Immunology* Online).

We next examined the functional role of basophils in an epicutaneously sensitized food allergy model. To this end, we temporally depleted basophils from mice using two different antibodies, anti-FcεRI antibody (MAR-1) (23) and anti-CD200 receptor like-3 antibody (Ba103) (43), by intra-peritoneal injection during the sensitization phase (Fig. 4A and B). Both antibodies efficiently depleted basophils (Supplementary Figure 5, available at *International*

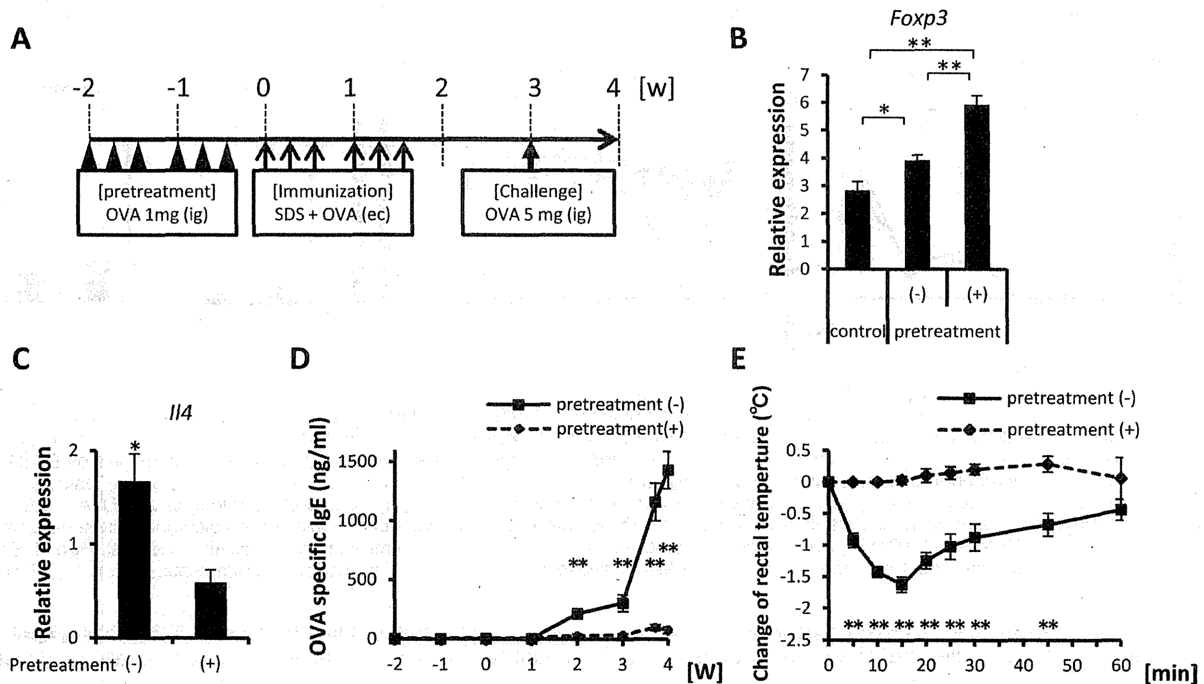

Fig. 2. Early oral exposure to OVA prevents epicutaneous sensitization. Mice were epicutaneously sensitized and orally challenged with OVA in the presence or absence of pre-treatment by oral OVA exposure. (A) Experimental schema. (B and C) Inguinal lymph nodes were obtained from mice at day 20 of epicutaneous sensitization with OVA or PBS in the presence or absence of pre-treatment with oral OVA exposure. Total RNAs were extracted and subjected to quantitative PCR analysis for the expression of *Foxp3* (B) and *Il4* (C). (D) Sera were collected weekly from mice with epicutaneous OVA sensitization in the presence or absence of pre-treatment to oral OVA exposure. OVA-specific IgE levels in the sera were determined by ELISA. (E) Changes in rectal temperature of mice after oral challenge were measured at the indicated time points. Data represent mean ± SEM from three to eight mice of two independent experiments. $^{**}P < 0.01$, $^{*}P < 0.05$.

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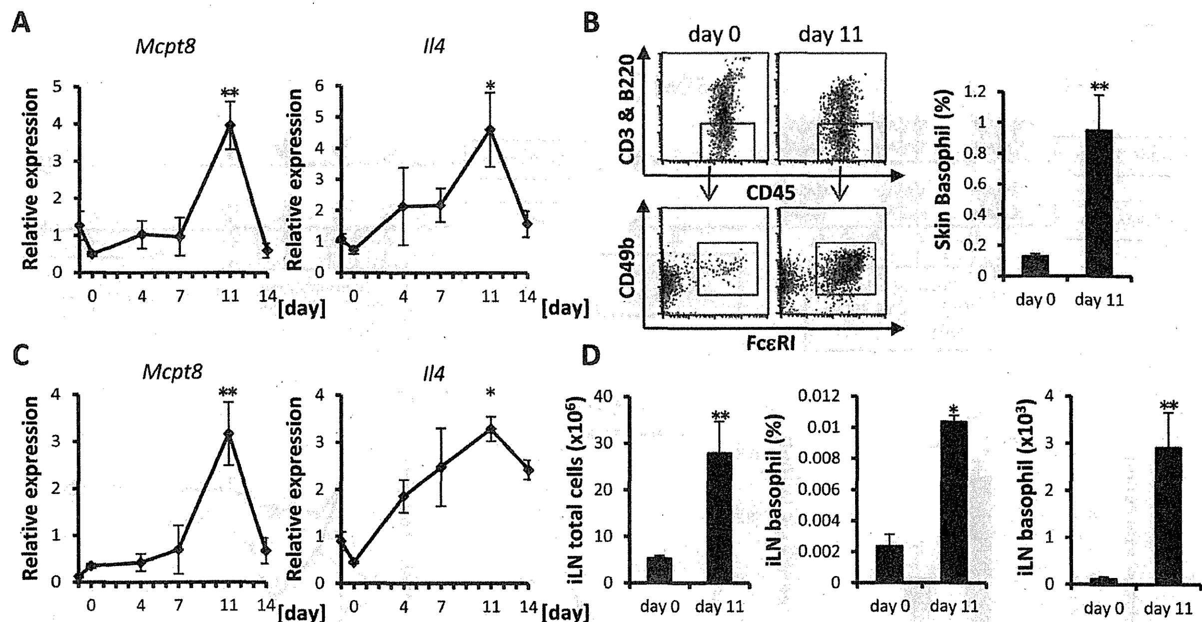

Fig. 3. Basophils are recruited to the skin and inguinal lymph nodes (iLNs) after epicutaneous sensitization with OVA. Mice were epicutaneously sensitized with OVA as in Fig. 1(A). (A and C) Skin (A) and inguinal lymph nodes (C) were obtained from mice at indicated time points of epicutaneous sensitization with OVA. Total RNAs were extracted and subjected to quantitative PCR analysis for the expression of *Mcpt8*, *Il4* and *Actb*. (B and D) Skin (B) and iLNs (D) were obtained from mice at day 0 and 11 of epicutaneous sensitization with OVA. Expression of CD49b and FcεRI in $CD45^+CD3^-B220^-$ cells was examined. Representative flow cytometry plots (B) and quantified graphs (B and D) are shown. Data represent mean ± SEM from three mice. $^{**}P < 0.01$, $^{*}P < 0.05$.

Immunology Online). The elevation of *Il4* mRNA expression in the inguinal LNs (Fig. 4C and D) and serum OVA-specific IgE levels (Fig. 4E and F) were markedly abrogated in basophil-depleted mice. Furthermore, basophil-depleted mice did not show decreased core-body temperature after oral antigen challenge (Fig. 4G and H). Basophil-depleting antibody treatment potentially affects mast cell function. However, mast cell-deficient $Kit^{W/W\text{-}v}$ and the control $Kit^{+/+}$ mice produced comparable levels of OVA-specific IgE in response to epicutaneous OVA sensitization, indicating that basophils, but not mast cells, are responsible for skin-mediated initiation of T_h2 responses (Supplementary Figure 6, available at *International Immunology* Online). Thus, basophils are essential for the skin-mediated induction of T_h2 responses and food allergy development. However, basophil numbers were unchanged in the small intestine following the induction of food allergy (Supplementary Figure 7, available at *International Immunology* Online), suggesting the specific involvement of the cells in the sensitization phase in the skin.

TSLP is essential for skin-mediated food antigen sensitization

Next, we sought to explore cytokines involved in the development of cutaneously sensitized food allergy. TSLP is an epithelia-derived T_h2-inducing cytokine that may have a central role in T_h2 initiation in the skin-basophil pathway (28–30). Epicutaneous sensitization with OVA induced the prompt up-regulation of TSLP mRNA (Fig. 5A) and protein (Fig. 5B) levels. Basophil recruitment in the skin (Fig. 5C) and inguinal LNs (Fig. 5D) after OVA sensitization was completely abrogated in TSLP-receptor-deficient *Crlf2*$^{-/-}$ mice. Furthermore, *Il4* mRNA up-regulation in inguinal LNs (Fig. 5E), and serum OVA-specific IgE production (Fig. 5F) were completely defective in *Crlf2*$^{-/-}$ mice. Consistently, *Crlf2*$^{-/-}$ mice did not develop anaphylaxis by oral challenge (Fig. 5G). Thus, *Crlf2*$^{-/-}$ mice showed a defect in the initiation of T_h2 responses in the epicutaneously sensitized food allergy model.

IL-33 is dispensable for sensitization, but is essential for effector phase responses

IL-33 is another epithelia-derived pro-T_h2 cytokine (31). As for TSLP, the mRNA (Fig. 6A) and protein (Fig. 6B) levels of IL-33 showed a prompt increase in the skin after the epicutaneous application of OVA. However, basophil numbers in the skin (Fig. 6C) and inguinal LNs (Fig. 6D) were comparable between *Il33*$^{-/-}$ and WT mice. Although *Il33*$^{-/-}$ mice showed decreased *Il4* mRNA levels (Fig. 6E), serum OVA-specific IgE levels were comparable between *Il33*$^{-/-}$ and WT mice (Fig. 6F). Thus, IL-33 may be partially involved in skin-mediated T_h2 development, but is dispensable for inducing systemic IgE responses. However, even though the IgE levels were high, *Il33*$^{-/-}$ mice were completely protected from oral challenge-induced core-body temperature decrease (Fig. 6G). In addition, mice treated with soluble ST2 (blockade of IL-33 signaling) at the effector phase were completely protected from oral challenge-induced anaphylaxis (Supplementary Figure 8, available at *International Immunology* Online). These results demonstrate that IL-33

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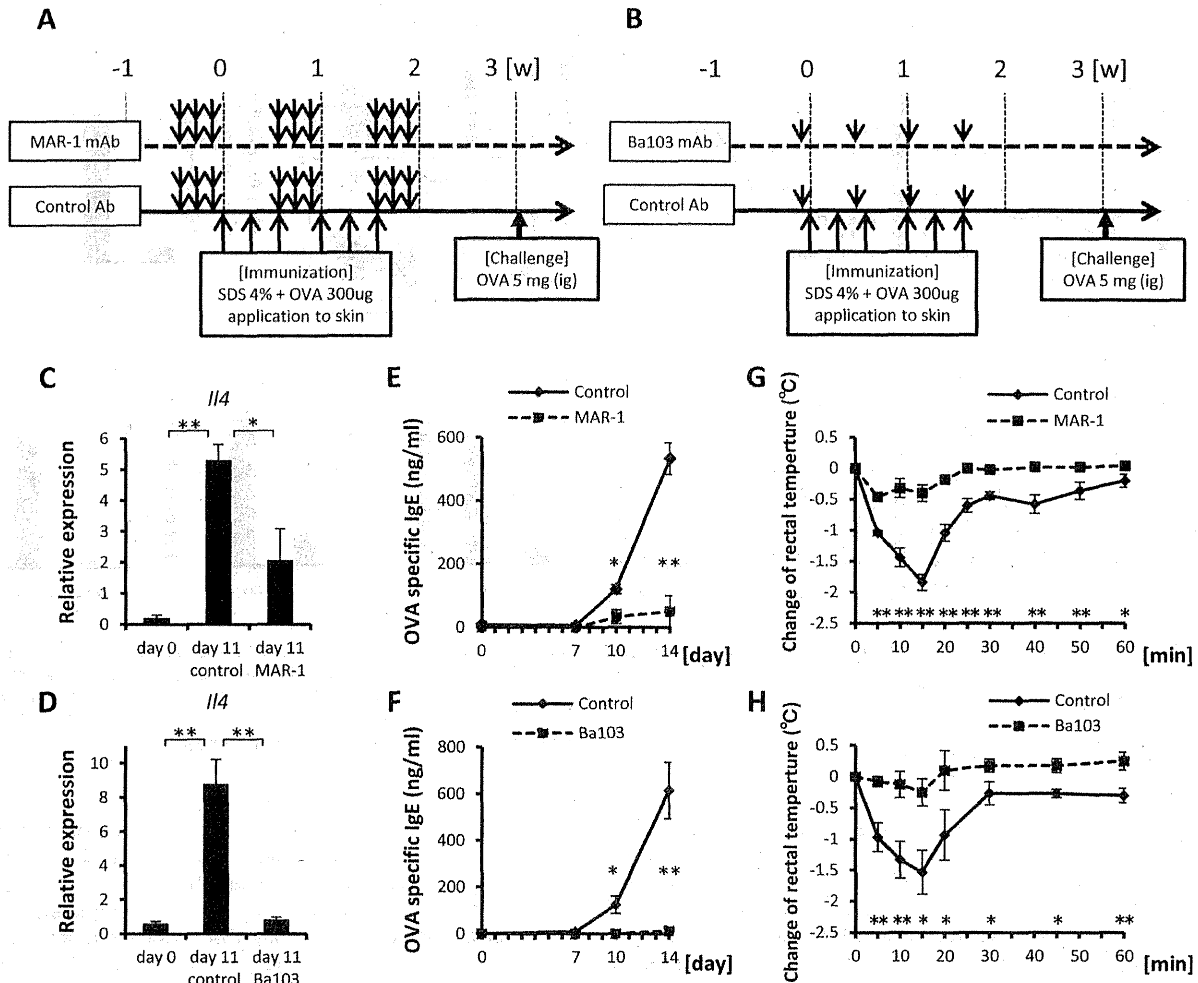

Fig. 4. Basophil depletion at the sensitization phase prevents mice developing food allergy. Basophil-depleted mice were epicutaneously sensitized and orally challenged with OVA. (A and B) Experimental schema. (C and D) Mice depleted with basophils using MAR-1 mAb (C) or Ba103 mAb (D) were epicutaneously sensitized with OVA. Total RNAs were extracted from inguinal lymph nodes at day 0 and 11 and subjected to quantitative PCR analysis for the expression of *Il4* and *Actb*. (E and F) Sera from mice depleted with basophils using MAR-1 mAb (E) or Ba103 mAb (F) were collected at the indicated time points of epicutaneous sensitization with OVA. OVA-specific IgE levels were determined by ELISA. (G and H) Mice depleted with basophils using MAR-1 mAb (G) or Ba103 mAb (H) were epicutaneously sensitized and orally challenged with OVA. Changes in rectal temperature of mice after oral challenge were measured at the indicated time points. Data represent mean ± SEM from four mice of two independent experiments. $^{**}P < 0.01$, $^{*}P < 0.05$.

has a more important role in the effector phase in the gut rather than the sensitization phase in the skin.

Taken together, TSLP is essential for local basophil recruitment in response to the application of antigens to the skin and the subsequent induction of systemic T_h2 responses. In contrast, although IL-33 is not essential for IgE production induced by cutaneous antigen sensitization, IL-33 has a pivotal role in the effector phase of food allergy.

Discussion

Here, we studied the pathophysiology of mice with food allergy initially sensitized to the food antigen by a cutaneous route. The mice developed symptoms closely resembling human food allergy patients with an IgE-dependent reduced core-body temperature and serum leakage in both the skin and intestine. We showed that basophils and TSLP have central roles in the development of food allergy by initiating cutaneous T_h2-type sensitization. In contrast, IL-33 was not involved in the cutaneous sensitization but was essential for inducing anaphylaxis after oral challenge.

Skin is considered a major route for antigen sensitization in food allergy (7, 8). In this study, we demonstrated that when an antigen was exposed to barrier-disrupted skin, T_h2-type immune responses were evoked resulting in the development of food allergy. In contrast, following oral exposure to the antigen, antigen-specific oral tolerance was induced and prevented skin-mediated antigen sensitization

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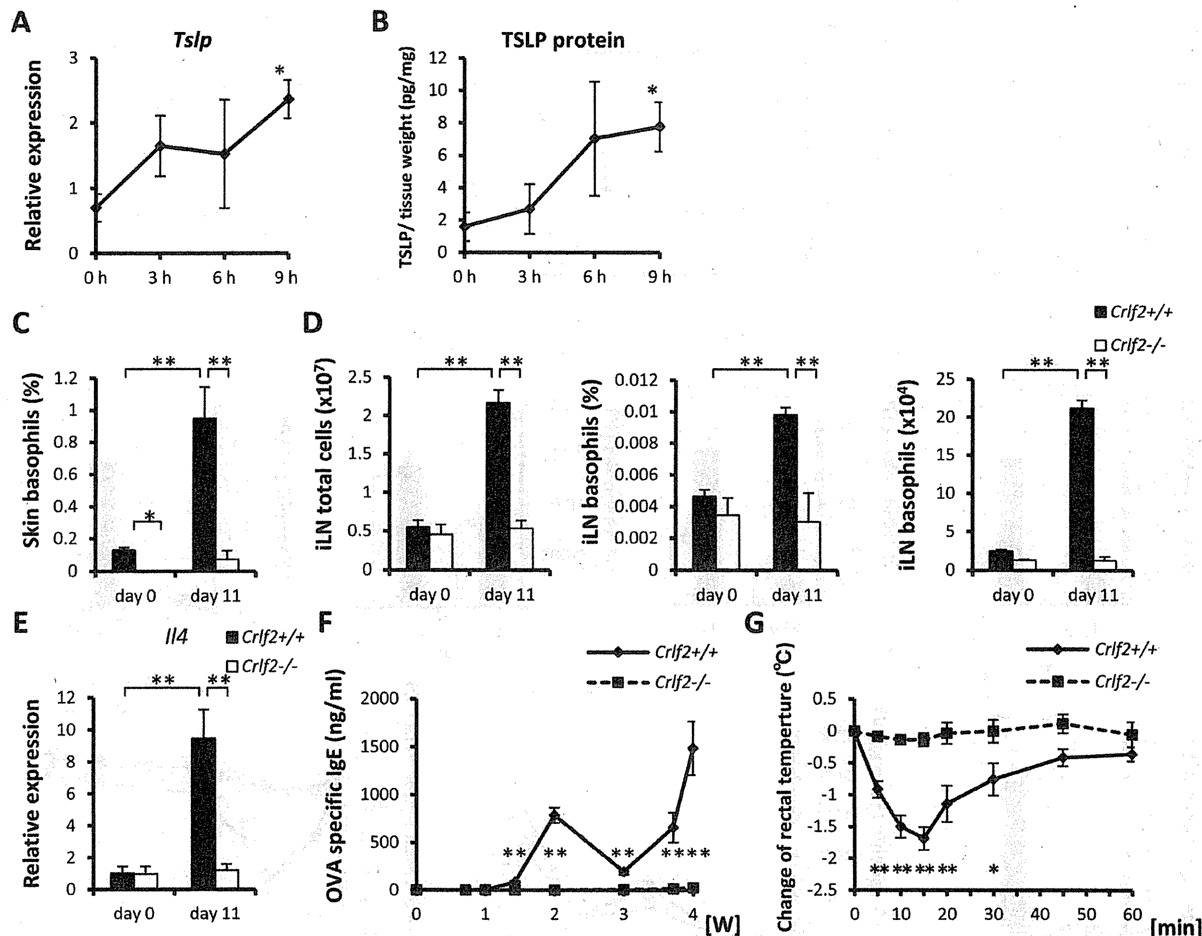

Fig. 5. TSLP is essential for cutaneous antigen sensitization and development of food allergy. (A and B) Skin was obtained from mice at the indicated time points after epicutaneous application with OVA. (A) Total RNAs were extracted and subjected to quantitative PCR analysis for the expression of *Tslp* and *Actb*. (B) Total proteins were extracted and TSLP content was determined by ELISA. (C–G) WT and *Crlf2*$^{-/-}$ mice were epicutaneously sensitized and orally challenged with OVA as in Fig. 1(A). (C and D) Skin (C) and inguinal lymph nodes (iLNs) (D) were obtained from WT and *Crlf2*$^{-/-}$ mice at day 0 and 11 following epicutaneous sensitization with OVA. The presence of basophils ($CD45^+CD3^-B220^-CD49b^+Fc\varepsilon RI^+$) was examined. (E) iLNs were obtained from WT and *Crlf2*$^{-/-}$ mice at day 0 and 11 following epicutaneous sensitization with OVA. Total RNAs were extracted and subjected to quantitative PCR analysis for the expression of *Il4* and *Actb*. (F) Sera from WT and *Crlf2*$^{-/-}$ mice were collected at the indicated time points of epicutaneous sensitization. OVA-specific IgE levels were determined by ELISA. (G) Changes in rectal temperature of WT and *Crlf2*$^{-/-}$ mice after oral challenge were measured at the indicated time points. Data represent mean ± SEM from six to seven mice. $^{**}P < 0.01$, $^{*}P < 0.05$.

and the subsequent development of food allergy. Previously, experts recommended families with high-risk food allergy infants (based on a family history of atopy) to avoid consuming common food allergens, such as peanuts, milk or eggs, during the first 3 years to prevent food antigen sensitization (9). However, recent studies question the beneficial effect of delaying the introduction of solid foods that are considered allergic (12, 13). Epidemiologic studies showed that the delayed consumption of a food increases the risk for developing food allergy (14, 44). Here, our animal study supports the theory that early consumption of a food antigen prevents future sensitization to the antigen. Thus, the introduction of tolerance and reduction of food allergy development could be achieved by early oral exposure of food antigens. Furthermore, skin is a major target site for the prevention of food antigen sensitization, especially in barrier-disrupted individuals.

The important roles of basophils and TSLP in the initiation of T_h2-type immune responses, especially in the skin, are becoming clear. Recently, Otsuka *et al.* (28) described a potential reason for the controversial basophil APC functions. In their epicutaneous antigen sensitization model, basophils functioned as APCs and were sufficient for initiating T_h2 responses when the antigen was a hapten or peptide. However, DCs were essential for T_h2 initiation to protein antigens, while basophils augmented T_h2 differentiation in an *in vitro* culture. Other groups also proposed a basophil-DC cooperation model and described the skin-based, basophil- and DC-mediated T_h2-inducing immune cascade (29, 30). Epicutaneous application of a vitamin D analog (29) or subcutaneous injection of

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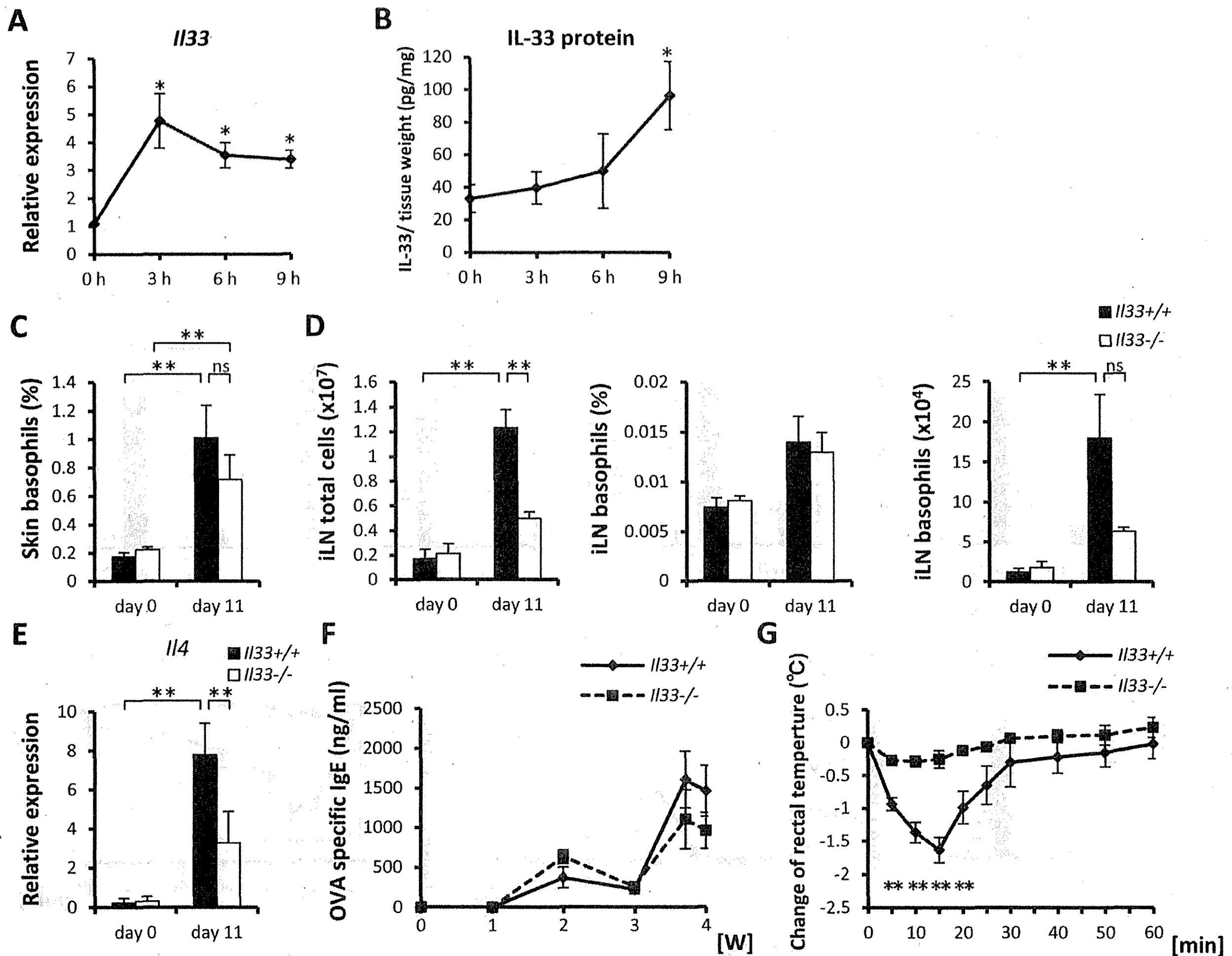

Fig. 6. IL-33 is dispensable for cutaneous antigen sensitization but essential for the development of anaphylaxis. (A and B) Skin was obtained from mice at the indicated time points after epicutaneous application with OVA. (A) Total RNAs were extracted and subjected to quantitative PCR analysis for the expression of *Il33* and *Actb*. (B) Total proteins were extracted and IL-33 content was determined by ELISA. (C–G) WT and *Il33*$^{-/-}$ mice were epicutaneously sensitized and orally challenged with OVA as in Fig. 1(A). (C and D) Skin (C) and nodes (iLNs) (D) were obtained from WT and *Il33*$^{-/-}$ mice at day 0 and 11 following epicutaneous sensitization with OVA. The presence of basophils (CD45$^+$CD3$^-$B220$^-$CD49b$^+$FcεRI$^+$) was examined. (E) iLNs were obtained from WT and *Il33*$^{-/-}$ mice at day 0 and 11 following epicutaneous sensitization with OVA. Total RNAs were extracted and subjected to quantitative PCR analysis for the expression of *Il4* and *Actb*. (F) Sera from WT and *Il33*$^{-/-}$ mice were collected at indicated time points of epicutaneous sensitization. OVA-specific IgE levels were determined by ELISA. (G) Changes in rectal temperature of WT and *Il33*$^{-/-}$ mice after oral challenge were measured at the indicated time points. Data represent mean ± SEM from five to six mice of two independent experiments. $^{**}P < 0.01$, $^{*}P < 0.05$, ns, not significant.

papain (30) induced local TSLP production, which activated DCs to up-regulate OX40 ligand and migrate into draining LNs. In turn, the DCs activated T cells cooperatively with basophils. Basophils skew the immune response toward T_h2 by secreting IL-4. In the present study, basophil-depleted or *Crlf2*$^{-/-}$ mice were completely defective in T_h2 development against epicutaneously sensitized antigens. Basophils in the inguinal LNs from OVA-sensitized mice produced more IL-4 than in naive LNs. Accordingly, in the case of skin-mediated sensitization to protein antigens, although the TSLP-DC pathway may play a central role in T cell activation, basophils are essential for skewing the response toward T_h2, as they are indispensable IL-4 producers. Thus, skin is a unique tissue where basophils have a pivotal role in the initiation of optimal T_h2 responses. In addition to localized T_h2 responses in the skin, we showed that skin basophils and TSLP pathways are essential for evoking systemic IgE responses and the development of food allergy.

The role of TSLP in food allergy was previously unclear. Although a genetic epidemiological study predicted an association between TSLP and food antigen sensitization (45), the cytokine was dispensable in gut-mediated food antigen sensitization in mice (36, 46). Therefore, TSLP may participate in food allergy by mediating skin-, but not gut-, based antigen sensitization. During the preparation of this manuscript, the important roles of basophils and TSLP in a cutaneously sensitized food

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allergy model were reported (47). Consistent with our results here, the depletion of basophils or TSLP in the sensitization phase protected mice from developing systemic IgE responses and oral challenge-induced anaphylaxis (47). Taken together, basophil- and TSLP-mediated immunological pathways can be targets for preventing the development of food allergy, especially for high-risk populations such as eczematous infants.

IL-33 is an epithelial cell-derived pleiotropic cytokine whose receptor is expressed on variety of cells including T cells, DCs, basophils and mast cells (31). Although our data showed the partial involvement of IL-33 in the initiation of T_h2 responses in the skin, *Il33*$^{-/-}$ mice developed serum OVA-specific IgE comparable to WT mice. Thus, skin derived IL-33 is not important for inducing systemic IgE responses. However, *Il33*$^{-/-}$ mice and mice treated with soluble ST2 were completely protected from oral challenge-induced anaphylaxis. Interestingly, some individuals with high serum food allergen-specific IgE have no clinical evidence of food allergy (5). This suggests that IgE alone is not sufficient for inducing anaphylaxis and that other factor(s) are required (19), such as IL-33. IL-33 signaling might have an essential role in gut-mediated food allergen sensitization, elicited by intra-gastric application of peanut allergen together with cholera toxin (36). Moreover, IL-33 amplifies IgE cross-linking-mediated mast cell degranulation (37, 38), and thus can enhance anaphylaxis (38). In this study, because anaphylaxis was induced by a single challenge of the antigen and was an immediate reaction, amplifying mast cell degranulation rather than augmenting T_h2 expansion likely explains the role of IL-33 in our system.

Because polymorphisms in the gene encoding ST2/IL-33R are significantly linked to AD prevalence, the IL-33-ST2 pathway might be a risk factor for AD (48). In addition, although it is controversial, ectopic expression of IL-33 in keratinocytes could induce AD-like symptoms in mice (33, 49). As AD is closely associated with food allergy (15, 17), IL-33 could also be secondarily involved in skin-mediated food allergy development.

Here, we demonstrated the role of basophils and proallergic cytokines, TSLP and IL-33, in cutaneously sensitized food allergy. The mode of action of the cytokines in food allergy pathogenesis is not consistent with that previously reported in gut-mediated sensitization models (36, 46), thus the cytokines may have tissue- and context-specific roles. As the cause of food allergy can vary depending on the patient (1, 2), further studies are required to clarify the involvement of immune pathways in the distinct disease settings of food allergy. Our results suggest that basophil/TSLP pathways can be targeted to manage food allergy development prophylactically in presensitized high-risk individuals, such as eczematous infants. In addition, the IL-33 pathway could be a major target for postsensitized individuals to prevent anaphylaxis.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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Interferon γ and plasminogen activator inhibitor 1 regulate adhesion formation after partial hepatectomy

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Background: The pathophysiology of intra-abdominal adhesions has not been studied extensively. The aim of this study was to elucidate the molecular mechanisms underlying adhesion formation in a murine model and in patients undergoing hepatectomy.

Methods: Partial hepatectomy was performed using bipolar forceps in mice. Wild-type mice, antibodies to CD4 and interferon (IFN) γ, IFN-γ, natural killer T (NKT) cells and plasminogen activator inhibitor (PAI) 1 knockout (KO) mice were used. Recombinant hepatocyte growth factor (HGF) was tested for its ability to prevent adhesions. Liver specimens were obtained during surgery from patients undergoing hepatectomy. Adhesion formation was evaluated using a scoring system that ranged from 0 (no adhesions) to 5 (severe adhesions). Levels of IFN-γ and PAI-1 mRNA, and protein concentration of PAI-I were measured, and fluorescence immunostaining was performed.

Results: Adhesion formation depended on IFN-γ produced by NKT cells, and NKT KO mice developed few adhesions (mean(s.d.) 1·7(0·3) *versus* 4·6(0·4) in wild-type mice; $P = 0{\cdot}037$). In wild-type mice, the level of PAI-1 mRNA increased after hepatectomy, followed by a decrease in the tissue plasminogen activator (tPA) mRNA level. Adhesion formation was inhibited completely in PAI-1 KO mice (0(0) *versus* 4·1(0·8) in wild-type mice; $P = 0{\cdot}002$). HGF inhibited formation of abdominal adhesions after hepatectomy by reducing IFN-γ and PAI-1 levels, and increasing tPA levels compared with those in mice treated with phosphate-buffered saline ($P < 0{\cdot}001$, $P = 0{\cdot}002$ and $P = 0{\cdot}035$ respectively). In human liver specimens, NKT cells accumulated in the liver after hepatectomy, and PAI-1 expression was increased 5·25-fold ($P = 0{\cdot}030$).

Conclusion: IFN-γ is a key molecule for abdominal adhesion formation after hepatectomy, acting via the reciprocal balance of PAI-1 and tPA. This molecular mechanism may also regulate adhesion formation in patients following hepatectomy. HGF inhibited formation of adhesions by regulating IFN-γ and PAI-1, suggesting that it may be an important target for prevention of adhesions after hepatectomy.

Surgical relevance

Postoperative intra-abdominal adhesions can be asymptomatic or cause significant morbidity and mortality. Adhesion formation after hepatectomy has not been studied extensively. In the present study, the molecular mechanisms underlying intra-abdominal adhesions after hepatectomy were investigated in a murine model and in patients.

Interferon (IFN) γ produced by natural killer T cells is a key molecule for adhesion formation after hepatectomy in mice, acting via the reciprocal balance between plasminogen activator inhibitor (PAI) 1 and tissue plasminogen activator, the pivotal factors in fibrinolytic activity. This mechanism was also involved in the regulation of adhesions in human tissue samples. Hepatocyte growth factor (HGF) strongly inhibited adhesion formation by regulating IFN-γ and PAI-1.

These results indicate that IFN-γ and PAI-1 are possible therapeutic targets, and HGF could prevent postoperative adhesion formation after hepatectomy.

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Introduction

Hepatic resection has an evolving and well established role in the treatment of benign and malignant liver diseases. The demand for living-donor liver transplantation is increasing because of the evolving indications for liver transplantation and the shortage of deceased organ donors[1].

Postoperative intra-abdominal adhesions frequently occur after abdominal operations. They can result in intestinal obstruction, infertility, pain and considerable economic costs[2,3]. Adhesions after hepatic resection pose several specific problems, including the demands of a difficult second hepatectomy and postoperative complications owing to adhesion formation[4,5]. Prevention of adhesion formation could have a great impact on healthcare.

Several studies have investigated the mechanism of adhesion formation after abdominal surgery. T helper type 1 (Th1) cells were essential for the development of adhesions in a mouse model of intra-abdominal sepsis[6]. In one investigation[7] IFN-γ produced by natural killer T (NKT) cells was shown to play a pivotal role in differential regulation of plasminogen activator inhibitor (PAI) 1 and tissue plasminogen activator (tPA), and recombinant hepatocyte growth factor (HGF) protein strongly inhibited adhesion formation by decreasing IFN-γ production.

The mechanism of adhesion formation after hepatic resection is largely unknown. In this study the sequential molecular mechanisms of adhesion formation after hepatectomy were investigated in a mouse model and in patients undergoing partial hepatectomy (PH).

Methods

Female 7–9-week-old BALB/c, C57BL/6 (B6) and 129/Sv *Pai-1*$^{-/-}$ (PAI-1 knockout (KO)) mice, weighing 18–24 g, were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). 129/Sv PAI-1 KO mice were back-crossed to BALB/c PAI-1 KO mice (7th generation). BALB/c NKT cell-deficient (NKT KO), BALB/c *Ifng*$^{-/-}$ (IFN-γ KO) and PAI-1 KO mice were bred under specific pathogen-free conditions in the animal facilities of Hyogo College of Medicine. All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care Committee, Hyogo College of Medicine, and the manuscript was written according to the Animal Research: Reporting *In Vivo* Experiments (ARRIVE) guidelines[8].

Mouse model of surgical adhesion formation

Mice were anaesthetized with 0·15 ml (20 per cent v/v) pentobarbital sodium solution (10 mg/ml). An upper midline incision was made and the left lobe of the liver isolated. A part of the left lobe with a volume of 60–100 mg was removed (PH) by using the coagulation mode of bipolar forceps (10 W, 500 kHz, 100 Ω; MERA, Tokyo, Japan). After 7 days the mice were killed and examined by an observer blinded to the genotype and treatment to evaluate the degree of adhesion formation. Adhesion formation was evaluated using a standard scoring system that ranged from 0 (no adhesions) to 5 (severe adhesions) (*Fig. 1*)[7]. Liver biopsies were obtained from the remnant left lobe of the liver. For histopathological examination during adhesion progression, wild-type mice were killed at 1, 3, 5 and 7 days after PH (2 mice per group). In addition, mice treated with anti-CD4 antibody or anti-IFN-γ, and NKT KO, IFN-γ KO and PAI-1 KO mice were killed to examine the adhesion score 7 days after hepatectomy (5 mice per group).

According to a previous study[7], changes in cytokine mRNA expression occurred within 24 h after abdominal injury. To examine the mRNA expression of IFN-γ, PAI-1 and tPA after hepatectomy (relative to 18S rRNA[7]), BALB/c mice were killed every 3 h until 24 h after PH (5 per group). The relative mRNA levels of IFN-γ and PAI-1 were maximal at 3 and 6 h respectively, whereas the tPA level was lowest at 18 h after hepatectomy. Therefore, BALB/c, NKT KO and IFN KO mice were killed at 3, 6 and 18 h after hepatectomy (5 per group) to compare the relative mRNA level at 3 h for IFN-γ, 6 h for PAI-1 and 18 h for tPA.

Purified monoclonal antibodies against CD4 (clone GK1.5) and IFN-γ (clone R6A2) were prepared in the authors' laboratory and injected as described previously[7]. To confirm the suppression of $CD4^+$ T cells by use of anti-CD4 antibodies, fluorescence-activated cell sorting analysis of splenic lymphocytes was performed. $CD4^+$ T cells consist of conventional NK1.1 – $CD4^+$ T cells and NKT cells expressing αβ T cell receptor with invariant Vα14-Jα18[9]. The type of $CD4^+$ T cells that contribute to adhesion formation was investigated. NKT cells, if activated, function mainly to secrete cytokines such as IFN-γ and interleukin 4[9]. Based on a previous study[7], it was suggested that these cells secreted IFN-γ in this model, and IFN-γ mRNA levels from the remnant left lobe of the liver were measured using real-time polymerase chain reaction (PCR). HGF (kindly provided by H. Tsubouchi, Kagoshima University Graduate School of Medical and Dental Sciences) (0, 10, 20, 40 μg per mouse) in 200 ml of phosphate-buffered saline (PBS) was injected subcutaneously into mice immediately after the surgery. Control mice received PBS alone.

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