

Basophils are recruited to inflamed lungs and exacerbate memory Th2 responses in mice and humans

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Abstract

Background: Although the contribution of basophils as inducers or amplifiers of Th2 responses is still debated, prolonged basophil/CD4 T cell interactions were observed in lungs but not lymph nodes (LNs) of parasite-infected mice. However, the impact of basophils on the function of tissue CD4 effector T cells remains unknown.

Methods: Basophils were purified from the lungs of ovalbumin (OVA)-sensitized and OVA-challenged (OVA-immunized) mice or human peripheral blood for *in vivo* and *in vitro* functional studies. Pulmonary basophils were adoptively transferred to OVA-sensitized hosts to assess airway inflammation in bronchoalveolar lavage fluid (BALF) and Th2 responses in lung explants and draining LNs. Basophils were co-cultured with effector T cells or Ag-specific naïve T cells alone or in combination with dendritic cells (DCs); IL-4 production was determined by flow cytometry and ELISA.

Results: Basophils accumulated in lungs of OVA-immunized mice. Adoptive transfer of basophils to OVA-sensitized hosts enhanced lung IL-4 and IL-13 release while co-administration of OVA further aggravated airway inflammation and Th2 responses in LNs. Mechanistic *in vitro* studies revealed that pulmonary basophils interacted with lung CD4 effectors, in the absence of DCs, to increase T cell survival and Th2 cytokine expression at the single cell level but amplified OVA-loaded DC-driven Th2 differentiation. Finally, human basophils augmented *in vitro* IL-4 expression in effector memory CD4 T cells that include CCR2⁺ cells through IL-4 and TCR-independent pathways.

Conclusions: Basophils may worsen Th2 inflammatory disorders through direct interactions with pathogenic CD4 T cells as well as by enhancing DC-induced Th2 cell development.

Basophils account for only 0.5–1% of circulating leukocytes and have long been associated with allergic diseases and helminth infections (1–3). Basophils are short-lived circulating cells that differentiate and mature in the bone marrow and can be detected *in situ* in patients suffering of asthma, allergic rhinitis and various allergic skin diseases (4–7). They express the high-affinity receptor for IgE (FcεR1) and rapidly release histamine in airways upon reexposure to allergens. The paucity of basophils has hampered analysis of their precise role in chronic allergic or helminth diseases. The reports that basophils are antigen-presenting cells (APCs), which are

necessary and sufficient for Th2 priming, have received most attention (8–11). However, there is no clear consensus that basophils may trump dendritic cells (DCs) in their APC function and ability to prime naïve CD4 T cell differentiation into Th2 effectors in lymph nodes (LNs) (12–16). Some studies provide evidence that basophils isolated from LNs of immunized mice may cooperate with DCs to drive Th2 polarization (13, 17). By contrast, development of acute ovalbumin (OVA)-induced airway inflammation is not altered in *Mcpt8Cre-4 get* basophil-deficient mice (15) and intra-peritoneal (i.p.) administration of low numbers of basophils

isolated from LNs of house dust mite (HDM)-immunized mice fail to drive Th2 responses and airway inflammation upon HDM challenge (16).

Chronic allergic diseases are initiated and orchestrated by Th2 lymphocytes (18). Th2 effector cells are generated in draining LNs and recruited to airway where they mediate inflammation, tissue destruction, and remodeling (19, 20). To improve therapeutic interventions in chronic inflammatory disorders, it is crucial to understand the causative and exacerbation pathways that result in CD4 T cell recruitment and reactivation locally. In humans, lung resident DCs augment *in vitro* Th2 cytokine expression (21), whereas the function of airway basophils remains ill-defined. A recent report indicated that injection of CD200R3 mAb basophil-specific (clone BA103) at the time of sensitization, significantly reduced Th2 polarization and eosinophil recruitment to bronchoalveolar lavage fluid (BALF), suggesting a role for circulating basophils in the regulation of Th2 response (16). Also, murine basophils interact with activated CD4 T cells in the lungs of parasite-infected mice, a process that triggers IL-4 production by basophils (14). However, the impact of basophils on the function of CD4 effector T cells has not been examined, and whether these observations can be translated to human remains to be elucidated.

Here, we show that *in vivo* administration of murine pulmonary basophils exacerbates lung Th2 responses. *In vitro* functional studies in mice and humans indicate that basophils directly activate effector memory CD4 T cells to increase IL-4 production via a TCR-independent pathway.

Materials and methods

Human blood

All human subjects signed informed consent form approved by Institutional Ethic Research Committee (CHUM). Blood was collected in heparinized tubes.

Mice

Female BALB/c and DO11.10 TCR Tg mice (6–8 week old) were purchased from Charles River (Wilmington, MA, USA) and Jackson Laboratory (Bar Harbor, ME, USA) respectively and maintained under specific pathogen-free conditions. All experiments were approved by the ethics committee of CRCHUM and met the standards required by the Canadian Council on Animal Care.

Allergic experimental asthma model

BALB/c mice were immunized intra-peritoneally (i.p.) with 10 µg OVA (Grade V; Sigma, St. Louis, MO, USA) absorbed to 1 mg inject Alum (Pierce, Rockford, IL, USA) on days 0 and 5. On days 12, 16, and 20, mice were challenged for 30 min with a 0.5% OVA aerosol. These sensitized and challenged mice hereafter referred as OVA-immunized mice. For some experiments, 4 mg/kg of Dexamethasone Sodium Phosphate (Sandoz, Holzkirchen,

Germany) was injected i.p. daily from day 12 to day 20. BALF, mediastinal LNs and lungs were collected 24 h after last challenge (22).

Cell purification

Lung cell suspensions were prepared following enzymatic and mechanical dissociation. CD4⁺ T cells (CD4⁺IE[−]DX5[−]IgE[−]CD11c[−]), DCs (IE⁺CD11c⁺CD11b⁺IgE[−]), and basophils (IgE⁺DX5⁺) were purified from the lungs of naïve or OVA-immunized mice. Splenic naïve CD4⁺ T cells (CD4⁺CD62L^{high}CD44^{low}CD25[−]CD11c[−]IA/IE[−]) were isolated from DO11.10 mice. Cell purity was >99%.

Human T_{EM} (CD4⁺CD8[−]CD45RA[−]CD45RO⁺CD62L^{low}CD25[−]), CRTH2⁺ T cells (CD4⁺CXCR3[−]CRTH2⁺), and basophils (CD14[−]CD1c[−]FcεRI^{high}SIRP-α^{low}) were purified from peripheral blood of allergic and nonallergic donors. Cell purity was >99%.

Adoptive transfer of pulmonary basophils

BALB/c recipient mice were sensitized i.p. with OVA-Alum on day 0. These mice hereafter referred as OVA-sensitized hosts. At day 5, hosts were administered i.p. lung basophils isolated from OVA-immunized donor mice in the absence or presence of 10 µg OVA. For some control conditions, PBS or OVA alone was administered to OVA-sensitized recipient mice. On days 12–15, mice were challenged with OVA aerosol and killed 48 h after last challenge. BALF, mediastinal LNs, and lungs were collected 48 h after last challenge. LN cells (0.8 × 10⁶ cells/ml) were cultured for 72 h with 100 µg/ml OVA in RPMI 1640, with 10% FBS, 500 U/ml penicillin, 500 µg/ml streptomycin, 10 mM HEPES buffer, and 1 mM 2-mercaptoethanol. For lung explant cultures, right upper whole pulmonary lobe was cut into small pieces and cultured without Ag supplement for 24 h (22).

In vitro basophil/T cell co-cultures

(i) CFSE-labeled naïve CD4 Tg T cells (500 × 10³ cells/ml) were cultured in the presence of IL-3 (30 ng/ml), with or without 2 µg/ml OVA peptide (Peptides International, Louisville, KY, USA), in the absence or presence of pulmonary DCs and/or basophils (10 × 10³ cells/ml). Cell proliferation was assessed by CFSE dilution at day 4 or 7 as indicated. For cytokine expression, cells were re-stimulated at day 5 with phorbol 12-myristate 13-acetate (PMA)/ionomycin for 6 h in the presence of Brefeldin A (Calbiochem, San Diego, CA, USA) for last 3 h, fixed, and stained to determine IL-4 expression. (ii) FACS-sorted lung CD4 T cells (500 × 10³ cells/ml) were co-cultured with or without basophils (10–40 × 10³ cells/ml) in the presence of IL-3 (30 ng/ml) (PeproTech, Rocky Hill, NJ, USA) for 5 days. For cell proliferation, CFSE-labeled lung CD4 T cells were co-cultured with or without basophils and IL-3. The proportion of viable cells was determined using 7AAD and Annexin V staining that marks necrotic and apoptotic cells, respectively. At day 5, cells were re-stimulated with PMA/ionomycin for 6 h in the presence of Brefeldin

A (Calbiochem) for last 3 h, fixed, and stained to determine IL-4 and/or IL-13 expression. For some experiments, lung T cells were co-cultured with basophils in the presence of 10 µg/ml of anti-IL-4 (1B11 clone). (iii) Human T_{EM} (1×10^6 /ml) or CRTH2⁺ T cells (0.5×10^6 /ml) were co-cultured for 4 days in 96-well round-bottom plate, in RPMI 1640 with 10% FBS medium, with allogeneic or autologous basophils (ratio 5 : 1) in the presence of IL-3 (10 ng/ml) and IL-2 (100 U/ml) (R&D Systems, Minneapolis, MN, USA). For some experiments, T_{EM} and basophils were either separated using Transwell 96-well plate (Corning, Tewksbury, MA, USA) or co-cultured in the presence of 10 µg/ml anti-IL-4 (8F12 clone) or 10 µg/ml murine IgG1 (Sigma). Cells were re-stimulated with PMA/ionomycin for 6 h in the presence of Brefeldin A for last 3 h, fixed, and stained for IL-4 expression.

Flow cytometry analysis

After blocking with CD16/32 blocking antibody (2.4G2) or human IgG, cells were stained with the mAbs indicated in Tables S1 and S2.

Serum Ig measurement

Total and OVA-specific IgE levels were quantified in sera using ELISA. Anti-IgE (clone R35-72; BD Biosciences, San Jose, CA, USA) and biotin-labeled anti-IgE (clone R35-118; BD Biosciences) were used as capture and detection Abs, respectively.

Cytokine production

IL-4, IL-5, and IFN γ (BD Biosciences); IL-10, IL-13, and eotaxin (R&D Systems) release were measured by commercial ELISA kits.

Statistical analysis

Statistical analyses were conducted using GraphPad Software (La Jolla, CA, USA) (Student's *t*-test, Mann–Whitney *U*-test).

Results

Basophils accumulate in the lungs of mice with allergic airway inflammation

Basophils are associated with allergic diseases. Studies demonstrating that prolonged basophil/T cell interactions occur in the lungs but not in LNs (14) prompted us to examine the role of pulmonary basophils in allergic asthma. To this end, we used a steroid-sensitive experimental model of allergic asthma (22). As expected, BALF was predominantly infiltrated by eosinophils and neutrophils and airway inflammation correlated with elevated polyclonal and OVA-specific serum IgE (Fig. 1A). We next showed that basophils [IgE⁺DX5⁺(CD49b)] were mobilized in increased numbers to lungs of OVA-sensitized and OVA-challenged (OVA-

immunized) mice (Fig. 1B,C). Administration of dexamethasone during the OVA challenge period ameliorated airway inflammation, suppressed serum IgE levels, and curbed the accumulation of basophils in the lungs. There was no apparent difference in the phenotype of lung basophils in naïve and OVA-immunized mice (Fig. 1C). Noteworthy, MHC class II expression was undetectable on IgE⁺DX5⁺c-Kit⁺CD11c⁺ basophils in lungs (Fig. 1C), and unlike other lung CD11b⁺ myeloid cells (i.e., neutrophils, eosinophils, and DCs), basophils did not display CD172a (SIRP- α) (Figs 1C and S1). These data demonstrate that basophil recruitment to lung tissues correlates with IgE-associated airway allergic inflammation.

Administration of inflammatory lung basophils to OVA-sensitized mice exacerbates ongoing airway inflammation and Th2 responses

Earlier *in vivo* studies indicated that basophils are not the initiators of Th2 responses (15, 16). Here, we thought to investigate the ability of pulmonary basophils to amplify ongoing airway Th2 responses. As depicted in Fig. 2A, BALB/c recipient mice were immunized once i.p. with OVA-Alum (OVA-sensitized). At day 5, OVA-sensitized hosts were i.p. injected with low numbers (5×10^4 cells) of basophils purified from lungs of OVA-immunized donors instead of Alum, in the presence or absence of OVA. Notably, the isolation procedure did not compromise basophil viability and function because lung basophils produced significant amounts of IL-4 in response to IL-3 stimulation (Fig. S2). The numbers of injected basophils matched those observed in the lungs of asthmatic mice (Fig. 1B). In control experiments, PBS, OVA alone or basophils isolated from lungs of naïve donors were administered, together with OVA, to OVA-sensitized recipient mice. After a 7 days resting period, the hosts were challenged with OVA (aerosol) for four consecutive days. A single injection of basophils together with OVA augmented polyclonal IgE response but not OVA-specific IgE titers in sera (Fig. 2B). Pulmonary basophils isolated from OVA-immunized mice significantly increased recruitment of inflammatory cells to the BALF (Fig. 2C) and enhanced OVA-stimulated Th2 responses in mediastinal LNs (Fig. 2D). Furthermore, they strongly promoted the secretion of IL-4, IL-5, IL-13, IL-10 and eotaxin but not IFN- γ in lung explant cultures (Fig. 2E). Notably, basophils isolated from lungs of OVA-immunized mice tended to be superior to naïve lung basophils in promoting IL-4 and IL-13 expression locally and systemically (Fig. S3). In the absence of adjuvant, soluble OVA injected i.p. is directly taken up by resident DCs in mediastinal LNs (23). We show here that pulmonary basophils from OVA-immunized mice injected i.p. in the absence of OVA to OVA-sensitized hosts, exacerbated IL-4 and IL-13 expression in the lungs without promoting eosinophil recruitment in BALF, Th2 responses in the mediastinal LNs and polyclonal IgE responses, indicating that co-administration of Ag was required for basophils to augment systemic but not mucosal Th2 responses (Fig. 2F–H). Collectively, these data demonstrate that purified pulmonary basophils are functional

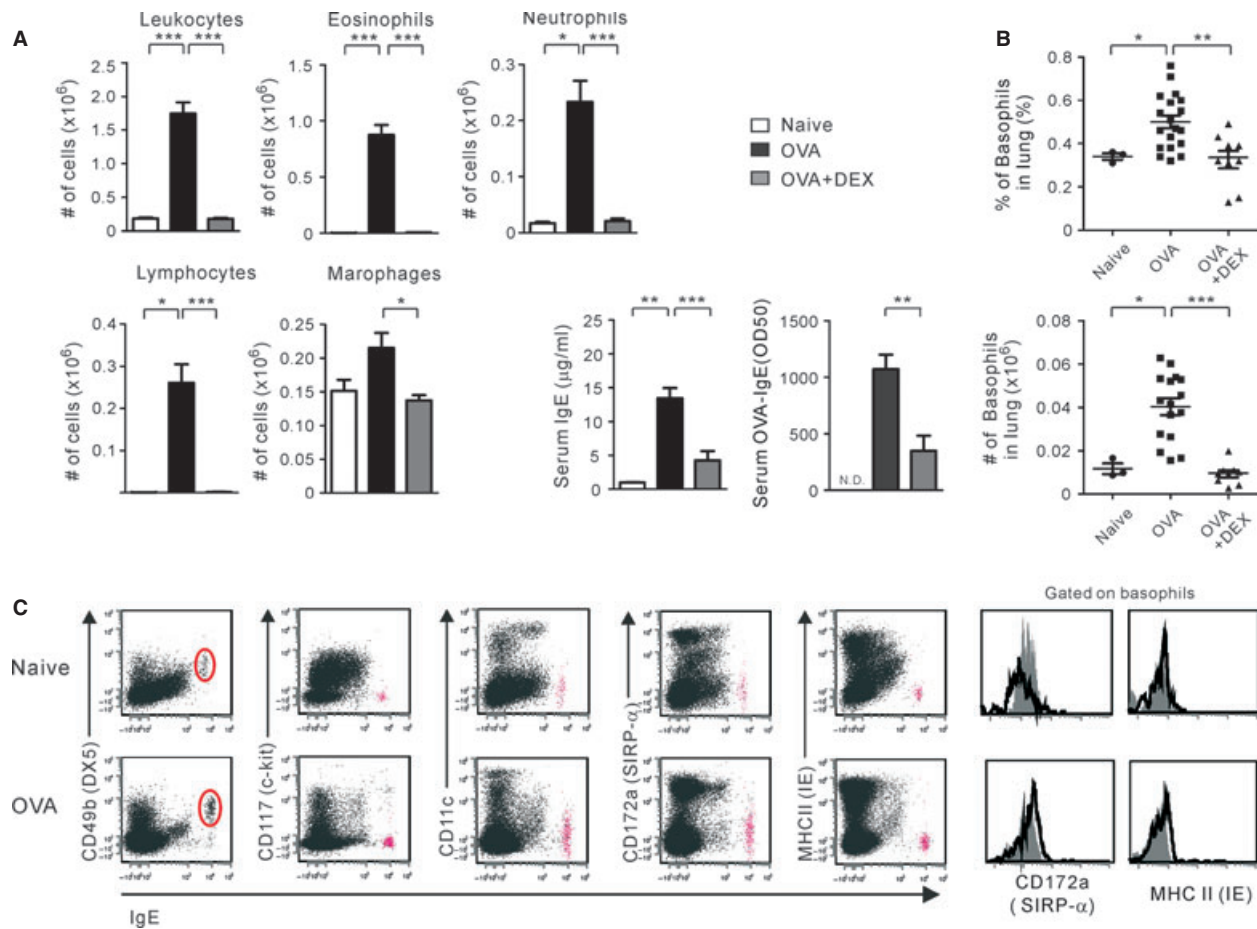


Figure 1 Basophils accumulate in lungs in an experimental model of allergic asthma. BALB/c mice were left untreated (naïve) or injected i.p. on days 0 and 5 with ovalbumin (OVA)-Alum. Mice were OVA-aerosol challenged on days 12, 16, and 20 and killed at day 21 (OVA-immunized mice). Some OVA-immunized mice were treated with dexamethasone (DEX) from day 12 to 20. (A) Differential BALF cell numbers, total IgE and OVA-specific IgE levels in sera. (B) Frequency and absolute numbers of basophils in lungs of naïve, OVA-immunized and

OVA + DEX mice. (C) Phenotype of lung basophils in naïve and OVA-immunized mice. Red dots correspond to basophils (IgE⁺DX5⁺ cells) and black lines to basophils stained with the isotype-matched control mAb. One representative data of at least three independent experiments. (A,B) Each data point represents a single mouse. Bars represent means of data from three pooled experiments ($n = 4-16$ mice/per group). * $P < 0.05$, *** $P < 0.01$, **** $P < 0.001$ as determined by Mann-Whitney U -test. BALF, bronchoalveolar lavage fluid.

and exacerbate ongoing Th2-associated airway inflammation. These results further suggest that basophils may amplify Th2 responses via an interaction with effector cells in the lungs while they act in concert with OVA-loaded DCs in LNs.

Lung basophils do not initiate Ag-specific naïve CD4 T cell proliferation but amplify DC-driven Th2 differentiation

The difficulty to isolate large numbers of purified lung basophils hampered studies aiming to track these cells *in vivo*. We postulated that i.p. injected pulmonary basophils, like DCs, have the potential to reach directly the mediastinal LNs. Indeed, basophils can migrate to LNs draining the sites of papain or helminth-injected mice (4, 16). We therefore investigated the APC function of purified pulmonary basophils and DCs *in vitro*. We found that pulmonary basophils, in

contrast to DCs, failed to induce proliferation of Ag-specific naïve DO11.10 T cells (TCR transgenic (Tg) T cells that recognize OVA peptide (323–339 residues) as measured by CFSE dilution (Fig. 3A). Importantly, highly purified Tg T cells did not divide when cultured in the presence of OVA without DCs (Fig. 3A) and vice versa in the presence of DCs without OVA peptide (Fig. 3B). These data demonstrate that Tg T cell preparation was not contaminated by DCs and that DCs, whether isolated from naïve or OVA-immunized mice, required to be loaded with OVA peptide to induce Ag-specific T cell priming. Basophils did no further increase naïve Tg T cell proliferation induced by inflammatory lung DCs (Fig. 3C). However, basophils augmented, in a dose-dependent manner, lung DC-driven T cell differentiation into IL-4-producing cells (Fig. 3D). We noticed that OVA-immunized basophil/DC predominated over naïve basophil/DC

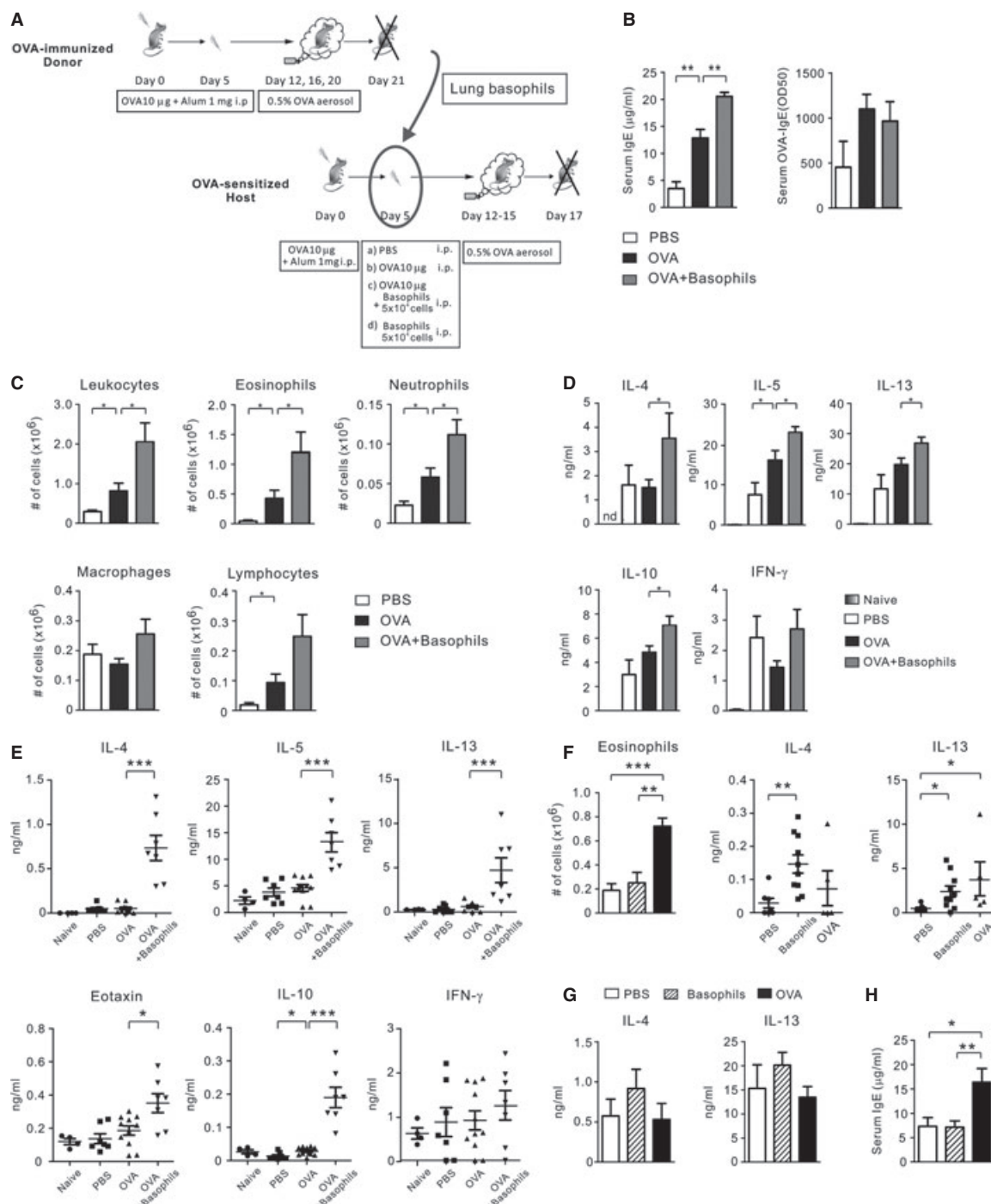


Figure 2 Adoptive transfer of lung basophils exacerbates airway inflammation and promotes Th2 cytokine expression. (A) BALB/c recipient mice were sensitized with ovalbumin (OVA)-Alum on day 0. At day 5, OVA-sensitized hosts were injected i.p. with either PBS, OVA or pulmonary basophils from OVA-immunized mice in the presence or absence of OVA. Recipient mice were challenged with OVA (aerosol) at day 12 for four consecutive days and killed at day 17. (B–E) PBS, OVA or basophils plus OVA. (F–H) PBS, basophils or OVA. (B,H) Total IgE and OVA-specific IgE levels in the sera of recipient animals. [C,F (left panel)] Differential BALF cell num-

bers. (D,G) Mediastinal LN cells (0.8×10^6) were stimulated with OVA for 3 days and cytokine production measured by ELISA. [E,F (right panels)] Lung explant cultures with no Ag stimulation. Cytokine and chemokine release measured in the culture supernatant after 24 h. (B,H) Bars represent means of data from three pooled experiments ($n = 7$ –11 mice/per group). Each data point represents a single mouse. ND, nondetectable. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as determined by unpaired Student's t -test (B,F) or Mann-Whitney U -test (C–F). BALF, bronchoalveolar lavage fluid; LN, lymph nodes.

combination to induce naïve Tg T cell differentiation into IL-4-producing cells (Fig. 3E) and IL-4 production (Fig. 3F). Thus, lung basophils, isolated from naïve or OVA-immunized mice, are not capable of inducing Ag-specific T cell primary response *in vitro*, whereas they cooperate with DCs to increase Ag-specific Th2 cell polarization.

Murine basophils interact with lung CD4 effector T cells to enhance IL-4 and IL-13

As adoptively transferred pulmonary basophils in the absence of OVA augmented local but not systemic Th2 responses, we hypothesized that they can be directly recruited into inflamed lungs to interact with lung CD4 effectors (18). Our data first indicated that lung basophils promoted *in vitro* cell survival

rather than proliferation of lung CD4 effectors (Fig. 4A). Furthermore, basophils significantly increased IL-4 and IL-13 expression in viable CD4 T cells in the absence of DCs and preferentially augmented in a dose-dependent manner the frequency of IL-4⁺/IL-13⁺ CD4 T cells (Fig. 4B,C). The increase in cytokine expression was IL-4 and Ag independent because it can be observed in CD4 T cells co-cultured with basophils in the presence of neutralizing antibody to IL-4 (Fig. 4D) as well as with pulmonary basophils isolated from HDM-sensitized and challenged mice (Wakahara K. and Sarfati M., unpublished observations). However, basophil-derived IL-4 mediated survival of lung CD4 effectors (Fig. 4E). These data indicate that lung basophils augment local Th2 responses via IL-4-dependent and IL-4-independent mechanisms.

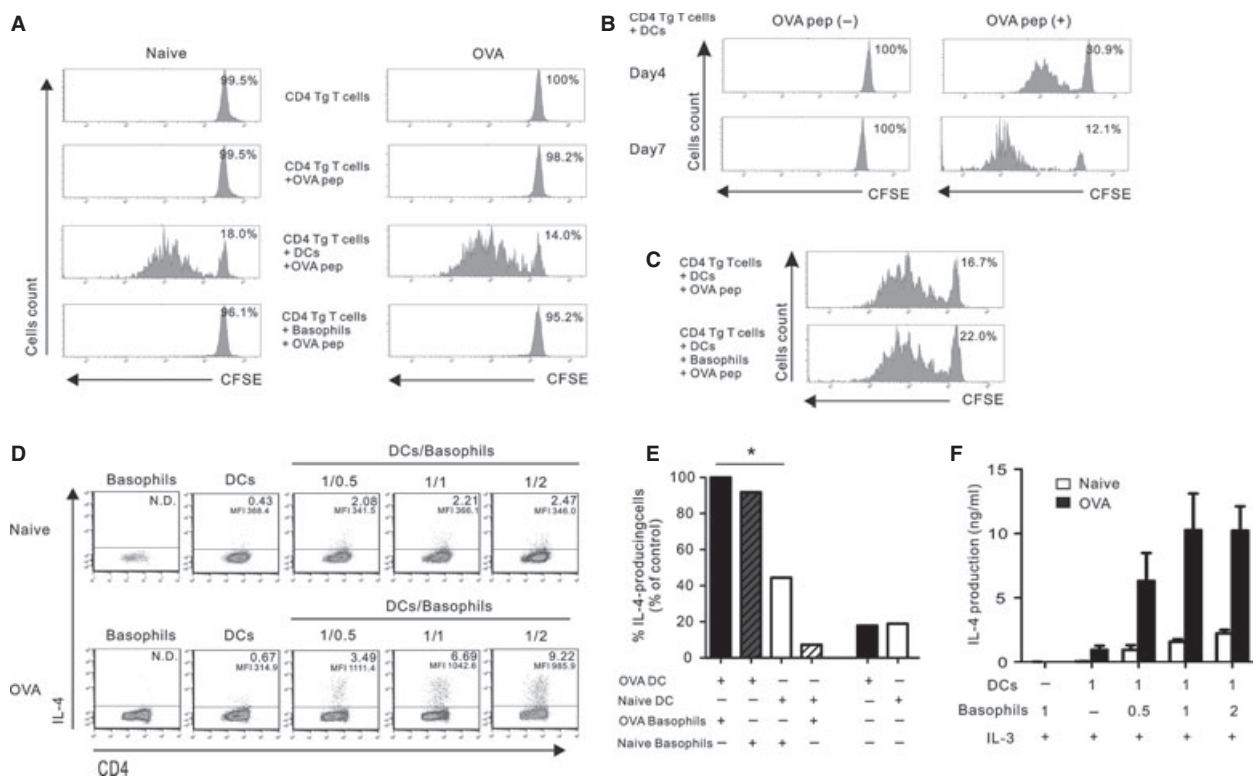


Figure 3 Lung basophils do not initiate Ag-specific Th cell priming but amplify inflammatory dendritic cells (DC)-driven Th2 cell differentiation. (A) Naïve CD4 Tg T cells (500 × 10³ cells/ml) were cultured in the presence of IL-3 with or without ovalbumin (OVA) peptide or co-cultured in the presence of IL-3 plus OVA peptide together with DCs or basophils (50/1 ratio) purified from lungs of naïve or OVA-immunized mice (one representative data out of three experiments). (B) Naïve CD4 Tg cells were co-cultured with DCs from OVA-immunized mice with or without OVA peptide (one representative data out of two experiments). (C) Naïve CD4 Tg cells were co-cultured with DCs from OVA-immunized mice in the presence or absence of basophils from OVA-immunized mice (one representative data out of two experiments). T cell proliferation was assessed by CFSE dilution at day 4 (A–C) and day 7 (B). (D–F) Naïve CD4 Tg T cells (500 × 10³ cells/ml) were co-cultured in the

presence of IL-3 and OVA peptide with DCs in the absence or presence of basophils, purified from lungs of naïve or OVA-immunized mice at a T/DC/basophil (50/1/0.5–2) cell ratio for 5 days. (D) T/basophil/DC co-cultures with cells isolated from naïve and OVA-immunized mice, respectively. IL-4 intracytoplasmic staining gated on CD4 (one representative data of three independent experiments, ND, nondetectable). (E) Frequency of IL-4 positive CD4 T cells in T/basophil/DC co-cultures as indicated. Data are represented as % of control response (T/DC/basophil (50/1/1) from OVA-immunized mice represent 100%). Data represent 2 (OVA-DC/naïve basophils or vice versa) and 5 (OVA-DC/OVA-basophils or naïve DC/naïve basophils) independent experiments. (F) IL-4 production as measured by ELISA (n = mean ± SEM of three independent experiments). *P < 0.05 as determined by unpaired Student's t-test.

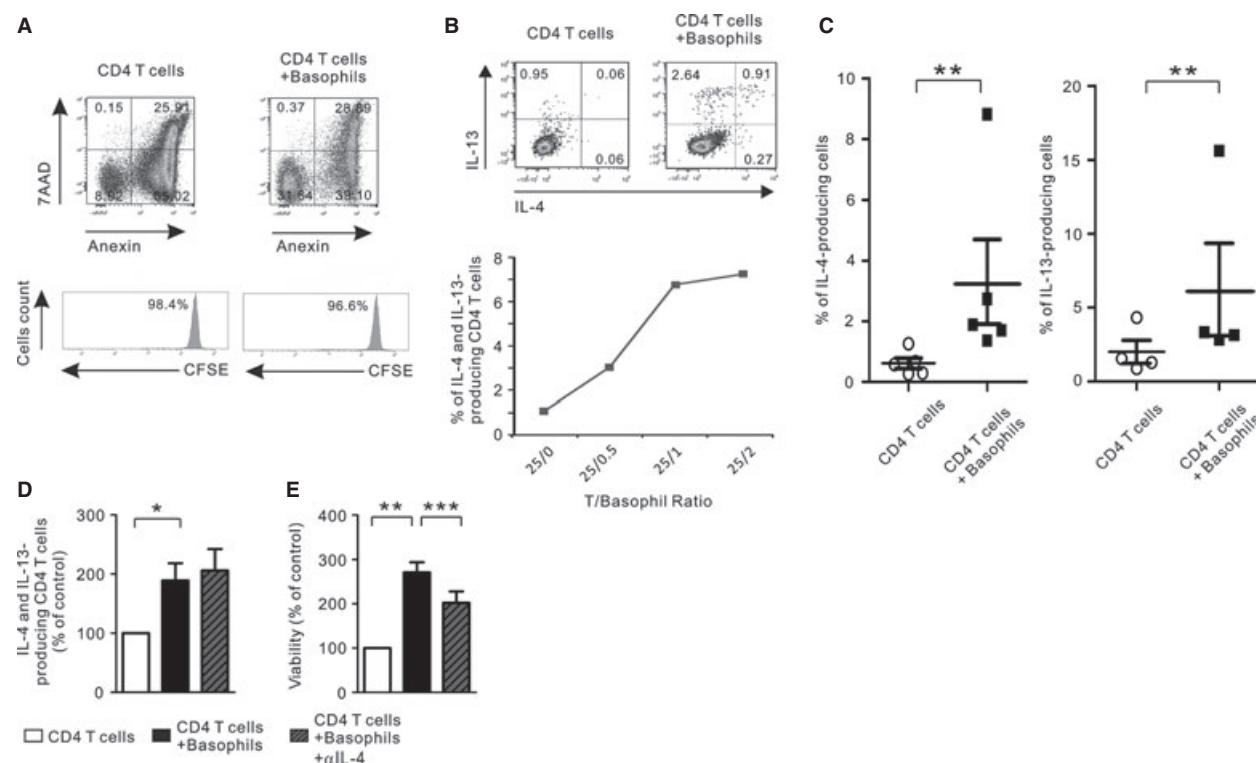


Figure 4 Pulmonary basophils directly interact with effector CD4 T cells to amplify IL-4 and IL-13 production. (A–E) Lung CD4 T cells (500×10^3 cells/ml) were co-cultured with lung basophils isolated from ovalbumin (OVA)-immunized mice in the presence of IL-3 but without OVA at a T cell/basophil (25/1) cell ratio for 5 days. (A) Cellular viability was evaluated by 7AAD and Annexin staining (upper panels) and cell proliferation by CFSE dilution (lower panels) (one representative data of three independent experiments). (B–D) Cytokine production was examined by intracytoplasmic staining. (B) Representative dot plot of IL-4 and IL-13-

producing CD4 T cells (upper panels) and dose–response curve of cytokine production (lower panel). Basophils were used at T cell/basophil (25/0–2). (C) Frequency of total IL-4 or IL-13-producing T cells at a 25/1 ratio ($n = 4$ –5 independent experiments). (D–E) T cell/basophil (25/1) co-cultures in the presence of anti-IL-4 neutralizing or isotype control mAbs. IL-4 and IL-13 producing cells (D) and cell viability (E). Data are represented as % of control response (lung CD4 T cells) ($n = 4$ independent experiments). * $P < 0.5$, ** $P < 0.01$, *** $P < 0.01$ as determined by paired Student's *t*-test.

Human basophils enhance IL-4 expression via direct interactions with CRTH2⁺ CD4 effector T cells

We next examined whether the ability of murine basophils to amplify CD4 effector memory responses is applicable to human basophils. We found that highly purified human basophils augmented IL-4 production by allogeneic as well as autologous peripheral blood effector memory T cells (T_{EM}) in the presence of IL-3 and IL-2 and in the absence of TCR stimulation (Fig. 5A). The circulating basophils were purified following a FACS-sorting strategy, which avoided IL-3R and MHC Class II ligation by the respective mAbs (Fig. S2).

CRTH2 characterizes a minority subset of circulating memory Th2 cells that comprised ~40% T_{EM} (24), and we showed that basophils significantly augmented IL-4 expression at the single cell level in autologous CRTH2⁺ T cells (Fig. 5B). The enhancement of IL-4 production in T_{EM} by basophils was not inhibited by neutralizing antibody to IL-4 (Fig. 5C) or inhibitors of IL-25 (data not shown). Indeed, basophils may express IL-25, and IL-25 promotes Th2

responses (25). Yet, the amplification of IL-4 expression was maximal when basophils were in direct contact with T_{EM} cells (Fig. 5D). However, OX40-Fc, CTLA4-Ig, and LFA-3-Ig did not inhibit the ability of basophils to augment Th2 responses (data not shown) (26).

Collectively, our data support a model in which basophils amplify memory Th2 responses by a process that requires direct interaction with effector CD4 T cells in humans and mice, *in vitro* and *in vivo*.

Discussion

Although recent studies reported that prolonged basophil/T cell contacts occur in lungs but not in LNs of parasite-infected mice, the functional consequences of these interactions on CD4 T cells were not examined (14). We demonstrate here the *in vitro* pro-inflammatory activity of basophils on autologous effect or CD4 T cells because basophils augmented the production of IL-4 at the single cell level by murine lung CD4 effectors as well as by human

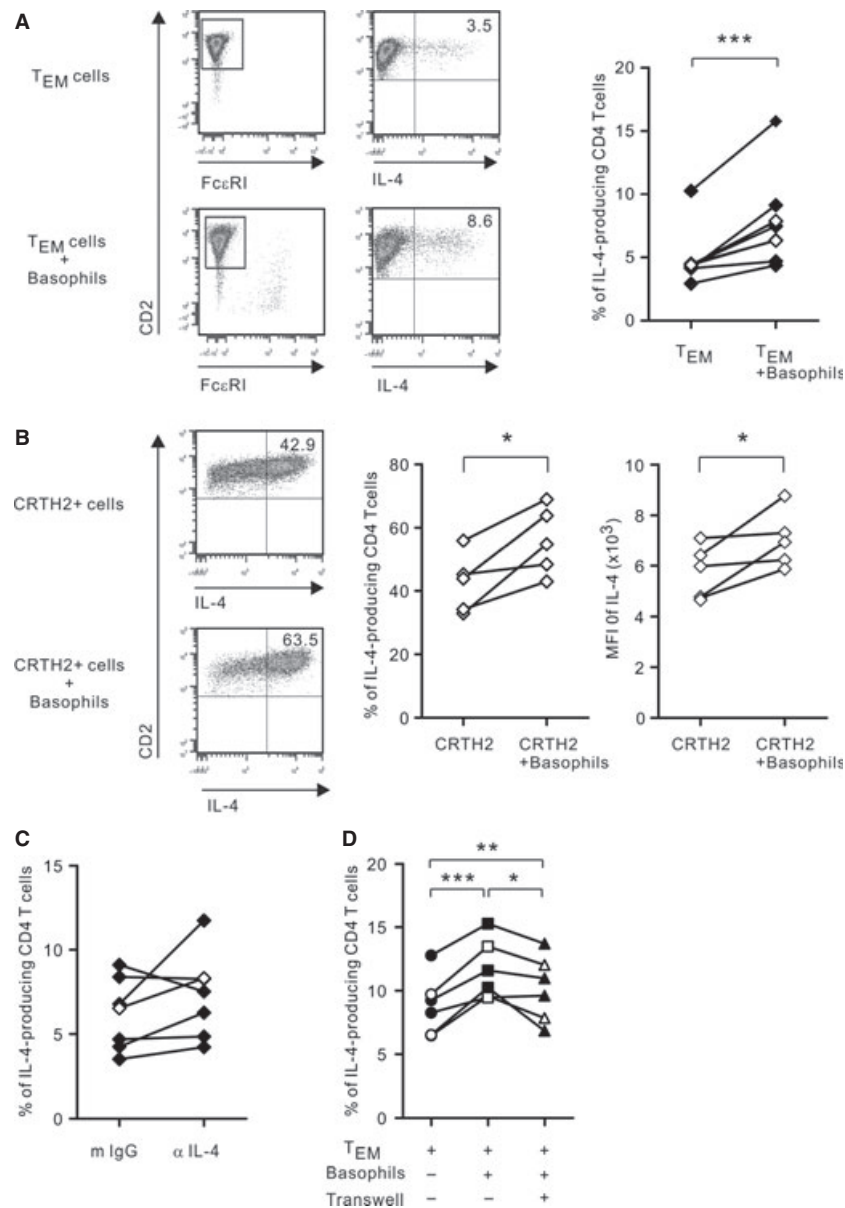


Figure 5 Human basophils directly interact with effector memory T cells to amplify IL-4 production. (A) Human effector memory T cells (T_{EM} ; $CD4^+CD8^-CD45RO^+CD45RA^-CD62L^{low}CD25^-$ cells) were co-cultured with or without autologous (white symbols) or allogeneic (black symbols) basophils (5/1 ratio) in the presence of IL-3 and IL-2. Proportion of IL-4-producing CD4 T cells was examined at day 4 by intra-cytoplasmic staining. (B) FACS-sorted CRTH2 $^+$ T cells

co-cultured with or without basophils in the presence of IL-3 and IL-2. Frequency of IL-4-producing CD4 T cells (left panel) and mean fluorescence intensity (MFI) of IL-4 expression (right panel). (C) T_{EM} were co-cultured with or without basophils in the presence of anti-IL-4 neutralizing or isotype control mAbs. (D) T_{EM} were separated from basophils using a Transwell insert. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as determined by paired Student's t -test.

effector memory CD4 T cells that include CRTH2 $^+$ T cells. We found that basophils were recruited to the lung in an experimental model of allergic asthma (22). Indeed, human basophils were identified in airways of allergic and asthmatic patients by immunohistochemistry (6, 7). Adoptive transfer of lung basophils to OVA-sensitized hosts augmented ongoing Th2 responses and airway inflammation. However, the physiological relevance of the present findings to allergic asthma

would be supported by the measurement of pulmonary function. Although the nature of the factors that attract basophils to inflammatory lungs remains to be clarified, MCs outnumbered basophils in inflamed airways, and MC-derived *PGD2* is a potent chemotactic factor for CRTH2-bearing cells that include basophils, Th2 lymphocytes, eosinophils, and IL-13-producing non-T non-B innate lymphoid cells (24, 27). Noteworthy, CRTH2 $^+$ T cells infiltrate asthmatic sputum (28).

The direct effects of basophils on CD4 memory T cells are presumably TCR-independent because they were observed in autologous system, without Ag supplementation and in absence of detectable levels of MHC class II on basophils. Although the precise mechanisms that govern the ability of basophils to activate memory CD4 T cell cytokine production remain to be elucidated, it may already be concluded that the augmentation of IL-4 expression in basophil/memory T cell co-cultures is partially contact dependent and IL-4 independent. Indeed, neutralizing IL-4 did not alter the ability of basophils to augment Th2 responses in effector CD4 memory T cells, which contrasts with the IL-4 dependency of DC/basophil cooperation for Ag-specific Th2 priming in mice (17). Given that murine-activated T cells stimulate IL-4 production by basophils (14), the functional interactions between these two types of cells appear to be bidirectional. In contrast, basophil-derived IL-4 can mediate lung CD4 effector T cell survival. In that regard, TNF-related ligand LIGHT and CX₃CR1 expression have been implicated in the maintenance and survival of Th2 memory cells in inflamed lungs, but the precise contribution of these molecules to basophil-mediated lung CD4 effector cell survival requires further investigations (29, 30).

Our present findings support the concept that pulmonary basophils not only contribute to CD4 effector T cell activation in peripheral tissues but may also cooperate with OVA-loaded DCs to induce a new round of naïve T cell differentiation in the mediastinal LNs (16). We show that injection of basophils in the absence of Ag augmented local Th2 responses, whereas co-administration of Ag was required to further amplify Th2 responses in LNs, corroborating our *in vitro* data showing that basophils amplified Th2 priming initiated by airway inflammatory DCs. This may explain the common observation that in the course of disease progression, allergic patients often become sensitized to a greater variety of antigens.

In conclusion, basophils represent previously unappreciated innate players recruited, together with eosinophils and

neutrophils, into inflamed lungs following allergen exposure to control the exacerbation of Th2-associated diseases via direct interaction with effector T cells.

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Author contributions

K.W. and M. S. designed the experiments; K.W., V.Q.V., N.B., P.B. and M.R. did the experiments; K.W., G.D. and M.S. analyzed the data and wrote the manuscript.

Conflict of interest

The authors declared no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Phenotype analysis of murine basophils.

Figure S2. FACS-sorting strategy to purify murine and human circulating basophils.

Figure S3. Adoptive transfer of naïve lung basophils exacerbates airway inflammation.

Table S1. Anti-mouse antibody.

Table S2. Anti-human antibody.

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Fig. S1

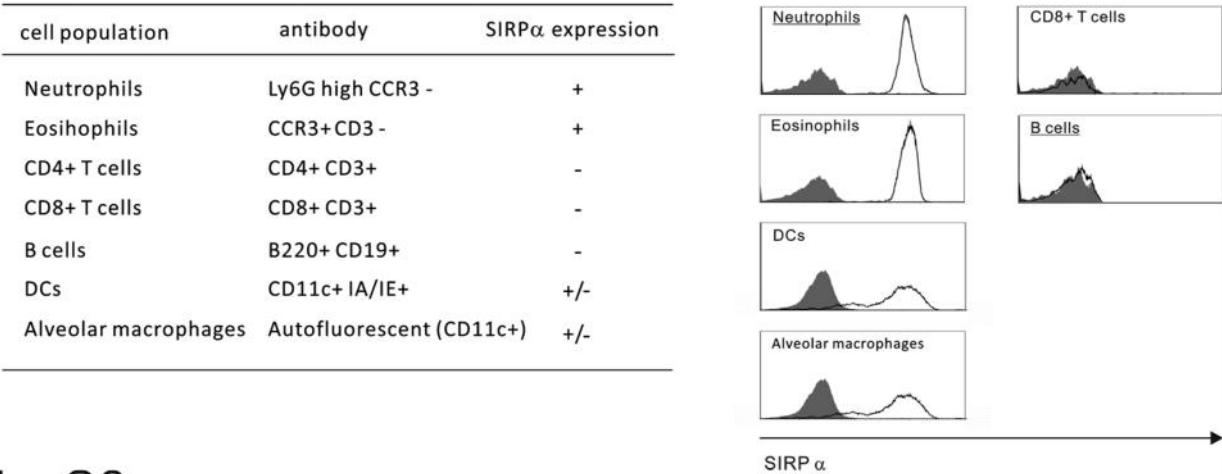
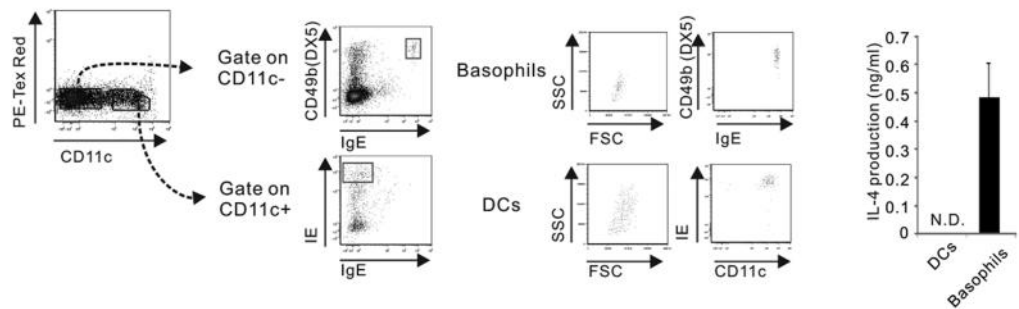


Fig. S2

a



b

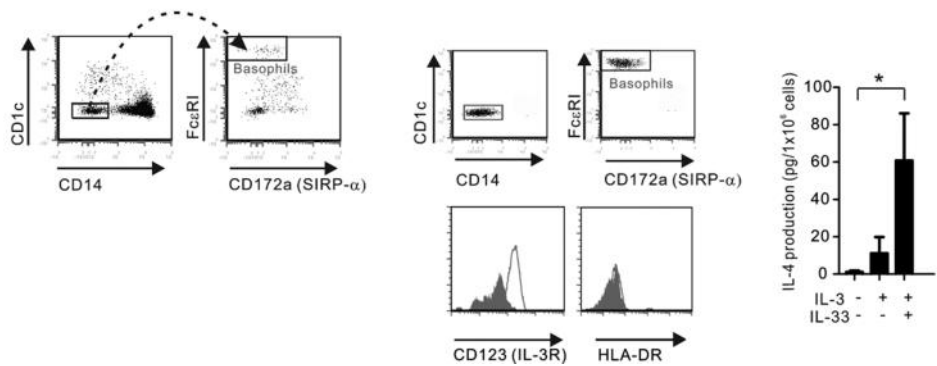
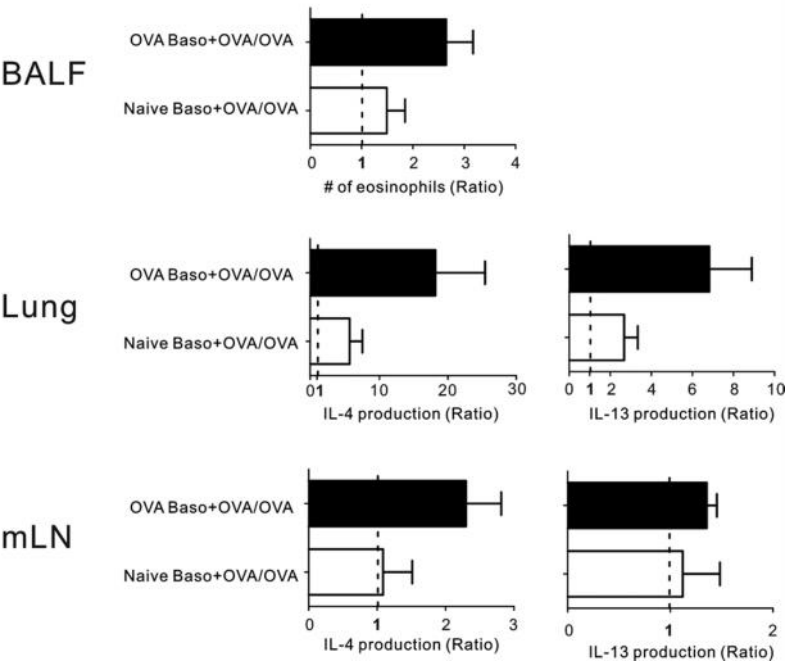


Fig. S3



Supplemental Table S1

Anti-mouse antibody

Antibody	Conjugate	clone	Company
anti-CD3	FITC	145-2C11	home
anti-CD4	APC-Cy7	RM4-5	BioLegend
anti-CD4	PE-Cy7	RM4-5	BioLegend
anti-CD8a	PE	53-6.7	BD biosciences
anti-CD11b	APC	M1/70	BioLegend
anti-CD11c	biotin	HL3	BD biosciences
anti-CD11c	PE-Cy7	N418	BioLegend
anti-CD49b (DX5)	FITC	DX5	BioLegend
anti-CD49b (DX5)	PE	DX5	BD biosciences
anti-CD62L	PerCP/Cy5.5	MEL-14	BioLegend
anti-CD117 (c-kit)	APC-Cy7	2B8	BioLegend
anti-CD172a (SIRP α)	APC	p84	BD biosciences
anti-CD193 (CCR3)	PE	83101	R & D
anti-DO11.10 TCR	PE	KJ1-26	BioLegend
anti-I-A/I-E	PE	M5/114.15.2	BD biosciences
anti-I-E	FITC	14-4-4S	home
anti-IgE	biotin	R35-118	BD biosciences
anti-Ly6G	FITC	IA8	BD biosciences
anti-IL-4	Alexa fluor 488	11B11	BioLegend
anti-IL-4	PE	11B11	BD biosciences
anti-IL-13	PE	eBio13A	eBioscience
anti-IL-13	Alexa fluor 647	eBio13A	eBioscience
Streptavidin	PerCP-Cy5.5		BioLegend
Streptavidin	APC		BioLegend
Streptavidin	PE		BD biosciences
blocking		2.4G2	BD biosciences

Supplemental Table S2

Anti-human antibody

Antibody	Conjugate	clone	Company
anti-CD1c	Alexa fluor 647	L161	BioLegend
anti-CD2	FITC	RPA-2. 10	BD Biosciences
anti-CD4	PE-Cy7	RPA-T4	BioLegend
anti-CD4	APC-Cy7	RPA-T4	BioLegend
anti-CD8a	PE	RPA-T8	BioLegend
anti-CD14	FITC	HCD 14	BioLegend
anti-CD25	APC	M-A251	BD Biosciences
anti-CD45RA	Alexa fluor 488	HI 100	BioLegend
anti-CD45RO	FITC	UCHL1	BioLegend
anti-CD45RO	PerCP/Cy5.5	UCHL1	BioLegend
anti-CD62L	PE-Cy7	DREG-56	BioLegend
anti-CD123	PerCP/Cy5.5	6H6	BioLegend
anti-CD172a (SIRP- α)	PE-Cy7	SE5A5	BioLegend
anti-CD203c	PE	FR3-16A11	Miltenyi Biotec
anti-CD294 (CRTH2)	PerCP/Cy5.5	BM16	BD Biosciences
anti-Fc ϵ R1 alpha	PE	AER-37	eBioscience
anti-IL-4	APC	MP4-25D2	BD Biosciences
anti-HLA-DR	FITC	L243	BD Biosciences
anti-HLA-DR	PerCP	L243	BioLegend