Papain Degrades Tight Junction Proteins of Human Keratinocytes *In Vitro* and Sensitizes C57BL/6 Mice via the Skin Independent of its Enzymatic Activity or TLR4 Activation

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Papain is commonly used in food, pharmaceutical, textile, and cosmetic industries and is known to induce occupational allergic asthma. We have previously shown that the papain-like cysteine protease Dermatophagoides pteronyssinus 1 from house dust mite exhibits percutaneous sensitization potential. We aimed here to investigate the potential of papain itself in epicutaneous sensitization. The effects of papain on tight junction (TJ) proteins were tested *in vitro* in human primary keratinocytes. Using C57BL/6 wild-type and Toll-like receptor 4 (TLR4)-deficient mice, we analyzed the sensitization potential of papain, its effects on the skin barrier, and immune cell recruitment. Our results show that papain affects the skin barrier by increasing transepidermal water loss, degrading TJ proteins and inducing vasodilation. When topically applied, papain exhibited a high epicutaneous inflammatory potential by recruiting neutrophils, mast cells, and CD3-positive cells and by induction of a T_H2-biased antibody response. However, its high potency for specific sensitization via the skin was TLR4 independent and, in spite of its capacity to degrade epidermal TJ proteins, does not rely on its enzymatic function. From our data, we conclude that papain has all features to act as a strong allergen via the skin.

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INTRODUCTION

Cysteine proteases like Dermatophagoides pteronyssinus (Der p) 1 and papain (proteins from house dust mite and papaya, respectively) are connected with IgE-mediated allergy (Roelandt *et al.*, 2008). Natural protease allergens of the peptidase C1 family, such as Der p 1, bromelain (Zentner *et al.*, 1997; Nettis *et al.*, 2001), actinidain (Mills *et al.*, 2004; Tuppo *et al.*, 2008), were modeled on papain and represent its structural and functional homologues (Mills *et al.*, 2004). In asthma models, the roles of cysteine proteases in T_H2-immune responses have been described (Wan *et al.*, 1999; Asokananthan *et al.*, 2002; Kouzaki *et al.*, 2009; Post *et al.*, 2012).

Papain is concentrated in the skin of papaya fruit and is commonly applied in pharmaceutical, food, cosmetic, and textile industry (Ramundo and Gray, 2008; Jeong and Hur, 2010). It is an ingredient in cosmetic exfoliants or bleaching cremes, although reports about allergic responses have been published (Santucci *et al.*, 1985; Niinimaki *et al.*, 1993; Reijula *et al.*, 1993; van Kampen *et al.*, 2005; Rosenthal and Blond, 2008).

In recent studies, papain was successfully used as a surrogate for exogenous proteases from mite allergen extracts (Miike and Kita, 2003; Kouzaki *et al.*, 2009; Cunningham *et al.*, 2012) or to substitute for endogenous proteases, such as mast cell tryptase (Reed and Kita, 2004; Cunningham *et al.*, 2012), reporting various strong immune responses to papain. Papain has been shown to induce degranulation of human eosinophils (Miike and Kita, 2003), trigger thymic stromal lymphopoietin release (Kouzaki *et al.*, 2009), and activate

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Abbreviations: Der p, Dermatophagoides pteronyssinus; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; TEWL, transepidermal water loss; TJ, tight junction; TLR4, Toll-like receptor 4; TRITC, tetramethylrhodamine; WT, wild type

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protease-activated receptor-2 (Liang *et al.*, 2012). In other studies, subcutaneous immunization with papain or bromelain in combination with a second antigen (e.g., ovalbumin) generated reactive oxygen species, leading to activation of dermal dendritic cells, basophils, and formation of IgE to the secondary antigen (Secor *et al.*, 2008; Sokol *et al.*, 2008, 2009; Tang *et al.*, 2010). The study by Tang *et al.* has further suggested a strong link between reactive oxygen species, oxidized phospholipids, Toll-like receptor 4 (TLR4)-, and TIR-domain-containing adapter-inducing interferon-β-based signaling in the induction of T_H2 responses after subcutaneous stimulation with papain.

Although valuable for studying immunological mechanisms, intradermal or subcutaneous sensitization models (Chambers *et al.*, 1998; Sokol *et al.*, 2008; Tang *et al.*, 2010) do not reflect the natural encounter to common allergens. However, truly epicutaneous sensitization models to relevant allergens are not well established so far. They often rely on artificial barrier disruption, and in most studies the food allergen ovalbumin has been used (Wang *et al.*, 2009; Lin *et al.*, 2012; Matsumoto and Saito, 2013; Savinko *et al.*, 2013). We recently succeeded establishing a percutaneous sensitization protocol for Der p 1 (Szalai *et al.*, 2012).

Considering the great structural homology with Der p 1 and its abundance and impact on daily exposure, we investigated in this study whether papain could be a relevant skin allergen. Hence, we studied its effect on the epidermal barrier, and its potential to trigger inflammation and an allergic response in an adjuvant-free epicutaneous sensitization mouse model. We analyzed recruitment of effector cells into the skin upon first encounter and after repeated epicutaneous papain applications and investigated the potential contribution of TLR4 and its cofactor lipopolysaccharide (LPS) by using TLR4-deficient C57BL/6 mice versus wild-type (WT) C57BL/6 mice.

RESULTS

TEWL is increased after treatment with papain

The epidermis, and more specifically the stratum corneum, represents an important barrier of the body that separates "inside" and "outside". Impairment of the epidermis leads to increased transepidermal water loss (TEWL), and proteases are known to affect the integrity of epithelia (Proksch *et al.*, 2008, 2009).

To test the effect of papain on the skin barrier, WT C57BL/6 mice were treated epicutaneously with 15 μg papain or in the presence of the cysteine protease inhibitor E-64 (which irreversibly blocks enzymatic activity (Varughese *et al.*, 1989)) on the back skin. TEWL was substantially increased 30 and 60 minutes after treatment with active papain, indicating an impairment of the epidermal barrier, whereas inactivated papain slightly elevated TEWL (Figure 1a). Controls treated only with buffer (distilled water) or E-64) also exhibited only minor increases in TEWL 30 minutes after treatment (Supplementary Figure S1A online).

Papain induces breakdown of tight junction proteins

Tight junctions (TJs) are cell–cell junctions controlling the epithelial paracellular pathway of molecules (Proksch *et al.*, 2008; Brandner, 2009; Kirschner *et al.*, 2010) and are essential for epithelial barrier integrity. To test the effect of papain treatment on TJs, we treated differentiated primary human keratinocytes with different doses of papain and analyzed cell lysates 30 minutes later by western blot.

Papain (at concentrations as low as 1 μg ml^{-1}) caused degradation of TJ proteins zonula occludens-1, claudin-4, and occludin in keratinocytes *in vitro* (Supplementary Figure S1

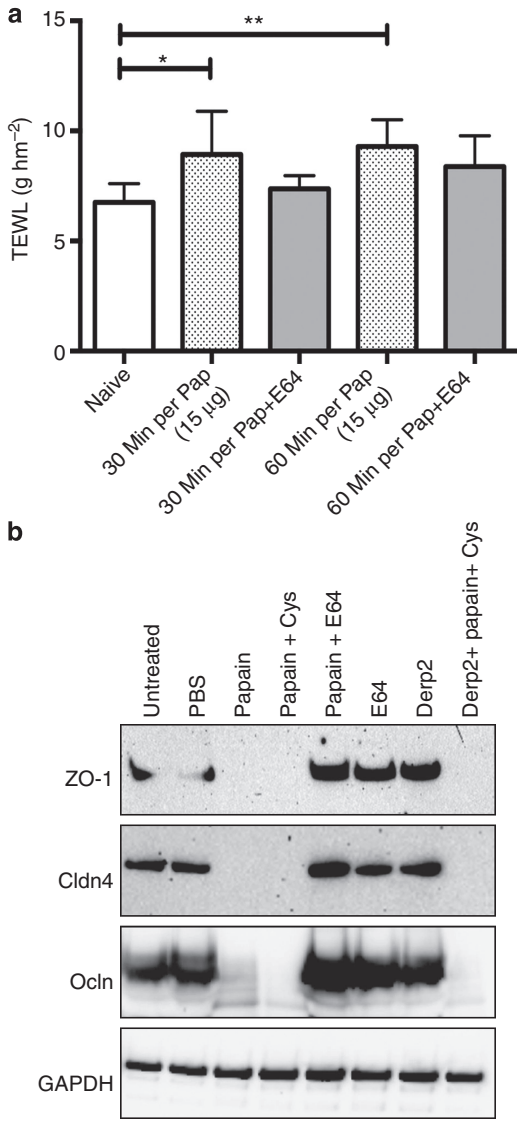

Figure 1. Immediate effects of papain on the skin barrier and tight junction proteins. (**a**) WT C57BL/6 mice ($n = 5$–8 per group) were treated topically with 15 μg active or E64-inactivated papain, and skin permeability was analyzed by measurement of TEWL. Median+Max./Min.; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (**b**) Primary keratinocytes from healthy human donors were treated with 1 μg ml^{-1} papain for 30 minutes, leading to breakdown of TJ proteins ZO-1, claudin-4, and occludin. Der p, Dermatophagoides pteronyssinus; max., maximum; min., minimum; PBS, phosphate-buffered saline; TEWL, transepidermal water loss; TJ, tight junction; ZO-1, zonula occludens-1, Cldn4, claudin-4; Ocln, occludin.

online), visible as a decrease in band intensity and degradation products for zonula occludens-1. Antibodies to claudin-4 and occludin failed to detect any degradation products, possibly because of epitope loss (data not shown).

Whereas additional papain activation with cysteine (for reactivation of thiol groups (Yasuhara *et al.*, 2001; Cunningham *et al.*, 2012)) did not significantly enhance enzymatic activity of papain, E-64 treatment blocked the degradation of TJ proteins by papain (Figure 1b). Of note, controls (untreated, phosphate-buffered saline (PBS), E-64, rDer p 2 as allergen control) did not degrade zonula occludens-1, claudin-4, or occludin (Figure 1b).

Epicutaneously applied papain induces a strong T_H2-biased antibody response

We next tested the epicutaneous sensitization potential of papain in a mouse model of allergic dermatitis (Szalai *et al.*, 2012).

WT C57BL/6 mice were sensitized and rechallenged (Supplementary Figure S2A–S2B online) with 15 μg papain or with controls—namely, E-64-inactivated papain, PBS, or allergen-control rDer p 2, which by its completely different molecular mechanism (acting via TLR4) was chosen to compare the mechanism and specificity of a papain-induced immune response. Papain-specific IgG1 and IgE antibodies were detectable after the third immunization, and a strong T_H2-biased antibody response had developed after the 4th immunization (Figure 2). Inhibition of enzymatic activity by E-64 treatment resulted in significantly decreased specific IgG1 and increased total IgE levels, whereas amounts of specific IgE were not influenced.

Using epidermal lysates of WT C57BL/6 mice, development of auto-antibodies due to breakdown of skin proteins by papain was analyzed by a dot-blot assay. We could not detect any immune response toward endogenous epitopes in sera pools of immunized mice (Supplementary Figure S3 online).

Papain challenge thickens the epidermis and induces mast cell infiltration

Skin sections were evaluated for epidermal layers and dermal thickening.

Papain-treated groups showed no increase in epidermal layers or increased dermal thickening, whereas hyperkeratinization characterized by additional epidermal layers could be observed in inactivated-papain-treated groups (Figure 3a). Numbers of eosinophils were not increased (data not shown), but mast cells tended to infiltrate the skin upon papain treatment. Notably, significantly more mast cells infiltrated the skin of mice sensitized with inactivated papain than of mice treated with papain active (Figure 3c).

Repeated application of papain triggers a moderate influx of $CD3^+$ T cells, Ly-$6G^+$, and $Lang^+$ cells into the epidermis and to some extent augments levels of CCL8 and numbers of $Lang^+$ cells in the dermis

The phenotype of T_H2 inflammation in the skin (Wang *et al.*, 2007; Jin *et al.*, 2009; Wang *et al.*, 2009) is characterized by

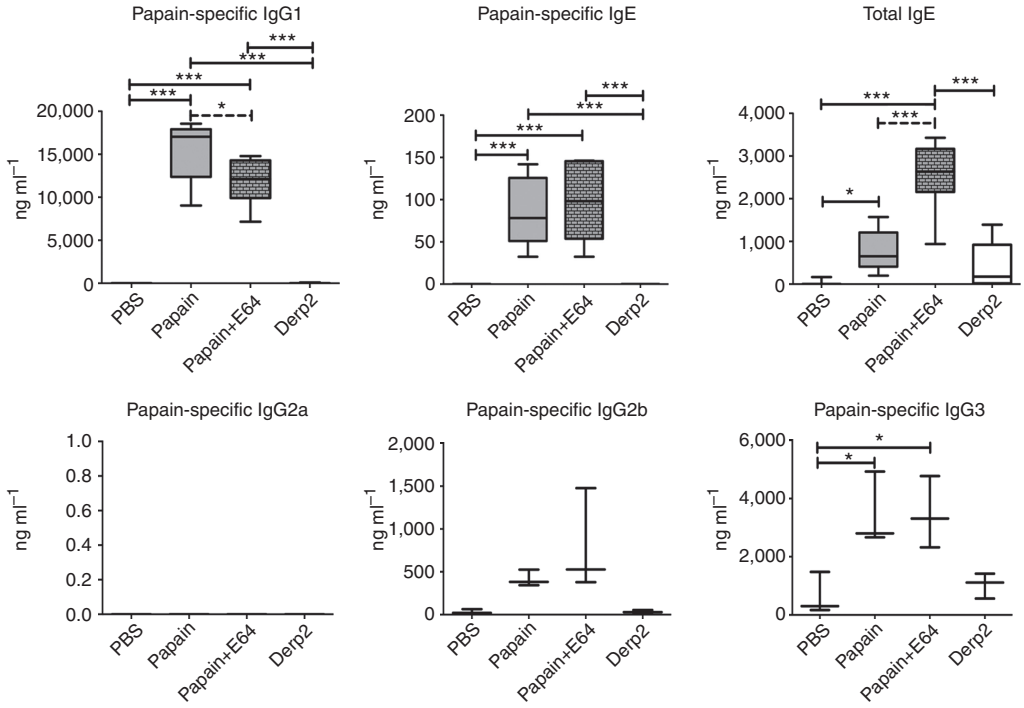

Figure 2. Papain induces specific T_H2-biased antibodies and high levels of total serum IgE, independent of enzymatic activity. Serum samples of all animals were collected before sensitization (naive; not shown) and after sensitization. Levels of papain-specific IgG1, IgE, and total IgE, as well as IgG2a, IgG2b, and IgG3, were determined by ELISA. Sera of all mice were tested separately in duplicates. Median+Max./Min.; $*P<0.05$; $**P<0.01$; $***P<0.001$. Der p, Dermatophagoides pteronyssinus; max., maximum; min., minimum.

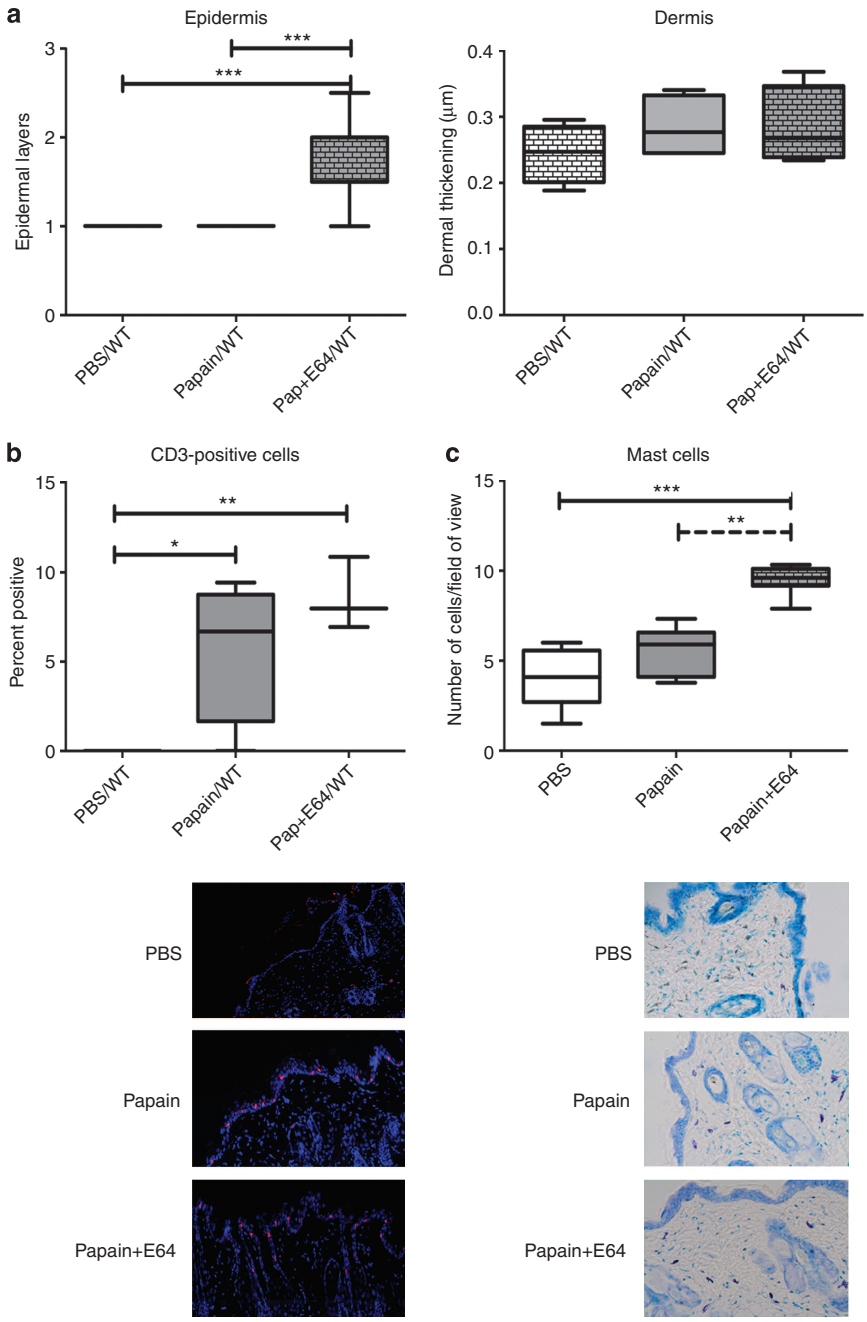

Figure 3. Alterations of skin structure and infiltrating cells after sensitization and re-challenge with papain. (**a**) Paraffin-embedded, H&E-stained skin samples of papain-sensitized and -challenged mice were evaluated for a number of epidermal layers and dermal thickening. (**b**) Immunofluorescence staining revealed protease activity–independent infiltration by $CD3^+$ T cells into the epidermis (left); ×20. (**c**) Paraffin-embedded, Giemsa-stained skin samples of papain-sensitized and -challenged mice were evaluated for mast cell numbers. Representative data from one mouse per group are shown below; ×40. Median+Max./Min.; $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. H&E, hematoxylin and eosin; PBS, phosphate-buffered saline.

cutaneous hyperplasia, hyperkeratinization, and cellular infiltrate consisting of $CD3^+$ T cells and eosinophils. Other cells being involved in the inflammatory response are neutrophils and—as major antigen-presenting cells in the skin—dendritic cells. Both, papain and inactivated papain, significantly increased the number of $CD3^+$ T cells infiltrating the epidermis (Figure 3b). Preliminary data, using flow cytometry, showed that active papain induces a mixed T-cell infiltration comparable to another allergen (Der p 2) in context with LPS (data not shown).

Papain but not inactivated papain showed a trend to attract more $Lang^+$ cells into the epidermis (Figure 4a), but $Ly\text{-}6G^+$ cells were more likely attracted into the epidermis by inactivated papain (Figure 4b).

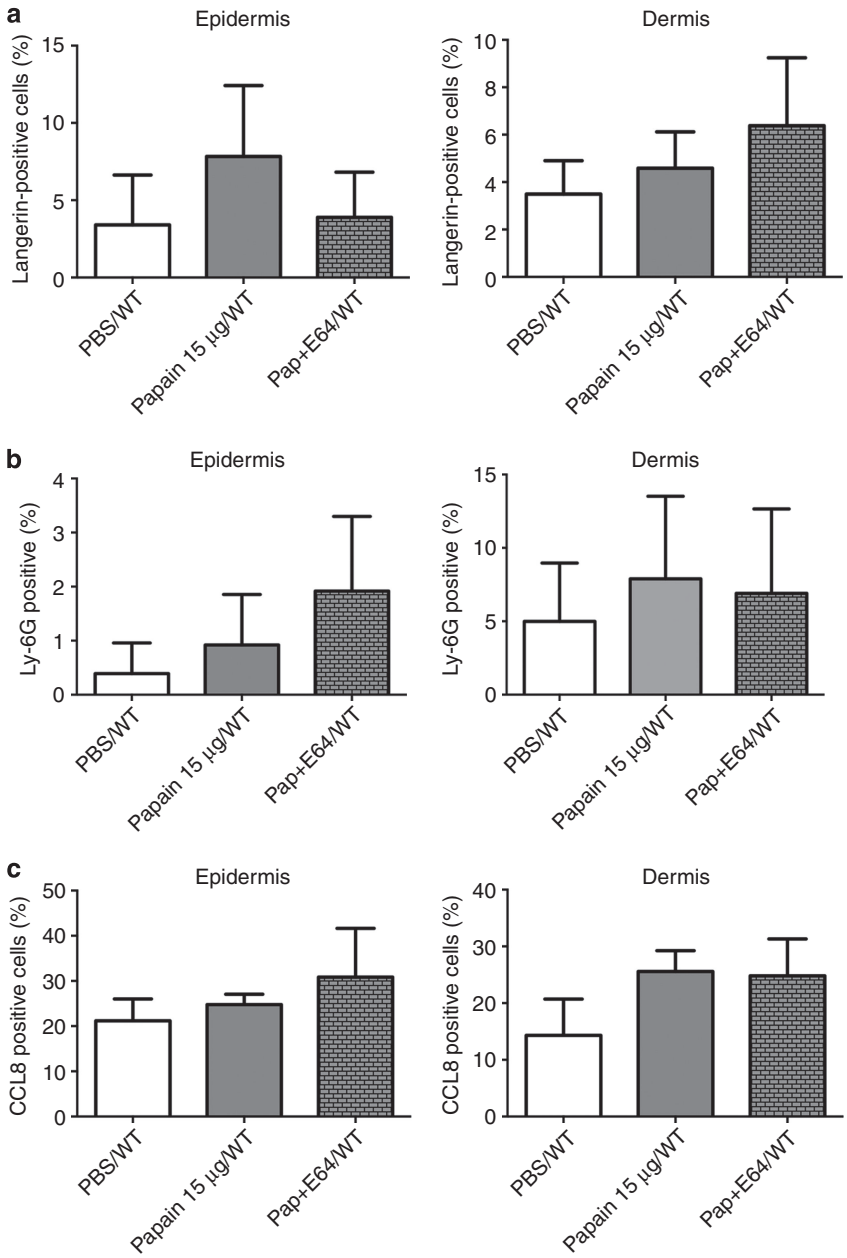

Figure 4. Evaluation of CCL8$^+$ cells and infiltration of dendritic cells and neutrophils in the skin. Skin sections were embedded in paraffin and stained ($n = 4$–5 per group) with anti-Langerin (**a**), and anti-Ly-6G (**b**) and anti-CCL8 antibody (**c**), scanned, and evaluated using Histoquest analysis program. The epidermis and dermis were evaluated separately. Median+Max./Min.; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Max., maximum; min., minimum; WT, wild type.

Several studies (Wang *et al.*, 2007; Dean *et al.*, 2008; Debes and Diehl, 2011; Islam *et al.*, 2011) underlined the CCR8-CCL8 (monocyte chemotactic protein-2) axis in chronic inflammatory processes in response to allergens. Both papain-treated groups displayed recruition of CCL8$^+$ cells into the epidermis and more pronounced, but still not significant, to the dermis (Figure 4c)

Papain treatment did not influence Ly-6G$^+$ cell numbers but produced increased Lang$^+$ cell numbers in the dermis. This response seemed to be stronger after application of E-64-inactivated papain (Figure 4a).

Papain increases vascular permeability and immediately recruits neutrophil granulocytes into the skin

Two-photon microscopy using Lys-eGFP (enhanced green fluorescent protein) mice enabled tracking of neutrophil granulocytes by *in-vivo* imaging. High molecular weight dextran tetramethylrhodamine (TRITC) (155 kDa) was administered intravenously to analyze a possible increase in vascular permeability induced by papain. Depilated ears were treated with papain (30 µg or 15 µg), inactive papain (inhibited by 25-molar E-64 (Cunningham *et al.*, 2012)), or sham control (PBS or 0.9%NaCl).

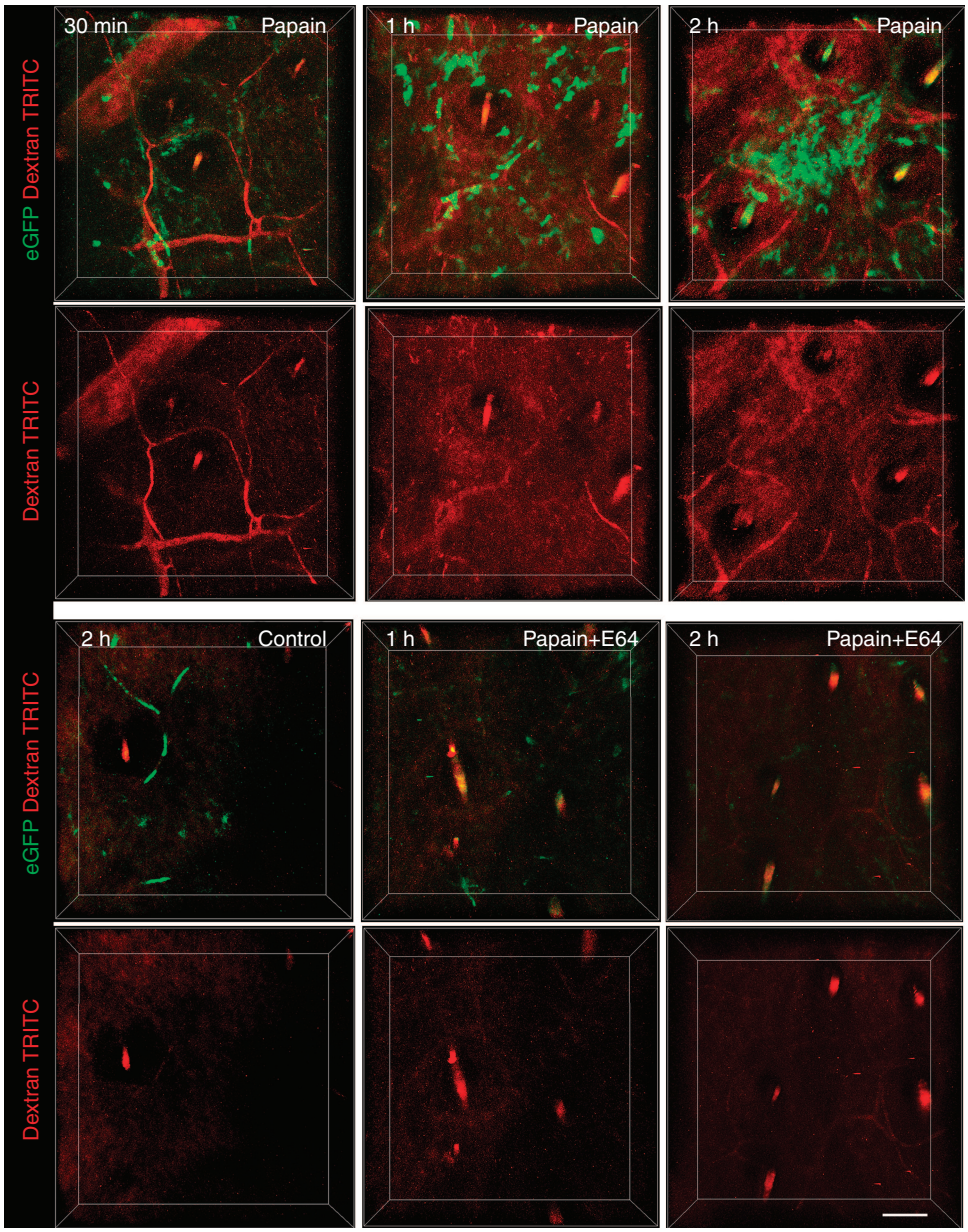

Figure 5. Papain recruits neutrophils into the skin and induces vascular leakage. Two-photon microscopy using Lys-eGFP mice. Dextran TRITC (155 kDa) was administered before treatment to analyze vascular leakage of papain (15 μg in 10 μl) or control allergen on ears. 30 minutes after application of papain, vascular leakage can be seen (excitation of dextran TRITC: 740 nm, eGFP: 940 nm). One–two hours after treatment, infiltration by neutrophils (GFP:490 nm) is induced by the enzymatically active papain but not by E-64-inhibited papain. PBS controls show no vascular leakage or neutrophil influx. PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine.

Active papain increased vascular permeability, starting 30 minutes after application, peaking at 120 minutes (Figure 5), and still visible after 165 minutes (data not shown), whereas inactivated papain or sham treatment did not induce vasodilation.

Neutrophils were strongly recruited into the skin by papain in concentrations of 15 μg starting 30–45 minutes after application and increasing even more over a time period of 2 hours (Figure 5). Inactivation of enzymatic activity or sham treatment neither increased vascular permeability nor induced recruitment of neutrophils into the skin (Figure 5).

Enzymatic activity of papain can be detected in exfoliants

Enzymes are frequently used in cosmetics. Cystein protease activity of papain and several cosmetic products in the presence or absence of E-64 was measured using the substrate Boc-Ala (Supplementary Table S1 online).

We detected enzymatic activity already in product concentrations of 100 μg ml^{-1} after 60-minute incubation with the substrate

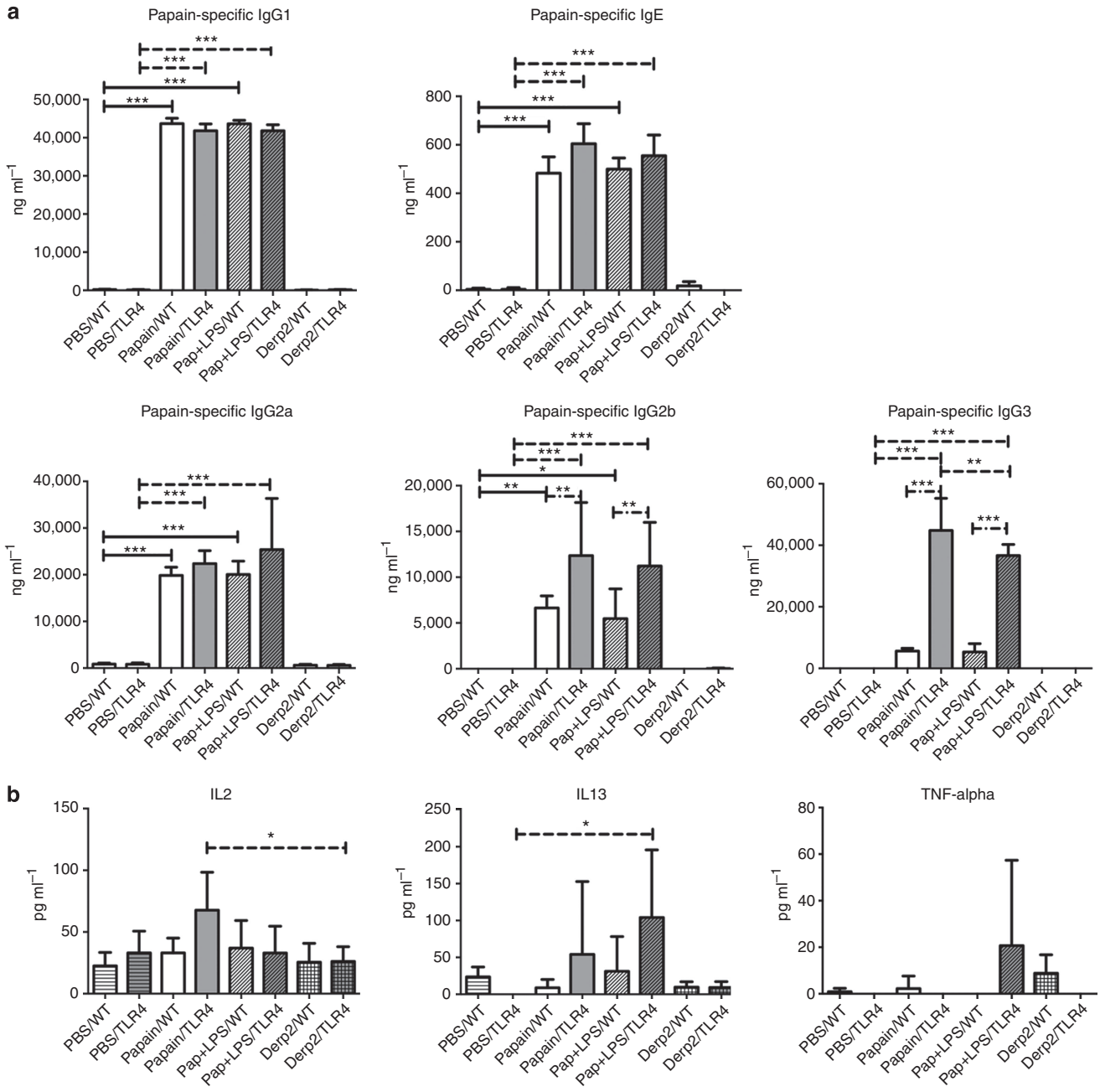

Figure 6. No role of lipopolysaccharide (LPS) and Toll-like receptor 4 (TLR4) in epicutaneous sensitization to papain—LPS or TLR4 deficiency does not alter papain-specific antibody response. WT and TLR4-deficient C57BL/6 mice were sensitized epicutaneously four times with papain or inactivated papain: PBS or rDer p 2. (**a**) Serum of mice was collected before the start of sensitization and after sensitization and specific antibody titers were determined in individual mice by ELISA. (**b**) Cytokine levels in ELISA. Median+Max./Min.; $*P<0.05$; $**P<0.01$; $***P<0.001$. Der p, Dermatophagoides pteronyssinus; max., maximum; min., minimum; PBS, phosphate-buffered saline; WT, wild type.

(Boc-Ala). For some samples, although propagated by the manufacturer, enzymatic activity was not traceable in our settings.

TLR4-activation is not important in epicutaneous sensitization to papain

Tang *et al.* (2010) proposed a crucial role for TLR4 activation and a TLR4-dependent T_H1 shift after subcutaneous sensitization with papain if LPS was co-applied.

Using papain alone or in combination with LPS, we compared the immune response between WT and TLR4$^{-/-}$ mice (Supplementary Figure S2A and S2C online).

In both mouse strains, papain triggered a high antibody response—all papain-treated mice had significantly elevated papain-specific IgG1 and IgE levels compared with controls. LPS did not alter the T_H2-bias of this response (Figure 6a).

$TLR4^{-/-}$ mice developed significantly higher specific IgG2b and IgG3 compared with WT C57BL/6 mice, but again independent of LPS content (Figure 6a), whereas specific IgG2a was equally induced in all papain-treated groups.

Evaluating the systemic immune response, IL-2 levels from stimulated splenocytes of sensitized WT mice were only slightly elevated compared with PBS controls. Splenocytes of $TLR4^{-/-}$ papain-immunized mice released significantly higher amounts of IL-2, whereas co-immunization with papain+ LPS resulted in significantly increased IL-13 production. Only minor tumor necrosis factor-α release was detectable (Figure 6b).

DISCUSSION

The relevance of proteolytic enzymes commonly used in industry is well documented, and their capacity to induce respiratory allergic reactions is known (Basketter *et al.*, 2012a, b). Specifically, sensitization and asthma after occupational exposure to the cysteine protease papain, such as in pharmaceutical industry or during silk production, are prevalent (Niinimaki *et al.*, 1993; van Kampen *et al.*, 2005; Rosenthal and Blond, 2008); also, case reports about dermatitis after the use of cosmetics containing papain (Bernstein *et al.*, 1984; Niinimaki *et al.*, 1993; Reijula *et al.*, 1993), as well as systemic reactions after ingestion of papaya juice (Iliev and Elsner, 1997), papaya cake (Quarre *et al.*, 1995), or papain-based meat tenderizer (Mansfield and Bowers, 1983), have been published. Papain is pharmaceutically used as a pro-digestive food supplement (Miller *et al.*, 2004; Buford *et al.*, 2009; Munasinghe *et al.*, 2010; Sharma *et al.*, 2011) and for sanitizing necrotic wounds (Weir and Farley, 2006; Ramundo and Gray, 2008). In spite of Food and Drug Administration warnings to stop marketing of unapproved products containing papain in 2008, papain is still widely applied as a cosmetic ingredient in exfoliants, dentifrices, shampoos, and other products.

In this study, we investigated the effect of topically applied papain on skin barrier function. In addition, we studied the immune responses against papain in the skin.

The immediate effects of papain on skin barrier and increased vascular leakage could be demonstrated by a significant increase in TEWL (Figure 1a) and by a rapid extravasation of dextran TRITC into papain-treated skin, respectively (Figure 5), probably via activation of protease-activated receptor-2 (Kouzaki *et al.*, 2009; Liang *et al.*, 2012) Cysteine proteases were previously shown to specifically act on TJ proteins of lung epithelia (Wan *et al.*, 1999; Kirschner *et al.*, 2010; Lambrecht and Hammad, 2012). Atopic patients frequently show impaired skin barrier; even in non-lesional skin, TEWL is increased compared with non-atopic patients (Proksch *et al.*, 2009). Enzymatic additives in, e.g., shampoos could therefore increase the risk for barrier defects and allergic responses. As we demonstrate, papain induces degradation of tight junctional proteins in human primary keratinocytes (Figure 1b). Our data thus indicate that the use of enzymes in cosmetics should be handled with care as their topical application can cause epidermal barrier impairment and thereby—like house dust mite allergen Der p 1 (Wan *et al.*, 1999)— papain might facilitate the sensitization to secondary allergens. Indeed, when we tested commercially available exfoliants, enzymatic activity was demonstrable in some (Supplementary Table S1 online) although not in all samples—we speculate that this might be a limitation of the method, where we tested specifically for cysteine protease activity and no other proteolytically active enzymes (data not shown), or due to a quenching of the signal by the cosmetic samples.

Our data suggest that topical formulation containing papain could permeabilize the skin barrier. Therefore, penetration of chemicals or fragrances present in the same formulation might be fragrances, resulting in a higher risk for induction of allergies against these substances.

Although the allergenic potential of papain is ascribed mainly to its proteolytic activity, E-64, an irreversible inhibitor of cysteine proteases, did not reduce the T_H2-biased immune response of papain (Figure 2). This is in contrast to previous reports (Chambers *et al.*, 1998; Tang *et al.*, 2010; Cunningham *et al.*, 2012). Notably, in our hands, the inactivated form of papain retained its significant sensitization capacity (Figure 2), although it could no longer degrade TJ-proteins or impair the skin barrier, nor induce acute inflammation. Therefore, enzymatic additives in cosmetics might present a risk factor even in the absence of enzymatic activity (Supplementary Table S1 online).

We also observed recruitment of neutrophils upon application of enzymatically active papain (Figure 5). In the *in vivo* imaging model, the inactive papain did not recruit neutrophilic granulocytes into the skin upon first encounter, whereas repeated application of inactive papain finally induced an increase in neutrophils ($Ly\text{-}6G^+$ cells) in the skin (Figure 4b). Also, other immune cells including $CD3^+$ cells (Figure 3b), mast cells (Figure 3c), and $CCL8^+$ cells were increased after repeated sensitization and rechallenge (Figure 4).

In addition to the enzyme-mediated effects of proteases through protease-activated receptor-2 (Asokananthan *et al.*, 2002), activation of TLR4 has been suggested in papain-induced allergic responses (Tang *et al.*, 2010). Overall, many studies propose a major role for endotoxins in eliciting allergic responses (Eisenbarth *et al.*, 2002; Agrawal *et al.*, 2003; Kuipers *et al.*, 2003; Hammad *et al.*, 2009; Trompette *et al.*, 2009; Hongjia *et al.*, 2010; Perros *et al.*, 2011). To test for the contribution of LPS as a cofactor and the activation of TLR4 in allergic sensitization, we treated WT mice with papain+LPS and tested our sensitization protocol in TLR4-deficient mice. In consideration of the fact that we did not detect LPS-mediated differences in WT mice together with our finding that $TLR4^{-/-}$ mice responded to papain comparably to WT mice (Figure 6) we rule out an LPS-mediated effect as a confounding factor. Which alternative immune receptors/pathways are involved here will be of interest in further studies.

In conclusion, the presented data suggest that papain impairs the skin barrier probably by degrading TJ-proteins. The increase in neutrophils, $CD3^+$ cells, mast cells, and CCL8-positive cells in skin exposed to active papain suggests that it acts in a pro-inflammatory manner. Whether this

pro-inflammatory effect is direct or indirect needs to be further explored.

As the induction of a T_H2-biased antibody response by papain via the skin was independent of its enzymatic activity, our data hence fully support the warnings of the Food and Drug Administration to reduce and carefully evaluate the addition of papain and related molecules to external or internal use in humans and animals.

MATERIALS AND METHODS

Animals

Female TLR4$^{-/-}$ mice ((Hoshino *et al.*, 1999; Stockinger *et al.*, 2004; Schellack *et al.*, 2006)) bred under SPF conditions according to FELASA (Federation of Laboratory Animal Science Associations) recommendations were obtained from Biomodels Austria (University of Veterinary Medicine Vienna, Himberg, Austria).

Female WT C57BL/6 mice were obtained from Charles River, Germany. Experiments were conducted according to the European Community rules for animal care, permission number BMWF-66.009/0170-II/10b/2009 of the Austrian Ministry of Science.

Female Lys-eGFP mice (background C57BL/6) were bred under SPF conditions according to FELASA recommendations, and experiments were conducted according to the European Community rules for animal care, permission number V242-7224.122-5 (2-1/11), of the German Ministry of Science.

Stimulation of human primary keratinocytes

Second passage primary keratinocytes from adult skin (19–37 years; $n=3$; Cell Systems, Troisdorf, Germany) were cultured in six-well-plates (Costar, Cambridge, MA) under confluent conditions for 4 days in a serum-free, keratinocyte growth medium, supplemented with 1.2 mM calcium (KGM-2, Lonza, Basel, Switzerland) at 37 °C and 5% CO_2. Subsequent stimulation of cells was performed with papain (1 μg ml^{-1}) alone or in combination with cysteine (20 μM), E-64 (1,1 μM) or Der p 2 (20 μg ml^{-1}), and Der p 2 alone. After 1 hour, cells were washed with PBS and lyzed in SDS-PAGE loading buffer.

Western blot analysis

Online repository

Measurement of TEWL

WT C57BL/6 mice were depilated on their backs using Veet creme sûpreme (Veet Sûprem'Essence Easy Spray, Reckitt Benckiser (Wallisellen, Switzerland) AG). Two days after depilation, papain (Sigma, Vienna, Austria; 30 μg per mouse or 15 μg per mouse) or papain+inhibitor E-64 (15 μg papain+25-fold-molar E64; Sigma), or controls such as distilled water or E-64 alone were applied onto depilated backs on an area of about 1.5 cm^2 using Q-tips. TEWL was measured using TewameterTM300 (Courage+Khazaka electronic GmbH, Cologne, Germany) before application and 30 minutes and 60 minutes after application.

Experimental epicutaneous sensitization model

Each group consisted of eight animals (age: 9 weeks), and results of two separate, independent experiments were compared.

Epicutaneous sensitization—backs of mice were carefully depilated using Veet-crème-sûpreme. After a skin recovery period of 2 days, 75 μl allergen per control solutions (Supplementary Figures S2B and S2C online) were applied onto filter discs (11 mm diameter) placed in 12 mm single chambers in Finn Chamber tape strips, on the back for 24 hours, four times in 3-week intervals (over a total of 12 weeks); blood samples were collected before the first and 14 days after each sensitization (Supplementary Figure S2A online).

Three weeks after the last sensitization, mice were challenged using the same doses as in sensitization but applied with Q-tip instead of tape. Six hours after the challenge, mice were killed and blood was collected by heart puncture. Samples of challenged and unchallenged skin were taken for histological analysis.

Monitoring antibody formation by ELISA

Total or allergen-specific (papain; or rDer p 2 (not shown; Szalai *et al.*, 2012)) IgG or IgE antibodies were analyzed using standard ELISA protocols (Supplementary Methods online).

Cytokine analysis

Online repository

Histological analysis

Paraffin-embedded sections were stained with hematoxylin and eosin and Giemsa using standard protocols (Szalai *et al.*, 2012); Online repository.

Immunohistochemistry

Tissues were deparaffinized and treated using 30 U ml^{-1} Proteinase K in PBS (Sigma) for 5 minutes at 37 °C, followed by standard protocols (Santa Cruz Biotechnology, ImmunoCruz ABC staining system kits; online repository). Primary antibodies were as follows: rat anti-mouse Ly-6G antibody 1:100 (Novus Biologicals, Littleton, CO, RB6-8C5), polyclonal rabbit anti-mouse CCL8/MCP-2 antibody 1:100 (antibody-online), and polyclonal rabbit anti-mouse Langerin antibody 1:250 (Novus Biological), controls: rat IgG2b, rabbit IgG. Primary antibodies were incubated over night at 4 °C (Ly-6G and CCL8) or for 2 hours at room temperature (Langerin).

Slides were embedded with Entellan® (Merck, Kenilworth, NJ) and evaluated using HistoQuest® cell analysis software from TissueGnostics, Vienna, Austria. In more detail, dermal (an area of at least 800 μm^2 per section per mouse) and epidermal regions (an area of at least 200 μm^2 per section per mouse) were determined manually for analysis (to separate the dermis and the epidermis and to avoid background due to hairfollicles) and cells were counted automatically by using modules "nuclei staining" and "cell & cytoplasm" (computer program–based automatic evalution/counting and calculation), thereby giving a percentage of positive cells to total cells.

Immunofluorescence

After deparaffinization, antigen retrieval (Proteinase K, Roche, Basel, Switzerland; 5 minutes, 37 °C), permeabilization (PBS/0.2%Tween20 for 5 minutes), and blocking (PBS/10% goat serum for 30 minutes at room temperature) anti-CD3-antibody (AbD Serotec, Puchheim, Germany; 1:50 in 5% goat serum/PBS) was incubated o/n at 4 °C. Next, sections were incubated with Alexa-Fluor-568-conjugated goat anti-rat antibody (LifeTechnologies, Vienna, Austria; 1:1,000 in PBS for 60 minutes at room temperature) and (4',6-diamidino-2-phenylindole; 1:2,000 in aqua dest.; for 10 minutes) and slides mounted with fluoromount-G (eBioscience, San Diego, CA). The number of T cells was counted manually within defined regions (in a blinded manner),

and the ratio to total cell number was finally calculated by using HistoQuest cell analysis software from TissueGnostics giving results in % of total cell number.

The Enzymatic activity assay

Cysteine protease activity was measured as described in (Jacquet *et al.*, 2000; Yasuhara *et al.*, 2001; Jacquet *et al.*, 2002; Takai *et al.*, 2002; Zhang *et al.*, 2009), with modifications using different concentrations of papain.

Commercially available exfoliants and standard dilutions of papain were activated for 15 minutes in 50 mM TRIS, pH 7, 1 mM EDTA, and 20 mM L-cysteine, at 37 °C, and then tested using 12.5 μM Boc-Gln-Ala-Arg-7-amido-4-methylcoumarinhydrochloride as substrate. Tests were performed in duplicates and with co-incubation of 50 μM cysteine-protease-specific inhibitor E-64.

Negative controls—activity buffer only, activity buffer+papain, activity buffer+substrate, activity buffer+E-64. Samples were measured after 30 minutes and 60 minutes using a microplate reader (Tecan, Groedig, Austria) at 355/460 nm. Enzymatic activity was calculated from means of duplicates by subtraction of mean of substrate control+(3 × standard-deviation of substrate control), using papain as a standard.

Effect of papain on influx of neutrophil granulocytes and vascular leakage by two-photon microscopy

Lys-eGFP mice (Faust *et al.*, 2000) were anesthetized and depilated on their ears. After retrobulbar injection of 200 μl of a 25 mg ml^{-1} TRITC-labeled Dextran (155 kDa; Sigma), 15 μg or 30 μg papain in 10 μl PBS was applied to the ear skin. Control ears were treated with 15 μg papain and 25-fold molar concentration of the protease inhibitor E64 (Sigma) or PBS. Z-stacks from the ear skin were recorded over time, and the occurrence of eGFP-positive cells and the extravasation of TRITC-dextrane were determined using a TriM Scope two-photon microscope (LaVision BioTec GmbH, Bielefeld, Germany) equipped with a XLUMPLFL 20 × W/0.95 Objective (Olympus, Hamburg, Germany). Excitation wavelength for visualization of eGFP was 940 nm and 740 nm for TRITC. Emitted light was detected by four wavelength separated PMTs (< 435 nm, 453–495 nm, 495–560 nm, and > 560 nm). Image processing was carried out using Imaris Software (Bitplane, Zurich, Switzerland).

Statistical analysis

All statistical tests were performed using one-way analysis of variance (comparing the mean of each group with the mean of each other group) with Tukey as the *post hoc* test. Data analysis and graphs with GraphPadPrism6-software (GraphPad, San Diego, CA). In graphs, median plus minimum and maximum is shown. Multiplicity adjusted *P*-values <0.05 were considered statistically significant. * indicates $P<0.05$, ** $P<0.01$, and *** $P<0.001$, otherwise no statistical significance was reached.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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