

A mechanism for the initiation of allergen-induced T helper type 2 responses

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Both metazoan parasites and simple protein allergens induce T helper type 2 (T_H2) immune responses, but the mechanisms by which the innate immune system senses these stimuli are unknown. In addition, the cellular source of cytokines that control T_H2 differentiation *in vivo* has not been defined. Here we showed that basophils were activated and recruited to the draining lymph nodes specifically in response to T_H2-inducing allergen challenge. Furthermore, we demonstrate that the basophil was the accessory cell type required for T_H2 induction in response to protease allergens. Finally, we show that basophils were directly activated by protease allergens and produced T_H2-inducing cytokines, including interleukin 4 and thymic stromal lymphopoietin, which are involved in T_H2 differentiation *in vivo*.

Bacterial, viral and fungal pathogens are evolutionarily distant from their host organisms and thus produce biochemically distinct macromolecules that can be sensed by a variety of pattern-recognition receptors of the host, such as Toll-like receptors (TLRs), Nod-like receptors and dectin-1. Activation of these receptors generally results in the induction of immune responses regulated by T helper type 1 (T_H1) cells and interleukin 17 (IL-17)-producing T helper (T_H-17) cells. In contrast, little is known about the innate immune recognition mechanisms and cell types involved in the initiation of T_H2 responses. T_H2-regulated immune responses are induced after helminth infection and are characterized by activation of eosinophils, mast cells and mucosal epithelial cells and production of immunoglobulin E (IgE)¹. It is notable and puzzling that T_H2 and IgE responses can also be induced by simple protein allergens, which seem to have little in common with complex parasites.

Many potent allergens, however, have intrinsic protease activity^{2–4}, and secreted proteases are essential for the infectious and reproductive cycles of helminths⁵. It is possible, therefore, that the innate immune system has evolved a detection mechanism based on sensing of the abnormal protease activity associated with helminth infection. The ability of protease allergens to trigger T_H2 responses could then be explained by their ability to trigger, unintentionally, the same pathway that has evolved to detect infection by helminths⁶.

The differentiation of naive T cells into T_H1 and T_H-17 effector T cells is controlled by cytokines produced by dendritic cells (DCs) as well as other accessory cell types. Thus, T_H1 differentiation is regulated by interferon- γ (IFN- γ) produced by natural killer cells recruited to the lymph nodes⁷ and by interleukin 12 (IL-12) produced by DCs after TLR activation⁸. T_H-17 differentiation is controlled by DC-derived IL-6 and transforming growth factor- β released from an unknown source and by IL-23 produced by DCs after dectin-1 activation^{9,10}. In contrast, the cell types and cytokines

involved in T_H2 differentiation *in vivo* are not well defined. IL-4 is essential for T_H2 differentiation¹¹, but the cellular source of IL-4 is not apparent.

Several possible cellular sources of IL-4 have been postulated; mast cells and basophils, for example, readily produce IL-4 after crosslinking of their Fc ϵ RI receptors¹². Fc ϵ RI crosslinking on basophils leads to T_H2 differentiation in a model of repeated exposure to an antigen¹³, and mice with more basophils show greater T_H2 differentiation in response to hapten immunization¹⁴. However, whether basophils are involved in T_H2 induction in the absence of antigen-specific IgE, as would occur during primary exposure to an antigen, is unknown.

In addition to the essential function of IL-4 in T_H2 differentiation, studies have identified an important function for thymic stromal lymphopoietin (TSLP) in T_H2-mediated immunity. TSLP is involved in lymphocyte development, but it can also be produced by epithelial cells, leading to T_H2 cell recruitment and allergic inflammation¹⁵. Stimulation of DCs with TSLP leads to upregulation of the costimulatory molecule OX40L and can promote T_H2 differentiation *in vitro*¹⁵. In addition, TSLP can contribute to T_H2 immunity by inhibiting T_H1 differentiation¹⁶. Finally, *in vitro* studies have shown that TSLP can act directly on T cells to promote T_H2 differentiation¹⁷. However, because TSLP is only known to be produced in peripheral tissues, such as epithelial cells, it is unclear whether it is involved in the initiation of T_H2 immune responses *in vivo*, which would require TSLP production in the lymph nodes at the time of activation of naive T cells.

Here we sought to identify mechanisms of innate immune control of T_H2 responses induced by primary exposure to an allergen. Although the protease activity of allergens has been shown to be required for the induction of T_H2 and IgE responses, the cellular target of that protease activity is unknown^{4,18,19}. We found that basophils directly responded to a model protease allergen, papain, by producing IL-4, TSLP and other T_H2-

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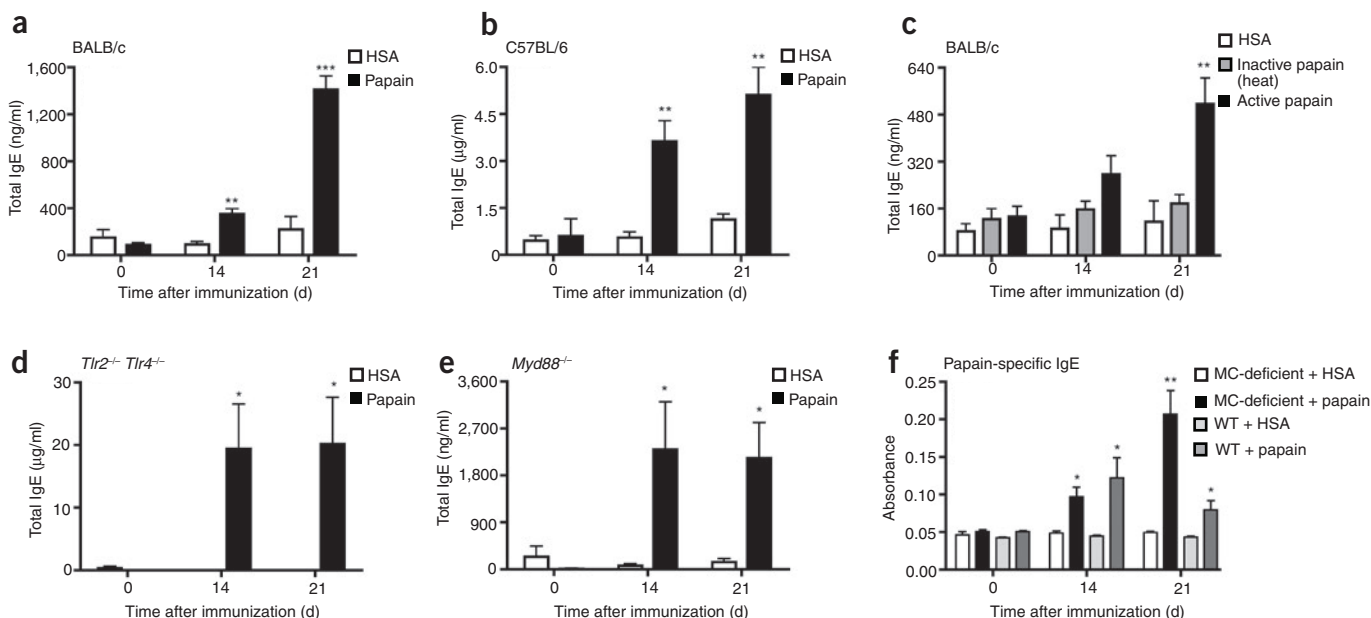


Figure 1 Active papain induces IgE secretion independently of TLR2, TLR4 or MyD88. (a,b) Serum IgE concentrations measured immediately before primary immunization (day 0), before secondary immunization (day 14), and at day 21 after immunization with HSA or active papain in BALB/c (a) or C57BL/6 (b) mice. (c) Serum IgE concentrations in BALB/c mice immunized with HSA, heat-inactivated papain (Inactive papain (heat)) or active papain. (d,e) Serum IgE concentrations in *Tlr2*^{-/-} *Tlr4*^{-/-} mice (d) and *Myd88*^{-/-} mice (e) immunized with HSA or active papain. (f) Antigen-specific IgE responses in mast cell-deficient (MC-deficient; *Ki*^{WNV}) and littermate control mice (WT) immunized with HSA or papain. ***, $P \leq 0.0001$; **, $P \leq 0.001$; *, $P \leq 0.01$; no asterisk, $P > 0.05$, relative to immunization with HSA (Student's *t*-test). Data are representative of at least three separate experiments, with five to ten mice (a–c) or three to five mice (d–f) analyzed at each time point (error bars, s.e.m.); d,e represent data combined from separate experiments.

associated cytokines and chemokines. Notably, basophils were transiently recruited to the draining lymph nodes in response to immunization with papain. Furthermore, basophils were essential for T_H2 responses induced by immunization with allergen. Finally, we found that TSLP was produced by basophils in the lymph nodes and was important in the initiation of T_H2 differentiation *in vivo* and *in vitro*.

RESULTS

T_H2 response to a protease allergen

Papain is a cysteine protease and a potent allergen associated with occupational allergy in humans²⁰ and the production of IgE and IgG1 and mast cell degranulation in mice^{6,19}. Immunization with papain without any additional adjuvants stimulated the production of IgE and IgG1 antibodies in BALB/c and C57BL/6 mice, whereas immunization with an inert and pure protein antigen, human serum albumin (HSA), did not (Fig. 1a,b and Supplementary Figure 1a online). Immunization with papain did not result in the production of IgM or IgG2 (Supplementary Figure 1b,c), which suggested that papain has specifically T_H2 adjuvant activity. Consistent with published reports¹⁹, this T_H2 -inducing effect was dependent on papain's protease activity. Heat or pharmacological inactivation of the protease activity of papain decreased or eliminated the induction of total and papain-specific IgE (Fig. 1c and Supplementary Figure 1d–f). The induction of IgE by papain did not depend on TLRs, as papain-induced IgE production was retained in mice lacking TLR2 and TLR4 or the TLR adaptor MyD88 (Fig. 1d,e). Although mast cells can degranulate in response to papain¹⁹, papain-induced IgE production did not depend on mast cells, as papain induced a robust IgE response in mast cell-deficient mice (Fig. 1f). Finally, IgE induction was not a unique property of papain, as immunization with bromelain, another protease allergen, also induced a strong IgE response (Supplementary Figure 1g).

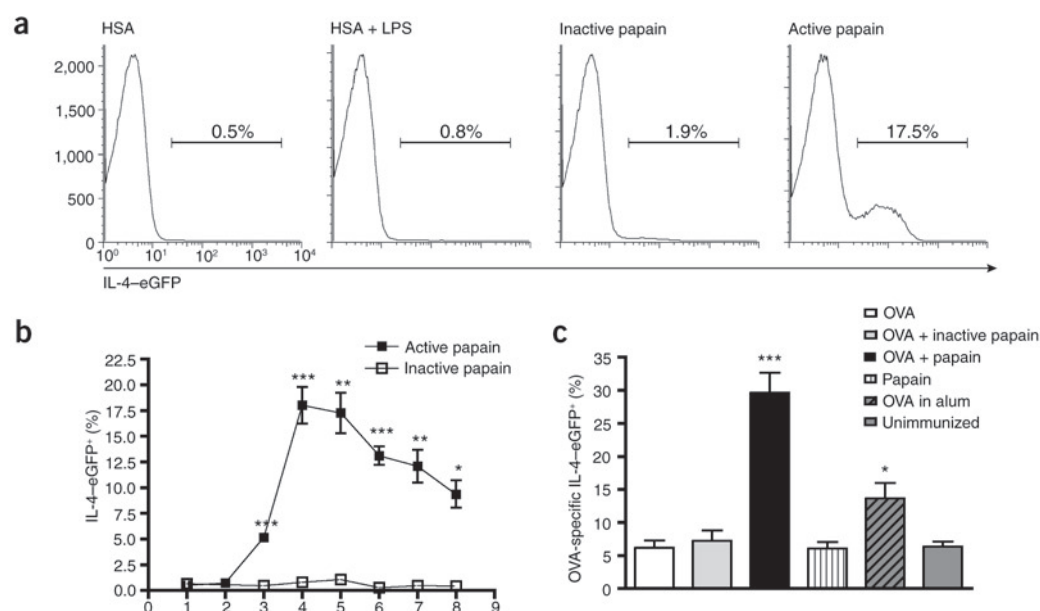
We next examined the function of protease activity in T cell responses to papain. To monitor T_H2 responses *in vivo*, we used '4get' mice, in which cells that activate the gene encoding IL-4 (*Il4*) can be visualized by tracking the expression of a bicistronic construct encoding enhanced green fluorescent protein (eGFP) 'knocked into' the *Il4* locus (IL-4-eGFP)²¹. Immunization of 4get mice with papain resulted in the induction of a T_H2 response, shown by accumulation of CD69^{hi} and CD44^{hi} IL-4-eGFP⁺ T_H2 cells in the draining lymph nodes (Fig. 2a). As described before^{21,22}, expression of IL-4-eGFP by T cells indicated genuine T_H2 differentiation, as shown by the production of IL-4, IL-5 and IL-13 after *in vitro* restimulation of IL-4-eGFP⁺ T cells (Supplementary Figure 2 online). This response peaked on day 4 after immunization and was dependent on the protease activity of papain (Fig. 2a,b). We did not find T_H2 differentiation in response to trypsin, collagenase or clostripain (data not shown), which indicated that certain classes of proteases are selectively able to induce T_H2 differentiation. To determine if protease activity had a true adjuvant effect, we reconstituted mice with ovalbumin (OVA)-specific 4get DO11.10 T cell receptor (TCR)-transgenic T cells and immunized them with OVA and papain. Active papain stimulated the production of OVA-specific T_H2 cells in an antigen-specific way, which indicated that protease activity had a T_H2 adjuvant effect that was independent of the antigenicity of papain (Fig. 2c). T_H2 differentiation in response to papain-OVA was twice as strong as that in response to OVA in alum, which demonstrated the robustness of papain adjuvant activity (Fig. 2c). Thus, the protease activity of papain is a potent inducer of T_H2 and IgE responses.

Papain-induced leukocyte migration

Activation of naive T cells requires DC maturation and migration into draining lymph nodes²³. Although DCs have not been shown to be

Figure 2 The protease activity of papain has adjuvant activity and leads to T_H2 cell differentiation on day 4 after immunization.

(a) IL-4-eGFP expression by $CD4^+DX5^-$ cells from 4get mice immunized with various stimuli (above plots). Numbers above bracketed lines indicate percent IL-4-eGFP⁺ cells. (b) IL-4-eGFP expression by $CD4^+DX5^-$ cells from 4get mice immunized with active or heat-inactivated papain. (c) IL-4-eGFP expression by $CD4^+$ cells transferred from DO11.10 $\times$ 4get mice into BALB/c mice, which were immunized with various combinations of OVA and papain (key); KJ1.26⁺ $CD4^+$ T cells were examined on day 4 after immunization, except for the OVA in alum condition, assessed at day 11 after immunization. Values in b,c indicate percent IL-4-eGFP⁺ cells of $CD4^+DX5^-$ cells (b) or KJ1.26⁺ $CD4^+$ cells (c). ***, $P \leq 0.0001$; **, $P \leq 0.001$; *, $P \leq 0.01$; no asterisk, $P > 0.05$, relative to immunization with papain (b) or OVA (c; Student's *t*-test). Data are representative of at least three experiments with two to three mice per group in each (error bars, s.e.m.).



sources of T_H2 -inducing cytokines, OX40L as well as the Notch ligands Jagged1 and Jagged2 on DCs have been shown to induce T_H2 differentiation^{24,25}. Subcutaneous immunization with active papain induced the accumulation of mature, $CD86^{hi}$ DCs in the draining lymph nodes, but immunization with inactive papain did not (Fig. 3a and Supplementary Figure 3a online). This DC migration was first apparent at 18 h after immunization (Supplementary Figure 3b). DC migration and maturation was not due to lipopolysaccharide contamination, as we used TLR4-deficient mice for these experiments. Although it has been linked to T_H2 differentiation²⁴, OX40L was not detectable on lymph node-resident or migrated DCs (data not shown). The DC subset that selectively accumulated in the draining lymph node was of dermal origin, characterized by a $CD11c^+CD8\alpha^-DEC-205^{int}CD11b^{hi}$ phenotype²⁶ (Fig. 3b

and Supplementary Figure 3a). Despite the *in vivo* effects of papain on DC migration and maturation, *in vitro* treatment of TLR4-deficient bone marrow-derived, splenic or lymph node-resident DCs with papain did not induce DC maturation, as assessed by upregulation of CD86, CD40, major histocompatibility complex class II or OX40L; nor did it induce transcription of Jagged1 or Jagged2 (Supplementary Figure 3c and data not shown). This disparity between the *in vivo* and *in vitro* effects of papain as well as the lack of expression of known T_H2 -inducing molecules suggested that an additional cell type was involved in the *in vivo* T_H2 responses induced by papain.

As IL-4 can contribute to T_H2 differentiation and is probably expressed together with other T_H2 -inducing signals, we examined the draining lymph nodes of 4get mice for the appearance of IL-4-eGFP⁺

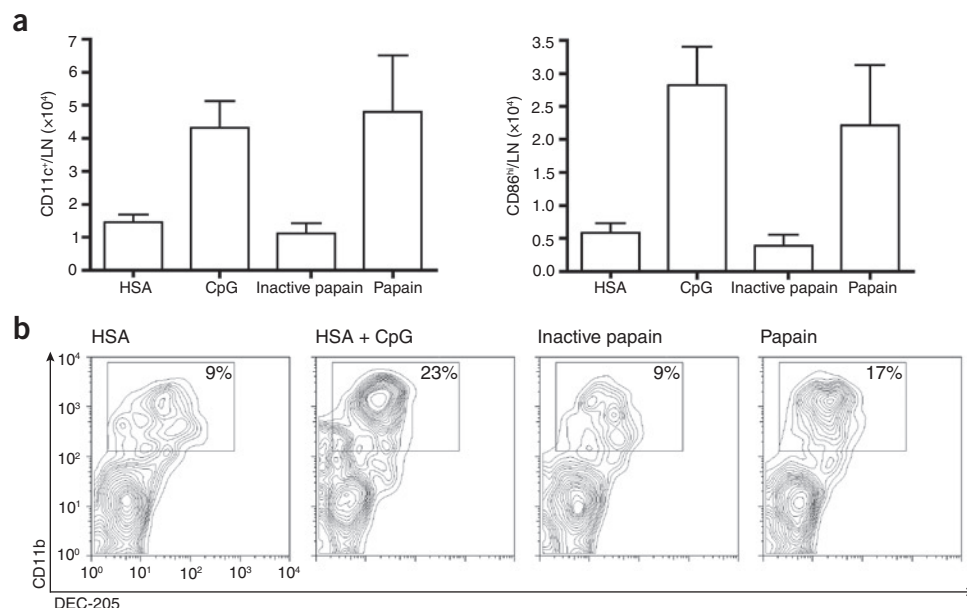


Figure 3 Proteolytically active papain induces the migration of $CD86^{hi}CD11c^+$ cells and dermal DCs into draining lymph nodes. (a) Live $CD11c^+$ cells (left) and $CD86^{hi}CD11c^+$ cells (right) in the popliteal lymph node (LN) at 22 h after subcutaneous footpad immunization with HSA, CpG, heat-inactivated papain or active papain, calculated by multiplication of percent $CD11c^+$ cells or $CD86^{hi}CD11c^+$ cells by total live cells. (b) Migration of dermal DCs in response to CpG and papain. Plots are gated on live $CD11c^+CD8\alpha^-$ cells. Numbers in outlined areas indicate gated percentage of total live $CD11c^+$ cells. Data are representative of more than three experiments (a, $n = 2-6$ mice per group; error bars, s.e.m.).

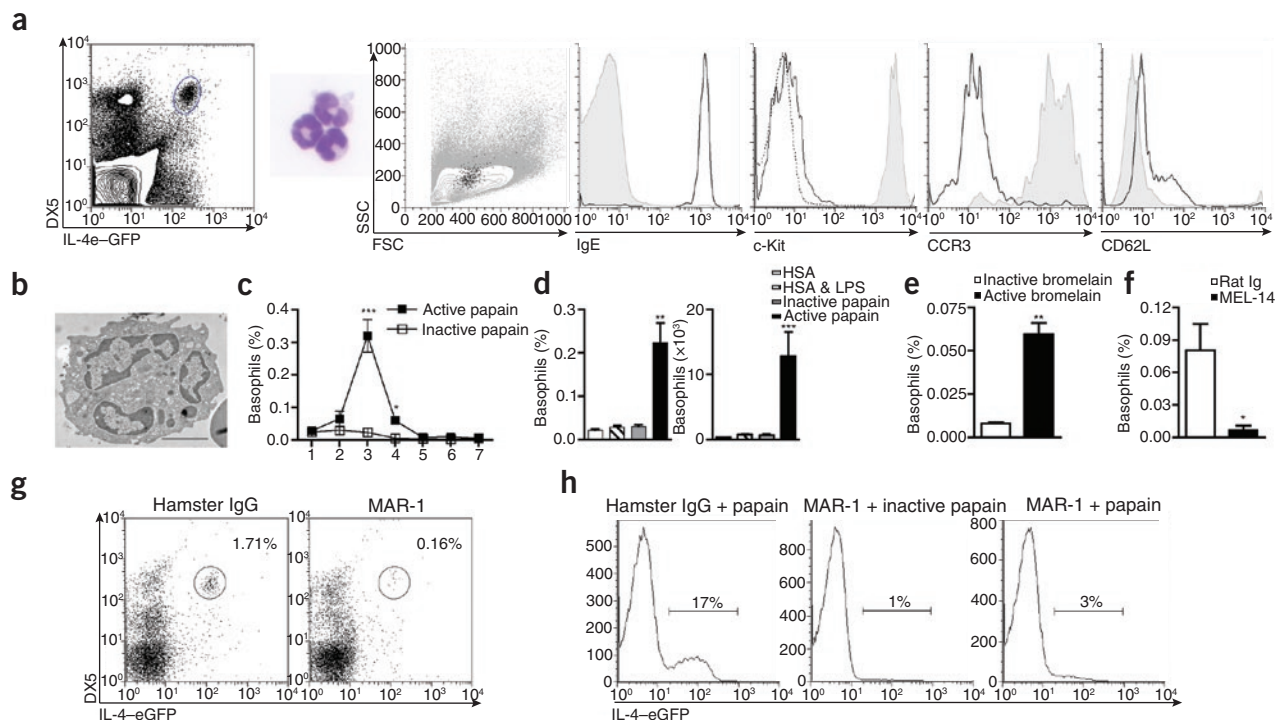


Figure 4 Basophils enter the draining lymph node after immunization with papain and are necessary for T_H2 differentiation. (a) Cytospin morphology, cell size and surface staining of DX5⁺IL-4-eGFP⁺ cells in lymph nodes after immunization with papain. Open histograms, DX5⁺IL-4-eGFP⁺ cells; filled gray histograms, total lymph node cells (side scatter (SSC) and forward scatter (FSC), and IgE, isotype control (CD62L), peritoneal mast cells (c-Kit) and eosinophils (CCR3); dotted lines, lymph node cells. Original magnification (middle image), $\times 400$. (b) Electron microscopy of sorted lymph node DX5⁺IL-4-eGFP⁺ cells 3 d after immunization with papain. Scale bar, 2 μ m. (c) Lymph node basophils at various times after immunization. (d) Lymph node basophils at 3 d after immunization. (e) Lymph node basophils after immunization with bromelain. (f) Lymph node basophils in mice treated with rat immunoglobulin or MEL-14 and immunized with papain. Values in c–f are percent of total live cells. (g) Depletion of basophils from 4get peripheral blood leukocyte samples at 3 d after treatment with hamster IgG or MAR-1. Numbers beside outlined areas indicate percent DX5⁺IL-4-eGFP⁺ cells. (h) T_H2 differentiation in basophil-depleted 4get mice. Numbers above bracketed lines indicate percent gated cells of CD4⁺DX5⁺ cells. ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; no asterisk, $P > 0.05$, relative to immunization with inactive protease (c–e) or HSA (d) or isotype control treatment (f; Student's *t*-test). Data (error bars, s.e.m.) are representative of five (a,c), four (b), six (d), three (e,f) or ten (g,h) experiments.

cells in response to immunization with papain. We identified a subset of DX5⁺CD3⁺CD4⁺CD19⁺CD11c^{lo}IL-4-eGFP⁺ cells in the draining lymph nodes 3 d after immunization with papain (Fig. 4a). These cells appeared in response to papain, and further examination showed they were FcεRI⁺IgE⁺c-Kit⁺CCR3⁺, which identified them as basophils (Fig. 4a and Supplementary Figure 4a online). This was consistent with their morphology determined by cytospin analysis as well as by electron microscopy (Fig. 4a,b). Basophil recruitment was transient and peaked at day 3, 1 d before the peak of T_H2 differentiation noted in the same immunization conditions (Figs. 2b and 4c). Notably, recruitment of basophils was induced by proteolytically active papain but not by HSA, lipopolysaccharide or inactive papain (Fig. 4c,d). Immunization with active bromelain also induced basophil recruitment 3 d after immunization, but immunization with inactive bromelain did not (Fig. 4e). Lymph node basophils had higher surface expression of CD62L and entered the lymph node by a CD62L-dependent mechanism that was inhibited by blockade with the CD62L-specific antibody MEL-14 (Fig. 4f). In addition, we detected basophils in the popliteal lymph node by immunofluorescence only in response to proteolytically active papain (Supplementary Figure 5a online). Basophils were localized in the T cell zone of the lymph node, among T cells but not among B cells (Supplementary Figure 5b). Thus, we found that a well known IL-4-producing cell type, the basophil, is specifically recruited to the draining lymph nodes during the initiation of allergen-induced T_H2 responses.

Basophils in allergen-induced T_H2 differentiation

Basophils constitute 1–2% of peripheral blood leukocytes and can enter peripheral tissues in response to nematode infection^{12,27}, but their function in innate activation of the adaptive immune response is unknown. Basophils are not normal constituents of the lymph node. Because of their unexpected recruitment to the draining lymph node after immunization with papain, we hypothesized that basophils may be involved in papain-induced T_H2 responses *in vivo*. Genetic models of basophil deficiency do not exist at present, so to test the function of basophils, we developed a protocol for basophil depletion with the MAR-1 antibody, which is specific for FcεRIα. During steady-state conditions in mice, FcεRI is present exclusively on basophils and mast cells. Because MAR-1 specifically targets the nonsignaling α-chain of the FcεRI complex, MAR-1 did not activate IL-4 production from basophils *in vitro* but did block subsequent staining with MAR-1 (Supplementary Figure 4a,b). Injection of MAR-1 led to depletion of over 90% of peripheral blood basophils by day 3 after the final MAR-1 treatment, and the depletion lasted for at least 8 d (Fig. 4g). Treatment with MAR-1 produced depletion of basophils in all organs examined (Supplementary Figure 4c–e). However, injection of MAR-1 did not result in depletion of skin or intraperitoneal mast cells (Supplementary Figure 4e). We subcutaneously immunized basophil-depleted mice with papain and analyzed T_H2 differentiation *in vivo* 4 d and 8 d later. T_H2 responses were abolished in mice depleted of basophils but not in mice

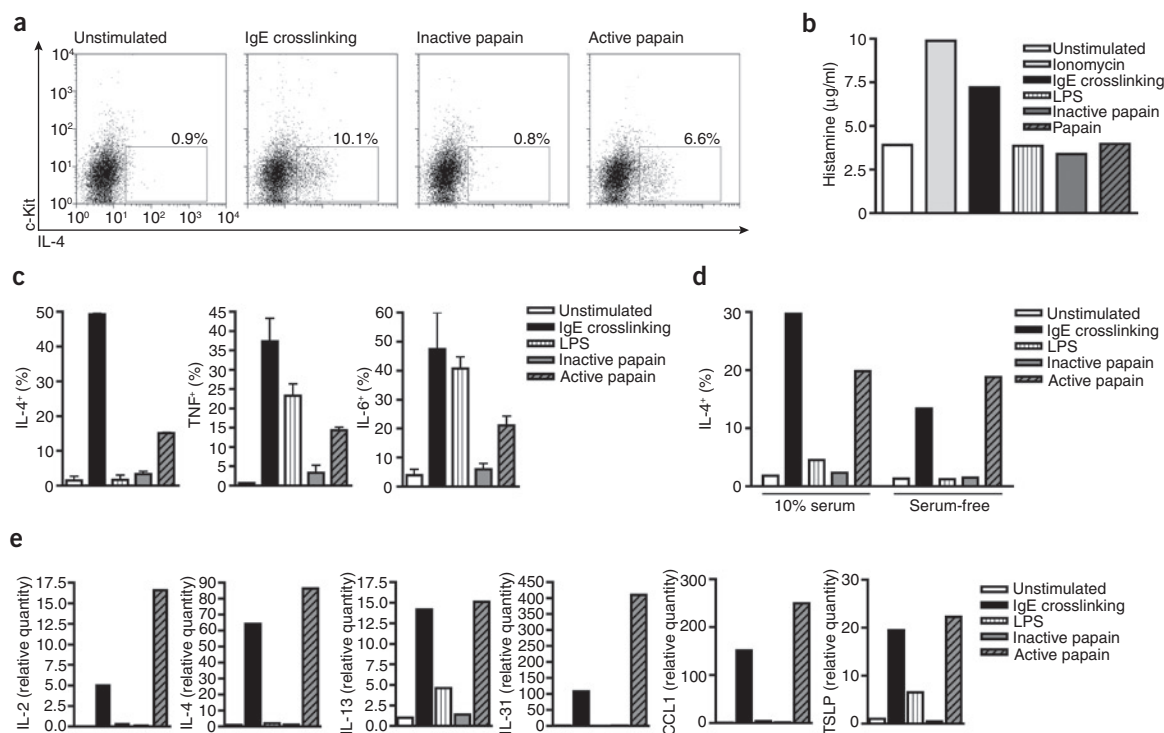


Figure 5 The protease activity of papain induces the production of IL-4, tumor necrosis factor and IL-6 in bone marrow-derived basophils. **(a)** IL-4 production by basophils purified from *in vitro*-stimulated bone marrow cultures (above plots) and stained for intracellular cytokines 6 h later. **(b)** Histamine release by basophils 24 h after stimulation. **(c)** Intracellular cytokine staining of basophil-enriched cultures at 6 h after stimulation. **(d)** IL-4 production by basophil-enriched cultures with or without serum, measured by intracellular cytokine staining. **(e)** Quantitative PCR of transcript expression at 4 h after stimulation of basophils purified from *in vitro* bone marrow cultures, relative to expression in unstimulated cells. Percentages in **a**, **c**, **d** indicate that of gated populations among total basophil populations. Data (error bars, s.e.m.) are representative of at least three experiments (**a**, **b**, **d**, **e**) or triplicate samples in one experiment (**c**).

treated with control antibody (Fig. 4h and Supplementary Figure 4f). In contrast, mast cell-deficient mice showed no defect in IgE production or basophil migration in response to immunization with papain (Fig. 1f and Supplementary Figure 4g). Therefore, we conclude that basophils have an essential and nonredundant function in the development of T_H2 responses to a protease allergen *in vivo*.

To determine whether papain was acting directly or indirectly on basophils, we analyzed basophil responses to papain *in vitro*. The methods available for producing basophils from bone marrow progenitors give rise to both basophils and mast cells²⁸. To specifically examine the effects of papain stimulation on basophils, we sorted cells by flow cytometry to purify basophils from the bone marrow cultures to over 99% purity. Like human basophils²⁹, purified mouse basophils produced IL-4 in response to IgE crosslinking or papain stimulation but not in response to inactive papain (Fig. 5a). Basophil activation is associated with both cytokine production and secretion of inflammatory mediators such as histamine and leukotriene C₄ (ref. 12). As expected, ionomycin and crosslinking of cell surface IgE induced histamine secretion from purified basophils (Fig. 5b). However, papain elicited IL-4, IL-6 and tumor necrosis factor, but not histamine, from basophils, which indicated that papain activated basophils by a pathway distinct from ionomycin and FcεRI (Fig. 5b,c). Furthermore, the activation of basophils by papain was not affected by the absence of serum, which indicated that serum-derived factors were not involved (Fig. 5d). Therefore, papain directly activates basophils, but the identity of the papain-activated sensor expressed by basophils remains unknown.

Basophils secrete T_H2-promoting cytokines

Papain strongly induced the expression of transcripts encoding IL-2, IL-4, IL-13, IL-31, TSLP and the chemokine CCL1, which have all been associated with T_H2 cell differentiation and recruitment, in purified basophils^{11,15,24,25,30,31} (Fig. 5e). Papain did not induce transcription of IL-25, OX40L or the Notch ligands Jagged1 or Jagged2 (data not shown), but it did induce robust production of IL-4 mRNA and protein (Fig. 5e). IL-4 is well known to be important in T_H2 differentiation *in vivo* and *in vitro*, but the relevant cellular source of IL-4 remains unclear¹¹. Blockade of IL-4 receptor α-chain (IL-4Rα) *in vivo* impaired T_H2 differentiation induced by immunization with papain (Supplementary Figure 6a online). Consistent with other reports³², T cell-derived IL-4 was sufficient for T_H2 differentiation in an experimental system that relies on transfer of TCR-transgenic T cells (Supplementary Figure 6b). However this result could be explained by the abnormally high frequency of antigen-specific T cells that could compensate for the lack of exogenous IL-4 (ref. 33) and thus does not exclude the possibility that basophil-derived IL-4 may contribute to T_H2 differentiation *in vivo* in wild type mice. Given the potent effect of IL-4 on T_H2 differentiation, this is a likely possibility.

TSLP is an IL-7-like cytokine with reported involvement in lymphocyte development, T cell homeostasis and allergic responses¹⁵. As papain treatment induced TSLP transcription in basophils *in vitro* (Fig. 5e), we first sought to determine if TSLP was indeed produced *in vivo* by lymph node basophils after immunization with papain. In accordance with quantitative PCR data, we found that 31.6% ± 8.5% of lymph node basophils expressed TSLP protein (Fig. 6a). This was probably underestimate, as we examined basophils immediately after isolation and without stimulation in the

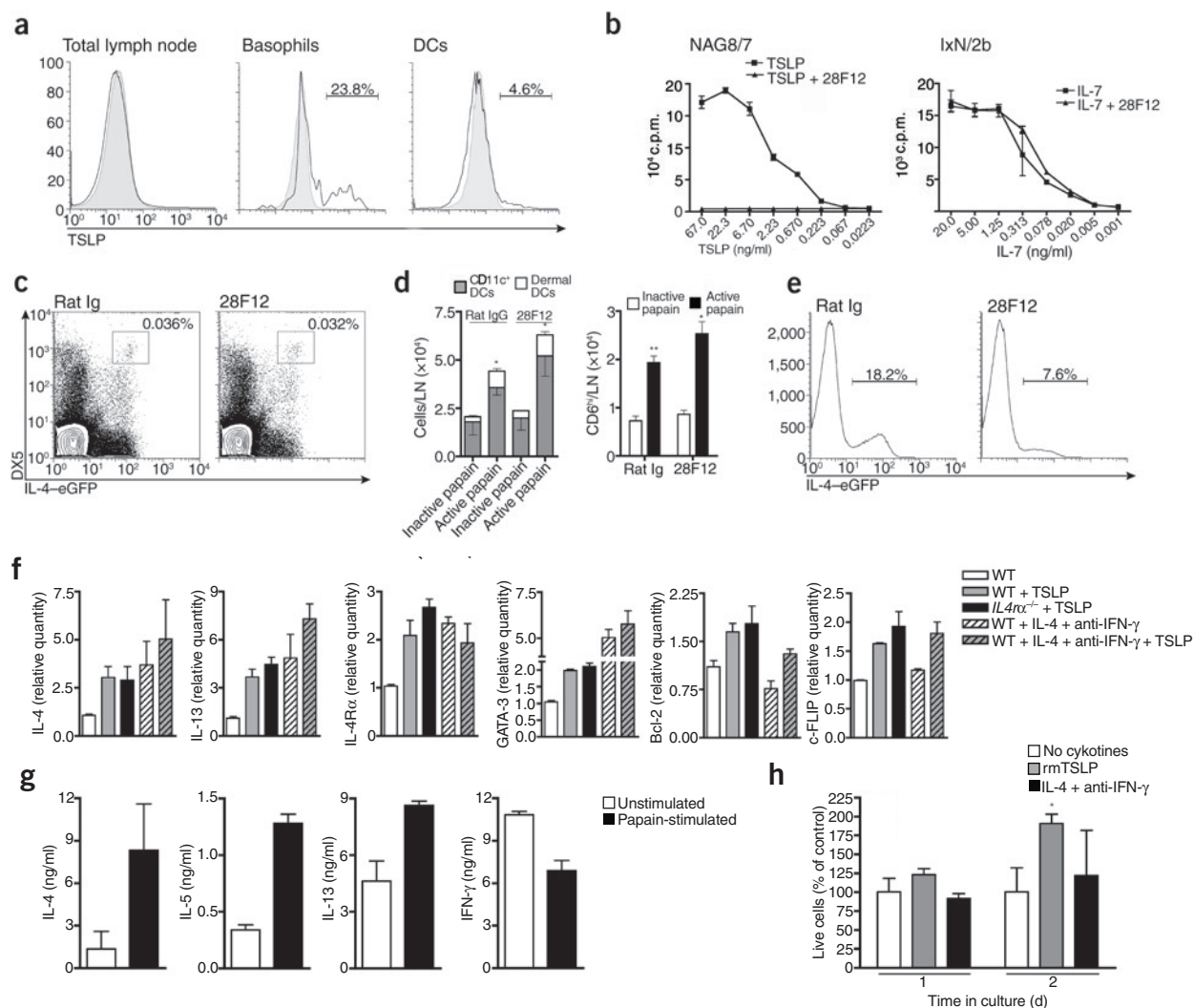


Figure 6 TSLP is required for basophil-mediated T_H2 differentiation. **(a)** Intracellular TSLP staining in total lymph node cells, basophils and DCs (open histograms) 3 d after immunization with papain. Shaded histograms, isotype control (left, right) or TSLP staining in peripheral blood basophils (middle). Numbers above bracketed lines indicate gated population among $CD4^+DX5^-$ cells. **(b)** Effect of 28F12 on the TSLP-dependent growth of NAG8/7 cells and the IL-7-dependent growth of IxN/2b cells. **(c,d)** Papain-induced migration of basophils **(c)** or DCs **(d)** to the lymph node after TSLP neutralization *in vivo*. **(e)** Papain-induced T_H2 differentiation after TSLP neutralization *in vivo*. Numbers above bracketed lines indicate gated population among $CD4^+DX5^-$ cells. **(f)** Quantitative PCR of transcript expression in naive $CD4^+$ T cells (IL-4, IL-13 and GATA-3) and total $CD4^+$ T cells (IL-4Rα, Bcl-2 and c-FLIP) stimulated *in vitro*, relative to expression in wild-type cells. WT is BALB/c; *IL4*^{-/-} mice were used in IL-4Rα experiments. **(g)** Cytokine production after restimulation of naive T cells activated by unstimulated or papain-stimulated basophils. **(h)** Survival of $CD4^+$ T cells after stimulation, presented as live cells from culture with cytokine versus culture without added cytokines (control). rmTSLP, recombinant mouse TSLP. **, $P \leq 0.01$; *, $P \leq 0.05$, immunization with inactive papain versus corresponding antibody treatment (Student's *t*-test). Data are representative of at least three experiments (error bars, s.e.m.).

presence of brefeldin A. As expected, we detected TSLP expression only in the allergen-activated basophils in the lymph node and not in unactivated peripheral blood basophils of papain-injected mice or unimmunized mice (**Fig. 6a**). Notably, lymph node TSLP production was specific to basophils, as we did not detect substantial TSLP in other cell types in the lymph node, including DCs (**Fig. 6a**). In the setting of chronic allergic inflammation, TSLP production in peripheral tissues has been associated with effector T_H2 responses^{15,16,34}. However, our identification of basophil-derived TSLP in the draining lymph node led us to hypothesize that TSLP may also be important in the initiation of T_H2 responses *in vivo*.

Because TSLP may influence T cell development³⁵, we developed a TSLP-neutralizing monoclonal antibody (28F12) to evaluate the function of TSLP specifically during the initiation of T_H2 responses *in vivo*. The 28F12 antibody effectively neutralized the TSLP-dependent proliferation of the mouse fetal liver-derived pre-B cell line NAG8/7 but not the IL-7-

dependent proliferation of the mouse pre-B cell line IxN/2b (refs. 36,37; **Fig. 6b**). To address the function of TSLP in T_H2 differentiation *in vivo*, we injected mice intravenously with 28F12 and analyzed their response to immunization with papain. Despite its reported involvement in DC maturation, TSLP neutralization did not inhibit papain-induced maturation or migration of basophils or DCs (**Fig. 6c,d**). However, *in vivo* neutralization of TSLP led to considerable inhibition of T_H2 differentiation (**Fig. 6e**). As basophils are the only cell type that produced TSLP in the draining lymph node and as TSLP neutralization inhibited T_H2 differentiation without affecting DC maturation or migration (**Fig. 6a,c-d**), these data indicate that basophil-derived TSLP is necessary for T_H2 differentiation.

$CD4^+$ T cells activated *in vitro* by TCR stimulation in the presence of exogenous IL-2, TSLP and neutralizing antibodies to IFN-γ produce IL-4 (ref. 17). However, because IL-2 can promote T_H2 differentiation³⁸, we sought to determine whether TSLP is directly involved in T_H2 differentiation.

Using a similar T cell-activation protocol, but without the addition of exogenous IL-2 or neutralizing antibodies to IFN- γ , we found that TSLP directly induced many genes associated with T_H2 differentiation. Activation of naive T cells in the presence of TSLP led to the production of T_H2-specific cytokines and the transcription factor GATA-3, which is essential for T_H2 differentiation³⁹ (Fig. 6f and Supplementary Figure 6c). IL-4R α was also upregulated in T cells in the presence of TSLP (Fig. 6f). These observations suggested that basophil-derived TSLP can promote the T_H2 differentiation of naive T cells. Indeed, papain-stimulated basophils promoted the T_H2 differentiation of naive T cells activated *in vitro* (Fig. 6g). Thus, TSLP and IL-4 are probably the critical basophil-derived signals required for T_H2 differentiation.

Finally, TSLP promoted T_H2 cell survival. TSLP, but not IL-4, led to transcription of the prosurvival factors Bcl-2 and c-FLIP⁴⁰ (Fig. 6f). This correlated with more live cells at early time points in TSLP-supplemented cultures (Fig. 6h). This seemed to be due to greater cell survival, as TSLP did not induce significantly more proliferation relative to that in standard T_H2 differentiation conditions or with TCR stimulation alone ($P > 0.98$; Supplementary Figure 6d). Thus, TSLP promoted naive T cell competence for T_H2 differentiation and survival.

These results collectively suggest a model of T_H2 differentiation by a protease allergen (Supplementary Figure 7 online). In this model, allergen proteases or proteases secreted by helminths cleave a host-derived sensor. This sensor is produced by basophils and probably other cell types. After cleavage of this sensor, basophils become activated to produce IL-4, TSLP and other T_H2-promoting cytokines, leading to T_H2 differentiation.

DISCUSSION

Activation of the adaptive immune response is generally dependent on innate immune recognition of the infectious agent(s). How the innate immune system recognizes both complex metazoan parasites and simple protein allergens to induce similar T_H2 responses has been a longstanding question. One possible solution to this conundrum is that the protease activity of allergens or proteases secreted by parasites may be sensed by the host innate immune system⁶. As proteases are essential for the infectious cycle of helminths, this innate sensing strategy may have evolved to deal with the complexity of parasites. Protease allergens, in contrast, probably trigger this same pathway because of shared enzymatic activity and specificity. Indeed, the protease activity of several allergens has been shown before to be essential for the induction of T_H2 and IgE responses^{4,18,41}. However, the mechanism and the physiologically relevant cellular target linking this protease activity with the induction of T_H2 differentiation have remained unidentified. The protease allergen Der p 1 has been shown to influence the activity of DCs, T cells and B cells *in vitro*^{41,42}. We did not find such effects *in vivo* and, as none have been described to induce T_H2 differentiation or IgE production, their *in vivo* relevance remains unclear (data not shown)⁴¹. Here we have identified basophils as a cell type that can be directly targeted by protease allergens with immunologically relevant consequences, such as the production of T_H2-promoting cytokines and induction of T_H2 differentiation *in vivo*.

Basophils have been reported to be involved in the effector response to helminths⁴³, memory responses⁴⁴, chronic allergic inflammation¹³ and IL-4 production in an IgE-dependent way^{28,45}. Because they are able to produce IL-4, it has been hypothesized that basophils may be involved in initiating T_H2-mediated immunity by providing an early source of IL-4 for T_H2 differentiation¹². However, this function has been indicated only by observations based on the activation of basophils through Fc ϵ RI-bound antigen-specific IgE in the setting of chronic allergic inflammation or helminth infection in mice with IL-3-mediated basophilia^{13,14}. Here we have provided evidence that basophils were activated and recruited to the draining lymph nodes in response to protease allergens and were required

for the induction of T_H2 responses to these allergens. Whether basophils are activated before or after lymph node recruitment remains unclear. We were unable to find basophils in the skin before or after immunization with papain and we did not detect activated basophils in the peripheral blood after such immunization (data not shown). Furthermore, the precise mechanism underlying their recruitment to the draining lymph node is not known. Basophils express many chemokine receptors, including CCR1, CCR2 and CXCR4, but which chemokine(s) is (are) responsible for the entry of basophils into the draining lymph node remains to be determined. Notably, basophil activation and recruitment occurred before the induction of the adaptive immune response, emphasizing the function of the basophil as an innate initiator of T_H2 responses to protease allergens.

In addition to their essential function in T_H2 response initiation, allergen-activated basophils produced IL-4 and TSLP. Although the critical function of IL-4 in T_H2 differentiation is well established, the cellular source of IL-4 during T_H2 differentiation *in vivo* remains unclear because the accessory cell type capable of supplying IL-4 for naive T cells in the lymph nodes has not been identified before our study here. T cell-derived IL-4 is sufficient for T_H2 differentiation *in vivo* after transfer of TCR-transgenic T cells³². Whether IL-4 produced by T cells is sufficient in conditions of normal T cell precursor frequency is not yet apparent. By analogy with T_H1 and T_H-17 responses, it seems likely that a critical lineage-specifying cytokine would be produced by cells of the innate immune system in response to the appropriate innate immune sensing pathway. Given the potent effect of IL-4 on T_H2 differentiation, it is reasonable to suggest that basophil-derived IL-4 is important in the induction of T_H2 responses.

TSLP is another T_H2-associated cytokine that we found here to be produced by basophils and to be involved in allergen-induced T_H2 differentiation *in vivo*. TSLP is produced in peripheral tissues, where it is important in the effector stages of T_H2 immunity and in allergic inflammation^{15,16,34}. In addition, TSLP acts directly on naive T cells, which suggests involvement of this cytokine in the induction of T_H2 responses¹⁷. However, it is unclear how TSLP produced in peripheral tissues may affect the activation of naive T cells. One possibility is that epithelial cell-derived TSLP acts on DCs to induce their migration to the lymph nodes and perhaps their 'programming' for T_H2 response induction, as shown before for human DCs¹⁵. However, we were unable to detect TSLP protein production in keratinocytes after immunization with papain (data not shown), and DC migration and maturation were unaffected by TSLP neutralization. Therefore, at least in the context of the response to protease allergens, basophils in the lymph nodes are an important source of TSLP during the initiation of T_H2 responses. Peripheral sources of TSLP may be more relevant for the control of effector stages of T_H2-mediated immunity in both physiological⁴⁶ and pathological¹⁵ conditions.

TSLP can promote the T_H2 differentiation of naive T cells *in vitro* by many mechanisms. TSLP stimulation *in vitro* resulted in the induction of transcripts encoding IL-4, IL-13, IL-4R α and GATA-3, and this induction was not dependent on IL-4 production or signaling by T cells, as T cells lacking IL-4 or IL-4R α retained TSLP-mediated gene induction. Thus, TSLP may promote naive T cell competence for T_H2 differentiation in a way analogous to the function of natural killer cell-derived IFN- γ in T_H1 differentiation⁷. Both cytokines are derived from accessory cell types and act on naive T cells to promote their competence for a particular differentiation fate through the induction of the appropriate cytokine receptor subunit. Finally, TSLP also upregulated the expression of antiapoptotic genes in T cells and promoted the survival of T_H2 cells. These and additional effects of TSLP on T_H2 responses *in vivo* will need to be investigated further in future studies.

Our findings collectively support a model for the innate initiation of T_H2 responses based on the sensing of protease activity of allergens and

helminths^{6,47}. In this model, a host-derived sensor of proteolytic activity is cleaved by parasite or allergen proteases. This sensor, once cleaved, activates cells of the innate immune system to induce a T_H2 response. This includes, but presumably is not limited to, basophil activation and recruitment to the draining lymph node, where basophils produce IL-4 and TSLP, which promote T_H2 differentiation. Our findings indicate that in addition to their effector functions, basophils also act as the sensors of protease allergens and possibly helminths, suggesting new therapeutic pathways for the treatment of atopic disease.

Notably, that strategy of innate immune recognition is distinct from pattern recognition in that it does not depend on the detection of molecular structures uniquely associated with pathogens. Instead, it is based on the detection of enzymatic activity associated with the presence of parasites. A similar principle is involved in innate immune recognition in plants and in fungal recognition in *Drosophila*^{48,49}. It would be useful to determine whether this strategy is also used in the innate sensing of fungal and protozoan pathogens in mammals.

METHODS

Mice. Animals were bred and maintained at the Yale Animal Resources Center at Yale University and all animal experiments were done with the approval of and in accordance with regulatory guidelines and standards set by the Institutional Animal Care and Use Committee of Yale University. BALB/c, C57BL/6, TLR4d BALB/c (C.C3-*Tlr4*^{ps-d}), *Il4*^{-/-} (BALB/c-*Il4*^{tm2Nnt/J}), mast cell-deficient (WBB6F1/J-*Kir*^W/*Kir*^{W-v}) and 4get mice were from Jackson Laboratories. *Tlr4*^{-/-}*Tlr2*^{-/-} mice were backcrossed two to three times and *Myd88*^{-/-} mice were backcrossed over six times onto the C57BL/6 background. *Il4ra*^{-/-} and DO11.10 × 4get mice were provided by K. Bottomly (Yale University).

Reagents and antibodies. Papain and E64 were from Calbiochem. Purified 2.4G2, antibody to FcεRIα (anti-FcεRIα; MAR-1), allophycocyanin-conjugated anti-CD11c (N418), phycoerythrin-conjugated anti-CD86 (GL1), allophycocyanin-conjugated DX5, biotin-conjugated DX5, allophycocyanin-conjugated anti-c-Kit (2B8), and fluorescein isothiocyanate-conjugated anti-I-A-I-E (M5/114.15.2) and anti-IL-4 (11B11) were from eBioscience. Anti-IL-4Rα (M1), biotin-conjugated anti-CD11b (M1/70), and phycoerythrin-conjugated anti-CD4 (GK1.5), anti-CD8α (53-6.7), anti-IL-4 (11B11), anti-tumor necrosis factor (MP6-XT22) and anti-IL-6 (MP5-20F3) were from BD Pharmingen. Fluorescein isothiocyanate-conjugated anti-DEC-205 (NLDC-145) was from Serotec. Rat anti-mouse TSLP (152614) was from R&D Systems. Biotin-conjugated KJ1.26 was provided by K. Bottomly (Yale University). A histamine enzyme-linked immunosorbent assay kit (IBL) was used for analysis of histamine. E64-inactivated papain was reduced with 5 mM cysteine-HCl (Sigma), followed by the addition of E64 in a 100-molar excess relative to papain. After incubation, inactivated papain was dialyzed for removal of excess E64, protein concentrations were measured with the Micro BCA Protein Assay Kit (Pierce) and then activity was tested by immunoglobulin cleavage. Alternatively, papain was heat-inactivated by being heated to 100 °C for 10 min.

Generation of 28F12 antibody. Sprague-Dawley rats were repeatedly immunized with purified mouse TSLP and alum, and their spleens were used for the generation of hybridomas by standard methodologies. Hybridomas were screened for the production of TSLP-specific antibodies by enzyme-linked immunosorbent assay. NAG8/7 cells were stimulated with various amounts of TSLP and 1 μg/ml of 28F12; IxN/2b cells were stimulated with various amounts of mouse IL-7 and 1 μg/ml of 28F12.

Immunization, depletion and cell transfer. Mice were immunized subcutaneously in the rear footpads with 50 μl PBS into which 50 μg HSA (Sigma), papain (active, heat-inactivated or E64-inactivated), bromelain (Calbiochem) or OVA was dissolved, with or without 10 μg lipopolysaccharide (Sigma) or CpG dinucleotide. For immunization with OVA in alum, OVA dissolved in PBS was mixed at a ratio of 1:1 with Imject alum (Pierce) and mice were immunized with 50 μg OVA in 20 μg alum. For basophil depletion, mice were immunized intravenously with 100 μg MAR-1 at 90 h and 72 h before subcutaneous immunization with antigen and adjuvant. CD4⁺ DO11.10 T cells were positively selected with CD4 microbeads followed by purification by magnetic-activated cell separation (Miltenyi). Purified CD4⁺ cells

(5×10^5 to 1×10^6) were transferred into recipient mice. For TSLP neutralization, mice were injected intravenously with 250 μg 28F12 concurrent with subcutaneous immunization with papain.

Enzyme-linked immunosorbent assay. Serum samples were obtained immediately before antigen immunization on days 0 and 14. Final serum samples were obtained at 21 d. Total IgE, IgG1, IgG2 and IgM were detected with anti-mouse IgE (R35-118), anti-mouse IgG1 (A85), anti-mouse IgG2 (R2-40) and anti-mouse IgM (II/41; all from BD Pharmingen). Anti-TNP IgE (557080) and purified IgG1 κ-chain (557273) were used as controls (BD Pharmingen). Papain (50 μg/ml) and E64 (100× molar excess) were plated together to coat plates overnight. After plates were blocked with 1% (wt/vol) BSA, serum was plated at multiple dilutions and papain-specific IgE was detected with biotinylated anti-mouse IgE (R35-118). Absorbance values at a serum dilution of 1:25 were used to indicate papain-specific IgE.

Bone marrow cultures. For the production of DCs, bone marrow was cultured for 5 d in media supplemented with granulocyte-macrophage colony-stimulating factor (Peprotech). Cultures were then stimulated for 18 h with heat-inactivated or active protease (100 μg/ml) or with CpG dinucleotide (5 μM). For the production of mast cells and basophils, erythrocyte-depleted mouse bone marrow was initially seeded at density of 5×10^6 cells/ml, followed by replating every 3–4 d at a density of 1×10^6 cells/ml for a total of 12 d culture in RPMI medium supplemented with IL-3 (30 ng/ml; Peprotech) and 10% (vol/vol) FCS. Samples were enriched for basophils by positive selection of DX5⁺ cells with magnetic-activated cell sorting. Cultures were stimulated with ionomycin (500 ng/ml; Calbiochem), lipopolysaccharide (100 ng/ml; Sigma) or heat-inactivated or active protease (100 μg/ml). Activation by IgE crosslinking was accomplished by incubation with mouse IgE (10 μg/ml) followed by incubation with anti-mouse IgE (10 μg/ml; R35-118).

T cell culture. Total splenic CD4⁺ T cells were isolated by positive selection of CD4⁺ T cells with magnetic-activated cell sorting. Naive splenic CD4⁺ T cells were isolated by sorting of CD4⁺CD62L⁺ splenocytes with flow cytometry. T cells were cultured on plates coated with anti-CD3 (145-2C11) and anti-CD28 (37.51; 2 μg/ml; BD Pharmingen) and were activated in the presence of recombinant mouse TSLP (30 ng/ml), recombinant mouse IL-4 (30 ng/ml; R&D Systems) and anti-IFN-γ (5 μg/ml; R4-6A2; BD Pharmingen). For basophil-T cell cocultures, basophils were activated for 4 h with papain (100 μg/ml), were mixed with naive CD4⁺ T cells and were incubated for 3 d. CD4⁺ T cells were then purified by positive selection of CD4⁺ T cells with magnetic-activated cell sorting and were restimulated for 3 d on plates coated with anti-CD3 and anti-CD28.

Flow cytometry and sorting. Lymphocytes and hepatic leukocyte, splenocyte and peripheral blood leukocyte samples depleted of erythrocytes were first blocked with 2.4G2 for elimination of Fc receptor-mediated antibody binding. Cells were then incubated for 20 min on ice with antibodies. Cells were analyzed on a FACSCalibur (BD Biosciences) and data were analyzed with FloJo software (TreeStar). Samples were sorted on a MoFlo (BD Biosciences) at 30 p.s.i. and basophils were selected as c-Kit^{Gr-1}DX5⁺ (anti-Gr-1: RB6-8C5). A Cytofix/Cytoperm Plus kit with GolgiPlug (BD Pharmingen) was used for intracellular cytokine staining according to the manufacturer's instructions. Sorted basophils were made to adhere to slides with Cytospin and were stained with Diff-Quick (VWR). Transmission electron microscopy of sorted basophils was done by the Yale Center for Cellular and Molecular Imaging.

Immunofluorescence. Lymph nodes were fixed overnight at 4 °C in periodate-lysine-paraformaldehyde fixative. Organs were then incubated in sucrose solutions of increasing percentages for cryoprotection, then were frozen in optimum cutting temperature compound (Tissue-Tek), and sections 6–8 μm in thickness were cut on a Kryostat (Leica) and were kept at –20 °C. Sections were rehydrated in PBS, were blocked in PBS containing 0.1% (vol/vol) Tween-20 and 5% (wt/vol) BSA, then were stained with fluorescein isothiocyanate-conjugated anti-GFP (600-402-215; Rockland), MAR-1 and anti-CD4 (GK1.5) or anti-B220 (RA3-6B2; eBioscience). Streptavidin-Alexa Fluor 594 (S-32356) and Alexa Fluor 647-conjugated anti-rat (A-21247 Molecular Probes) were used as secondary antibodies. Vectashield mounting medium (Vector Laboratories) was used to prevent fading of staining while samples were being examined.

Note: Supplementary information is available on the Nature Immunology website.

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