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Basophils Function as Antigen Presenting Cells for an Allergen-Induced T_H2 Response

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Abstract

T_H2 mediated immune responses are induced upon infection with multicellular parasites and can be triggered by a variety of allergens. Mechanisms of induction and the antigen-presenting cells involved in 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, while dendritic cells were dispensable, antigen presentation by basophils was necessary and sufficient for allergen-induced activation of T_H2 responses *in vitro* and *in vivo*. Thus, basophils function as antigen presenting cells for T_H2 differentiation in response to protease allergens.

Introduction

Different $CD4^+$ T helper cell effector lineages control host defenses against distinct classes of pathogens. T_H1 cells provide protective immunity against intracellular bacterial, viral and protozoan pathogens, T_H-17 cells regulate host defense against extracellular bacterial and fungal pathogens, and T_H2 cells orchestrate immunity against multicellular parasites, including helminths, which are mostly extracellular pathogens[1]. 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 responses[1].

Although the basic aspects of activation of T_H1 and T_H-17 immune responses are well characterized, the mechanisms of induction of T_H2 immune responses remain obscure. To a large extent this reflects our 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 (TLRs) and Dectin-1, detect

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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 cells[6]. In addition to presenting antigens derived from phagocytosed or endocytosed pathogens, DCs produce other necessary signals for naive $CD4^+$ T cell activation and differentiation into the appropriate T_H1 or T_H-17 effector lineage[4,5,7]. Importantly, DCs that present antigens to naive T cells, also provide co-stimulatory molecules and produce cytokines (such as interleukin 12 (IL-12), IL-23 and IL-6) that control T_H1 and T_H-17 differentiation[8,9].

This scenario, however, 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 play important roles in parasites' infection cycles and can have immunogenic activity for the induction of T_H2 responses[10,12,13]. Another notable distinction between activation of T_H1 and T_H-17 responses and T_H2 responses is that DCs do not produce the cytokines that are known to be important for T_H2 differentiation, including IL-4 (http://www.signaling-gateway.org/molecule/query?afcsid=A001262) and thymic stromal lymphopoietin (TSLP; http://www.signaling-gateway.org/molecule/query?afcsid=A002363). We recently reported that basophils are recruited to the lymph nodes during the primary immune response to protease allergens and schistosome soluble egg antigen (SEA) [14]. There they produce T_H2 promoting cytokines, including IL-4 and TSLP, and play an essential role in the initiation of T_H2 responses[14]. This finding suggested 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 naive $CD4^+$ T cell activation in the lymph nodes. Although we observed DC migration to the draining lymph node after papain immunization, the identity of the antigen-presenting cell (APC) for T_H2 induction *in vivo* has not been established.

Here we investigated the roles of basophils and DCs in the initiation of T_H2 responses, and found that DCs were neither required nor sufficient for the induction of a T_H2 response by a protease allergen papain. Both *in vitro* and *in vivo*, basophils were capable of antigen presentation and induction of T_H2 differentiation of naive $CD4^+$ T cells. Our data indicated that basophils were the relevant APCs for T_H2 induction by papain. We demonstrate that antigen presentation by basophils was necessary and sufficient for T_H2 induction to a protease allergen *in vitro* and *in vivo*.

Results

Basophils induce T_H2 differentiation *in vitro*

We had previously shown that basophils were essential for *in vivo* T_H2 differentiation in response to papain immunization[14]. However, the precise identity of the APC responsible

for the induction of the T_H2 response to papain remained unclear. DCs are not directly activated by papain *in vitro*[14]. Furthermore, papain treated DCs were unable to induce T_H2 differentiation *in vitro* (data not shown), suggesting that either 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 these questions, we used an *in vitro* system of T_H2 differentiation using purified ovalbumin (OVA) specific $CD4^+$ T cells from DO11.10 x 4get mice (in which IL-4 mRNA expression is reported by enhanced green fluorescence protein, eGFP[15]), bone marrow-derived basophils (BMBs) and bone marrow-derived dendritic cells (BMDCs). In accordance with a previous report using a similar culture system[16], co-culture of BMDCs, BMBs and $CD4^+$ T cells 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 (IFN-γ) production, under 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. Surprisingly, despite our previous observations 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 different subsets of *ex vivo* purified DCs: splenic DCs, DCs sorted from draining (popliteal) lymph nodes after papain immunization and DCs from non-draining (brachial and cervical) lymph nodes. We found no role for any of those DC subsets in *in vitro* T_H2 differentiation (Fig. 1b). In fact, we found no role 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, IL-10 was produced by T_H2 cells after *in vitro* differentiation as measured by upregulation of *Il10* gene expression (Supplementary Fig. 1 online). *In vitro* T_H2 differentiation was strongly enhanced upon basophil activation by papain, although unstimulated basophils could also support T_H2 activation to a lesser extent (Fig. 1d), presumably because of some level of spontaneous activation caused by tissue culture conditions.

Antigen presentation by basophils via MHC class II has not been previously described. Therefore, we further examined whether basophils functioned as APCs by presenting antigens through the classical MHC class II pathway, or whether they simply provided cytokines (for example, IL-4) necessary for T_H2 differentiation. To assess this, we studied T_H2 differentiation in BMBs and splenic $CD4^+$ T cell co-cultures 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), indicating that basophils activated and induced T_H2 differentiation via the classical MHC class II dependent pathway. Finally, to rule out contamination of the *in vitro* culture system with alternative APCs or mast cells, we co-cultured highly purified (>99%) populations of BMBs and OVA specific splenic $CD4^+$ T cells (Supplementary Fig. 2a–c online). T_H2 differentiation was not due to

contaminating APCs or mast cells (Fig. 1f). Thus, basophils appeared 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 cadre 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 systems[16]. In accordance with this, we found that T_H2 differentiation was largely dependent on IL-4 production by basophils (Fig. 2). T_H2 differentiation was greatly reduced in cultures involving *Il4*$^{-/-}$ BMBs. Thus, basophils appear 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 following stimulation with papain *in vitro* and *in vivo*[14]. However, basophils have not been previously reported 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 (http://www.signaling-gateway.org/molecule/query?afcsid=A000657), which controls expression of several key components of the MHC class II antigen presentation pathway[17-19]. Upon papain stimulation, but not IgE crosslinking, CIITA was induced in basophils (Fig. 3a). The expression of *Ciita* mRNA in basophils was less than that in DCs but equivalent to that seen in macrophages, cells that are known to be capable of antigen presentation via MHC class II (Fig. 3a). In mice, CIITA expression is controlled by three 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 non-hematopoietic cells, such as thymic epithelium[20,21]. Examination of promoter specific expression revealed 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). Interestingly, induction of CIITA and of its targets, MHC class II associated genes, was specific to basophil activation by active papain, whereas IgE cross-linking 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 IFN-γ in myeloid cells[21], lack of induction of MHC class II genes by IgE crosslinking indicate 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 enter the popliteal lymph nodes transiently, three days after subcutaneous papain immunization in the rear footpad[14]. We found that these lymph node basophils expressed abundant MHC class II molecules (Fig. 3c). Lymph node basophils also expressed high amounts of the co-stimulatory molecules CD40 and CD86, as 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

were increased on lymph node basophils compared to 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 co-stimulatory molecules was equivalent regardless of papain immunization or where the basophils were isolated: peripheral blood, spleen, 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 expressed lower amounts of steady state MHC class II, presumably secondary to a more immature state of development (Supplementary Fig. 3b). As opposed 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 increased numbers of MHC class II expressing cells (Fig. 3d,e). Finally, papain activated, OVA peptide ($OVA_{323–339}$) pre-treated basophils were capable of forming immunological synapses with T cells after 60 minutes of co-culture, as measured by co-clustering of MHC class II and the T cell receptor at the point of basophil-T cell contact (Fig. 3f)[22,23].

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 capable of presenting peptide antigens to $CD4^+$ T cells, leading to T_H2 differentiation. We next tested whether basophils were capable of endocytosing, processing and presenting soluble proteins. Basophils were capable of endocytosing ovalbumin coupled to fluorescein isothiocyanate (OVA-FITC; 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 pre-activated with papain and then extensively washed before co-culture with OVA and $CD4^+$ T cells. Interestingly, although basophils are capable of uptake, processing and presentation of a soluble protein antigen, they were inefficient in taking up particulate antigens. Compared to DCs, basophils were far less efficient in phagocytosis of fluorescently labeled 2 μm latex beads after 4 hour or overnight co-culture (Fig. 4c and data not shown). Thus, basophils appear to be specifically capable of presenting 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 illustrated that basophils can function as APCs for 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 asked whether DCs are necessary or sufficient for the activation of the T_H2 response by papain *in vivo*. While 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 removing the site of injection several hours after immunization[24]. 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

hours after immunization. In mice that underwent removal of the injection site, T_H2 differentiation was still induced and basophil recruitment to the lymph nodes retained, albeit decreased (Fig. 5a,b). Thus, migration of skin dendritic cells was not necessary for T_H2 differentiation in response to papain immunization. Of note, since T_H2 differentiation after papain immunization is dependent on basophils, the observed decrease in T_H2 differentiation is likely secondary to decreased basophil migration in mice that underwent removal of the injection site. This decrease in basophil migration, in turn, is likely a result of a functionally decreased dose of papain in the draining lymph node because of the early time-point for removal of the injection site, which was chosen to confidently rule out migration of DCs or other peripheral antigen capturing cells. Soluble antigens have been shown to be taken up by conduit-associated dendritic cells in the T cell zone of draining lymph nodes starting at 90 minutes after subcutaneous injection[25]. Thus, removal of the injection site and the remaining depot of antigen at 120 minutes likely reduced amount of injected papain in the draining lymph node. Regardless, the observation that basophil migration and T_H2 differentiation is retained after removal of the injection site indicates that the response does not require antigen capture at peripheral sites. Instead, this 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 utilized the CD11c-diphtheria toxin receptor (DTR)-GFP system in which $CD11c^+$ cells express DTR and can be selectively depleted by diphtheria toxin (DT) injection[26,27]. Basophils do not express CD11c and therefore would not be affected by DT expression[28] (data not shown). Bone marrow chimeras were established by transferring CD11c-DTR-eGFP bone marrow into BALB/c recipients. Chimerism was assessed by GFP expression in $CD11c^+$ cells and chimeras were depleted of $CD11c^+$ cells after injection of diphtheria toxin (Fig. 6a). Depletion of DCs by DT injection had no effect on basophil migration in response to papain immunization (Fig. 6b). After transfer of OVA-specific DO11.10 $CD4^+$ T cells, mice were immunized with OVA, OVA plus papain, or OVA plus lipopolysaccharide (LPS) to induce no differentiation, T_H2, or T_H1 differentiation, respectively. Restimulation of $CD4^+$ T cells with OVAp *in vitro* illustrated that while T_H1 differentiation by OVA plus LPS was lost when $CD11c^+$ cells were depleted by DT, 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 this finding in a different system, we used the CD11c $A\beta^b$ (CD11c-IABB) strain of mice in which MHC class II expression is restricted to $CD11c^+$ cells[29]. Of note, reconstitution of MHC class II expression in CD11c-IABB mice is not complete. DC subsets with low endogenous CD11c expression (plasmacytoid DCs and Langerhans cells) remain MHC class II negative[29,30]. However, MHC class II expression on $CD11b^{hi}$ DCs (which migrate into the draining lymph node after papain immunization) is reconstituted, although at decreased levels in CD11c-IABB mice as compared to wild type mice[29]. Notably, this same DC subset migrated in response to both papain and LPS[14]. 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 (OTII) $CD4^+$ T cells into C57BL/6 or CD11c-IABB mice, followed by OVA plus LPS immunization led to equivalent T_H1 differentiation, based on IFN-γ production on *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 antigen presenting cells *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, on the other hand were necessary and sufficient for T_H2 differentiation *in vitro*, and as we showed previously, they are 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 whether basophils can present antigens *in vivo* for T_H2 induction remained unclear. To address these questions, we developed a method of basophil transfer. Basophils have a short life span and poor survival following purification, which prevented their study in adoptive transfer experiments. To circumvent this limitation, we used BMBs derived from *Bcl2* transgenic mice[31] to improve post-transfer survival. MHC class II sufficient basophils were transferred into wild-type or *Ciita*$^{-/-}$ or I-A^b-deficient (*H2-Ab1*$^{-/-}$) mice following the protocol outlined in Supplementary Fig. 4. Interestingly, antigen loaded, MHC class II positive basophils were able to mediate the papain induced T_H2 response in MHC class II-deficient (*Ciita*$^{-/-}$ mice or *H2-Ab1*$^{-/-}$) mice (Fig. 7 and data not shown). Because in these mice basophils are the only cells expressing MHC class II molecules, we conclude that basophils were sufficient for antigen presentation to $CD4^+$ T cells *in vivo*.

Discussion

Initiation of T_H2 immune responses differs from T_H1 and T_H-17 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 manner as bacteria, viruses and fungi – pathogen classes that elicit T_H1 and T_H-17 responses. While the source of antigens presented by DCs for T_H1 and T_H-17 induction is generally a phagocytosed pathogen, helminths are too large to be internalized by the APCs for antigen processing and presentation. Therefore, the primary source of antigens for T_H2 responses is likely to be 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_H-17 responses, the DCs that present antigens also produce T_H1 and T_H-17 inducing cytokines, including IL-12 and IL-6. However, DCs do not produce T_H2 inducing cytokines, such as IL-4 and TSLP. Therefore, 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 T_H 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*, invoking the necessity of an accessory cell or an alternative APC. Collectively, these and other differences between T_H1 or T_H-17 responses on the one hand, and T_H2 responses

on the other hand, suggest that there may be fundamentally different pathways involved in initiation of these arms of adaptive immunity.

Although basophils are primarily appreciated for their role as type-2 effector cells, they have been shown to be essential in IgG mediated systemic anaphylaxis[32], and recent discoveries have underscored their importance in the induction and regulation of the adaptive immune response. Basophils play an integral role in 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]. On the other hand, basophils have also been reported to regulate the T_H1 and T_H2 balance and to specifically inhibit T_H1 differentiation[16,36]. Additionally, cytokine production and CD40L expression by basophils has been suggested to be involved in regulating the antibody response[37-40]. Finally, basophils have been shown to play an important role as antigen-capturing cells via antigen specific IgE bound to their surface via FcεRI[41]. However, whether they are capable of antigen capture during the primary response (in the absence of antigen specific IgE) or antigen presentation, was unknown.

In our previous study, we demonstrated that while papain had no direct effect on DCs *in vitro*, it potently activated basophils inducing them to express and secrete several T_H2 promoting signals, including IL-2, IL-4, IL-13 and TSLP. In response to papain administration, basophils were recruited from the circulation to the lymph nodes where they produced IL-4 and TSLP, which are involved in T_H2 differentiation. Basophils, and basophil-derived TSLP, were required for the papain induced activation of T_H2 responses *in vivo*[14]. These findings suggested 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 this possibility and found that DCs played no discernable role 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 co-stimulatory 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 likely to be 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 directly travels with lymph to the draining lymph node without requiring capture by antigen presenting cells at peripheral sites. This 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 that basophils were not only necessary for T_H2 induction by papain, but specifically that antigen presentation by basophils was sufficient for the initiation of T_H2 responses both *in vitro* and *in vivo*. Therefore, basophils are not simply accessory cells that provide cytokines for T_H2 differentiation, but they also are essential APCs for T_H2 induction. Importantly, basophils were also independently found to function as APCs in two models of helminthes infections (D. Artis, personal communication; K. Nakanishi, personal communication), suggesting that

basophils may play a predominant role as APCs in T_H2 immunity in physiological and pathological settings. Thus, basophils appear to play multiple roles in the regulation of type-2 immunity to helminths, and in the induction of T_H2 responses to protease allergens[42].

It is important to note, however, that T_H2 responses are heterogeneous and can be induced by multiple, seemingly unrelated, pathways. For example, low doses of inhaled LPS can trigger T_H2 responses in the lung in a TLR4-dependent manner[43,44]. Der p 2 was recently shown to function as an allergen due to its ability to bind LPS and to mimic the function of MD-2, a component of TLR4 receptor complex[45]. 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 responses[51]. SEA 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 phagocytosed[53]. This 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. Accordingly, there is unlikely to be one mechanism accounting for the activity of different classes of allergens. The challenge for the future studies, therefore, is to unravel the full spectrum of mechanisms and pathways involved in physiological and pathological initiation of T_H2 responses.

Methods

Mice

Animals were bred and maintained at the Yale Animal Resources Center at Yale University. All animal experiments were performed 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*$^{Lps\text{-}d}$), *Ciita*$^{-/-}$, *H2-Ab1*$^{-/-}$, OT-II, DO11.10, BALB/c CD11c-DTR-eGFP, *Il4*$^{-/-}$ and 4get mice were purchased from Jackson laboratories. DO11.10 X 4get mice were provided by K. Bottomly (Yale University). H-2^k-*Bcl2* tg mice were provided by I. Weissman (Stanford University).

In vitro T_H2 Differentiation

MACS or FACS sorted CD4$^+$ T cells plated at 1×10^6 cells/ml in the presence of indicated ratio of BMDCs or BMBs in RPMI with 10% 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. M5/114.15.2 was added to culture at 20 ng/ml.

Immunizations, Depletions and Cell Transfer

Mice were immunized subcutaneously in rear footpads with 50 μg papain or 2.5 μg LPS with or without 50 μg of OVA (Worthington) in 50μl PBS. BALB/c CD11c-DTR-GFP chimeras were established as previously described[27]. 1×10^6 MACS purified CD4$^+$ T cells were transferred in T cell transfers. Chimeras were injected with 60 ng DT (Sigma) intraperitoneally on days 0, 2, and 5 and CD4$^+$ DO11.10 T cells were transferred by IV

injection on day 3. Popliteal lymph nodes were harvested on day 7 for *in vitro* restimulation. For IABB experiments, MACS purified OTII $CD4^+$ (CD11c-IABB and C57BL/6 recipients) and C57BL/6 $CD4^+$ (CD11c-IABB recipients only) cells were intravenously transferred. Mice were intraperitoneally immunized the following day with 100 μg OVA with 500 μg papain or 10 μg LPS; spleens were harvested 4 days later for *in vitro* restimulation. All in vitro restimulations followed standard protocols and ELISA used reagents listed above. For ear injections, 50 μg of papain in 10 μl PBS was injected into the distal pinna, which was excised 2 h later in indicated conditions. Basophil migration and T_H2 differentiation were assayed 3 and 4 days later, respectively, in the ipsilateral cervical lymph nodes.

Adoptive transfer of basophils

Procedure executed as in Supplementary Fig. 4. Of note, MACS sorted *Bcl2* tg BMBs were were cultured for 2 h with or without 2.5 μg/ml of OVA peptide (Keck). After peptide loading, cells were thoroughly washed 3 times and 2×10^7 basophils were transferred intravenously into recipient mice followed by intraperitoneal immunization with 500 μg papain.

Bone Marrow Dendritic Cell and Basophil Cultures

Briefly BMDCs were derived from bone marrow cultures of 0.7×10^6 cells/ml were cultured for 5 days in GM-CSF. BMBs were derived from bone marrow cultures of 5×10^6 cells/ml, which were replated every 3-4 days at 1×10^6 cells/ml for 10 days culture in 30 ng/ml IL-3 (Peprotech) supplemented standard media. Basophils were enriched as indicated. Cultures were stimulated by ionomycin (500 ng/ml, Calbiochem), LPS (100 ng/ml, Sigma), heat inactivated or active protease (100 μg/ml). Activation by IgE cross-linking was performed by first incubating with mouse IgE (10 μg/ml) followed by incubation with anti-mouse IgE (10 μg/ml).

Uptake Assays

2×10^6 MACS purified splenic B cells, BMDCs or BMBs were incubated at 4 °C or 37 °C for 3h or overnight with 100 μg/ml of OVA-FITC (Invitrogen) or 10 μl of fluorescent yellow latex (sulfate modified polystyrene) beads/ml (Sigma). After incubation, cells were harvested, thoroughly washed and FACS analyzed.

Flow Cytometry and Sorting

Cells were incubated with indicated antibodies at 4 °C for 20 min. For MHC class II staining on basophils, cells were stained with unconjugated antibody to MHC class II, washed and a species specific secondary antibody was used for detection. Cells were analyzed on a FACSCalibur Flow Cytometer (BD Biosciences) and data was analyzed using FloJo software (Tree Star). For sorting, samples were run on a MoFlo cell sorter (BD Biosciences) at 30 psi and were selected as in Supplementary Fig. 2a-c. MACS sorting of cells was by positive selection using the following microbeads: BMBs, DX5 microbeads; DCs, CD11c microbeads; $CD4^+$ cells, L3T4 microbeads; B cells, 6D9 microbeads.

Immunofluorescence

Paraformaldehyde fixed (1.6%), saponin permeabilized and 10% BSA blocked basophils on Alcian Blue coated coverslips were stained as indicated. Synapse formation proceeded as above, but basophils were treated with $CD4^+$ DO11.10 cells (1:10 ratio) for 60 min before adherence to coverslips. After MHC Class II staining, coverslips were fixed in 1% paraformaldehyde and stained for TCRβ. Vectashield (Vector) mounting medium was used to prevent fading.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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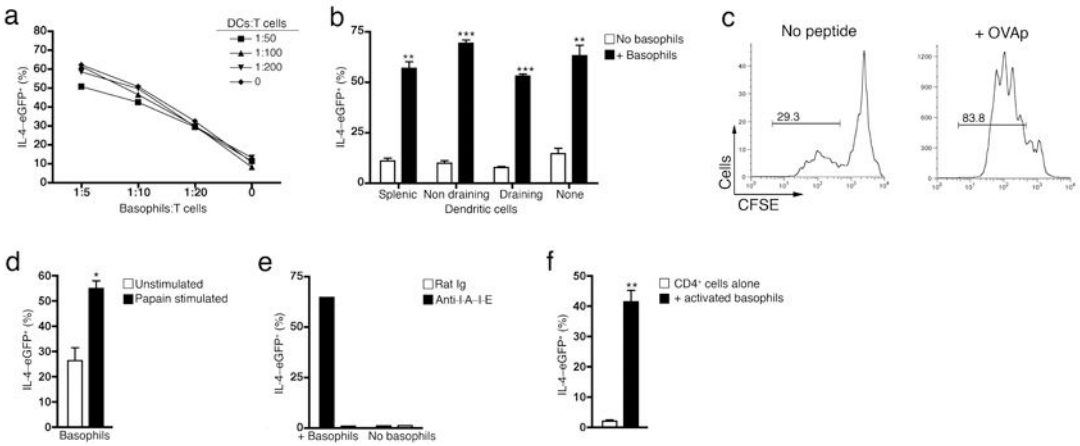

Figure 1. Basophils, but not dendritic cells, are necessary for T_H2 differentiation *in vitro*

(a) Splenic $CD4^+$ cells from DO11.10 x 4get mice were co-cultured with OVAp and indicated ratios of MACS purified BMBs and BMDCs from Balb/c mice. The percentage of IL-4-eGFP$^+$, out of total $CD4^+$ cells, was measured after three days. (b) $CD4^+$ cells as in (a) were mixed with DCs from the indicated sites in papain immunized C3H.T4d mice (draining = popliteal lymph node and non draining = brachial lymph node), in the presence (black) or absence (white) of BMBs. (c) Proliferation of CFSE labeled $CD4^+$ cells after co-culture with BMBs. Percentage is of proliferating cells out of total live $CD4^+$ cells. (d) Splenic $CD4^+$ as in (a) were mixed with unstimulated (white bar) or papain stimulated (black bar) BMBs. (e) T_H2 differentiation in the presence of blocking antibodies to MHC class II. (f) T_H2 differentiation in cultures with FACS purified DO11.10 x 4get $CD4^+MHCII^-$ cells and FACS purified BALB/c BMBs. Unless noted, BMBs were stimulated with papain prior to co-culture. p values were calculated using the Student's t test, comparing conditions with or without basophils or with or without papain stimulation. *, $p<0.01$; **, $p<0.001$; ***, $p<0.0001$. Data shown are representative of at least three independent experiments.

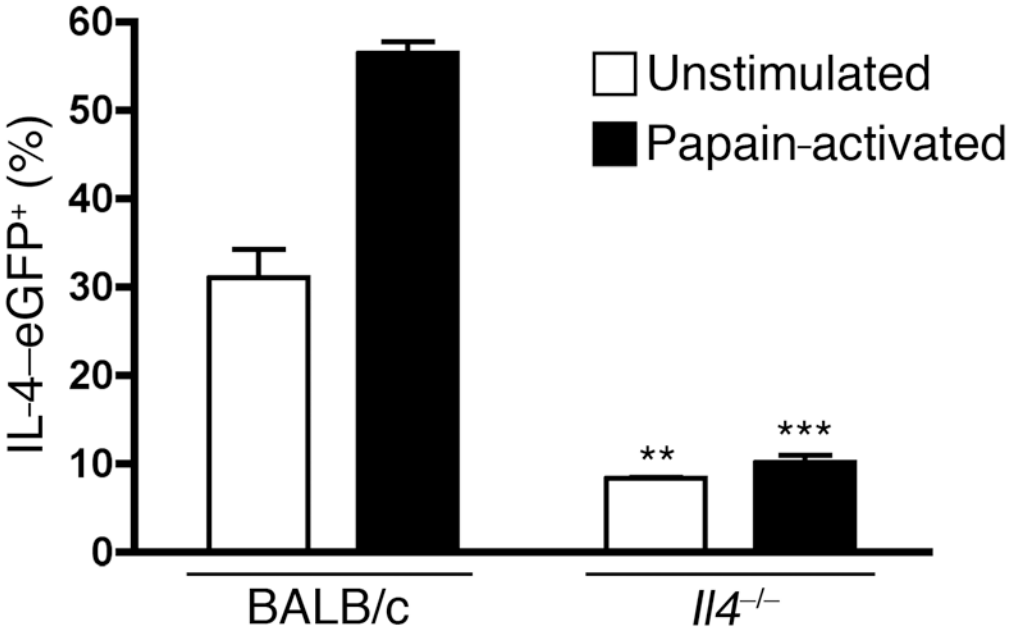

Figure 2. Basophil mediated T_H2 differentiation *in vitro* is dependent upon IL-4 and TSLP

Unstimulated (white bars) or papain stimulated (black bars) BMBs from IL-4 sufficient (BALB/c) or deficient (*Il4*$^{-/-}$) mice were co-cultured with splenic CD4$^+$ cells from DO11.10 x 4get mice and OVAp. T_H2 differentiation was assessed by the percentage of IL-4-eGFP$^+$ CD4$^+$ T cells after 3 days of co-culture. p values were calculated using the Student's t test, comparing *Il4*$^{-/-}$ conditions to corresponding BALB/c conditions. *, $p<0.01$; **, $p<0.001$; ***, $p<0.0001$. Data shown are representative of five experiments.

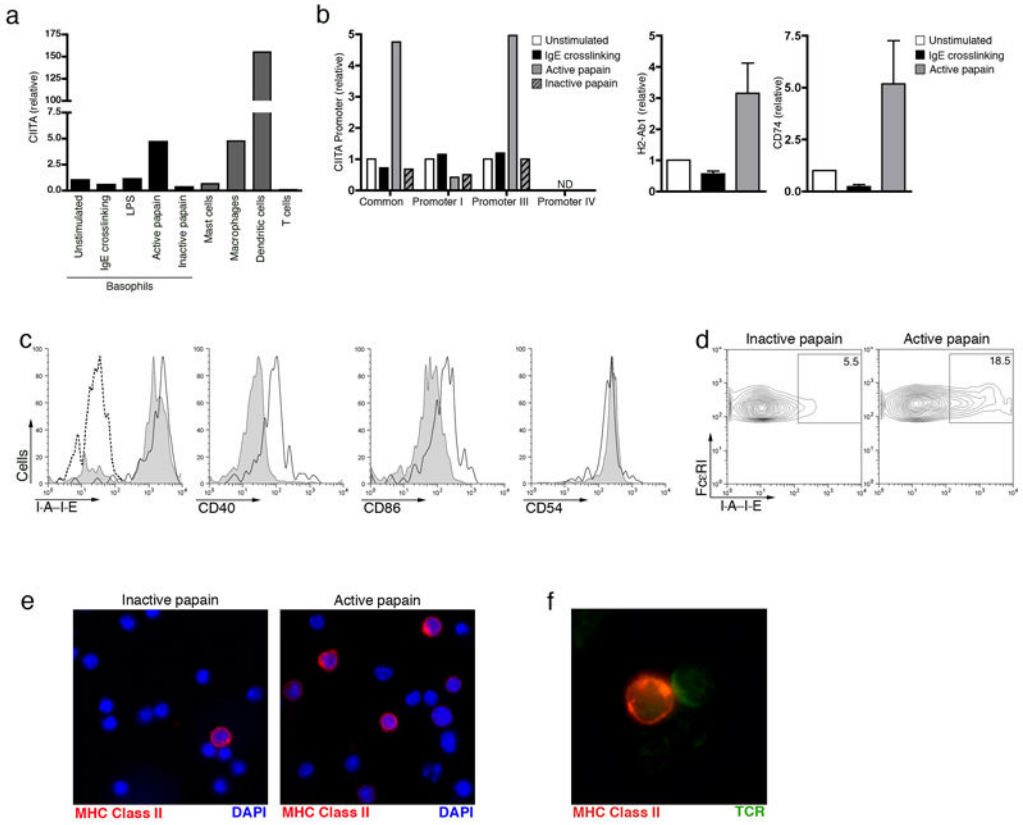

Figure 3. Basophils express and upregulate MHC Class II after papain stimulation and are capable of forming synapses with T cells *in vitro*

(a) Q-PCR expression of *Ciita* in BMBs activated for 4 hours *in vitro* as indicated compared to expression in various other hematopoetic cells. Mast cell, macrophage and DC RNA was isolated from bone marrow derived cell cultures. T cell RNA was derived from FACS sorted splenic $CD3^+CD4^+$ cells. Q-PCR expression of MHC Class II and related genes in BMBs from BALB/c mice activated for 4 hours as indicated (b). (c) FACS staining of lymph node basophils (black histogram) versus peripheral blood basophils (gray shaded histogram) three days after papain immunization. Dashed histogram in I-A—I-E stain indicates isotype control. (d) Mast cell depleted cultures of BMBs were stimulated with inactive or active papain. I-A—I-E expression is shown on live basophils cells after stimulation. Percentages shown are of the gated population out of live basophils. (e) Immunofluorescence of MHC Class II expression in BMBs after stimulation with inactive or active papain. MHC Class II (red), DAPI (blue); 20x magnification. (f) Immune synapse formation 60 minutes after co-culture of papain activated BMBs (BALB/c) and splenic $CD4^+$ T cells (DO11.10). TCRβ (green), MHC Class II (red); 100x magnification. Data shown are representative of at least four independent experiments. ND indicates no transcript detected.

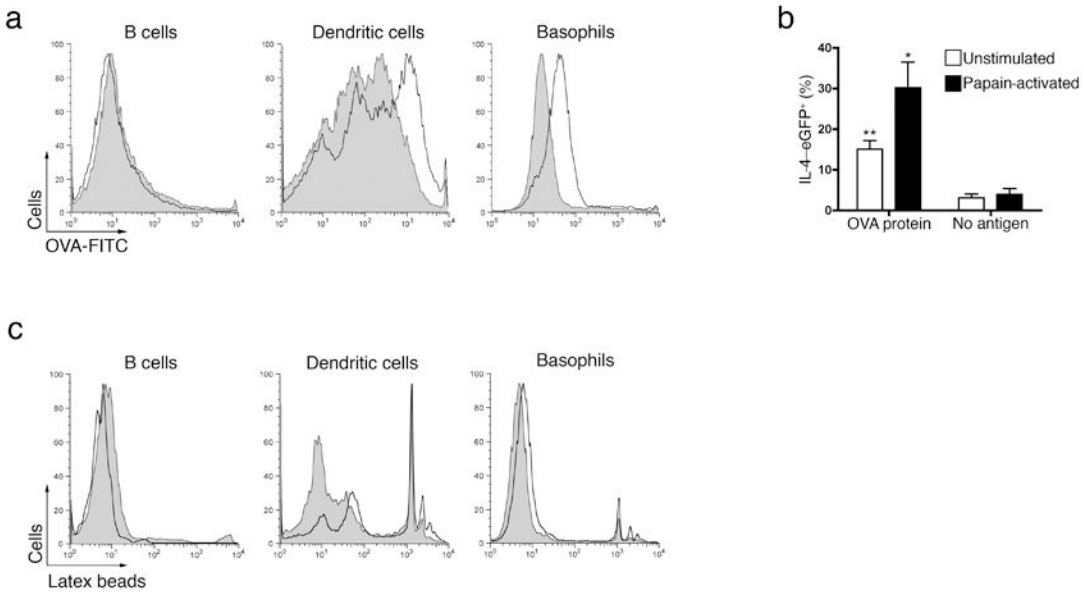

Figure 4. Basophils are capable of endocytosis of soluble, but not particulate antigens

(a) Endocytosis of soluble OVA-FITC after 3 hour culture by B cells, BMDCs or BMBs *in vitro* at 37°C (black lined histogram) or at 4°C (gray shaded histogram). (b) Endocytosis of soluble proteins by basophils leads to functional antigen presentation as measured by T_H2 differentiation. Unstimulated (white bars) or papain stimulated (black bars) BMBs were co-cultured with splenic $CD4^+$ T cells from DO11.10 x 4get mice in the presence or absence of OVA protein. T_H2 differentiation is calculated as a measure of IL-4-eGFP$^+$ $CD4^+$ T cells three days after co-culture. (c) Phagocytosis of fluorescently labeled 2μm latex beads after overnight culture by B cells, BMDCs or BMBs *in vitro* at 37°C (black lined histogram) or at 4°C (gray shaded histogram). p values were calculated using the Student's t test, comparing conditions with or without OVA protein. *, $p<0.01$; **, $p<0.001$. Data shown are representative of at least three independent experiments.

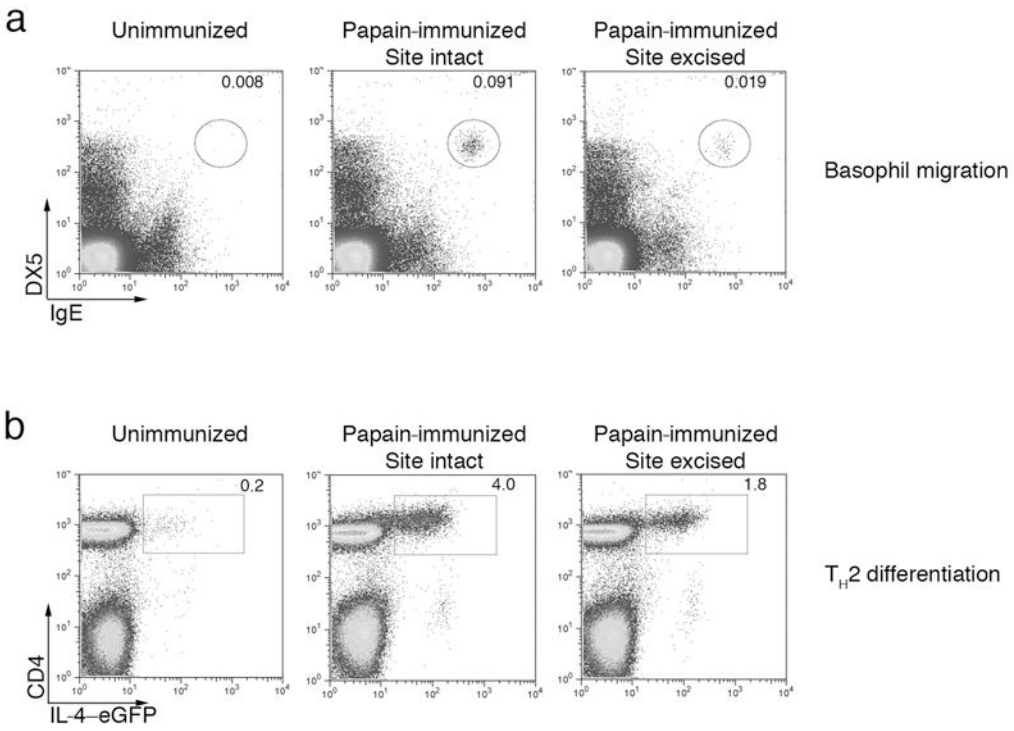

Figure 5. Migratory DCs are not necessary for basophil migration and T_H2 differentiation after papain immunization

4get mice were immunized with 50μg of active papain in 10μl of PBS in the distal pinna. The injection site was either removed or left intact 2 hours after immunization. Basophil migration (a) and T_H2 differentiation (b) in the ipsilateral cervical lymph node was assessed 3 and 4 days later, respectively. Percentages shown are that of the gated population out of total live cells from the cervical lymph node. FACS plots and percentages shown are representative of three independent experiments.

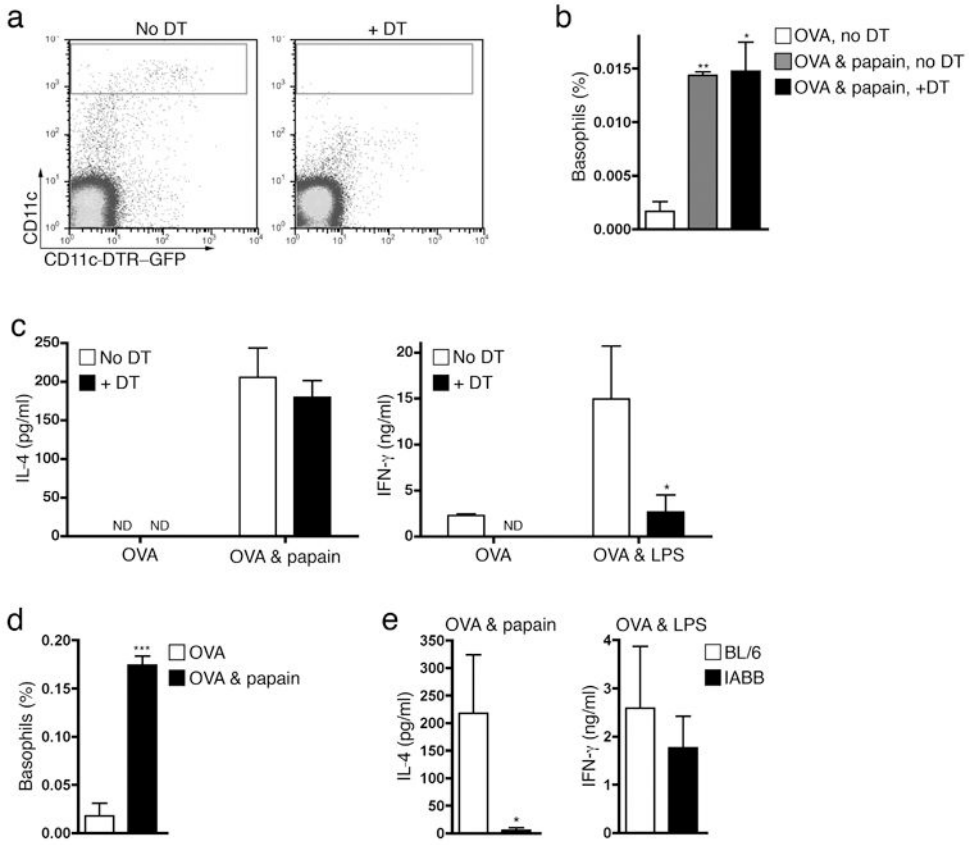

Figure 6. DCs and DC derived MHC Class II is not required for T_H2 differentiation after papain immunization

DC depletion after DT injection of CD11c-DTR-GFP → BALB/c bone marrow chimeras (a). (b) Basophil migration in chimeras after OVA immunization without DT depletion (white bar) or after OVA & Papain immunization without (gray bar) or with (black bar) DT depletion of DCs. Values are percentage of live cells. (c) T cell differentiation (based on cytokine production after *in vitro* restimulation) in chimeras after immunizations with (black bars) or without (white bars) DT injection. Chimeras first received $CD4^+$ splenic T cells from DO11.10 mice and were immunized as indicated. (d) Basophil migration in CD11c-IABB mice after OVA (white bar) or OVA & papain (black bar) immunization. (e) T cell differentiation, assessed as in (c), after OVA & papain or OVA & LPS immunization in CD11c-IABB (IABB) mice (black bars) or BL/6 mice (white bars). OT-II $CD4^+$ T cells were transferred into CD11c-IABB mice, which were subsequently immunized as indicated. p values were calculated using the Student's t test, comparing conditions to No DT injection (b & c), OVA immunization (d) or to wild type mice (e). *, $p<0.01$; **, $p<0.001$; ***, $p<0.0001$. ND indicates none detected. Data shown are representative of at least three independent experiments.

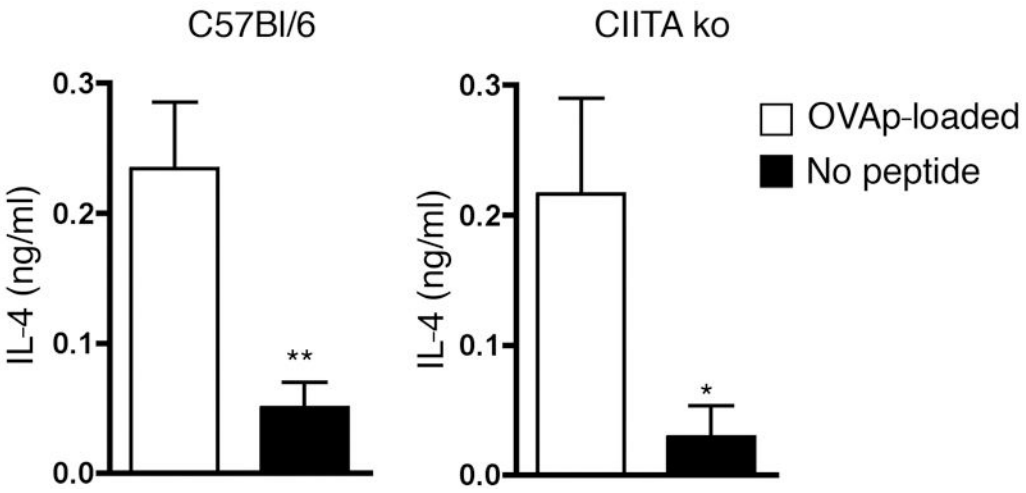

Figure 7. Antigen presentation by basophils is sufficient for T_H2 differentiation

T_H2 differentiation as measured by T cell IL-4 production on *in vitro* restimulation of $CD4^+$ T cells isolated four days after transfer of antigen coated (white bars) or uncoated (black bars) BMBs into BL/6 or CIITA ko mice. p values were calculated using the Student's t test, comparing antigen loaded conditions to no peptide conditions. *, $p<0.01$; **, $p<0.001$. Data shown are representative of three independent experiments.