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Epicutaneous Allergic Sensitization by Cooperation between Allergen Protease Activity and Mechanical Skin Barrier Damage in Mice

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Abstract (199 words)

Allergen sources such as mites, insects, fungi, and pollen contain proteases. Airway exposure to proteases induces allergic airway inflammation and IgE/IgG1 responses via IL-33-dependent mechanisms in mice. We herein examined the epicutaneous sensitization of mice to a model protease allergen, papain, the effects of tape-stripping, which induces epidermal barrier dysfunction, and the atopic march upon a subsequent airway challenge. Papain painting on ear skin and tape-stripping cooperatively promoted dermatitis, the skin gene expression of proinflammatory cytokines and growth factors, upregulation of serum total IgE, and papain-specific IgE/IgG1 induction. Epicutaneous sensitization induced Th2 and Th17 differentiation in draining lymph nodes. Ovalbumin and protease inhibitor-treated papain induced no or weak responses, while the co-administration of ovalbumin and papain promoted ovalbumin-specific IgE/IgG1 induction. Wild-type and IL-33-deficient mice showed similar responses in the epicutaneous sensitization phase. The subsequent airway papain challenge induced airway eosinophilia and maintained high papain-specific IgE levels in an IL-33-dependent manner. These results suggest that allergen source-derived protease activity and mechanical barrier damage such as that caused by scratching cooperatively promote epicutaneous sensitization and skin inflammation, and that IL-33 is dispensable for epicutaneous sensitization, but is crucial in the atopic march upon a subsequent airway low-dose encounter with protease allergens.

Keywords: Protease allergen, mechanical barrier damage, epicutaneous sensitization, atopic march, IL-33

Abbreviations:

Ab: antibody

AD: atopic dermatitis

AREG: amphiregulin

DLN: draining lymph node

e.c.: epicutaneous, epicutaneously

EGF: epidermal growth factor

E64-papain: papain treated with the irreversible protease inhibitor E64

HB-EGF: heparin-binding EGF-like growth factor

HDM: house dust mite

ILC2: group 2 innate lymphoid cells

OVA: ovalbumin

SC: stratum corneum

ST2: an IL-33 receptor subunit also known as IL1RL1

TJ: tight junction

TS: tape-stripping

TSLP: thymic stromal lymphopoietin

^{-/-} : -deficient

Introduction

Allergic diseases are multifactorial, heterogeneous disorders caused by interactions between environmental and genetic factors. With a major breakthrough in the genetics of AD (Palmer et al., 2006), skin barrier dysfunction has emerged as a critical predisposing factor in the initiation and exacerbation of atopic dermatitis (AD) and, interestingly, as a risk factor for asthma in patients with a previous history of eczema, *i.e.* features of the “atopic march” (Dharmage et al., 2014; Spergel and Paller, 2003; Lack, 2008). Thus, epicutaneous (e.c.) exposure to allergens via barrier-disrupted skin is considered an important route of sensitization towards AD and other allergic diseases such as asthma and food allergies in the atopic march (De Benedetto et al., 2012; Leung and Guttman-Yassky, 2014).

Allergen sources, such as house dust mites (HDMs), insects, fungi, and pollen, produce or contain proteases, the proteolytic activities of which may be involved in the pathogenesis of allergies (Takai and Ikeda, 2011; Hammad and Lambrecht, 2008; Shakib et al., 2008). These proteases are frequently allergens, recognized by IgE in sensitized patients. The papaya fruit-derived occupational protease allergen, papain, and HDM major allergens Der f 1 and Der p 1 belong to the same family of cysteine proteases (Barrett and Rawlings, 2001; Thomas, 2015). Papain was recently used as a model protease allergen mimicking those contained in allergen sources (Sokol et al., 2008; Cunningham et al., 2012; Kamijo et al., 2013; Iida et al., 2014).

In murine models of airway inflammation induced by papain inhalation without an adjuvant, IL-33 (Cayrol and Girard, 2014; Kamijo et al., 2013; Oboki et al., 2010), which is released by airway epithelia damaged by a protease attack, and IL-33-responsive group 2 innate lymphoid cells (ILC2) (Halim et al., 2014; Halim et al., 2012) play essential roles in the induction of Th2 differentiation and lung eosinophilia. A relationship has also been

suggested between IL-33 and AD (Shimizu et al., 2005; Savinko et al., 2012; Salimi et al., 2013; Imai et al., 2013). Despite recent advances in our understanding of the roles of protease allergens upon airway exposure, their involvement in e.c. allergic sensitization remains unclear.

We recently reported a murine model of e.c. sensitization to papain via intact skin, in which specific IgE/IgG1 production was induced in a manner dependent on the protease activity of papain (Iida et al., 2014). We herein further analyzed responses to e.c. sensitization in the presence or absence of tape-stripping (TS), which removes the outermost layer of the epidermis, the stratum corneum (SC), and reduces epidermal barrier function, and compared responses between wild-type and IL-33-deficient (IL-33^{-/-}) mice in the e.c. sensitization phase and the atopic march upon subsequent airway exposure to a low dose of papain.

Results

E.c. administration of papain and TS cooperatively promoted skin responses

Papain solution was applied to both sides of one ear lobe with or without TS on Days 0, 3, 7, and 11. We used TS, which minimally damages ear skin, to affect barrier function, evaluated as increased transepidermal water loss in pilot experiments (**Supplementary Figs. S1 and S2**). The administration of papain after TS markedly induced ear swelling (**Fig. 1a**) and significantly affected the appearance of the ear (**Supplementary Fig. S3**), whereas papain or TS alone induced a small or no change.

The time course of histology after the administration of papain onto the ear treated with TS was analyzed (**Fig. 1b** and **Supplementary Fig. S4**). Damage to the epidermis, inflammation, and swelling of the dermis were observed within 12 hours of the first administration plus TS (**Fig. 1b** and **Supplementary Fig. S4, 12 h**). After the exacerbation of inflammation (24 h), the epidermis was repaired, and this was associated with hyperplasia (72 h). More severe damage to the epidermis and inflammation were observed 12 hours after the second administration of papain plus TS (84 h). The thickened epidermis was repaired 24 hours after the second application (96 h), and this was associated with intercellular edema. Thus, the repeated e.c. administration of papain after TS caused epidermal damage, inflammation, and dermal swelling, and led to epidermal repair with hyperplasia and intercellular edema.

A quantitative real-time PCR analysis revealed that the combined treatment with papain and TS induced the highest gene expression of proinflammatory cytokines (IL-1 β , IL-6, TNF- α , and IL-1 α) and epidermal growth factor (EGF) homologues [amphiregulin (AREG) and heparin-binding EGF-like growth factor (HB-EGF)] in ear lobes 24 hours after the first treatment (**Fig. 1c** and **Supplementary Fig. S5a**) and also 24 hours after the fourth treatment,

except for IL-1 α and HB-EGF (**Fig. 1d** and **Supplementary Fig. S5b**). The treatment with papain alone increased IL-1 β and IL-6 gene expression after the fourth treatment (**Fig. 1d** and **Supplementary Fig. S5b**).

E.c. administration of papain and TS cooperatively promoted antibody (Ab) responses

On day 11, papain plus TS significantly elevated serum total IgE and papain-specific IgE/IgG1 levels (**Fig. 2a-c, Day 11**). On day 17, papain without TS also delayed elevations in papain-specific IgG1 levels (**Fig. 2c, Day 17**). Total and papain-specific IgE levels decreased by Day 17.

Draining lymph nodes (DLN) were collected on Day 12 and restimulated for cytokine production by papain-specific T cells. DLN cells were restimulated with papain treated with the irreversible protease inhibitor E-64 (E64-papain) to avoid possible stimulatory effects via protease activity, but not T cell receptors. The restimulation of DLN cells obtained from papain-administered mice with E64-treated papain significantly induced the release of Th2 (IL-4, IL-5, and IL-13) and Th17 cytokines (IL-17A, and IL-17F) (**Fig. 2d**). Thus, the administration of papain with or without TS induced Th2/Th17 differentiation in DLNs.

Responses induced by the e.c. administration of papain were dependent on protease activity

We determined whether the enzyme activity of papain contributed to the induction of dermatitis and Ab production in this model. E64-papain administered after TS did not induce significant ear swelling (**Fig. 3a**) and the production of IgE/IgG1 was less than that with intact papain (**Fig. 3b-d**). Histological findings showed that E64-papain did not induce eosinophil-associated inflammation or thickening of the epidermis, both of which were

induced by papain (**Fig. 3e** and **Supplementary Fig. S6**, upper panels).

Taken together with the ear swelling results (**Fig. 1a** and **Supplementary Fig. S7a**), the administration of papain, even without TS, induced dermatitis (**Fig. 3e** and **Supplementary Fig. S6**, lower panels) and the production of IgE/IgG1 (**Supplementary Fig. S7b-d**), the latter of which we reported recently (Iida et al., 2014); however, these changes were less severe or delayed more than those induced by TS. Thus, the more or less severe dermatitis induced by papain with or without TS, respectively, was dependent on the cysteine protease activity of papain.

Papain exhibited adjuvant activity to induce Ab responses against a co-administered antigen in the model of e.c. sensitization with TS

We evaluated the effects of papain as an adjuvant in the e.c. sensitization model with TS. The administration of chicken ovalbumin (OVA) alone did not induce significant ear swelling (**Fig. 4a**) or dermatitis (**Fig. 4b** and **Supplementary Fig. S8**). The co-administration of OVA and papain induced ear swelling and dermatitis as well as papain-specific IgE/IgG1, similar to the administration of papain alone (**Fig. 4a, d, e**). The co-administration of OVA and papain induced higher levels of OVA-specific IgG1 than the administration of OVA alone (**Fig. 4g**) and slightly higher levels of OVA-specific IgE (**Fig. 4f**). Thus, papain exhibited the adjuvant activity of Ab responses against a co-administered antigen with no protease activity.

IL-33 was dispensable for responses in the e.c. sensitization phase to papain with TS

In order to examine the role of IL-33 in the present model, in which mice were e.c. sensitized with papain after TS, we compared responses between wild-type and IL-33^{-/-} mice (**Fig. 5**).

Papain plus TS induced a similar degree of ear swelling, skin inflammation, and thickening of

the epidermis between wild-type and IL-33^{-/-} mice (**Fig. 5a, b** and **Supplementary Fig. S9**). Total IgE and papain-specific IgE/IgG1 levels were also similar between wild-type and IL-33^{-/-} mice (**Fig. 5c-e**). Thus, IL-33 was dispensable for dermatitis and IgE/IgG1 responses in the present model of e.c. sensitization to papain with TS, being different from IL-33-dependent, papain-induced allergic airway inflammation models.

The treatment with papain, TS, or their combination did not increase the gene expression of three epithelial-derived Th2-inducing cytokines: IL-33, thymic stromal lymphopoietin (TSLP), and IL-25, in ear lobes 24 hours after the first or fourth treatment in wild-type mice (**Supplementary Fig. S10**).

IL-33 was crucial to the atopic march induced by airway exposure to a low dose of papain after e.c. sensitization with TS

We investigated the contribution of IL-33 to the atopic march induced by the subsequent inhalation of papain after e.c. sensitization. We modified the method of papain administration; *i.e.* papain solution was applied to both sides of both ear lobes after TS 3 times using cellophane tape. Similar to the results obtained from the administration of papain to one ear lobe after TS 5 times using surgical tape (**Fig. 5c-e**), Ab production was similar between wild-type and IL-33^{-/-} mice on Day 12 (**Fig. 6a**). After e.c. sensitization, mice were intranasally challenged with a low dose of papain (2.5 µg), which did not induce significant Ab responses or lung eosinophilia in mice without e.c. presensitization to papain. High total and papain-specific IgE levels were maintained or enhanced after the intranasal challenge in wild-type, but not IL-33^{-/-} mice e.c. presensitized with 1 mg/ml of papain (**Fig. 6a, b**). The intranasal challenge with the low dose of papain induced eosinophilic airway inflammation in wild-type, but not IL-33^{-/-} mice e.c. presensitized to papain (**Fig. 6c**).

Discussion

Exposure to allergens through the skin is considered an important route of sensitization in not only AD, but also the atopic march, the natural progression of allergic diseases in which the development of AD predates that of food allergies, asthma, or allergic rhinitis. We herein demonstrated that, in mice, the combination of the protease activity of the antigen e.c. administered and mechanical barrier damage by TS cooperatively and markedly induced dermatitis and antigen-specific IgE/IgG1 production, which were absent or slower with the e.c. administration of non-protease antigens. E.c. sensitization enables mice to show atopic march responses upon an airway challenge even with a lower dose of the protease allergen that does not induce responses in mice without e.c. presensitization. IL-33 was dispensable in the e.c. sensitization phase, but was essential to the atopic march caused by an encounter with low doses of the allergen in the airways.

Roles of protease allergens and mechanical skin barrier damage

In most previous studies using murine models of e.c. sensitization to OVA, HDM, and adjuvants, Th2-skewed immune responses were achieved after various barrier-disrupting methods including shaving, TS, patch applications with an occlusive dressing that may result in maceration, leading to barrier disruption, and repeating the procedures for longer periods (De Benedetto et al., 2012). In the present study, shaving or an occlusive dressing was not performed and the sensitization period was short. Environmental proteases cause the breakdown of even the rigid skin barrier (Nakamura et al., 2006; Jeong et al., 2008; Hirasawa et al., 2010; Iida et al., 2014). The combination of the e.c. administration of papain and TS markedly enhanced dermatitis and accelerated IgE/IgG1 responses. The critical permeability function of the skin is mediated by the SC, which is the primary physical barrier against the

penetration of environmental antigens and stimuli via skin, while the second physical barrier is the tight junction (TJ) barrier, just beneath the SC (De Benedetto et al., 2012). Allergen source-derived proteases including papain are known to degrade TJ molecules (Takai and Ikeda, 2011; Wan et al., 1999; Wan et al., 2001; Runswick et al., 2007; Stremnitzer et al., 2015). Removal of the SC by mechanical injury such as scratching and subsequent disruption of the TJ barrier by allergen protease activity may cooperatively reduce total skin physical barrier function, thereby facilitating the e.c. penetration of protease allergens and co-existing non-protease allergens towards allergic sensitization.

Proteases and TS induce disruptions to the physical skin barrier and stimulate cellular responses. Protease allergens were previously shown to stimulate or affect the functions of various cell types to cause proinflammatory or allergic responses including the release of cytokines and chemokines in epidermal keratinocytes (Kato et al., 2005; Ogawa et al., 2008; Kato et al., 2009), airway epithelial cells (King et al., 1998; Adam et al., 2006; Kouzaki et al., 2009), and basophils (Phillips et al., 2003; Sokol et al., 2008; Kamijo et al., 2013; Kamijo et al., in press), the accumulation of inflammatory cells (Hirasawa et al., 2010; Oboki et al., 2010), airway hyper-responsiveness (Grunstein et al., 2005; Balenga et al., 2015), Th2-skewed sensitization (Chambers et al., 1998; Gough et al., 1999; Kikuchi et al., 2006; Sokol et al., 2008; Tang et al., 2010; Kamijo et al., 2013; Iida et al., 2014), and others (Takai and Ikeda, 2011; Hammad and Lambrecht, 2008; Shakib et al., 2008). TS removes the SC, which enables epidermal Langerhans cells to capture environmental protein antigens (Kubo et al., 2009). Disruption of the TJ barrier by protease activity may facilitate antigens to reach interstitial spaces, thereby increasing the chances of encounters with dermal dendritic cells. TS can induce proinflammatory responses (Onoue et al., 2009; Oyoshi et al., 2010). A previous study reported that TS of the ear skin of naïve mice caused the marked infiltration of

leukocytes including the precursors of Langerhans cells into hair follicles (Nagao et al., 2012); in the present study, the e.c. administration of papain to the tape-stripped ear skin of naïve mice induced cellular infiltration within 12 hours and markedly upregulated proinflammatory cytokine and growth factor gene expression within 24 hours. Furthermore, a recent study demonstrated vascular leakage and the recruitment of neutrophils by the application of papain to intact ear skin (Stremnitzer et al., 2015), indicating the inflammatory potential of TS and e.c. protease administration.

Shaving and TS of the back skin of mice was shown to upregulate the expression of TSLP (Oyoshi et al., 2010), a cytokine that contributes to Th2 differentiation in e.c. sensitization to OVA mediated by Langerhans cells (Nakajima et al., 2012) or to a low molecular weight hapten mediated by dermal dendritic cells (Oyoshi et al., 2010). However, the TSLP-TSLP receptor pathway appeared to be dispensable in the present model of e.c. papain administration with TS (unpublished data). Allergen sources such as HDM, which may contain another class of protease, serine proteases, along with papain-like cysteine proteases, have been used in some murine models of e.c. sensitization or AD, and barrier disruption activates endogenous serine proteases causing skin inflammation (Takai and Ikeda, 2011; De Benedetto et al., 2012; Leung and Guttman-Yassky, 2014; Moniaga et al., 2013; Lee et al., 2014). The cellular and molecular mechanisms underlying skin and Ab responses in our protease-dependent e.c. sensitization models and their modifications need to be investigated further.

Th2 and Th17 differentiation in e.c. protease-allergen sensitization

Although the Th2 subset appears to be a key factor for allergic inflammation, the contribution of other subsets has also been suggested (Werfel et al., 1996; Leung and Guttman-Yassky,

2014). In the present study, e.c. sensitization to papain induced the differentiation of antigen-specific Th2 and Th17 cells. The differentiation of antigen-specific Th1, Th2, and Th17 cells has been reported in models of e.c. sensitization to OVA (He et al., 2007; Oyoshi et al., 2009), and e.c. sensitization induced more distinct Th17 skewing than that with intraperitoneal sensitization (He et al., 2007). T cells specific to HDM major allergens in AD were recently shown to have Th2, Th17, and Th2/Th17 phenotypes (Roesner et al., 2015).

No IL-33-dependency in the e.c. protease-allergen sensitization phase

IL-33 and ST2, a subunit of the IL-33 receptor, are expressed in the skin of AD patients (Shimizu et al., 2005; Savinko et al., 2012; Salimi et al., 2013). ILC2 cells have also been detected and single nucleotide polymorphisms in the ST2 gene have been associated with AD (Shimizu et al., 2005). Furthermore, the overexpression of IL-33 in the skin of mice plays a role in the activation of ILC2 and onset of AD-like inflammation (Imai et al., 2013). In airway sensitization models, inhaled papain caused the release of IL-33 in the airways, which, in response to the protease attack, caused robust airway eosinophilia and IgE/IgG1 production (Kamijo et al., 2013), in which the IL33-ILC2 axis was critical for the induction of Th2 differentiation and eosinophil infiltration (Halim et al., 2014). However, IL-33^{-/-} mice showed similar responses to wild-type mice in the e.c. sensitization phase. Since AD is a complex disease that includes multiple clinical phenotypes, we cannot exclude the potential contribution of IL-33 to ongoing AD or in murine models with different experimental settings. Nevertheless, our results suggest that protease activity, with the aid of mechanical barrier damage, efficiently elicited e.c. allergic sensitization without the significant contribution of IL-33, at least in the initiation step of e.c. sensitization. Recent studies reported that ST2 deficiencies did not reduce cellular infiltration in skin e.c. sensitized to OVA with the adjuvant,

staphylococcal enterotoxin B (Savinko et al., 2013), and IL-33 deficiencies did not attenuate OVA-specific IgE levels induced in the e.c. sensitization phase to OVA in shaved skin treated with the detergent, sodium lauryl sulfate (Muto et al., 2014).

IL-33 dependency in the atopic march upon subsequent inhalation of low-dose papain

E.c. papain sensitization enabled allergic airway inflammation in mice upon airway exposure to even a low dose (2.5 µg), and IL-33 was essential to this atopic march. Total and papain-specific IgE levels were maintained or enhanced after the airway challenge in wild-type, but not IL-33^{-/-} mice e.c. presensitized to papain. This IL-33 dependency may be attributed to ILC2 (Halim et al., 2014) and pathogenic memory Th2 cells (Endo et al., 2015), both of which release cytokines such as IL-5 and IL-13 in response to IL-33 to cause airway eosinophilia and promote Th2 development.

Two distinct sequential steps contribute to the atopic march in the e.c. sensitization-airway challenge model, *i.e.* the IL-33-independent step in the initial e.c. sensitization phase and IL-33-dependent step in the airway challenge. The first step may involve the differentiation of Th subsets including Th2 and Th17 cells and Ab responses, while the second step may be mediated by IL-33-responsive cells in the airway, which include ILC2, Th2, and/or other cell types (Cayrol and Girard, 2014; Endo et al., 2015). E.c. presensitization with 1 mg/ml papain resulted in more severe lung eosinophilia and an IgE boost upon an airway challenge than e.c. presensitization with 10 mg/ml papain, suggesting that the e.c. application of high-dose papain involves inducing not only sensitization but also, in part, immunological tolerance.

Dependency on allergen protease activity

In contrast to the present study, Stremnitzer et al. (Stremnitzer et al., 2015) recently reported that e.c. papain sensitization was independent of protease activity. Although causes for the discrepancy between our study and theirs remain unknown, the application method, application site, and time schedule for e.c. sensitization markedly differed. The method of the E64 treatment of papain also differed. They administered a mixture of papain and excess E64. In the present study, papain was reacted with excess E64 after the addition of *L*-cysteine, and *L*-cysteine and unbound free E64 were removed by dialysis after the reaction (**Online Methods**). We administered the preparation of the covalent complex between papain and E64.

Conclusions

The results of the present study suggest that allergen source-derived protease activity and mechanical barrier damage such as that caused by scratching cooperatively promoted e.c. allergic sensitization and skin inflammation including AD, that e.c. sensitization significantly promoted the atopic march induced by a subsequent airway encounter with low doses of papain, and also that IL-33 was dispensable to the initial e.c. sensitization to papain, indicating distinct mechanisms between the skin and airways, but was crucial in the atopic march in the airways. Blocking the release of IL-33 upstream or downstream may be a promising strategy to prevent allergic airway inflammation even after the establishment of e.c. sensitization, which enhances sensitivity to the inhalation of minute amounts of air-borne allergens.

The e.c. OVA sensitization model (Wang et al., 1996; Spergel et al., 1998) and its modified versions, have been used in many investigations on the mechanisms underlying e.c. sensitization and the atopic march. We consider the protease activities of allergens and mechanical barrier damage to have dual roles *i.e.* disruption of barrier function and induction

of cellular responses in different manners, and, thus, when combined, e.c. sensitization and dermatitis may be cooperatively promoted. The e.c. protease-allergen sensitization model and its modification may assist in elucidating the mechanisms underlying e.c. sensitization to environment allergen sources, many of which exhibit protease activity.

Materials and Methods

Mice. Mice were used in accordance with the guidelines of the institutional committee on animal experiments after institutional approval of experiments. For further information on mice and reagents see **Online Methods**.

E.c. administration of antigens to mice. The method originally described for sensitization to HDM extract (Gao et al., 2004) was modified as follows. A total of 12.5 μ l of 10 mg/ml of papain (except for **Fig. 6**), E64-treated papain, or OVA was applied to both sides of the surface of the right ear (250 μ g/25 μ l/animal) or both ears of each female C57BL/6N mouse with or without TS five or six times using surgical tape (21N, Nichiban, Tokyo, Japan), and this procedure was repeated twice per week for 2 weeks for a total of 4 times. In some experiments (**Fig. 6**), TS was performed three times using cellophane tape (Cellotape, Nichiban). For further information on mice and reagents see **Online Methods**.

Measurement of ear thickness and histology. Ear thickness was measured using a dial thickness gauge (G-1A, Ozaki, Tokyo, Japan). Portions of the ear lobe were fixed with 20% neutral formalin, embedded in paraffin and sectioned. Sections were stained with hematoxylin-eosin.

Quantitative real-time PCR. Ear lobes excised were subjected to real-time quantitative PCR. The mRNA levels of the target gene were normalized to the gene expression levels of glyceraldehyde-3-phosphate dehydrogenase and are shown as relative expression levels to the control group. For further information see **Online Methods**.

ELISA for serum total IgE and antigen-specific Abs and restimulation of DLN cells.

Serum total IgE, papain- or OVA-specific IgG1 were measured by ELISA. DLNs were collected from mice the day after the last e.c. administration and were stimulated with E64-treated papain or medium alone for 96 hours. For further information see **Online Methods**.

Intranasal challenge. E.c. sensitized mice were intranasally challenged with a low dose of papain (2.5 µg/50 µl) 4 and 7 days after the last e.c. administration. Sera and bronchial alveolar lavage fluids were collected 4 days after the last intranasal administration and analyzed as described previously (Kamijo et al., 2013).

Statistical analysis. One-way ANOVA with the Tukey *post hoc* test or the Mann-Whitney *U* test (2-tailed) was used. A value of $p < 0.05$ was regarded as significant.

Conflict of interest: There is no conflict of interest declared.

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Figure Legends

Fig. 1. Epicutaneous administration of papain and tape-stripping cooperatively promoted dermatitis. Papain was painted [Days 0, 3, 7, and 11 (10 in **c** and **c**)] onto ears with (*TS+*) or without (*TS-*) tape-stripping. **(a)** Ear swelling. Data represent the mean $\pm$ SD of four mice per group. $*P < 0.05$ vs *Vehicle* and $\#P < 0.05$ vs *Papain (TS-)* by the Mann-Whitney *U* test. **(b)** Histology after the first (12 *h*, 24 *h*, and 72 *h*) and second administration (84 *h* and 96 *h*: 12 and 24 hours after Day 3, respectively). Scale bar = 50 μ m except for 72 *h* (100 μ m). **(c, d)** Gene expression 24 hours after the first (**c**) and last administration (**d**). $*P < 0.05$ by ANOVA and $\#P < 0.05$ vs the vehicle alone by the Mann-Whitney *U* test. *TS*: tape-stripping.

Fig. 2. Epicutaneous administration of papain induced IgE/IgG1 responses and differentiation of Th2 and Th17 cells. Papain was applied to the surface of the right ear with (*TS+*) or without (*TS-*) tape-stripping (Days 0, 3, 7, and 11). **(a-c)** Serum antibody response. $*P < 0.05$ vs *Vehicle* and $\#P < 0.05$ vs *Papain (TS-)* by the Mann-Whitney *U* test. Data represent the mean $\pm$ SD of four or eight mice per group. *TS*: tape-stripping. **(d)** Cytokine release in draining lymph node cells recovered on Day 12 and restimulated *in vitro* with E64-treated papain. $*P < 0.05$ vs without restimulation by the Mann-Whitney *U* test. Data represent the mean $\pm$ SD of four mice per group.

Fig. 3. Dermatitis and IgE/IgG1 responses depended on the protease activity of epicutaneously administered papain. Papain or that treated with E64 (*E64-Papain*), which was prepared as described in **Online Methods**, was applied to the surface of the right ear with tape-stripping (*TS+*) (Days 0, 3, 7, and 11). **(a)** Ear swelling. **(b-d)** Serum antibody response.

* $P < 0.05$ vs *Vehicle* and # $P < 0.05$ vs *E64-Papain* by the Mann-Whitney U test. Data represent the mean $\pm$ SD of four mice per group. (e) Histology (Day 12). Scale bar = 50 μ m. TS: tape-stripping.

Fig. 4. Papain showed adjuvant activity in antibody responses against co-administered OVA. OVA was administered alone or with papain to the surface of the right ear with tape-stripping (Days 0, 3, 7, and 11). (a) Ear swelling. (b) Histology. Scale bar = 50 μ m. Representative data are shown. (c-g) Serum antibody response. * $P < 0.05$ vs *Vehicle* and # $P < 0.05$ vs *OVA* by the Mann-Whitney U test. Data represent the mean $\pm$ SD of four mice per group. TS: tape-stripping.

Fig. 5. IL-33 was dispensable for responses in the model of epicutaneous sensitization to papain. Papain was applied to the surface of the right ear with tape-stripping in wild-type C57BL/6N and IL-33^{-/-} mice (Days 0, 3, 7, and 11). (a) Ear swelling. (b) Histology. Scale bar = 50 μ m. (C-E) Serum antibody response. * $P < 0.05$ vs *Vehicle (Wild-type)* by the Mann-Whitney U test. ns: not significantly different. Data represent the mean $\pm$ SD of four or five mice per group, except for one group (*Vehicle, IL-33^{-/-}*; n = 2). TS: tape-stripping.

Fig. 6. IL-33 contributed to atopic march and maintenance of IgE levels induced by papain inhalation after epicutaneous sensitization. (a) Serum antibody levels on Day 12 after epicutaneous papain sensitization. Papain (1 or 10 mg/ml) was applied to the surface of both ears with tape-stripping in wild-type C57BL/6N and IL-33^{-/-} mice (Days 0, 3, 7, and 10). (c and d) Serum antibody levels (b) and allergic airway inflammation (c) on Day 20 after the

intranasal challenge with papain. A low dose of papain (2.5 μ g) was intranasally administered (twice: Days 14 and 17) to mice presensitized with papain. * $P < 0.05$ by the Mann-Whitney U test. Data represent the mean $\pm$ SD of 5-7 mice per group. *i.n.*: intranasal administration.











