

Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response

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T helper type 2 (T_H2)-mediated immune responses are induced after infection with multicellular parasites and can be triggered by a variety of allergens. The mechanisms of induction and the antigen-presenting cells involved in the activation of T_H2 responses remain poorly defined, and the innate immune sensing pathways activated by parasites and allergens are largely unknown. Basophils are required for the *in vivo* induction of T_H2 responses by protease allergens. Here we show that basophils also function as antigen-presenting cells. We show that although dendritic cells were dispensable for allergen-induced activation of T_H2 responses *in vitro* and *in vivo*, antigen presentation by basophils was necessary and sufficient for this. Thus, basophils function as antigen-presenting cells for T_H2 differentiation in response to protease allergens.

Different CD4⁺ helper T cell effector lineages control host defenses against distinct classes of pathogens. T helper type 1 cells (T_H1 cells) provide protective immunity to intracellular bacterial, viral and protozoan pathogens; interleukin 17 (IL-17)-producing T helper cells (T_H-17 cells) regulate host defense against extracellular bacterial and fungal pathogens; and T_H2 cells orchestrate immunity to multicellular parasites, including helminths, which are mostly extracellular pathogens¹. Inappropriate activation of the three arms of adaptive immunity can lead to different types of immunopathologies, including autoimmunity in the case of T_H1 and T_H-17 responses and allergies in the case of T_H2 responses¹.

Although the basic aspects of the activation of T_H1 and T_H-17 immune responses are well characterized, the mechanisms of the induction of T_H2 immune responses remain obscure. To a large extent this reflects a lack of understanding of the mechanisms of innate immune recognition of 'type 2 pathogens'. In the case of T_H1 and T_H-17 immunity, several classes of pattern-recognition receptors, including Toll-like receptors and dectin-1, detect bacterial, viral and fungal pathogens through the recognition of conserved molecular structures characteristic of each pathogen class^{2–5}. These pattern-recognition receptors are expressed on, among other cell types, dendritic cells (DCs), where they control their activation, migration to the lymph nodes and presentation of pathogen-derived antigens to naive T cells⁶. In addition to presenting antigens derived from phagocytosed or endocytosed pathogens, DCs produce other signals necessary for the activation and differentiation of naive CD4⁺ T cells into the appropriate T_H1 or T_H-17 effector lineage^{4,5,7}. Notably, DCs that present antigens to naive T cells also provide costimulatory molecules and produce cytokines (such as IL-12, IL-23 and IL-6) that control T_H1 and T_H-17 differentiation^{8,9}.

However, the scenario outlined above may not apply to the initiation of T_H2 responses. First, unlike bacterial, fungal and viral pathogens, parasitic worms are far too large to be phagocytosed by DCs or any other phagocytes. Therefore, in contrast to the situation with T_H1 and T_H-17 cells, the source of antigens presented to T_H2 cells is unlikely to be phagocytosed pathogens. One possibility is that a source of antigens for T_H2 cells is the proteins shed or excreted by helminths^{10,11}. These proteins include cysteine proteases that are important in parasites' infection cycles and can have immunogenic activity for the induction of T_H2 responses^{10,12,13}. Another notable distinction between the activation of T_H1 and T_H-17 responses and T_H2 responses is that DCs do not produce the cytokines known to be important for T_H2 differentiation, including IL-4 (A001262) and thymic stromal lymphopoietin (TSLP; A002363). Basophils have been shown to be recruited to the lymph nodes during the primary immune response to protease allergens and schistosome soluble egg antigen¹⁴. There they produce T_H2-promoting cytokines, including IL-4 and TSLP, and are essential in the initiation of T_H2 responses¹⁴. Such findings suggest that basophils function as accessory cells for T_H2 differentiation, at least in response to protease allergens such as papain, by producing T_H2-promoting cytokines at the site of the activation of naive CD4⁺ T cells in the lymph nodes. Although migration of DCs to the draining lymph node after papain immunization has been reported, the identity of the antigen-presenting cell (APC) for T_H2 induction *in vivo* has not been established.

Here we investigate the functions of basophils and DCs in the initiation of T_H2 responses and find that DCs were neither required nor sufficient for the induction of a T_H2 response by the protease allergen papain. Both *in vitro* and *in vivo*, basophils were able to present antigen and induce T_H2 differentiation of naive CD4⁺ T cells. Our data indicate that basophils are the relevant APCs for T_H2

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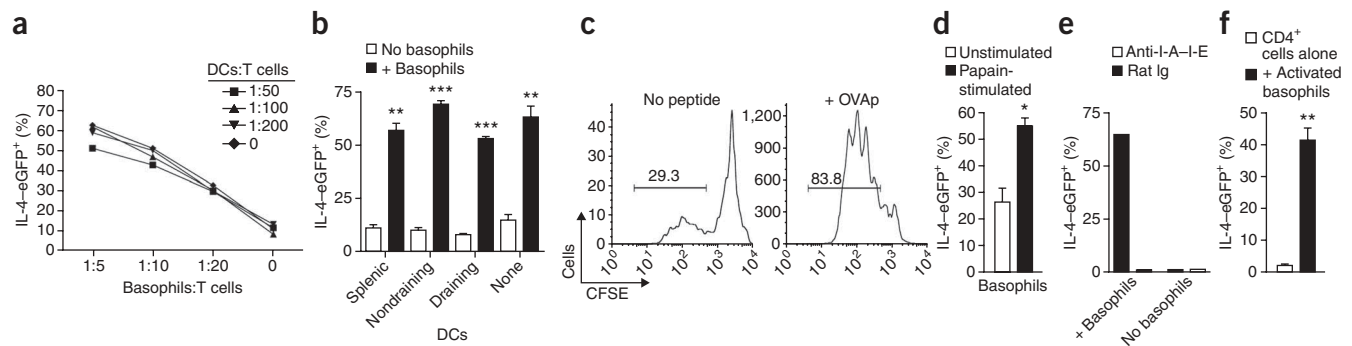


Figure 1 Basophils are necessary for T_H2 differentiation *in vitro*, but DCs are not. **(a)** IL-4-eGFP⁺ T cells among total splenic CD4⁺ T cells from DO11.10 $\times$ 4get mice, cultured for 3 d together with OVA and various ratios of BMBs and BMDCs purified from BALB/c mice by magnetic-activated cell sorting. **(b)** IL-4-eGFP⁺ cells among CD4⁺ cells as described in **a**, mixed with DCs obtained from various sites in papain-immunized Toll-like receptor-4-deficient BALB/c mice, in the presence (+ Basophils) or absence (No basophils) of BMBs. Nondraining, brachial lymph node; draining, popliteal lymph node. **(c)** Proliferation of cytosolic dye CFSE-labeled CD4⁺ cells after culture together with BMBs. Numbers above bracketed lines indicate percent proliferating cells among total live CD4⁺ cells. **(d)** IL-4-eGFP⁺ cells among splenic CD4⁺ cells prepared as described in **a**, then mixed with unstimulated or papain-stimulated BMBs. **(e)** T_H2 differentiation in the presence of blocking antibodies to MHC class II. **(f)** T_H2 differentiation in cultures of DO11.10 $\times$ 4get CD4⁺ MHC class II-negative cells and BALB/c BMBs purified by flow cytometry. Unless noted otherwise, BMBs were stimulated with papain before coculture. *, $P < 0.01$; **, $P < 0.001$; and ***, $P < 0.0001$, with versus without basophils (**b,f**), or with versus without papain stimulation (**d**; Student's *t*-test). Data are representative of at least three independent experiments (error bars (**b,d,f**), s.e.m.).

induction by papain. We demonstrate that antigen presentation by basophils was necessary and sufficient for T_H2 induction in response to a protease allergen *in vitro* and *in vivo*.

RESULTS

Basophils induce T_H2 differentiation *in vitro*

Basophils have been shown to be essential for *in vivo* T_H2 differentiation in response to papain immunization¹⁴. However, the precise identity of the APC responsible for the induction of the T_H2 response to papain has remained unclear. DCs are not directly activated by papain *in vitro*¹⁴. Furthermore, here we found that papain-treated DCs were unable to induce T_H2 differentiation *in vitro* (data not shown), which suggests either that an accessory cell type is required, in addition to DCs, or that DCs are not the relevant APC for T_H2 differentiation, at least in response to papain.

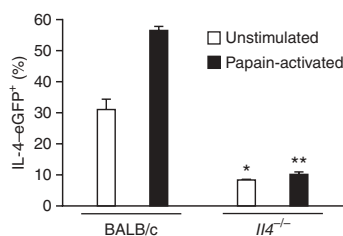
To address those issues, we used an *in vitro* system of T_H2 differentiation with purified ovalbumin (OVA)-specific CD4⁺ T cells from DO11.10 $\times$ 4get mice (in which *Il4* mRNA expression is 'reported' by enhanced green fluorescence protein (eGFP)¹⁵), bone marrow-derived basophils (BMBs) and bone marrow-derived DCs (BMDCs). In accordance with a published report using a similar culture system¹⁶, culture of BMDCs, BMBs and CD4⁺ T cells together in the presence of antigen led to T_H2 differentiation, as measured here by IL-4-eGFP expression in CD4⁺ T cells (**Fig. 1a**). T cell expression of IL-4-eGFP has been shown to correlate well with actual production of IL-4, IL-5 and IL-13 and lack of interferon- γ production in standard culture conditions and after papain immunization *in vivo*^{14,15}. Thus, we used IL-4-eGFP expression here as a reliable marker of T_H2 differentiation. Unexpectedly, despite the previous observation that DCs migrate to the draining lymph node after papain immunization *in vivo*¹⁴, this T_H2 differentiation *in vitro* was dependent on basophils but showed no dependence on DCs (**Fig. 1a**). To assess whether other DC subtypes were the relevant APCs, we studied *in vitro* T_H2 differentiation in the presence of various subsets of *ex vivo*-purified DCs: splenic DCs, DCs sorted from draining (popliteal) lymph nodes after papain immunization and DCs from nondraining (brachial and cervical) lymph nodes. We found no function for any of those DC subsets in *in vitro* T_H2 differentiation (**Fig. 1b**). In fact, we found

no function for DCs as APCs *in vitro*; instead, T_H2 differentiation was dependent only on the presence of basophils (**Fig. 1b**), which also supported robust T cell proliferation (**Fig. 1c**). In addition to the previously described cytokine profile of these IL-4-eGFP⁺ cells^{14,15}, IL-10 was produced by T_H2 cells after *in vitro* differentiation, as measured by upregulation of *Il10* expression (**Supplementary Fig. 1** online). *In vitro* T_H2 differentiation was strongly enhanced after basophil activation by papain, although unstimulated basophils could also support T_H2 activation to a lesser extent (**Fig. 1d**), presumably because of some amount of spontaneous activation caused by tissue culture conditions.

Antigen presentation by basophils via major histocompatibility complex (MHC) class II has not been described before, to our knowledge. Therefore, we further examined whether basophils functioned as APCs by presenting antigens via the classical MHC class II pathway or whether they simply provided cytokines (such as IL-4) necessary for T_H2 differentiation. To assess this, we studied T_H2 differentiation in cocultures of BMBs and splenic CD4⁺ T cells in the presence or absence of antibodies blocking MHC class II. As before, T_H2 differentiation was dependent on the presence of basophils (**Fig. 1e**). However, this T_H2 differentiation was completely lost in the presence of MHC class II-blocking antibody (**Fig. 1e**), which indicated that basophils activated and induced T_H2 differentiation through the classical MHC class II-dependent pathway. Finally, to rule out the possibility of contamination of the *in vitro* culture system with alternative APCs or mast cells, we cultured highly purified (>99%) populations of BMBs and OVA-specific splenic CD4⁺ T cells together (**Supplementary Fig. 2a–c** online). T_H2 differentiation was not due to contaminating APCs or mast cells (**Fig. 1f**). Thus, basophils seemed to be able to present antigen via MHC class II and to induce T_H2 activation and differentiation *in vitro*.

We next sought to determine whether *in vitro* T_H2 differentiation was mechanistically similar to *in vivo* T_H2 differentiation. Basophils produce a group of cytokines after exposure to papain *in vitro*¹⁴. One such cytokine, IL-4, has been reported to be necessary for T_H2 differentiation in similar *in vitro* culture systems¹⁶. In accordance with that, we found that T_H2 differentiation was dependent mainly on IL-4 production by basophils (**Fig. 2**). T_H2 differentiation was much

Figure 2 Basophil-mediated T_H2 differentiation *in vitro* is dependent on IL-4. T_H2 differentiation of unstimulated or papain-stimulated BMBs from IL-4-sufficient mice (BALB/c) or IL-4-deficient mice ($Il4^{-/-}$), cultured together with splenic $CD4^+$ cells from DO11.10 $\times$ 4get mice and OVA_p, assessed as the percentage of IL-4-eGFP $^+$ $CD4^+$ T cells after 3 d. *, $P < 0.001$ and **, $P < 0.0001$, $Il4^{-/-}$ versus BALB/c (Student's *t*-test). Data are representative of five experiments (error bars, s.e.m.).



lower in cultures that contained $Il4^{-/-}$ BMBs. Thus, basophils seem to 'instruct' T_H2 differentiation via IL-4 production *in vitro*.

Basophils express MHC class II and costimulatory molecules

Basophils produce the T_H2 -inducing cytokines IL-4 and TSLP after stimulation with papain *in vitro* and *in vivo*¹⁴. However, to our knowledge, basophils have not been reported before to express MHC class II molecules. We therefore examined MHC class II expression and its regulation in basophils. Expression of MHC class II is dependent on the transcriptional regulator CIITA (A000657), which controls the expression of several key components of the MHC class II antigen-presentation pathway^{17–19}. After papain stimulation, but not after crosslinking of immunoglobulin E (IgE), CIITA was induced in basophils (Fig. 3a). The expression of *Ciita* mRNA in

basophils was less than that in DCs but equivalent to that in macrophages, cells known to be able to present antigen via MHC class II (Fig. 3a). In mice, CIITA expression is controlled by three of four separate promoters used by specific cell types: promoter I is used in myeloid cells (macrophages and conventional DCs); promoter II is inactive in mice but directs CIITA expression in T cells in humans and other species; promoter III controls CIITA expression in B cells and plasmacytoid DCs; and promoter IV is active in nonhematopoietic cells, such as thymic epithelium^{20,21}. Examination of promoter-specific expression showed that in basophils, *Ciita* was transcribed from promoter III (Fig. 3b). CIITA expression was accompanied by transcriptional upregulation of MHC class II and the invariant chain CD74 (Fig. 3b). Notably, induction of CIITA and of its targets, MHC class II-associated genes, was specific to basophils activated by active papain, whereas IgE crosslinking did not induce their expression (Fig. 3b). Therefore, although *Ciita* promoter III is inducible by IL-4 in B cells, just as promoter I is inducible by interferon- γ in myeloid cells²¹, lack of induction of MHC class II genes by IgE crosslinking indicates that additional stimuli other than IL-4 are necessary for CIITA induction in basophils. This upregulation of MHC class II transcripts in papain-activated basophils was accompanied by induction of MHC class II proteins by papain *in vivo* and *in vitro* (Fig. 3c–e).

Basophils transiently enter the popliteal lymph nodes 3 d after subcutaneous immunization of papain in the rear footpad¹⁴. We found that these lymph node basophils had abundant expression of MHC class II molecules (Fig. 3c). Lymph node basophils also had high expression of the costimulatory molecules CD40 and CD86, as

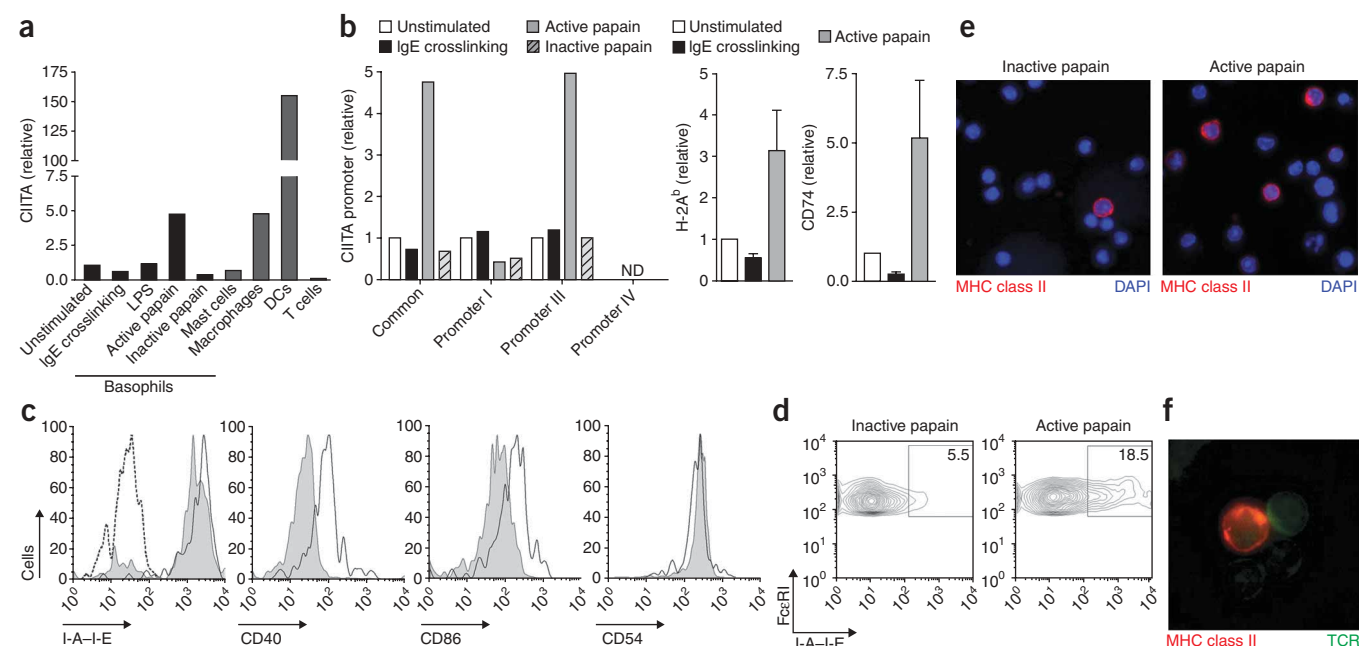


Figure 3 Basophils express and upregulate MHC class II after papain stimulation and can form synapses with T cells *in vitro*. (a) Quantitative PCR analysis of *Ciita* expression in BMBs activated for 4 h *in vitro* (stimuli, horizontal axis) and in various other hematopoietic cells, presented relative to the expression in unstimulated cells, set as 1. Mast cell, macrophage and DC RNA was isolated from bone marrow-derived cell cultures; T cell RNA was derived from splenic $CD3^+CD4^+$ cells sorted by flow cytometry. (b) Quantitative PCR analysis of the expression of MHC class II promoters and related genes in BALB/c BMBs activated for 4 h (stimuli in key), presented relative to the expression in unstimulated cells, set as 1. Common, shared (common) promoter segments; ND, not detected. (c) Flow cytometry staining of lymph node basophils (solid lines) and peripheral blood basophils (shaded histograms) 3 d after papain immunization. Dashed line (I-A-I-E stain), isotype-matched control antibody. (d) I-A-I-E expression on live basophils after stimulation of mast cell-depleted cultures of BMBs with active or inactive papain. Numbers in outlined areas indicate percent of the gated population of live basophils. (e) Immunofluorescence analysis of MHC class II expression (red) in BMBs after stimulation with inactive or active papain. Blue, DAPI (DNA-intercalating dye). Original magnification, $\times 20$. (f) Formation of the immune synapse 60 min after coculture of papain-activated BMBs (BALB/c) and splenic $CD4^+$ T cells (DO11.10). Green, T cell antigen receptor- β (TCR); red, MHC class II. Original magnification, $\times 100$. Data are representative of at least four independent experiments (error bars (b), s.e.m.).

well as CD54 (Fig. 3c). Expression of MHC class II, CD40, CD86 and CD54 was detectable and equivalent on the peripheral blood basophils from both papain-immunized and unimmunized mice (Supplementary Fig. 3a,b online), but surface expression of MHC class II, CD40 and CD86 was higher on lymph node basophils than on peripheral blood basophils after papain injection (Fig. 3c and Supplementary Fig. 3b). Except for being upregulated on lymph node basophils after papain immunization, expression of the costimulatory molecules was equivalent regardless of papain immunization or the location from which the basophils were isolated: peripheral blood, spleen or bone marrow (Supplementary Fig. 3a,c). The same was true for MHC class II expression, with the exception of bone marrow basophils (Supplementary Fig. 3b). Basophils isolated from the bone marrow had lower steady-state expression of MHC class II, presumably secondary to a more immature state of development (Supplementary Fig. 3b). In contrast to the nearly uniform expression of MHC class II on basophils in unimmunized mice *in vivo*, few unactivated BMBs expressed MHC class II *in vitro* (Fig. 3d,e). In accordance with quantitative PCR data, papain stimulation led to larger numbers of cells expressing MHC class II (Fig. 3d,e). Finally, papain-activated basophils pretreated with OVA peptide (amino acids 323–339; OVAp) were able to form immunological synapses with T cells after 60 min of coculture, as measured by clustering of MHC class II and the T cell antigen receptor together at the point of basophil–T cell contact^{22,23} (Fig. 3f).

Basophils endocytose, process and present soluble antigens

Our data thus far indicated that basophils expressed MHC class II both *in vivo* and *in vitro* and were able to present peptide antigens to CD4⁺ T cells, leading to T_H2 differentiation. We next tested whether basophils were able to endocytose, process and present soluble proteins. Basophils were able to endocytose ovalbumin coupled to fluorescein isothiocyanate (Fig. 4a). This endocytosis was followed by antigen processing and presentation, as assayed by basophil-driven T_H2 activation *in vitro* (Fig. 4b). This ability of basophils to take up and process OVA was not due to any direct effects of papain on OVA, as basophils were preactivated with papain and then extensively washed before culture together with OVA and CD4⁺ T cells. Notably, although basophils were able to take up, process and present a soluble protein antigen, they were inefficient in taking up particulate antigens. Compared with DCs, basophils were far less efficient in the phagocytosis of fluorescence-labeled 2-μm latex beads after coculture for 4 h or overnight (Fig. 4c and data not shown). Thus, basophils seem to be specifically able to present soluble antigens.

DCs are not essential for T_H2 differentiation *in vivo*

Basophils are required for the induction of the T_H2 response by papain *in vivo*, and our data so far showed that basophils can function as APCs for the activation of naive T cells and their differentiation into T_H2 cells *in vitro*. Furthermore, *in vitro*, DCs were unable to induce and were not required for T_H2 activation after papain stimulation. We therefore sought to determine whether DCs are necessary or sufficient for activation of the T_H2 response by papain *in vivo*. Although basophils are not normally present in the skin, DCs pick up antigens at peripheral sites and migrate to the draining lymph node, where they present the antigens to T cells to initiate the immune response. The function of these migratory DCs can be assayed by removal of the site of injection several hours after immunization²⁴. Therefore, to address whether skin-resident DCs were necessary for antigen presentation or antigen delivery, we immunized mice in the ear with papain and then removed or retained the injection site 2 h after immunization. In mice that underwent removal of the injection site, T_H2 differentiation was still induced and recruitment of basophils to the lymph nodes was retained, albeit to a lesser extent (Fig. 5). Thus, migration of skin DCs was not necessary for T_H2 differentiation in response to papain immunization. Of note, as T_H2 differentiation after papain immunization was dependent on basophils, the observed decrease in T_H2 differentiation was probably secondary to less basophil migration in mice that underwent removal of the injection site. This diminished basophil migration, in turn, was probably a result of a functionally lower dose of papain in the draining lymph node because of the early time point for removal of the injection site, which we chose to confidently rule out the possibility of migration of DCs or other peripheral antigen-capturing cells. Soluble antigens have been shown to be taken up by conduit-associated DCs in the T cell zone of draining lymph nodes starting at 90 min after subcutaneous injection²⁵. Thus, removal of the injection site and the remaining depot of antigen at 120 min probably resulted in less injected papain in the draining lymph node. Regardless of the possible differences in papain dose, the observation that basophil migration and T_H2 differentiation were retained after removal of the injection site indicates that the response does not require antigen capture at peripheral sites. Instead, it indicates that free, soluble papain enters the draining lymph node with the lymph; there, it may be captured by resident DCs or by basophils.

Next, to address whether migratory or resident DCs were necessary for *in vivo* T_H2 differentiation, we used the CD11c–diphtheria toxin receptor (DTR)–eGFP system, in which CD11c⁺ cells express DTR and can undergo selective depletion by injection of diphtheria toxin^{26,27}. Basophils do not express CD11c and therefore would not be affected

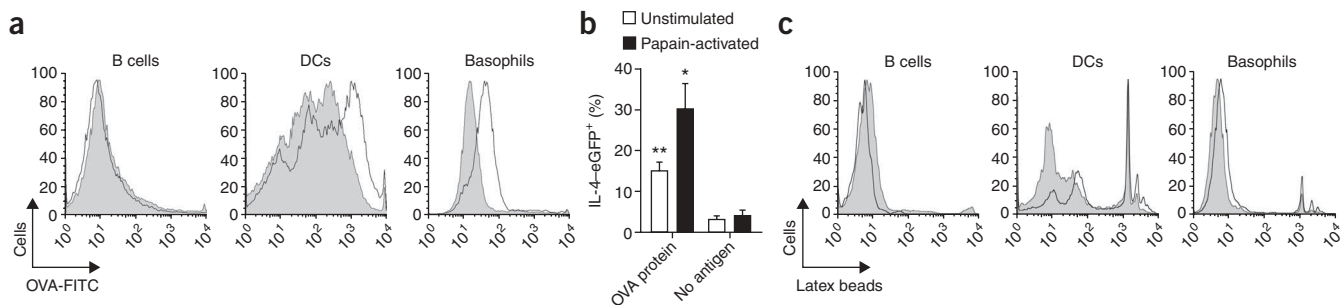


Figure 4 Basophils are able to endocytose soluble antigens but not particulate antigens. (a) Endocytosis of soluble ovalbumin coupled to fluorescein isothiocyanate (OVA-FITC) by B cells, BMDCs or BMBs after 3 h of culture *in vitro* at 37 °C (solid lines) or at 4 °C (shaded histograms). (b) T_H2 differentiation by unstimulated or papain-stimulated BMBs cultured for 3 d together with splenic CD4⁺ T cells from DO11.10 × 4get mice in the presence or absence (No antigen) of OVA protein, assessed as IL-4-eGFP⁺ CD4⁺ T cells. *, $P < 0.01$ and **, $P < 0.001$, with versus without OVA protein (Student's *t*-test). (c) Phagocytosis of fluorescence-labeled 2-μm latex beads by B cells, BMDCs or BMBs after overnight culture *in vitro* at 37 °C (solid lines) or at 4 °C (shaded histograms). Data are representative of at least three independent experiments (error bars (b), s.e.m.).

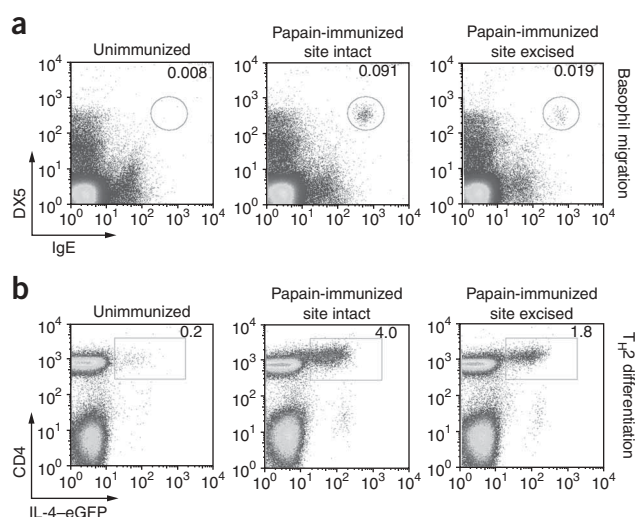


Figure 5 Migratory DCs are not necessary for basophil migration or T_H2 differentiation after papain immunization. Basophil migration (**a**) and T_H2 differentiation (**b**) in the ipsilateral cervical lymph node at 3 d and 4 d, respectively, after immunization of 4get mice with 50 μ g active papain in 10 μ l PBS in the distal pinna; the injection site was either removed (excised) or left intact 2 h after immunization. Numbers above outlined areas indicate percent of the gated population among total live cells. DX5, anti-CD49b. Data are representative of three independent experiments.

by expression of diphtheria toxin²⁸ (data not shown). We established bone marrow chimeras by transferring CD11c-DTR-eGFP bone marrow into BALB/c recipients. We assessed chimerism by eGFP expression in CD11c⁺ cells, and we depleted chimeras of CD11c⁺ cells by injecting diphtheria toxin (**Fig. 6a**). Depletion of DCs by injection of diphtheria toxin had no effect on basophil migration in response to papain immunization (**Fig. 6b**). After transferring OVA-specific DO11.10 CD4⁺ T cells into the mice, we immunized them with OVA, OVA plus papain or OVA plus lipopolysaccharide (LPS) to induce no differentiation, T_H2 differentiation or T_H1 differentiation, respectively. Restimulation of CD4⁺ T cells with OVA *in vitro* showed that although T_H1 differentiation induced by OVA plus LPS was lost after depletion of CD11c⁺ cells by injection of diphtheria toxin, T_H2 differentiation was unaffected by DC depletion (**Fig. 6c**). Thus, DCs were not required for activation of the T_H2 response by papain *in vivo*.

To confirm and extend the finding reported above in a different system, we used the CD11c A β^b (CD11c-IABB) strain of mice, in which MHC class II expression is restricted to CD11c⁺ cells²⁹. Of note,

reconstitution of MHC class II expression in CD11c-IABB mice is incomplete. DC subsets with low endogenous CD11c expression (plasmacytoid DCs and Langerhans cells) remain MHC class II negative^{29,30}. However, MHC class II expression is reconstituted on CD11b^{hi} DCs (which migrate into the draining lymph node after papain immunization), although to a lesser extent in CD11c-IABB mice than in wild-type mice²⁹. Notably, this same DC subset migrates in response to both papain and LPS¹⁴. Thus, if MHC class II expression on the migrating DCs remained defective, we would expect to see defects in both T_H1 and T_H2 differentiation. Limiting MHC class II expression to DCs had no effect on basophil migration in response to papain immunization (**Fig. 6d**). Transfer of OVA-specific TCR-OT-II.2a (OT-II) CD4⁺ T cells into C57BL/6 or CD11c-IABB mice, followed by immunization with OVA plus LPS, led to equivalent T_H1 differentiation, assessed on the basis of interferon- γ production after *in vitro* restimulation (**Fig. 6e**). However, activation of T_H2 differentiation by papain was lost in CD11c-IABB mice (**Fig. 6e**). Thus, despite the fact that basophils are capable of normal migration and cytokine production in CD11c-IABB mice, restricting MHC class II expression to DCs prevented activation of the T_H2 response by papain.

Basophils are APCs *in vivo*

The data so far indicated that DCs were neither necessary nor sufficient for activation of the T_H2 response by papain *in vitro* and *in vivo*. Basophils, in contrast, were necessary and sufficient for T_H2 differentiation *in vitro*, and, as shown above, they were necessary for the papain-induced T_H2 response *in vivo*. However, whether the requirement for basophils *in vivo* is due to their APC function and

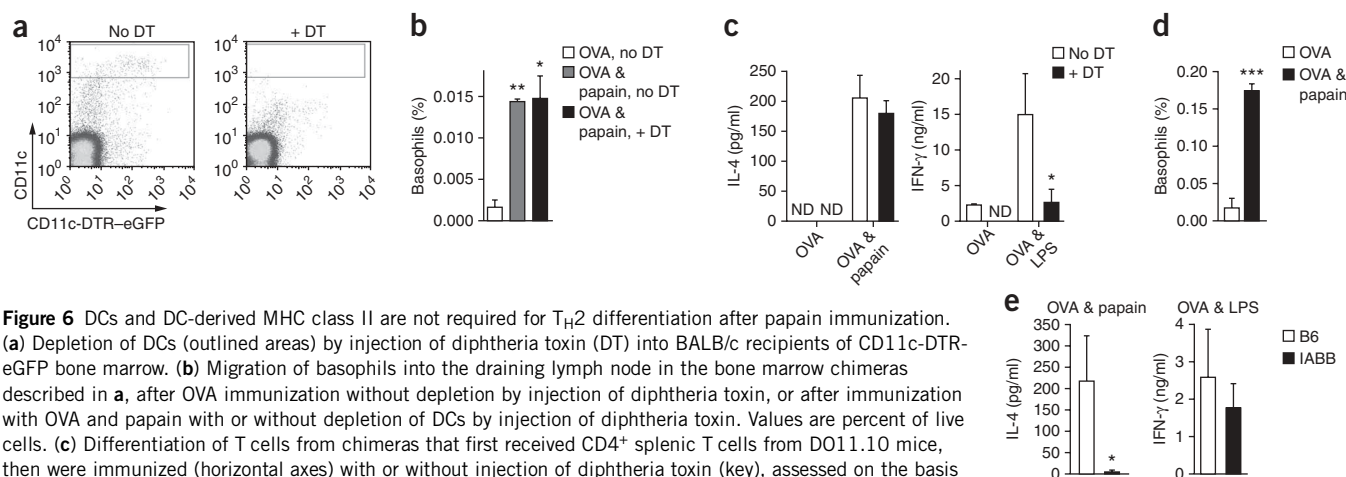
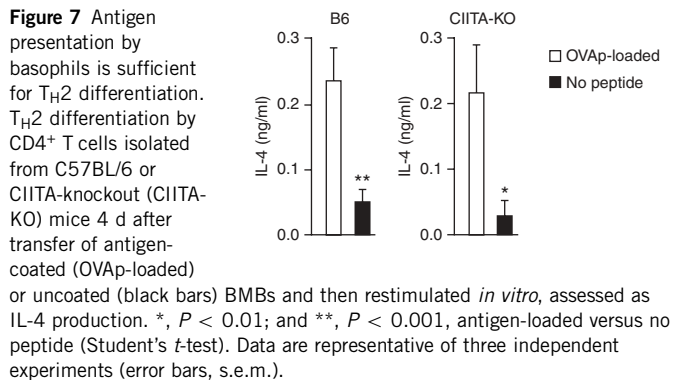


Figure 6 DCs and DC-derived MHC class II are not required for T_H2 differentiation after papain immunization. (**a**) Depletion of DCs (outlined areas) by injection of diphtheria toxin (DT) into BALB/c recipients of CD11c-DTR-eGFP bone marrow. (**b**) Migration of basophils into the draining lymph node in the bone marrow chimeras described in **a**, after OVA immunization without depletion by injection of diphtheria toxin, or after immunization with OVA and papain with or without depletion of DCs by injection of diphtheria toxin. Values are percent of live cells. (**c**) Differentiation of T cells from chimeras that first received CD4⁺ splenic T cells from DO11.10 mice, then were immunized (horizontal axes) with or without injection of diphtheria toxin (key), assessed on the basis of cytokine production after *in vitro* restimulation. (**d**) Migration of basophils into the draining lymph node in CD11c-IABB mice after immunization with OVA or with OVA and papain. (**e**) Differentiation of T cells from CD11c-IABB (IABB) mice or C57BL/6 (B6) mice after immunization with OVA and papain or with OVA and LPS, assessed as described in **c**; OT-II CD4⁺ T cells were transferred into CD11c-IABB mice before immunization. *, $P < 0.01$; **, $P < 0.001$; and ***, $P < 0.0001$, compared with no diphtheria toxin (**b,c**), OVA immunization (**d**) or wild-type mice (**e**; Student's *t*-test). Data are representative of at least three independent experiments (error bars (**b-e**), s.e.m.).



whether basophils can present antigens *in vivo* for T_H2 induction remained unclear. To address those issues, we developed a method of basophil transfer. Basophils have a short life span and poor survival after purification, which prevents their study in adoptive-transfer experiments. To circumvent that limitation, we used BMBs derived from mice transgenic for the antiapoptotic protein Bcl-2 (ref. 31) to improve survival after transfer. We transferred MHC class II-sufficient basophils into wild-type mice, *Ciita*^{-/-} mice and I-A^b-deficient mice (protocol, **Supplementary Fig. 4** online). Notably, antigen-loaded MHC class II-positive basophils were able to mediate the papain-induced T_H2 response in MHC class II-deficient mice (*Ciita*^{-/-} or I-A^b-deficient mice; **Fig. 7** and data not shown). Because in these mice, basophils are the only cells that express MHC class II molecules, we conclude that basophils were sufficient for antigen presentation to $CD4^+$ T cells *in vivo*.

DISCUSSION

The initiation of T_H2 immune responses differs from T_H1 and T_H17 responses in several ways. First, a major pathogen class that elicits T_H2 responses, helminth parasites, is unlikely to be handled by the host APCs in the same way as bacteria, viruses and fungi, the pathogen classes that elicit T_H1 and T_H17 responses. Although the source of antigens presented by DCs for T_H1 and T_H17 induction is generally a phagocytosed pathogen, helminths are too large to be internalized by the APCs for antigen processing and presentation. Therefore, the main source of antigens for T_H2 responses is probably the soluble antigens shed or excreted by helminths. Likewise, most allergens are soluble proteins and are presumably similarly endocytosed by the APCs. Second, in the case of T_H1 and T_H17 responses, the DCs that present antigens also produce T_H1 - and T_H17 -inducing cytokines, including IL-12 and IL-6. However, DCs do not produce T_H2 -inducing cytokines, such as IL-4 and TSLP. Therefore, the induction of T_H2 responses may require either an accessory cell type to provide cytokines or an alternative (non-DC) APC to present antigen and provide helper T cell-differentiating cytokines. Finally, the T_H2 -inducing innate immune signals and their receptors are not well defined. Papain is a potent inducer of T_H2 responses *in vivo*, but it does not activate DCs *in vitro*, which indicates the necessity of an accessory cell or an alternative APC. Collectively, these and other differences between T_H1 or T_H17 responses and T_H2 responses suggest that there may be fundamentally different pathways involved in initiation of these arms of adaptive immunity.

Although basophils are appreciated mainly for their function as type 2 effector cells, they have been shown to be essential in IgG-mediated systemic anaphylaxis³², and published discoveries have emphasized their importance in the induction and regulation of the

adaptive immune response. Basophils are integral to the induction of the T_H2 -mediated immune response after immunization with protease allergens and have been shown to be an important source of primary IL-4 after helminth infection^{33–35}. However, basophils have also been reported to regulate the T_H1 - T_H2 balance and to specifically inhibit T_H1 differentiation^{16,36}. Additionally, cytokine production and expression of the ligand for the costimulatory molecule CD40 by basophils has been suggested to be involved in regulating the antibody response^{37–40}. Finally, basophils have been shown to serve an important function as antigen-capturing cells through antigen-specific IgE bound to their surface through the receptor FcεRI (ref. 41). However, whether they are able to capture antigen during the primary response (in the absence of antigen-specific IgE) or antigen presentation has remained unknown.

It has been shown that although papain has no direct effect on DCs *in vitro*, it potently activates basophils, inducing them to express and secrete several T_H2 -promoting signals, including IL-2, IL-4, IL-13 and TSLP¹⁴. In response to papain administration, basophils are recruited from the circulation to the lymph nodes, where they produce IL-4 and TSLP, which are involved in T_H2 differentiation. Basophils and basophil-derived TSLP are required for the papain-induced activation of T_H2 responses *in vivo*¹⁴. Such findings suggest that basophils may function as accessory cells, aiding DCs in T_H2 induction by producing the cytokines involved in T_H2 differentiation. Here we investigated that possibility and found that DCs had no discernable function in T_H2 induction by papain *in vitro* or *in vivo*, whereas basophils were both necessary and sufficient for papain-induced T_H2 responses *in vitro* and *in vivo*. Basophils have all the characteristics required of a T_H2 -inducing APC: they respond directly to the T_H2 inducer (in this case, papain), they produce T_H2 -inducing cytokines, they express MHC class II and costimulatory signals, they inducibly migrate to the T cell zones of draining lymph nodes, and they can endocytose, process and present soluble proteins, which, as discussed above, are probably the main source of antigens for T_H2 induction.

Indeed, our analyses of the APC involved in T_H2 induction by papain *in vitro* and *in vivo* have demonstrated that DCs are neither necessary nor sufficient for papain-induced T_H2 differentiation. Papain travels directly with lymph to the draining lymph node without requiring capture by APCs at peripheral sites. That observation may explain how basophils, which are not located in normal (uninfected) skin, are able to access and then present soluble antigens such as papain. Furthermore, we found not only that basophils were necessary for T_H2 induction by papain but specifically that antigen presentation by basophils was sufficient for the initiation of a T_H2 response both *in vitro* and *in vivo*. Therefore, basophils are not simply accessory cells that provide cytokines for T_H2 differentiation but also are essential APCs for T_H2 induction. Notably, basophils have also been found independently to function as APCs in two models of helminth infection (D. Artis, personal communication, and K. Nakanishi, personal communication), which suggests that basophils may be the main APCs in T_H2 immunity in physiological and pathological settings. Thus, basophils seem to serve many functions in the regulation of type 2 immunity to helminths and in the induction of T_H2 responses to protease allergens⁴².

It is important to note, however, that T_H2 responses are heterogeneous and can be induced by many, seemingly unrelated, pathways. For example, low doses of inhaled LPS can trigger T_H2 responses in the lung in a Toll-like receptor 4-dependent way^{43,44}. Der p 2 has been shown to function as an allergen because of its ability to bind LPS and to mimic the function of MD-2, a component of the Toll-like receptor 4 complex⁴⁵. Alum promotes T_H2 responses by activating the NALP3

inflammasome, presumably in myeloid cells^{46–50}. Chitin induces type 2 inflammation by acting on alternatively activated macrophages and may also promote T_H2 immune responses⁵¹. Soluble egg antigen has at least some components that activate DCs *in vitro*⁵². Finally, antigens endocytosed by mast cells can be indirectly presented *in vitro* by conventional APCs after the mast cell itself has been phagocytosed⁵³. Such diversity of T_H2-inducing pathways is presumably reflected in the functional diversity of allergens that can trigger them by mimicking the activity of the intended inducers of a particular pathway. Thus, unlike T_H1 and T_H-17 immunity, T_H2 immunity may not follow one unifying model. It follows that there is unlikely to be one mechanism that accounts for the activity of different classes of allergens. The challenge for future studies, therefore, is to delineate the full spectrum of mechanisms and pathways involved in the physiological and pathological initiation of T_H2 responses.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureimmunology/>.

Accession codes. UCSD-Nature Signaling Gateway (<http://www.signaling-gateway.org>): A001262, A002363 and A000657.

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

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AUTHOR CONTRIBUTIONS

C.L.S. and R.M. designed the experiments; C.L.S., N.-Q.C., S.Y. and S.A.N. did the experiments; C.L.S. and R.M. analyzed the data and wrote the manuscript; and T.M.L. provided CD11c-IABB mice.

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ONLINE METHODS

Mice. Animals were bred and maintained at the Yale Animal Resources Center at Yale University. All animal experiments were done with approval by 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^{Lps-d}), *Ciita*^{-/-}, I-A^b-deficient (*H2-Ab1*^{-/-}), OT-II, DO11.10, BALB/c CD11c-DTR-eGFP, *Il4*^{-/-} and 4get mice were from Jackson Laboratories. DO11.10 × 4get mice were provided by K. Bottomly. H-2^k-*Bcl2*-transgenic mice were provided by I. Weissman.

In vitro T_H2 differentiation. CD4⁺ T cells were sorted by magnetic-activated cell sorting or flow cytometry and were plated at a density of 1×10^6 cells per ml in the presence of various ratios of BMDCs or BMBs in RPMI medium with 10% (vol/vol) FCS, standard supplements and IL-3 (30 ng/ml) for basophil survival. If not otherwise noted, BMBs were plated at a ratio of 1:5 with CD4⁺ T cells. The antibody M5/114.15.2 (anti-MHC class II; eBioscience) was added to cultures at a concentration of 20 ng/ml.

Immunization, depletion and cell transfer. Mice were immunized subcutaneously in the rear footpads with 50 µg papain or 2.5 µg LPS with or without 50 µg OVA (Worthington) in 50 µl PBS. BALB/c CD11c-DTR-eGFP chimeras were established as described²⁷. CD4⁺ T cells (1×10^6) purified by magnetic-activated cell sorting were transferred in T cell transfers. Chimeras were injected intraperitoneally with 60 ng diphtheria toxin (Sigma) on days 0, 2 and 5, and CD4⁺ DO11.10 T cells were transferred by intravenous injection on day 3. Popliteal lymph nodes were collected on day 7 for *in vitro* restimulation. For experiments with CD11c-IABB mice, OT-II CD4⁺ cells (CD11c-IABB and C57BL/6 recipients) and C57BL/6 CD4⁺ cells (CD11c-IABB recipients only) purified by magnetic-activated cell sorting were transferred intravenously. Mice were immunized intraperitoneally the next day with 100 µg OVA plus 500 µg papain or 10 µg LPS; spleens were collected 4 d later for *in vitro* restimulation. All *in vitro* restimulations followed standard protocols and enzyme-linked immunosorbent assays used reagents listed above. For ear injection, 50 µg papain (Calbiochem) in 10 µl PBS was injected into the distal pinna, which was excised 2 h later. Basophil migration and T_H2 differentiation were assayed 3 d and 4 d later, respectively, in the ipsilateral cervical lymph nodes.

Adoptive transfer of basophils. This procedure was done as described in **Supplementary Figure 4**. Of note, *Bcl2*-transgenic BMBs sorted by magnetic-activated cell sorting were cultured for 2 h with or without 2.5 µg/ml of OVA peptide (Keck). After peptide loading, cells were washed thoroughly three times and 2×10^7 basophils were transferred intravenously into recipient mice, followed by intraperitoneal immunization with 500 µg papain.

Bone marrow DC and basophil cultures. BMDCs derived from bone marrow cultures of 0.7×10^6 cells per ml were cultured for 5 d in granulocyte-macrophage colony-stimulating factor. BMBs were derived from bone marrow cultures at a density of 5×10^6 cells per ml, which were replated every 3–4 d at a density of 1×10^6 cells per ml for 10 d of culture in standard media supplemented with IL-3 (30 ng/ml; Peprotech). Basophils were enriched by magnetic-activated cell sorting or flow cytometry. Cultures were stimulated with ionomycin (500 ng/ml; Calbiochem), LPS (100 ng/ml; Sigma) or heat-inactivated or active papain (100 µg/ml). Activation by IgE crosslinking was done by incubation with mouse IgE (10 µg/ml; 27-74; Pharmingen-BD Biosciences), followed by incubation with antibody to mouse IgE (10 µg/ml; R35-118; BD Biosciences).

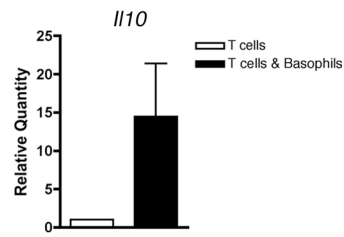
Uptake assays. Splenic B cells, BMDCs or BMBs purified by magnetic-activated cell sorting (2×10^6 cells) were incubated for 3 h or overnight at 4 °C or 37 °C with 100 µg/ml of ovalbumin coupled to fluorescein isothiocyanate (Invitrogen) or 10 µl fluorescent yellow latex beads per ml (sulfate-modified polystyrene beads; Sigma). After incubation, cells were collected, were thoroughly washed and were analyzed by flow cytometry.

Flow cytometry and sorting. Cells were incubated for 20 min at 4 °C with the appropriate antibodies. For staining of MHC class II on basophils, cells were stained with unconjugated antibody to MHC class II, then were washed and a species-specific secondary antibody was used for detection. Cells were analyzed on a FACSCalibur (BD Biosciences) and data were analyzed with FlowJo software (TreeStar). For sorting, samples were run on a MoFlo cell sorter (BD Biosciences) at a pressure of 30 psi and were selected as described in **Supplementary Figure 2a–c**. Cells were sorted by magnetic-activated cell sorting by positive selection with the following microbeads: BMBs, DX5 microbeads; DCs, CD11c microbeads; CD4⁺ cells, L3T4 microbeads; and B cells, 6D9 microbeads (Miltenyi).

Immunofluorescence. Basophils were fixed with 1.6% (vol/vol) paraformaldehyde, were made permeable with saponin and were blocked with 10% (wt/vol) BSA, then were placed on Alcian blue-coated coverslips and stained. Synapse formation proceeded as described above, but basophils were treated with CD4⁺ DO11.10 cells (ratio, 1:10) for 60 min before adherence to coverslips. After MHC class II staining, coverslips were fixed in 1% (vol/vol) paraformaldehyde and were stained for T cell antigen receptor-β. Vectashield mounting medium (Vector) was used to prevent fading.

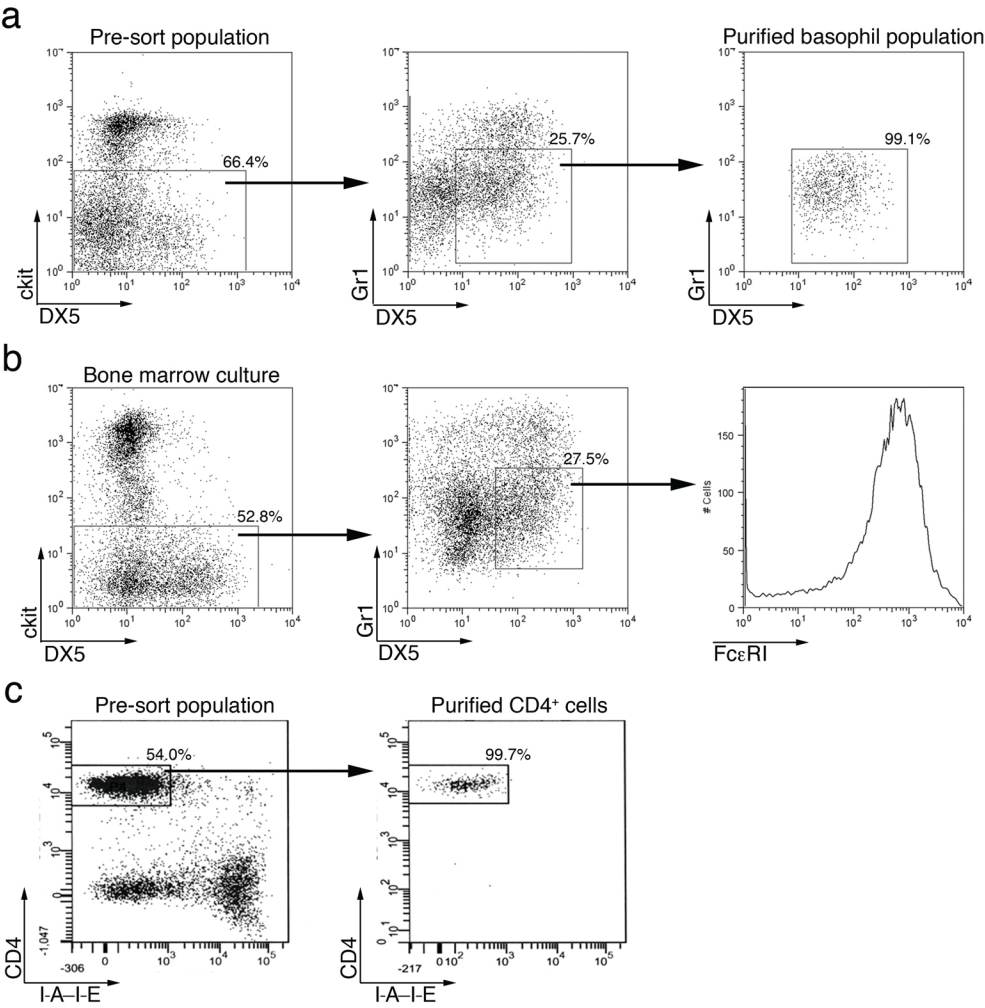
Additional methods. Information on reagents and antibodies and quantitative PCR analysis is available in the **Supplementary Methods** online.

Supplementary Figure 1



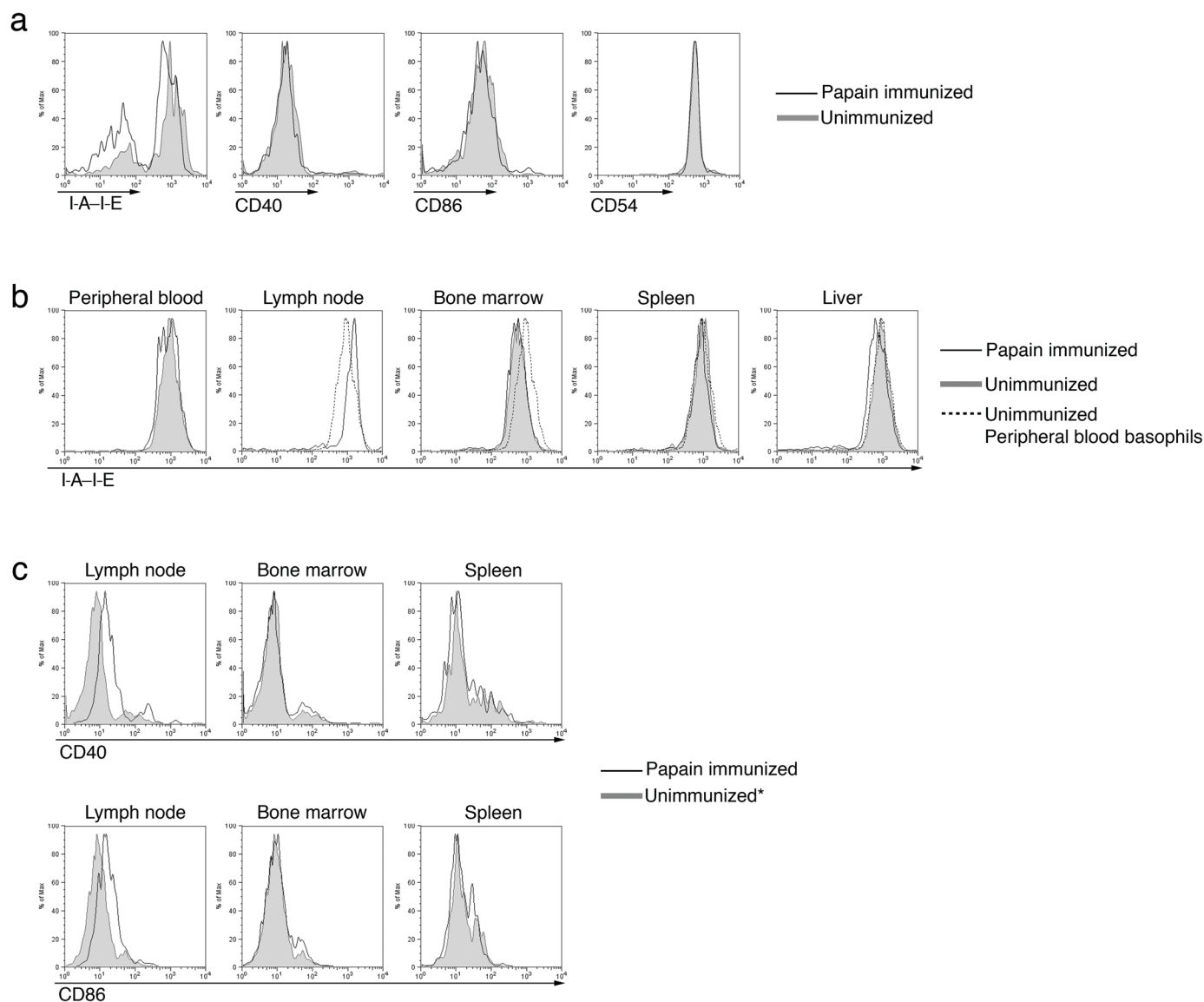
Supplementary Figure 1: *Il10* gene expression from CD4⁺ T cells after *in vitro* co-culture with BMBs. Q-PCR analysis of *Il10* gene expression from DO11.10 splenic CD4⁺ cells after positive selection from total splenocytes (T cells), or after 3 days of co-culture with BMBs (T cells & Basophils) in the presence of OVAp. Data shown is representative of two independent experiments.

Supplementary Figure 2



Supplementary Figure 2: FACS sorting of BMBs and splenic CD4⁺ cells.
(a) Day 10 cultures of bone marrow derived mast cell and basophil cultures were stained with ckit, DX5 and Gr1. Cultures were first sorted for ckit⁺ cells to remove mast cells. Cultures were then sorted for DX5⁺Gr1⁺ cells to specifically select for basophils. (b) Expression of FcεRI on basophils sorted as in (a). (c) Pre and post sort FACS staining showing purification of CD4⁺I-A-I-E⁺ splenocytes from DO11.10 x 4get mice. The purified populations show FACS staining of cells immediately after sorting to gauge purity of sort. All FACS plots shown are first gated on live cells and percentages given are out of total live cells for the shown sub-population.

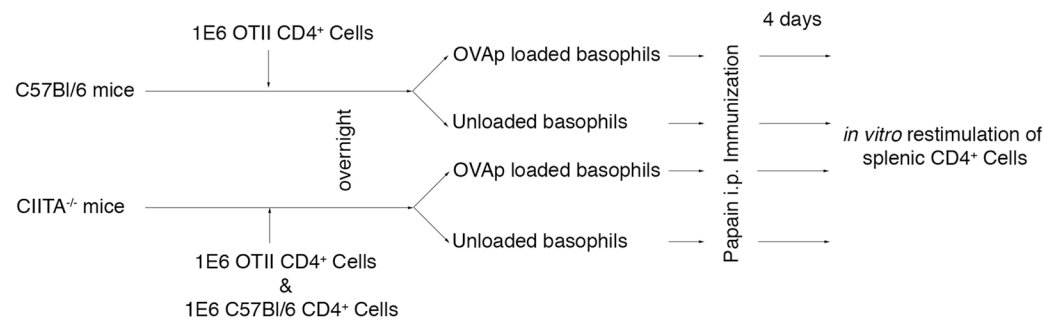
Supplementary Figure 3



Supplementary Figure 3: Expression of MHC Class II and costimulatory molecules on basophils.

(a) MHC Class II, CD40, CD86 and CD54 expression on peripheral blood basophils 3 days after papain immunization (black histogram) compared to those from unimmunized mice (shaded gray). (b) MHC Class II expression on basophils from peripheral blood, lymph nodes, bone marrow, spleen or liver. Basophils from the specified organs in mice 3 days after subcutaneous papain immunization (black histogram) are compared with those from unimmunized mice (shaded gray histogram) and peripheral blood basophils from unimmunized mice (dashed histogram). (c) Expression of CD40 and CD86 in basophils from the lymph node, bone marrow and spleen. Basophils from the specified organs in mice 3 days after subcutaneous papain immunization (black histogram) are compared with those from unimmunized mice (shaded gray histogram). (*) Because basophils are not found in the lymph nodes of unimmunized mice, papain immunized lymph node basophils (black histogram) are compared to basophils from the bone marrow of unimmunized mice (shaded gray histogram). To specifically target splenic and liver basophils, papain immunization in these samples was done via the intraperitoneal route, while all other immunization were performed via the subcutaneous route. Histograms represent live cell gated, IgE⁺DX5⁻ cells. Data shown are representative of at least 3 independent experiments.

Supplementary Figure 4



Supplementary Figure 4: Experimental scheme for basophil transfer
Procedure for basophil transfer into CIITA ko or wild type mice.

Supplementary Methods

Reagents and Antibodies. Papain was purchased from Calbiochem. Unlabeled 2.4G2, unlabeled MHC class II (I-A—I-E) (M5/114.15.2), CD49b-allophycocyanin (APC) (DX5), Gr1-fluorescein isothiocyanate (FITC) (RB6-8C5), anti-rat IgG-APC, c-kit-APC and -biotin (2B8), and anti-DO11.10-biotin (KJ1-26) were purchased from eBioscience. CD86-phycoerythrin (PE) (GL1), CD40-PE (3/23), CD49b-biotin (DX5), TCR β -FITC (H57-597), CD54-biotin (3E2), I-A, I-E-FITC (2G9), CD11c-APC (HL3), CD4-PE (GK1.5) were purchased from BD Pharmingen. Papain was heat inactivated by heating to 100 °C for 10 min. MACS sorting microbeads were purchased from Miltenyi Biotech: anti-NK (DX5), anti-CD4 (L3TA), anti-CD11c (N418), anti-CD19 (6D9) and anti-biotin (Bio3-18E7). ELISA reagents were as follows: purified anti-mouse IL-4 (11B11), IFN- γ (R4-6A2), biotinylated IL-4 (BVD6-24G2) were purchased from BD Pharmingen. Biotinylated IFN- γ (R4-6A2) was purchased from eBioscience. Recombinant IL-4 (R&D Systems) and IFN- γ (Peprotech) were used as standards. Immunofluorescence antibodies listed above and anti-rat AlexaFluor647 and anti-FITC-AlexaFluor488 (Molecular Probes).

QPCR Analysis. 1–2 x 10⁶ MACS sorted BMBs were stimulated via protocols described below for 4 h. Using standard protocols RNA was prepared using RNABee (Tel-Test) and cDNA was prepared using Superscript III (Sigma). QPCR was performed using the MX3000P (Stratagene) and standard protocols utilizing SYBR green reagent (Qiagen). All CIITA primers were based on published primer sets and are listed 5'-3'. CIITA Common, FWD: AAGCAGGACAGAAGCCTCAGAA, REV: GCTTCCTGTGCTTTGAGTCCAT. CIITA Promoter I, FWD: AGCACTGCCATGAACCACTTC, REV: AGAAGCCCAGGCAGGTGC. CIITA Promoter III, FWD: TGGTTCCTGGCCCTTCTG, REV:

CAGATCCATGGTGGCACACA. CIITA Promoter IV, FWD:

CCCCAGCCTCACTTGTTATTCA, REV: TCCATGGTGGCACACAGACT. H2-Ab1, FWD:

TCTACCCAGCCAAGATCAAAGT , REV: CCCATTCCTAATAAGCTGTGTGG. CD74,

FWD: GCCCATACTGGGCAACCGC, REV: CGGTGTACAGAGCTCCACGGC. T cell IL-10

analysis was done as above using RNA derived from MACS purified CD4⁺ T cells with or

without 3 days of co-culture with BMBs and OVAp. IL-10 primers used were, FWD:

GCTCTTACTGACTGGCATGAG; REV: CGCAGCTCTAGGAGCATGTG.