Antigen Protease Activity on Intact or Tape-Stripped Skin Induces Acute Itch and T Helper Sensitization Leading to Airway Eosinophilia in Mice

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Respiratory allergen sources such as house dust mites frequently contain proteases. In this study, we demonstrated that the epicutaneous application of a model protease antigen, papain, onto intact or tape-stripped ear skin of mice induced acute scratching behaviors and T helper (Th)2, Th9, Th17/Th22, and/or Th1 sensitization in a protease activity–dependent manner. The protease activity of papain applied onto the skin was also essential for subsequent airway eosinophilia induced by an intranasal challenge with low-dose papain. With tape stripping, papain-treated mice showed barrier dysfunction, the accelerated onset of acute scratching behaviors, and attenuated Th17/Th22 sensitization. In contrast, the protease activity of inhaled papain partially or critically contributed to airway atopic march responses in mice sensitized through intact or tape-stripped skin, respectively. These results indicated that papain protease activity on epicutaneous application through intact skin or skin with mechanical barrier damage is critical to the sensitization phase responses, including acute itch and Th sensitization and progression to the airway atopic march, whereas dependency on the protease activity of inhaled papain in the atopic march differs by the condition of the sensitized skin area. This study suggests that exogenous protease-dependent epicutaneous mechanisms are a target for controlling allergic sensitization and progression to the atopic march.

Keywords: Murine model, Protease antigen, Acute itch, Th sensitization, Atopic march

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INTRODUCTION

Biological activities derived from allergen sources (Jacquet and Robinson, 2020) or skin surface–residing *Staphylococci* (Geoghegan et al, 2018), such as protease activity, promote the sensitization and exacerbation of allergic diseases. These exogenous protease antigens disrupt airway epithelial and epidermal barriers and stimulate various types of cells directly or indirectly. In asthma models, they induce innate-type allergic inflammation (Halim et al, 2012; Oboki et al, 2010) and also promote the induction of antigen-specific T helper (Th)2-type responses and adaptive-type allergic airway inflammation (Halim et al, 2014; Kamijo et al, 2013) to which the IL-33–dependent activation of group 2 innate lymphoid cells contributes. Previous studies also examined subcutaneous or intradermal protease antigen sensitization models (Kamijo et al, 2021, 2016b; Perner et al, 2020; Sokol et al, 2008; Tang et al, 2010). However, the mechanisms underlying protease-induced epicutaneous (e.c.) sensitization remain largely unknown.

Papain is an occupational allergen that belongs to the same cysteine protease family as house dust mite major group 1 allergens (Takai and Ikeda, 2011) and *Staphylococci* cysteine proteases (Cau et al, 2021; Williams et al, 2020). Previously, some models of e.c. sensitization through ear skin using papain as a model protease antigen in C57BL/6 mice were reported (Iida et al, 2014; Ochi et al, 2017; Shimura et al, 2016), showing that papain-specific IgE/IgG1 production and the tape stripping (TS)–dependent exacerbation of skin inflammation were dependent on the protease activity of papain. The development of allergic diseases, such as asthma, rhinitis, and food allergy, after earlier e.c. presensitization to allergens is clinically known as the atopic march, a natural history of allergic diseases. In a model of the atopic march, allergic inflammation subsequently induced by the inhalation of low-dose papain after e.c. presensitization through TS-treated skin was dependent on the protease activity of papain used in an intranasal (i.n.) challenge (Nishioka et al, 2018). However, the dependencies of the other responses on papain protease activity remain unclear in these models. For example, the protease dependency of Th differentiation, for which the Th2 and Th22 subsets are

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Abbreviations: BALF, bronchial alveolar lavage fluid; DLN, draining lymph node; e.c., epicutaneous; i.n., intranasal; Th, T helper; TS, tape stripping

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known to clinically contribute to atopic dermatitis and Th17 to some subtypes of atopic dermatitis (Czarnowicki et al, 2019), has not yet been examined in models with sensitization through intact (the intact model) (Iida et al, 2014) or TS-treated (the TS model) (Nishioka et al, 2018; Shimura et al, 2016) ear skin.

An intradermal or intraepidermal injection of papain has been shown to induce acute itch in humans and mice (Aliotta et al, 2022; Arthur and Shelley, 1955). A recent study reported that an itch mediator released from skin sensory neurons after an intradermal injection of papain was responsible for the initiation of Th2 differentiation in lymph nodes (Perner et al, 2020). However, to the best of our knowledge, the capacity of papain applied onto the skin surface but not through an injection to induce itch remains unknown. In this study, using models of the e.c. application of papain, we examined the dependency of skin-draining lymph node (DLN) Th differentiation and subsequent airway inflammation on the protease activity of epicutaneously applied papain and the capacity of papain to induce itch. We also compared the dependency of the airway atopic march between the intact and TS models on the protease activity of papain used for an i.n. challenge.

RESULTS AND DISCUSSION

Th cell differentiation in mice sensitized to papain through intact or tape-stripped skin

A previous study described Th cytokine production in antigen-restimulated DLN cells in the intact and the TS models (Shimura et al, 2016). To investigate the models more precisely, we modified them with the application of higher volumes of papain to a wider ear skin area, which improved the Th cytokine responses. We epicutaneously applied papain (10 mg/ml) onto the ear skin surface with or without TS at 3- or 4-day intervals (Figure 1a). The papain application caused increased transepidermal water loss in the TS model but not the intact model (Figure 1b). Skin DLN cells stimulated in vitro with protease inhibitor—treated papain produced a variety of Th cytokines (Th2: IL-4, IL-5, and IL-13; Th9: IL-9; Th17/Th22: IL-17A and IL-22; and Th1: IFN-γ), confirming previous (Shimura et al, 2016) or very recent (Yoshimura et al, 2023) reports. We found that TS attenuated the production of Th17/Th22 and Th1 cytokines (Figure 1c).

To explore the cellular sources of cytokines, skin DLN cells (Figure 1d) were also stimulated in vitro with phorbol myristate acetate and ionomycin in the presence of a protein transport inhibitor for 6 hours and subjected to intracellular staining with antibodies specific to IL-4, IL-17A, and IFN-γ (Figure 1e and f). The results showed that Th cells ($CD3^+$ $CD4^+$ cells) were the main IL-4 source and one of the cellular sources of IL-17A and IFN-γ in papain-sensitized mice, being consistent with cytokine responses in antigen-restimulated cells, which were generally considered to be responses of antigen-specific Th cells to the exogenously added antigen (Figure 1c). In contrast to the TS-induced attenuation of IL-17A and IFN-γ production in antigen-restimulated cells (Figure 1c), TS did not significantly affect cell numbers of $IL\text{-}17A^+$ and $IFN\text{-}\gamma^+$ Th cells. This discrepancy may be attributed to nonspecific potent activity of phorbol myristate acetate and ionomycin to stimulate cells.

$CD3^+$ $CD4^-$ cells, including $CD8^+$ cytotoxic T cells and γδT cells, and $CD3^-$ cells with signature transcription factor expression of RORγt or T-bet, including innate lymphoid cells and NK cells (Spits et al, 2013) were sources of IL-17A or IFN-γ equivalent or superior to Th cells (Figure 1f). Small numbers of $CD3^+$ $CD4^-$ cells were positively stained with anti—IL-4 antibody; however, the cell types for this are unknown, and we cannot exclude the possibility that it was an artifact.

Protease activity of epicutaneously applied papain was essential for differentiation of the Th subsets

In the intact and TS models with papain sensitization through ear skin, the protease dependency of Th differentiation has not been examined. We analyzed the capacity of the protease inhibitor—treated papain, that is, E64-papain, which retains the tertiary structure and T-/B-cell epitopes of papain but lacks protease activity (Nishioka et al, 2018), to induce the responses (Figure 2a). Papain protease activity contributed to the transepidermal water loss increase along with ear swelling in the TS model (Figure 2b) and the increases in the skin DLN cell number and Th cytokine responses in antigen-restimulated DLN cells in both models (Figure 2c and d).

Next, we analyzed gene expression in ear specimens (Figure 2e and f). We demonstrated that papain protease activity induced upregulation of gene expression for Th17/Th22 and Th9 but not for Th2 and Th1 cytokines in the intact model (Figure 2f) and proinflammatory cytokines—IL-1β and IL-6—and Th cytokines—IL-4 and IFN-γ—in the TS model (Figure 2e and f). The TS-induced attenuation of Th17/Th22 gene expression (Figure 2e) was consistent with that of Th17/Th22 cytokine production in antigen-restimulated DLN cells (Figures 1c and 2d). We can only speculate that TS might decrease unknown factors, which promote Th17/Th22 and Th1 differentiation, and/or induce inhibitory factors. Upregulation of gene expression of IL-4 and IL-9 on the application of papain and/or E64-papain in the TS model suggests that TS might promote Th2 and Th9 e.c. sensitization against nonprotease antigens (Figure 2f).

The protease activity of epicutaneously applied papain was essential to induce airway atopic march responses

We also examined the contribution of the protease activity of epicutaneously applied papain to the progression of the airway atopic march. Mice presensitized through intact skin with 1 mg/ml papain showed the most significant airway eosinophilia after a subsequent i.n. challenge with low-dose papain (Suchiva et al, 2021). Therefore, we selected 1 mg/ml papain as the concentration for e.c. sensitization to investigate the contribution of the protease activity of papain to atopic march responses (Figure 3a). Mice presensitized with E64-papain with or without TS showed almost no responses for airway eosinophilia (Figure 3b) and weaker responses than papain-presensitized mice for Th2-attracting chemokine release (CCL17 and CCL22) into bronchial alveolar lavage fluid (BALF) (Figure 3c) and bronchial DLN cell Th2 and Th17 cytokine responses (Figure 3d). Mice presensitized with papain but not with E64-papain showed higher papain-specific IgE and IgG1 levels after the i.n. challenge (Figure 3e). These results demonstrated that the protease activity of papain used for e.c. sensitization through intact or

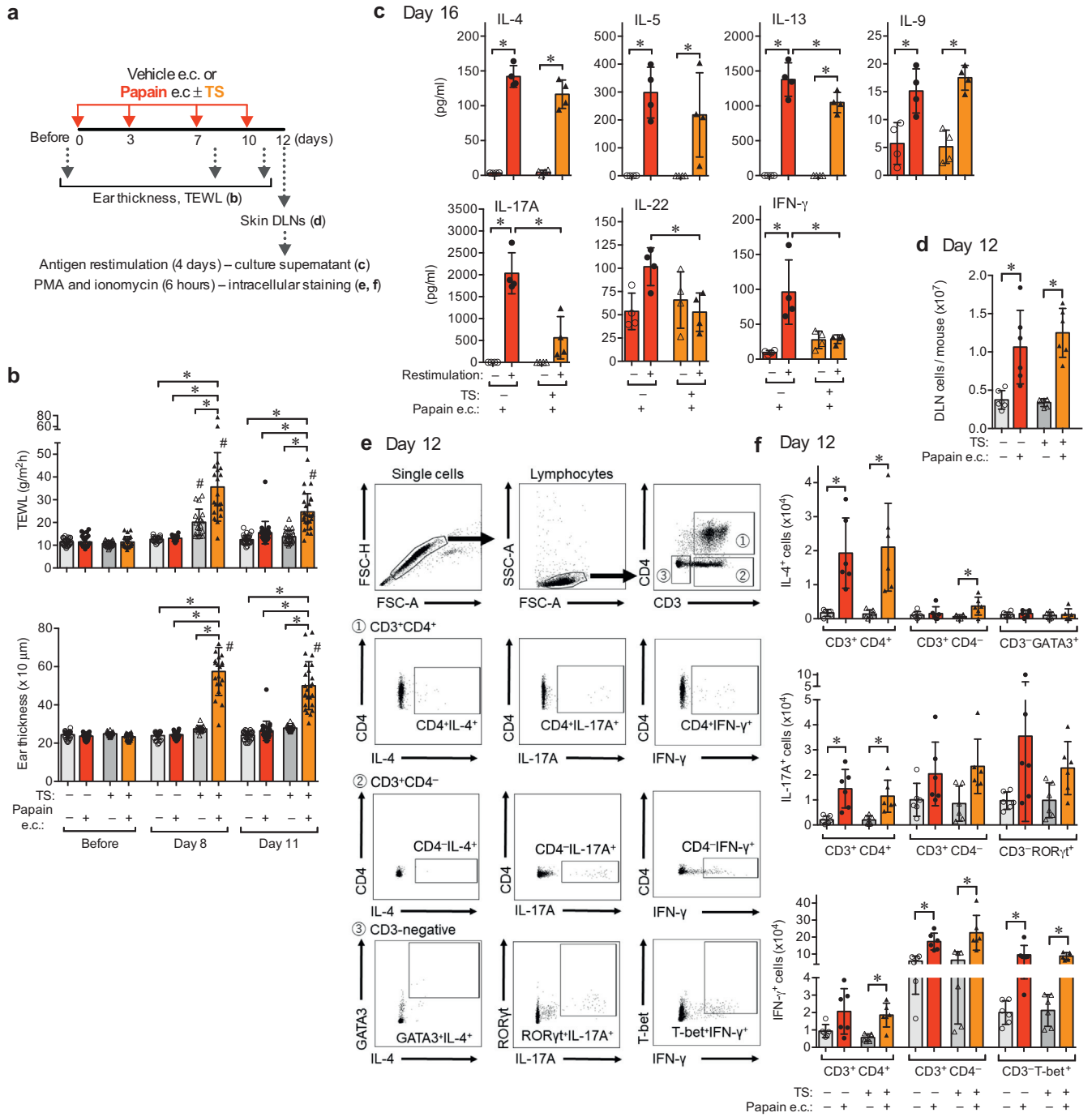

Figure 1. Th cell differentiation in mice sensitized to papain through intact or tape-stripped skin. Vehicle or papain (10 mg/ml) was applied onto intact or tape-stripped ear skin. (**a**) Timeline. (**b**) Ear thickness and TEWL (n = 18–30). (**c**) Cytokine responses in skin DLN cells restimulated with E64-papain for 4 days (4 wells). (**d**) Total DLN cell numbers recovered prior to the PMA/ionomycin stimulation and flow cytometric analysis (n = 6). (**e**) Gating strategies. Data shown are of a vehicle-treated mouse or mice showing the highest percentage for gated regions. (**f**) Flow cytometric analysis for Th cytokine–positive cells (n = 6). Data indicate means ± SD. Data are of results of (**b**) 3–5 or (**d, f**) 2 independent experiments or representative of (**c**) 2 or more independent experiments with similar results. $^{*}P < .05$ and $^{\#}P < .05$ versus (**b**) before by ANOVA among (**c**) the 4 data groups or (**d, f**) the 4 mouse groups. DLN, draining lymph node; e.c., epicutaneous; FSC-A, forward scatter area; FCS-H, forward scatter height; PMA, phorbol myristate acetate; SSC-A, side scatter area; TEWL, transepidermal water loss; Th, T helper; TS, tape stripping.

TS-treated skin was essential for progression to the airway atopic march.

We assumed that the Th2 cells contributed to the development of the airway atopic march because of significant eosinophil but little neutrophil infiltration into BALF in the models (Figure 3b). The dependency of airway eosinophilia on the protease activity of epicutaneously applied papain is not surprising because the papain-specific Th2 differentiation in the skin DLNs depended on the papain protease activity (Figure 2d). Bronchial DLN cell Th2 cytokine production was

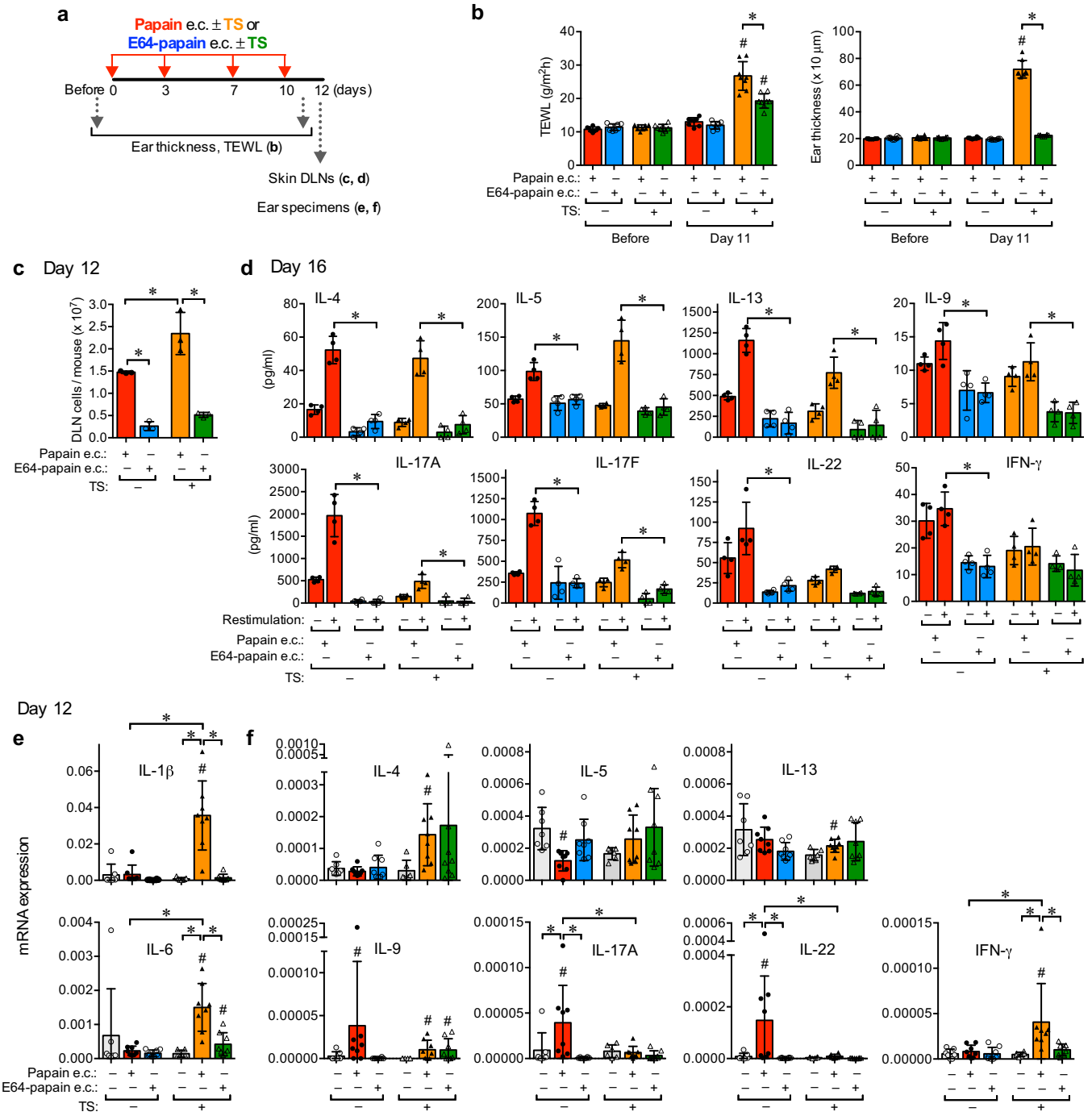

Figure 2. The protease activity of papain applied through intact or tape-stripped skin was essential to the differentiation of Th subsets. Papain or E64-papain (10 mg/ml) was applied onto intact or tape-stripped ear skin. (**a**) Timeline. (**b**) Ear thickness and TEWL (n = 8). (**c**) Total cell numbers of skin DLNs recovered. (**d**) Cytokine responses in skin DLN cells restimulated with E64-papain for 4 days (4 wells). (**e, f**) mRNA expression levels of (**e**) proinflammatory and (**f**) Th cytokines in ears relative to those of *GAPDH* (n = 6–8). Data indicate means ± SD. Data are representative of (**b, d**) 2 or more independent experiments with similar results or (**e**, **f**) results of 2 independent experiments. In **c**, each of the data points represents the average value in each of the independent experiments (n = 3). **P* < .05 by ANOVA (for **b**, **e**, and **f**). #*P* < .05 versus before by ANOVA (for **b**) or versus no antigen e.c. application by the Mann–Whitney *U* test (for **e** and **f**). DLN, draining lymph node; e.c., epicutaneous; TEWL, transepidermal water loss; Th, T helper; TS, tape stripping.

observed in mice presensitized to E64-papain, although it was lower than in papain-presensitized mice (Figure 3d), and we cannot exclude the possibility that an increase in times of e.c. application with E64-papain could lead to airway inflammation after the i.n. challenge.

A previous study reported that e.c. vaccination with papain or E64-papain through intact ear skin for 4 weeks showed prophylactic effects to attenuate airway eosinophilia and bronchial DLN cell Th2 and Th9 responses on higher-dose papain inhalation and that with papain but not with E64-papain led to

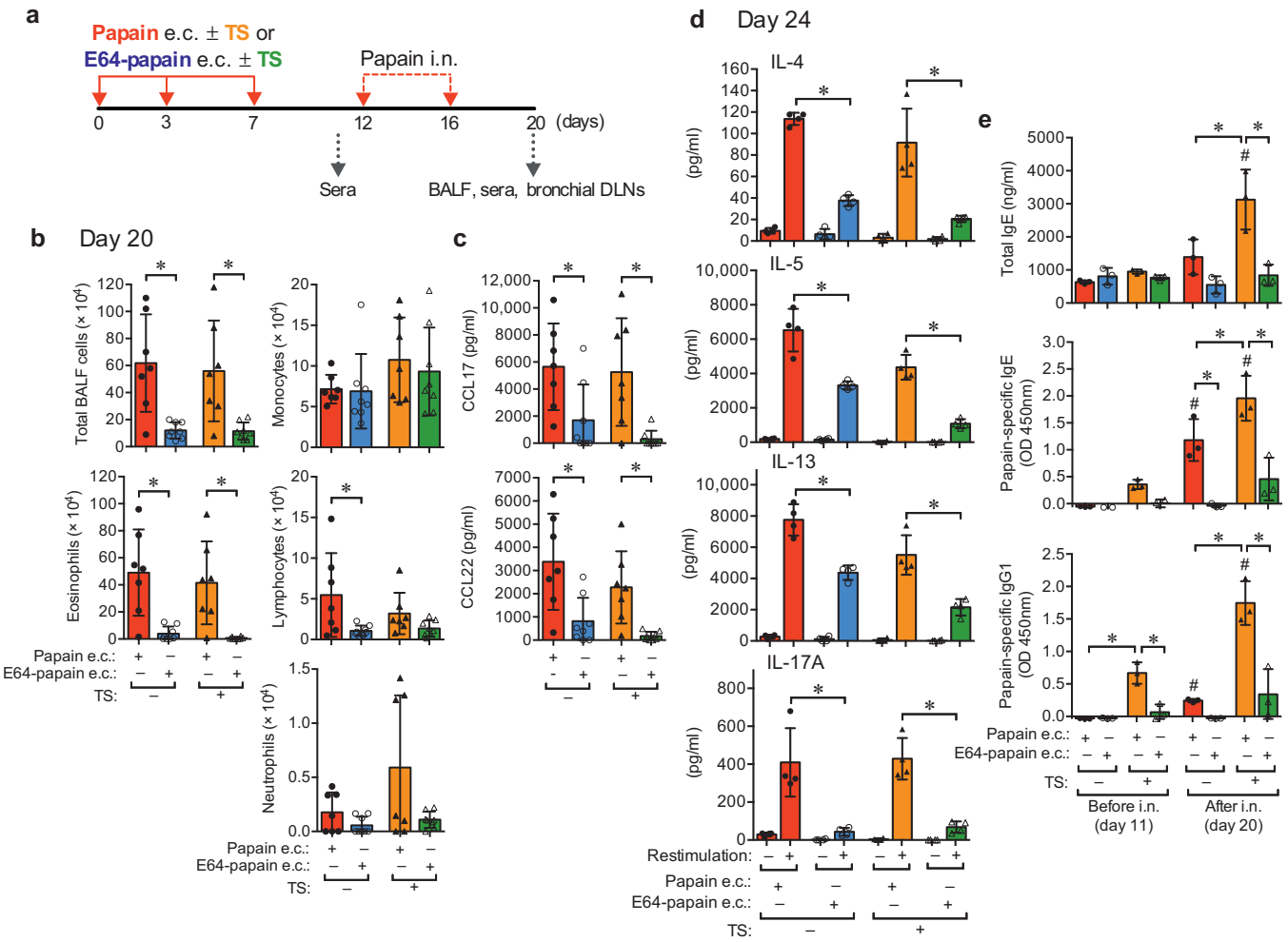

Figure 3. The protease activity of papain applied through intact or tape-stripped skin was essential to the induction of airway atopic march responses. Papain or E64-papain (1 mg/ml) was applied onto intact or tape-stripped ear skin. Mice were then intranasally administered papain (2.5 μg/mouse). (**a**) Timeline. (**b**) Airway inflammation (n = 7 or 8). (**c**) Th2-attracting chemokine release into BALF (n = 7 or 8). (**d**) Cytokine responses in bronchial DLN cells restimulated with E64-papain for 4 days (4 wells). (**e**) Serum antibodies (n = 3). Data indicate means ± SD. Data are representative of 2 or more independent experiments with similar results. *$P < .05$ by the Mann–Whitney U test (for **b** and **c**) or ANOVA (for **d** and **e**). #$P < .05$ versus before i.n. application by ANOVA (for **e**). BALF, bronchial alveolar lavage fluid; DLN, draining lymph node; e.c., epicutaneous; i.n., intranasal; Th, T helper; TS, tape stripping.

bronchial DLN cell Th17 responses (Kunimine et al, 2021). In contrast, this study demonstrated that short-term e.c. application with papain but not with E64-papain through intact ear skin led to airway eosinophilia on low-dose papain inhalation and that with E64-papain led to Th responses of bronchial DLN cells being attenuated for Th2 and markedly attenuated for Th17 (Figure 3d). Differential mechanisms of sensitization and tolerance, both of which were induced by e.c. exposure to the protease antigen, are yet to be elucidated.

The e.c. application of papain onto intact skin promoted acute itch

To evaluate the capacity of papain applied onto the skin surface to induce itch, we examined the ability of epicutaneously applied papain on intact ear skin to induce hind paw scratching behaviors (Figure 4a). One day after the fourth application of papain (0.1–10 mg/ml) through intact skin, mice showed scratching bouts equivalent to those before the first application, indicating that chronic itch was not induced (Figure 4b). We also evaluated the induction of acute itch by starting measurements immediately after the first and the fourth application of papain and continuing measurements for 3 hours (Figure 4c). The application of 10 mg/ml papain resulted in more scratching bouts than that of vehicle on the fourth application. The results shown in Figure 4b and c are for consecutive scratching bouts consisting of 4 or more strokes. Scratching occurred after waking from anesthesia, and short-term scratching bouts with a few strokes were more frequent than basal scratching in untreated mice or detergent-inducible chronic scratching, which was recently reported (Masutani et al, 2022). Reanalysis of the raw data showed more scratching bouts consisting of 3 or more strokes (Figure 4d) than bouts consisting of 4 or more strokes (Figure 4c). We also assessed the time courses for scratching behaviors consisting of 3 or more strokes (Figure 4e and f). The application of 10 mg/ml papain induced the strongest responses for bouts, strokes, and total times for scratching behaviors, and these responses were gradually enhanced and prolonged by repeated applications.

The application of vehicle induced slightly stronger responses than that of 0.1 mg/ml papain, and comparisons

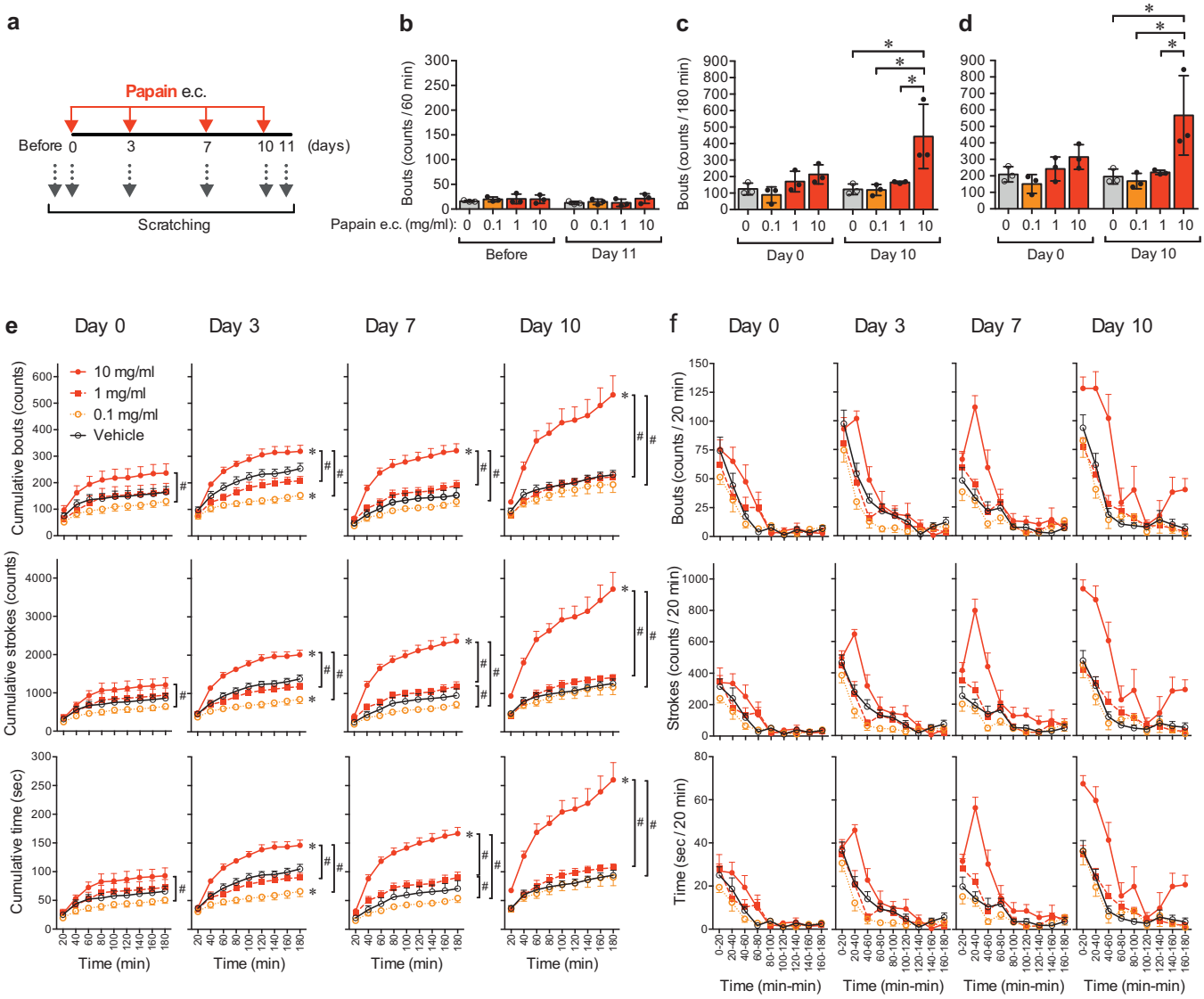

Figure 4. e.c. papain application onto intact skin promoted acute itch. Vehicle or papain (0.1−10 mg/ml) was applied onto intact ear skin. (**a**) Timeline. (**b**−**d**) Scratching bouts for (**b**) 60 min or (**c, d**) 180 min (n = 3). (**e, f**) Time course (**e**: cumulative values; **f**: values for each of the 20-min periods) (n = 7). Scratching behaviors with bouts consisting of 4 or more (**b, c**) or 3 or more (**d**−**f**) strokes are shown. In **c**−**f**, measurements were started immediately after the e.c. application. Data indicate means ± SD (for **b**−**d**) or SEM (for **e** and **f**). Data are representative of 2 independent experiments with similar results (for **b**−**d**) or results of 2 independent experiments (for **e** and **f**). *$P < .05$ by ANOVA (for **b**−**d**). *$P < .05$ versus vehicle and #$P < .05$ by ANOVA for the values obtained at 180 min (for **e**). No statistical analyses were conducted for **f**. e.c., epicutaneous; min, minute.

among the responses induced by the 3 concentrations of papain revealed the dose-dependent promotion of acute itch, suggesting that unknown properties that differed between the vehicle (PBS containing 0.5% [v/v] Tween 20) and protein-added ones affected these responses. In comparisons with the responses induced by 0.1 mg/ml papain, the application of 1 mg/ml papain showed significantly (Figure 4e, day 7, middle and bottom) or slightly stronger responses, whereas the application of 10 mg/ml papain induced significantly stronger responses even on the first application (Figure 4e, day 0). These results demonstrated that the e.c. application of papain onto even intact skin promoted acute but not chronic itch.

The protease activity of papain was essential for the promotion of acute itch, and TS accelerated its onset

We investigated the contribution of the protease activity of 10 mg/ml papain applied onto intact skin or TS-treated skin to itch responses (Figure 5a). The application of papain or E64-papain onto the skin surface with or without TS did not induce chronic itch (Figure 5b). The application of papain onto TS-treated skin induced earlier and more significant increases in acute itch responses (Figure 5c−e). By repeating applications, responses induced by papain in the intact model gradually increased to the level of those in the TS model. Increases in acute itch responses were dependent on papain protease activity. No significant differences were observed between responses induced by E64-papain and TS plus E64-papain, indicating that TS without papain protease activity did not increase acute itch responses.

These results demonstrated that the protease activity of papain applied onto intact or TS-treated skin was essential for the promotion of acute itch, and TS significantly accelerated the onset of protease activity−dependent acute itch, the latter

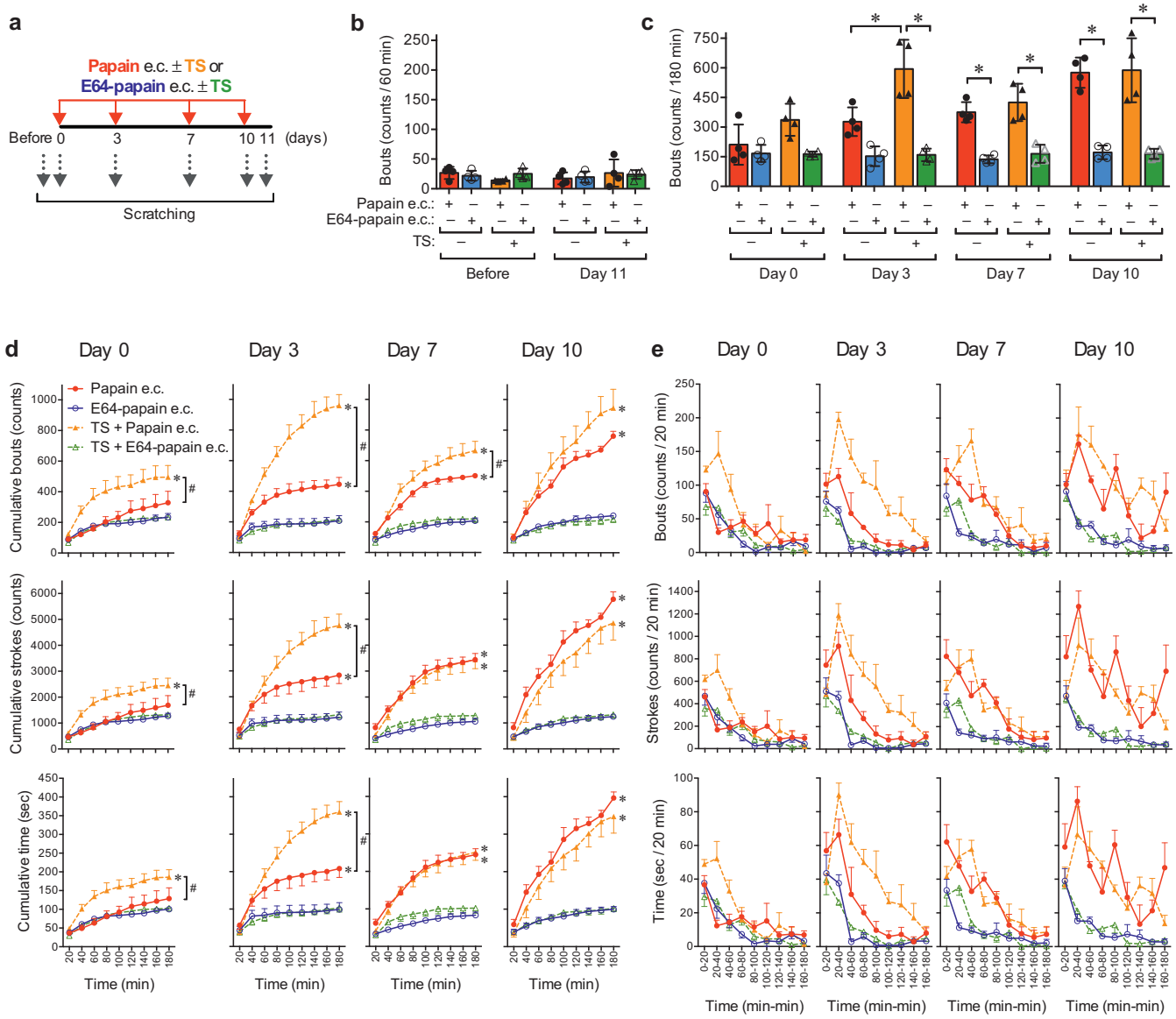

Figure 5. The protease activity of papain applied through intact or tape-stripped skin was essential for the promotion of acute itch, and TS accelerated its onset. Papain or E64-papain (10 mg/ml) was applied onto intact or tape-stripped ear skin. (**a**) Timeline. (**b, c**) Scratching bouts for 60 and 180 min, respectively. (**d, e**) Time course (**d**: cumulative values; **e**: values for 20 min). Scratching behaviors with bouts consisting of (**b, c**) 4 or more or (**d, e**) 3 or more strokes are shown. In **c**–**e**, measurements were started immediately after the e.c. application. Data indicate means ± SD (for **b** and **c**) or SEM (for **d** and **e**) (n = 4). Data are representative of 2 independent experiments with similar results. *$P < .05$ by ANOVA (for **b** and **c**). *$P < .05$ versus vehicle and #$P < .05$ by ANOVA for the values obtained at 180 min (for **d**). No statistical analyses were conducted for **e**. e.c., epicutaneous; min, minute; TS, tape stripping.

of which may be attributed to the removal of the stratum corneum (Shimura et al, 2016), facilitating the access of proteases to living epidermal cells. The exacerbation of acute scratching behaviors by the repeated application may involve sensory nerve elongation, and we cannot exclude the possible contribution of adaptive immunity through Th2-related cytokines to the promotion of itch (Agelopoulos et al, 2022; Yosipovitch et al, 2018).

A recent study reported that an itch mediator, substance P, released from skin sensory neurons after an intradermal injection of papain stimulated dendritic cells, resulting in the initiation of Th2 differentiation (Perner et al, 2020), whereas another study showed that substance P contributed to house dust mite–induced atopic dermatitis model through the stimulation of mast cells (Serhan et al, 2019). Further studies are needed to establish whether sensory neuron–derived mediators, such as substance P, contribute to Th sensitization in the e.c. sensitization models.

Different skin conditions for e.c. sensitization affected dependency levels of allergic airway inflammation on the protease activity of airway-challenged papain

The results described earlier indicated that the protease activity of papain used for e.c. sensitization on intact or TS-treated skin was essential for e.c. sensitization phase responses, including Th differentiation and acute itch (Figures 2 and 5), and the effector phase responses of the airway atopic march (Figure 3). We also examined the

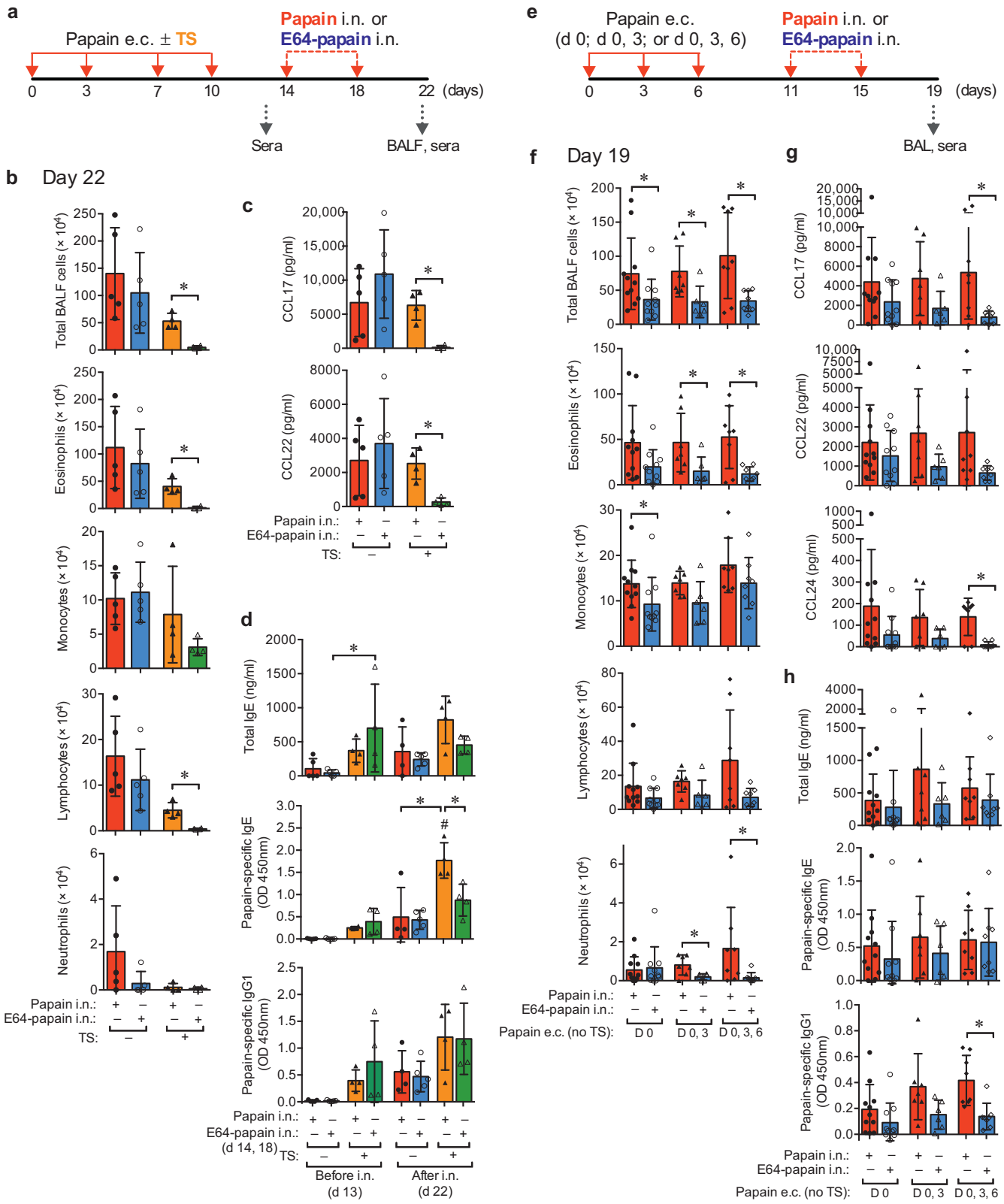

Figure 6. Different skin conditions for e.c. sensitization affected dependency levels of allergic airway inflammation on the protease activity of airway-challenged papain. Papain (1 mg/ml) was applied onto intact or tape-stripped ear skin. Mice were then intranasally administered papain or E64-papain (2.5 μg/mouse). **(a, e)** Timeline for **b**–**d** and **f**–**h,** respectively**. (b, f)** Airway inflammation. **(c, g)** Th2-attracting chemokine and **(g)** eosinophil-attracting chemokine release into BALF. (**d, h**) Serum antibodies. Data indicate means ± SD (for **b** and **c:** n = 5, 5, 4, and 4; d: n = 4 or 5; f–h: n = 11, 10, 7, 6, 8, and 8). Data are representative of 2 or more independent experiments with similar results. **P* < .05 by the Mann–Whitney *U* test (for **b**, **c**, **f**, and **g**) or ANOVA (for **d**). #*P* < .05 versus before i.n. application by ANOVA (for **d**). BALF, bronchial alveolar lavage fluid; e.c., epicutaneous; i.n., intranasal; Th, T helper; TS, tape stripping.

contribution of the protease activity of low-dose papain used in the i.n. challenge after e.c. presensitization to airway atopic march responses (Figure 6a). In mice subjected to e.c. presensitization through TS-treated skin, the i.n. challenge with E64-papain did not induce allergic airway inflammation (Figure 6b) or BALF Th2-attracting chemokine release (Figure 6c), confirming previous findings (Nishioka et al, 2018). The i.n. challenge with low-dose papain but not with E64-papain increased the serum levels of papain-specific IgE in the TS model (Figure 6d). However, unexpectedly, the i.n. challenge with papain and that with E64-papain similarly induced allergic airway inflammation and BALF Th2-attracting chemokine release in mice presensitized through intact skin (Figure 6b and c).

To examine responses in mice epicutaneously sensitized more lightly in the intact model, we examined mice with e.c. sensitization less than 4 times through intact skin (Figure 6e–h). In comparisons with the i.n. papain challenge, the i.n. E64-papain challenge resulted in the attenuation of airway eosinophilia (Figure 6f), the BALF release of Th2-attracting chemokines and the eosinophil chemotactic protein-2 (CCL24) (Kurowska-Stolarska et al, 2009) (Figure 6g), and papain-specific IgG1 levels (Figure 6h). However, the E64-papain challenge after the e.c. presensitization still induced the marked infiltration of eosinophils and chemokine release in some mice (Figure 6f and g) in contrast to no or negligible responses in mice epicutaneosuly sensitized through TS-treated skin (Figure 6b and c). These results indicated that different conditions in the skin area for e.c. sensitization affected the dependency levels of allergic airway inflammation on the protease activity of intranasally challenged papain.

Protease activity of inhaled antigens evokes protease-mediated alarm, such as release and activation of IL-33 (Cayrol et al, 2018; Chen et al, 2022; Halim et al, 2014; Kamijo et al, 2013), presumably through damage to the lung epithelium, such as disruption of the tight junction barrier (Takai and Ikeda, 2011). This could be the mechanism for the significant or partial dependency of the airway eosinophilia on the protease activity of inhaled papain in the TS (Figure 6b) (Nishioka et al, 2018) or intact model (Figure 6f), respectively. However, mechanisms for the airway inflammation on E64-papain inhalation in the intact model, which was equivalent to that on papain inhalation (Figure 6b) or still partially remained (Figure 6f), are yet to be elucidated.

In conclusion, in this study, we demonstrated that the protease activity of papain applied onto intact or tape-stripped ear skin of mice was essential to promote acute itch; the differentiation of Th subsets, including Th2, Th9, Th17/Th22, and/or Th1; and subsequent allergic airway inflammation. Papain protease activity also contributed to the barrier dysfunction in the TS model. Taken together with previous findings of protease activity–dependent IgE/IgG1 production and skin inflammation (Iida et al, 2014; Shimura et al, 2016), these results indicated that the protease activity of papain used for e.c. sensitization was essential for all e.c. sensitization phase responses. In contrast, the protease activity of papain used for the subsequent i.n. challenge was not always essential to induction of allergic airway inflammation in the effector phase of the atopic march in the intact model. Therefore, this study suggests that exogenous protease–dependent e.c. mechanisms, which largely remain unknown, are a potential target for controlling allergic sensitization and progression to the atopic march. In addition, we found TS-induced attenuation of production in antigen-restimulated skin DLN cells and ear tissue gene expression of Th17/Th22 cytokines.

A more detailed understanding of the mechanisms underlying the protease activity–dependent initiation of the Th responses and skin inflammation, which may be attributed not only to barrier disruption (Hirasawa et al, 2010; Nakamura et al, 2006; Stremnitzer et al, 2015) but also to the activation of various types of cells (Kamijo et al, 2021, 2016a; Perner et al, 2020; Serhan et al, 2019; Takai and Ikeda, 2011), will provide insights into allergic sensitization mechanisms after e.c. exposure to protease allergens or skin-colonized protease-producing *Staphylococci* that are relevant in atopic dermatitis and the atopic march. Very recently, Yoshimura et al (2023) reported another model with papain sensitization through detergent-treated skin, resulting in protease activity–dependent severe dermatitis with chronic itch and robust Th17/Th22 sensitization and protease activity–independent Th2 sensitization. Thus, the 3 models of the present intact and TS models and the detergent model show different features. Differential mechanisms among the models with different skin conditions for e.c. sensitization would be addressed in future studies.

MATERIALS AND METHODS

Antigens

Papain was purchased from Calbiochem. E64-papain, the covalent complex between the cysteine protease inhibitor E-64 (Peptide Institute) and papain, was prepared as previously described (Kamijo et al, 2013). Papain, which was incubated similarly to E64-treated papain (but without the addition of E-64) and dialyzed, was prepared and used for comparisons with E64-treated papain.

Mice

C57/BL6J female mice aged 7–12 weeks (Sankyo Lab Service) were maintained in a specific pathogen-free animal facility.

e.c. sensitization

The present models had a minor modification, namely, the application of higher volumes of papain to a wider ear skin area. Vehicle (PBS containing 0.5% [v/v] Tween 20), papain, or E64-papain was applied onto ear skin between 1 and 4 times with 3- or 4-day intervals with or without TS 3 times using cellophane tape (cellotape, Nichiban) before the e.c. application of the antigen. These solutions were applied with a micropipette to both sides of the surfaces of both ears and the dorsal hairless area at the base of the ear of lightly anesthetized mice (30 μl/ear, 60 μl/mouse). Aliquots of antigen solution were stored at −80 °C and thawed just before use.

Ear thickness, barrier dysfunction, and histology

The ear thickness of lightly anesthetized mice was measured using a dial thickness gauge (G-1A, Ozaki). Regarding barrier dysfunction, transepidermal water loss on the dorsal side of the ear lobe was measured using a VapoMeter (Delfin Technologies) in nail mode.

Scratching behaviors

Scratching behaviors with the hind paws were analyzed using the MicroAct system (Neuroscience) according to a previously

described method (Masutani et al, 2022) with modifications. Briefly, a cylindrical magnet was subcutaneously implanted into each of the insteps of mice. Each mouse was placed in an acryl chamber, which was surrounded by a detection coil set. After allowing 20–30 minutes for habituation, measurements were started. To assess acute itch, measurements were started immediately after the e.c. application and were continued for 60 or 180 minutes to assess chronic or acute itch, respectively. Scratching bouts, strokes, and the total time for scratching behaviors were assessed using software (ANIMA, Takeda LabDesign). In the setting of parameters to omit false-positive signals, the apparatus detected consecutive scratching behaviors consisting of 4 or more strokes as chronic itch or 3 or more strokes as acute itch.

Antigen restimulation of DLN cells

Sera and skin or bronchial DLNs (cervical or mediastinal lymph nodes, respectively) were collected and stimulated as previously described with modifications (Nishioka et al, 2018; Ochi et al, 2017). Briefly, skin and bronchial DLN cells were restimulated with medium alone or E64-papain (50 and 25 μg/ml, respectively) in 96-well round-bottomed culture plates for 96 hours (5×10^5 cells/200 μl/well). E64-papain was used as the antigen for the restimulation to avoid potential protease activity–dependent effects. DLN cells from mice in each of the mouse groups were pooled and stimulated in 3 or 4 wells.

Flow cytometry and antibodies

Ear skin-draining cervical lymph nodes were aseptically recovered. Single-cell suspensions were prepared by gently teasing apart the tissue in culture media (RPMI-1640 medium [Sigma] supplemented with 2 mM L-glutamine, 10% [v/v] heat-inactivated fetal bovine serum, 10 mM 4-(2-Hydroxyethyl)piperazine-1-ethane-sulfonic acid, 0.05 mM 2-mercaptoethanol, and antibiotics), that is, the homogenization of DLNs by sandwiching and crushing them between 2 glass slides and passage through EASYstrainer (70-μm pore size, Greiner Bio-One). After centrifugation at 300*g* at 4 °C for 5 minutes, cells were resuspended in ACK buffer at room temperature (2 ml, 2 minutes) to deplete red blood cells. After counting the cells, DLN cells were stimulated with phorbol myristate acetate (50 ng/ml) and ionomycin (1 μg/ml) in the presence of BD Golgi plug (BD Biosciences) for 6 hours.

The cell surface was stained with FITC- or phycoerythrin-Cy7–conjugated anti-mouse CD3 (clone: 145-2C11, BioLegend) and PerCP-Cy5.5–conjugated anti-mouse CD4 (clone: RM4-5, BD Biosciences). After surface staining, washing, fixation, and permeabilization with BD Cytofix/Cytoperm reagents (BD Biosciences), intracellular IL-4, IL-17A, IFN-γ, GATA-3, RORγt, and T-bet were stained with phycoerythrin-conjugated anti-mouse IL-4 (clone: 11B11, BD Biosciences) or phycoerythrin-conjugated rat IgG1 isotype control (BioLegend), allophycocyanin-conjugated anti-mouse IL-17A (clone: eBio17B7, Thermo Fisher Scientific) or allophycocyanin-conjugated rat IgG2a isotype control (BD Biosciences), FITC-conjugated anti-mouse IFN-γ (clone: XMG1.2, BD Biosciences) or FITC-conjugated rat IgG1 isotype control (BD Biosciences), eFluor 660–conjugated anti-mouse GATA-3 (clone: TWAJ, Invitrogen) or eFluor 660–conjugated rat IgG2b isotype control (Thermo Fisher Scientific), phycoerythrin-conjugated anti-mouse RORγt (clone: B2D, Invitrogen) or phycoerythrin-conjugated rat IgG1, and phycoerythrin-conjugated anti-human/mouse T-bet (clone: 4B10, BioLegend) or phycoerythrin-conjugated mouse IgG1 isotype control (BioLegend). Acquisition and analysis were performed using a FACSVerse cell sorter and FlowJo software (BD Biosciences).

qPCR

Ear lobes were excised and homogenized in TRI Reagent (Molecular Research Center) using TissueLyser II (Qiagen). Total RNA was extracted from homogenized skin or lung tissue specimens using an RNeasy Plus Micro Kit (Qiagen). First-strand cDNA was synthesized from total RNA using a ReverTra Ace qPCR RT Master Mix (Toyobo). Real-time quantitative PCR was performed with the TaqMan method using a StepOnePlus real-time PCR system (Applied Biosystems). The mRNA levels of the target genes were shown as values relative to the gene expression levels of the endogenous control (*GAPDH*). The following probes were purchased from Applied Biosystems: IL-1β (Mm00434228_m1), IL-6 (Mm99999064_m1), IL-4 (Mm00445259_m1), IL-5 (Mm99999063_m1), IL-13 (Mm00434204_m1), IL-9 (Mm00434305_m1), IL-17A (Mm00439618_m1), IL-22 (Mm00444241_m1), IFN-γ (Mm00801778_m1), and GAPDH (predeveloped TaqMan Assay Reagents Mouse GAPDH).

i.n. challenge and bronchial alveolar lavage

Epicutaneously sensitized mice with light anesthesia were subjected to an i.n. challenge with low-dose papain resolved in PBS (2.5 μg/50 μl/mouse) after the last e.c. administration twice with a 4-day interval. Sera and BALF (1 ml/mouse) were collected 4 days after the last i.n. challenge and analyzed as previously described (Maruyama et al, 2019).

ELISA

Serum total IgE and papain-specific antibodies were detected on plates, which were coated with 2 μg/ml anti-murine IgE mAbs or 10 μg/ml papain as previously described (Kamijo et al, 2021; Shimura et al, 2016; Takai et al, 2005) with modifications. Total IgE was measured by sandwich ELISA. Serum dilution factors for the detection of antigen-specific IgE, IgG1, IgG2b, and IgG2c were 50, 5000, 250, and 250, respectively. Absorbance at 450 nm, from which that at 570 nm was subtracted, was used as the signal. Cytokine and chemokine concentrations were measured with ELISA kits (R&D Systems), except for IL-9 (BioLegend).

Statistical analysis

The Student's *t*-test (two tailed), the Mann–Whitney *U* test (two tailed), or a one-way ANOVA with Tukey's posthoc test was used. A value of $P < .05$ was considered to be significant.

Ethics statement

All animal experiments were approved by the Committee on Animal Experiments of Juntendo University.

Data availability statement

No large datasets were generated or analyzed during this study. Minimal datasets necessary to interpret and/or replicate data in this paper are available upon request to the corresponding author.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: TT, TK, SK; Data Curation: TK, TT, SK, TY, YM, SS, KT, PS; Formal Analysis: TK, TT, SK, TY, YM, SS, KT, PS; Funding Acquisition: TT, SK, KO, SI; Investigation: TK, TT, SK, TY, YM, SS, KT, PS; Methodology: TK, TT, SK, TY, YM, SS, KT, PS; Project Administration: TT, TK, SK, KO, SI, HO; Supervision: TT, KO, SI; Visualization: TK, TT, SK; Writing - Original Draft Preparation: TK, TT, SK; Writing - Review and Editing: TT, TK, SK, TY, YM, SS, KT, PS, HO, KO, SI

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