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ARTICLE

Interleukin-33–activated basophils promote asthma by regulating Th2 cell entry into lung tissue

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Asthma is characterized by lung eosinophilia, remodeling, and mucus plugging, controlled by adaptive Th2 effector cells secreting IL-4, IL-5, and IL-13. Inhaled house dust mite (HDM) causes the release of barrier epithelial cytokines that activate various innate immune cells like DCs and basophils that can promote Th2 adaptive immunity directly or indirectly. Here, we show that basophils play a crucial role in the development of type 2 immunity and eosinophilic inflammation, mucus production, and bronchial hyperreactivity in response to HDM inhalation in C57Bl/6 mice. Interestingly, conditional depletion of basophils during sensitization did not reduce Th2 priming or asthma inception, whereas depletion during allergen challenge did. During the challenge of sensitized mice, basophil-intrinsic IL-33/ST2 signaling, and not FcεRI engagement, promoted basophil IL-4 production and subsequent Th2 cell recruitment to the lungs via vascular integrin expression. Basophil-intrinsic loss of the ubiquitin modifying molecule *Tnfaip3*, involved in dampening IL-33 signaling, enhanced key asthma features. Thus, IL-33–activated basophils are gatekeepers that boost allergic airway inflammation by controlling Th2 tissue entry.

Introduction

The prevalence of allergic diseases like asthma, hay fever, and eczema has increased dramatically over the past decades, affecting 300 million people worldwide (Braman, 2006). Allergic asthma is characterized by chronic eosinophilic inflammation of the airways, causing bronchial hyperreactivity, airway wall remodeling, and mucus overproduction with plugging (Hammad and Lambrecht, 2021; Schuijs et al., 2013). Overproduction of the Th2 cytokines IL-4, IL-5, and IL-13 is a hallmark of type 2 immunity and is tightly associated with eosinophilic airway inflammation, goblet cell metaplasia, bronchial hyperresponsiveness, vessel wall priming, and production of IgE by B cells (Lambrecht et al., 2019). Airway epithelial barrier cells and epithelial-derived cytokines also play an important role in driving allergic disease (Willart et al., 2012; Hammad and Lambrecht, 2015; Schuijs et al., 2015; Kabata et al., 2020). Studies in *Il-33*– and *Il1rl1*-deficient animals have shown that epithelial IL-33 promotes dendritic cell (DC) maturation, Th2 effector cell cytokine production, mast cell activation, and basophil activation (Liew et al., 2016; Siracusa et al., 2011; de Kleer et al., 2016). Polymorphisms within the *IL1RL1* gene locus that encodes for the IL-33R are strongly associated with increased susceptibility to develop asthma (Moffatt et al., 2010), and IL-33R engagement promotes activation of various immune cells in a MyD88-dependent manner (Kroeger et al., 2009; Schmitz et al., 2005; Kondo et al., 2008). In addition to Th2 adaptive immune cells, innate sources of IL-4, IL-5, and IL-13, like ILC2s, eosinophils, and basophils, can also contribute to eosinophilic inflammation (Schuijs and Halim, 2018; Voehringer et al., 2004; Kondo et al., 2008; Liang et al., 2011; Hammad and Lambrecht, 2021).

Basophils have long been implicated in the context of allergic diseases of the skin and airways and the basophil activation test is emerging as an important in vitro diagnostic biomarker for allergic diseases (Yamanishi et al., 2017; Schwartz et al., 2016; Karasuyama et al., 2017; Sullivan and Locksley, 2009). Basophils are also highly enriched in postmortem lung tissue of patients who have died from asthma as well as in bronchial biopsies and sputum of patients with eosinophilic type 2 asthma, where basophils are a prominent source of mRNA encoding type 2 cytokines (Macfarlane et al., 2000; Kepley et al., 2001; Gordon et al.,

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2016b). These findings suggest that basophils may contribute to the pathogenesis of chronic asthma in humans and could be specifically targeted, but more mechanistic studies are required to directly address this hypothesis. Basophils express the high-affinity receptor for IgE (FcεRI) and can degranulate upon allergen encounter and receptor crosslinking, leading to stored mediator release, cytokine production, and plasma extravasation (Cheng et al., 2015; Siracusa et al., 2013; Yamanishi et al., 2017; Karasuyama et al., 2017). Basophils have also been implicated in controlling adaptive immune responses upon encounter with allergens or large extracellular parasites like helminthes by providing an early source of IL-4 and IL-6 that can influence B cell isotype switching and polarize naïve CD4⁺ T cells (Pulendran and Artis, 2012; Sokol et al., 2008; Shan et al., 2018; Khodoun et al., 2004; Oh et al., 2007; Denzel et al., 2008; Peng and Siracusa, 2021). Several studies have even identified basophils as antigen-presenting cells that are necessary and sufficient for the induction of type 2 immunity during sensitization to allergens (Perrigoue et al., 2009; Sokol et al., 2009; Yoshimoto et al., 2009; Miyake et al., 2017), although follow-up studies have challenged this idea (Phythian-Adams et al., 2010; Hammad et al., 2010). Using basophil-depleting antibodies, IL-4–producing basophils were first proposed to contribute to Th2 cell differentiation in vivo in response to the protease allergen papain, yet these findings were later refuted when genetic models of basophil depletion were used (Sokol et al., 2008; Ohnmacht et al., 2010). Although basophil-derived IL-4 promotes the secretion of IL-5, IL-9, and IL-13 from ILC2s (Motomura et al., 2014), depletion of basophils did not lead to impairment of lung Th2 responses (Ohnmacht et al., 2010; Sullivan et al., 2011). The role of basophils in priming adaptive response to helminths is equally controversial and confounded by the genetic model used to deplete or target basophils; however, in sum, these studies have highlighted that in addition to IgE crosslinking, epithelial cytokines can be important drivers of basophil activation and that basophil functions are highly context and model dependent (Gordon et al., 2016b; Kroeger et al., 2009; Giacomin et al., 2012; Hill et al., 2012; Kim et al., 2014; Noti et al., 2014; Schneider et al., 2009; Chhiba et al., 2017).

Despite the undisputable effector functions of basophils, their precise contribution during the initiation or effector phase of adaptive mucosal inflammation remains incompletely defined, particularly in the context of HDM-driven asthma. We used different basophil depletion strategies and adoptive reconstitution models to demonstrate a previously unrecognized role for IL-33–activated basophils driving the effector phase of HDM-induced allergic airway inflammation by controlling the recruitment of adaptive Th2 cells to the lungs. Increased IL-33 sensitivity through loss of function of the asthma susceptibility gene *Tnfaip3* in basophils disrupts their homeostasis, thus enhancing the severity of type 2 immunity.

Results

Basophils are required for HDM-induced eosinophilic inflammation

To address the role of basophils in allergic airway inflammation driven by the relevant allergen house dust mite (HDM), mice were sensitized via the intratracheal (i.t.) route to 1 µg of house dust mite (HDM) or PBS as a control on day 0 and challenged intranasally (i.n.) between days 6 and 10 with 10 µg of HDM extract daily (Fig. 1 A). After challenge with HDM extracts and analysis at day 14, HDM-sensitized mice showed increased numbers of eosinophils and lymphocytes in the bronchoalveolar lavage fluid (BAL) compared with non-sensitized controls (Fig. 1 B). The increased BAL eosinophilia was accompanied by elevated Th2 cytokines IL-5 and IL-13 in mediastinal lymph node (MLN) cultures (Fig. S1 A). Using basophil reporter mice, in which YFP is expressed from the basophil-specific mast cell protease 8 gene promotor (*Mcpt8*$^{Cre/YFP}$ mice), we found increased numbers of basophils in the lungs after active sensitization and challenge with HDM (Fig. 1, C and D), consistent with previous reports (Hammad et al., 2010; Di et al., 2015). We could also identify lung CD49b⁺YFP⁺ basophils by confocal microscopy (Fig. S1 B) and found that after i.v. injection of a fluorescently labeled CD45 Ab immediately prior to analysis, most of the basophils were labeled (Fig. 1 E and Fig. S1 C). This suggests that upon HDM challenge most of the lung basophils persist at the endothelial boundary, as previously described in the ear vasculature (Cheng et al., 2015). The major effector functions of basophils have been linked to arming and crosslinking of IgE or IgG on their surface; therefore, we measured serum immunoglobulin levels of IgG1 and IgE and found both to be elevated after HDM sensitization (Fig. S1 D), indicating active B cell isotype switching which could be mediated by basophils (Sokol et al., 2008). In line with the increased serum IgE, we could also detect surface-bound IgE on lung basophils (Fig. 1 F).

To further investigate the role of basophils in HDM-induced asthma, we used *Mcpt8*-Cre BAC-transgenic animals (also known as *Mcpt8*Cre^{Voeh}) in which basophils are constitutively deleted due to Cre toxicity (Ohnmacht et al., 2010) and analyzed asthma development. Whereas actively sensitized and challenged littermate controls mounted robust airway responses characterized by an influx of airway eosinophils and lymphocytes, this response was severely hampered in basophil-deficient *Mcpt8*Cre^{Voeh} mice (Fig. 1 G), concomitant with strongly reduced IL-5 and IL-13 production from the MLN (Fig. 1 H). The monocyte and eosinophil-recruiting chemokines CCL2 and CCL24 were similarly reduced in the BAL of basophil-deficient mice (Fig. 1 I). Moreover, the levels of CCL5, CXCL9, and CXCL10 were also reduced (Fig. S1 E). Permanent basophil depletion was confirmed during the course of the experiment (Fig. 1 J). The absence of basophils also led to reduced serum concentration of HDM-specific IgG1 and IgE (Fig. 1 K). Basophil-deficient *Mcpt8*Cre^{Voeh} mice showed reduced bronchial hyperreactivity and constriction of the airways to high doses of methacholine compared with their littermate controls (Fig. 1 L). Asthma is characterized by extensive mucus plugging, which causes most asthma fatalities (Lambrecht et al., 2019; Aegerter and Lambrecht, 2023). Whereas WT mice had a marked increase in mucus production, *Mcpt8*Cre^{Voeh} mice showed substantially less mucus in the airways after active HDM sensitization and challenge (Fig. 1 M), a finding quantified by reduced abundance of *Muc5ac* mRNA, coding for gel-forming mucin in lung homogenates (Fig. 1 N). In all, these data imply that basophils play an

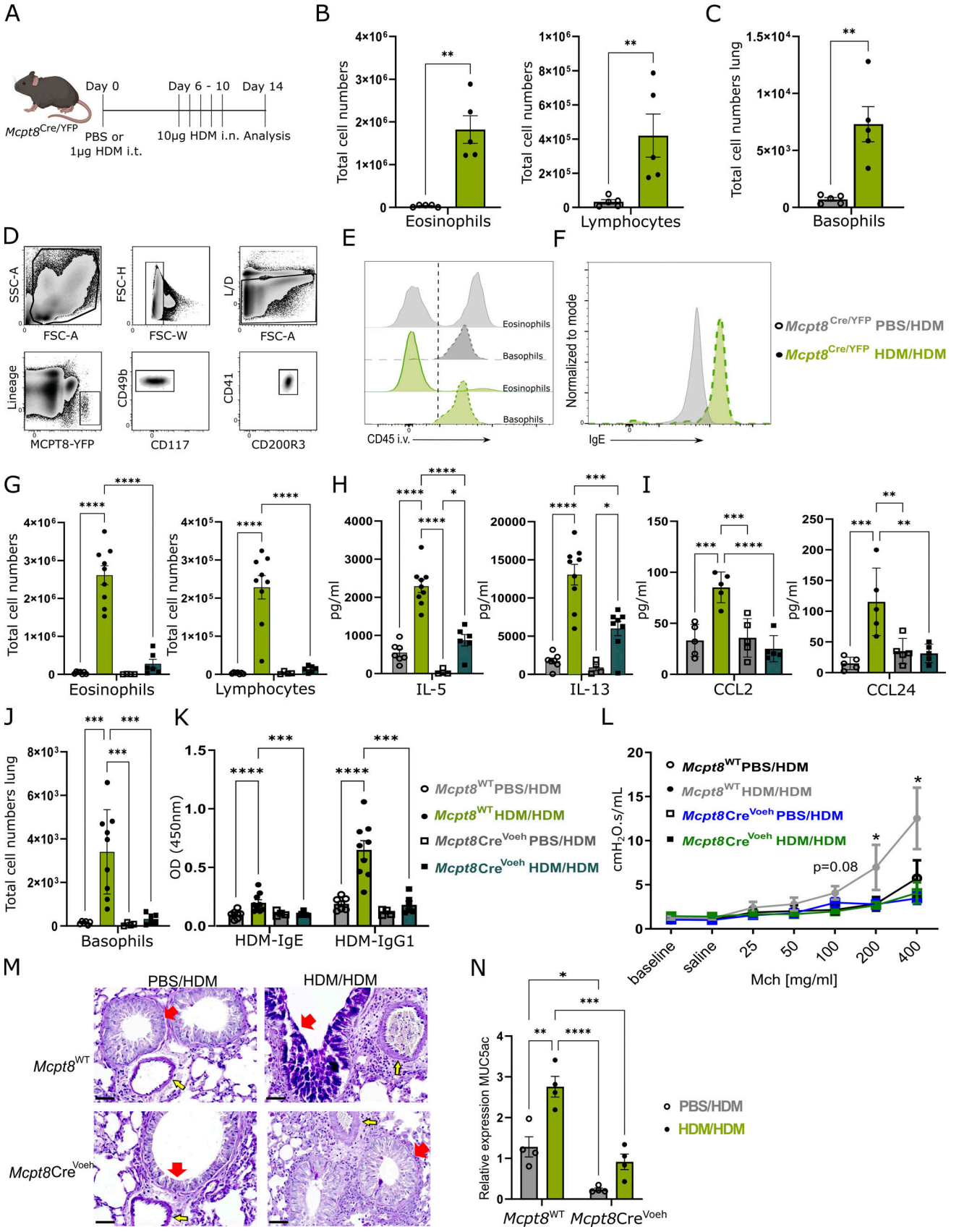

Figure 1. **Basophils are required for HDM-induced eosinophilic inflammation. (A)** *Mcpt8*$^{Cre/YFP}$ reporter mice were sensitized i.t. (1 µg) with HDM extract or PBS, as control, at day 0. Mice were then challenged i.n. with 10 µg HDM extract daily between day 6 and 10, and on day 14 BAL was performed and lungs were taken for further analysis. **(B)** Shown are the numbers of eosinophils and lymphocytes present in BAL as assessed by flow cytometry. **(C)** Basophil

numbers in the lung were measured by flow cytometry. **(D)** Gating strategy used for the identification of basophils by flow cytometry. **(E)** CD45 intravascular staining of basophils compared with eosinophils in lungs, as measured by flow cytometry. **(F)** Flow cytometric analysis of IgE binding to basophils. **(G)** Eosinophil and lymphocyte numbers in BAL are shown for *Mcpt8Cre*^{Voeh} or littermate controls. **(H)** Graphs show the amount of IL-5 and IL-13 in supernatant of restimulated MLN cells, as measured by ELISA. **(I)** Measurement of CCL2 and CCL24 chemokines in BAL supernatant of *Mcpt8*Cre^{Voeh} or littermate controls. **(J)** Flow cytometric analysis of lung basophils in *Mcpt8*Cre^{Voeh} mice and littermate controls. **(K)** Concentration of HDM-specific IgE and IgG1 in serum measured by ELISA. **(L)** Graph shows the increase in airway resistance of *Mcpt8*Cre^{Voeh} compared with littermate controls after PBS or HDM exposure. **(M)** PAS staining of PBS or HDM-challenged lungs, red arrows point to airways, and yellow arrows indicate vessels. Scale bar = 30 µm. **(N)** Normalized gene expression of MUC5ac. One representative of three independent experiments, all of which gave similar results (B–F, I, and L–N) with five mice per group and for (L) six mice per group, or pooled data of two independent experiments (G, H, J, and K), with 7–10 mice per group is shown. Data are means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Student's *t* test (B and C), ordinary ANOVA (G–J and L), or two-way ANOVA (K and N).

important role in the establishment of key features of HDM-induced allergic asthma.

Basophils are redundant for Th2 priming to inhaled HDM allergen

We next tested whether basophils are required during the Th2 sensitization or challenge phase of the lung mucosal allergic response. Contrary to Cre expression in *Mcpt8*Cre^{Voeh} mice, the levels of Cre in *Mcpt8*$^{Cre/YFP}$ mice are not toxic to basophil development, and Cre can be used to recombine floxed alleles (Sullivan et al., 2011). To create conditional basophil deleter mice, we crossed the *Mcpt8*$^{Cre/YFP}$ mice to *Rosa26*-^{iDTR} mice to generate *Mcpt8*-iDTR mice, in which conditional basophil depletion in the lung, MLN, and spleen could be obtained by intraperitoneal administration of 100 ng of diphtheria toxin (DTx) (Fig. S2, A and B). In *Mcpt8*-iDTR mice, basophils recovered from depletion within 3–4 days (Sullivan et al., 2011), and basophil numbers were confirmed to be unaffected during the challenge phase (Fig. S2 C). When basophils were depleted immediately prior to sensitization (Fig. 2 A), HDM-induced airway eosinophilia (Fig. 2 B), MLN cytokine production (Fig. 2 C), and serum immunoglobulins (Fig. 2 D) were identical as in non-basophil depleted animals. These data show that basophil depletion during the initial sensitization to allergens does not alter the subsequent Th2 immune response.

Basophils have been suggested to act as potential antigen-presenting cells providing a source of early polarizing IL-4 sufficient to induce or expand Th2 immunity, although it was shown that basophils in mice and humans express little MHCII on their surface, and acquire it by trogocytosis from DCs when they do (Hammad et al., 2010; Miyake et al., 2017). We measured ex vivo MHCII expression on the surface of basophils after HDM exposure. As described before (Miyake et al., 2017), we found that basophils displayed only low levels of MHCII on their cell surface, well below the levels observed on B cells and DCs (Fig. 2 E). We next sort-purified both CD11c$^+$ DCs and basophils from HDM-sensitized animals and in vitro cocultured them with Derp1-specific TCR-transgenic CD4$^+$ 1-DER T cells (Coquet et al., 2015) to see whether basophils would be able to induce primary T cell division. Whereas DCs induced 1-DER T cell proliferation, basophils were unable to do so (Fig. 2 F). However, the addition of basophils did result in a slight increase in OX40 expression on the expanding T cells (Fig. 2 G). To further explore the potential role of basophils as APCs for initiating CD4$^+$ T cell priming, we adoptively transferred CFSE-labeled 1-DER T cells into basophil-sufficient or *Mcpt8*Cre^{Voeh} basophil-deficient mice and exposed them to HDM. Analysis of CD4$^+$ 1-DER T cells 3 days after HDM exposure revealed that basophil deficiency did not reduce the number of T cell divisions (Fig. 2 H) nor did it influence MLN Th2 cytokine production (Fig. 2 I) induced by HDM. Next, we investigated the effect of restoration of *Mcpt8*Cre^{Voeh} basophil-deficient animals with cultured basophils (Siracusa et al., 2011). When given at the time of sensitization, intravenous add-back of 1 × 10^5 wild type (WT) basophils, derived from an IL-3 supplemented in vitro culture, was insufficient to restore defective airway type 2 immunity of basophil-deficient mice (Fig. 2 J). Finally, although basophils might affect DC functions, we observed that HDM-uptake by various DC subsets in the lungs and their migration to the MLN was unaffected in basophil-deficient animals (Fig. 2 K). Thus, the presence of basophils during sensitization is neither necessary nor sufficient to induce pulmonary eosinophilia to HDM.

Basophils exert their main function during lung allergen challenge

To study the role of basophils during the effector immune response to HDM allergen, we again turned to the conditional basophil depletion system. To this end, we injected *Mcpt8*-iDTR mice with PBS as a control or 100 ng DTx (i.p.) every other day selectively to deplete basophils only during the HDM challenge (Fig. S3 A). At day 3 after challenge, basophil-depleted *Mcpt8*-iDTR mice developed less eosinophilic inflammation as compared with basophil-sufficient animals (Fig. 3, A and B), concurrent with reduced Th2 cytokine production in the MLN (Fig. 3 C) and reduced serum HDM-specific IgE, but not IgG1 (Fig. 3 D). Although the *Mcpt8* gene is highly expressed in basophils but not in mast cells in steady state, lung challenge with allergens induces the *Mcpt8* gene in mucosal mast cells (Derakhshan et al., 2021), potentially confounding results in *Mcpt8*-iDTR mice. To ensure that the observed effects were specifically caused by the loss of basophils during the challenge phase of the allergic response, we used *Mcpt8*-iDTR (as in Fig. 3 A) and subsequently reconstituted basophils by adoptive transfer of 1 × 10^5 ex vivo cultured WT BM basophils just before challenge. The reduction in HDM-driven eosinophilic infiltration and lymphocyte influx seen in *Mcpt8*-iDTR mice were fully restored in mice receiving WT basophils at the time of challenge (Fig. 3 E and Fig. S3 B). As the secondary response to HDM depends on both DC activation and migration, as well as on the activation and recruitment of CD4$^+$ T cells, we next confirmed HDM uptake by various DC populations and observed reduced HDM-uptake by CD11b$^+$ cDC and moDC subsets (Fig. 3 F). To

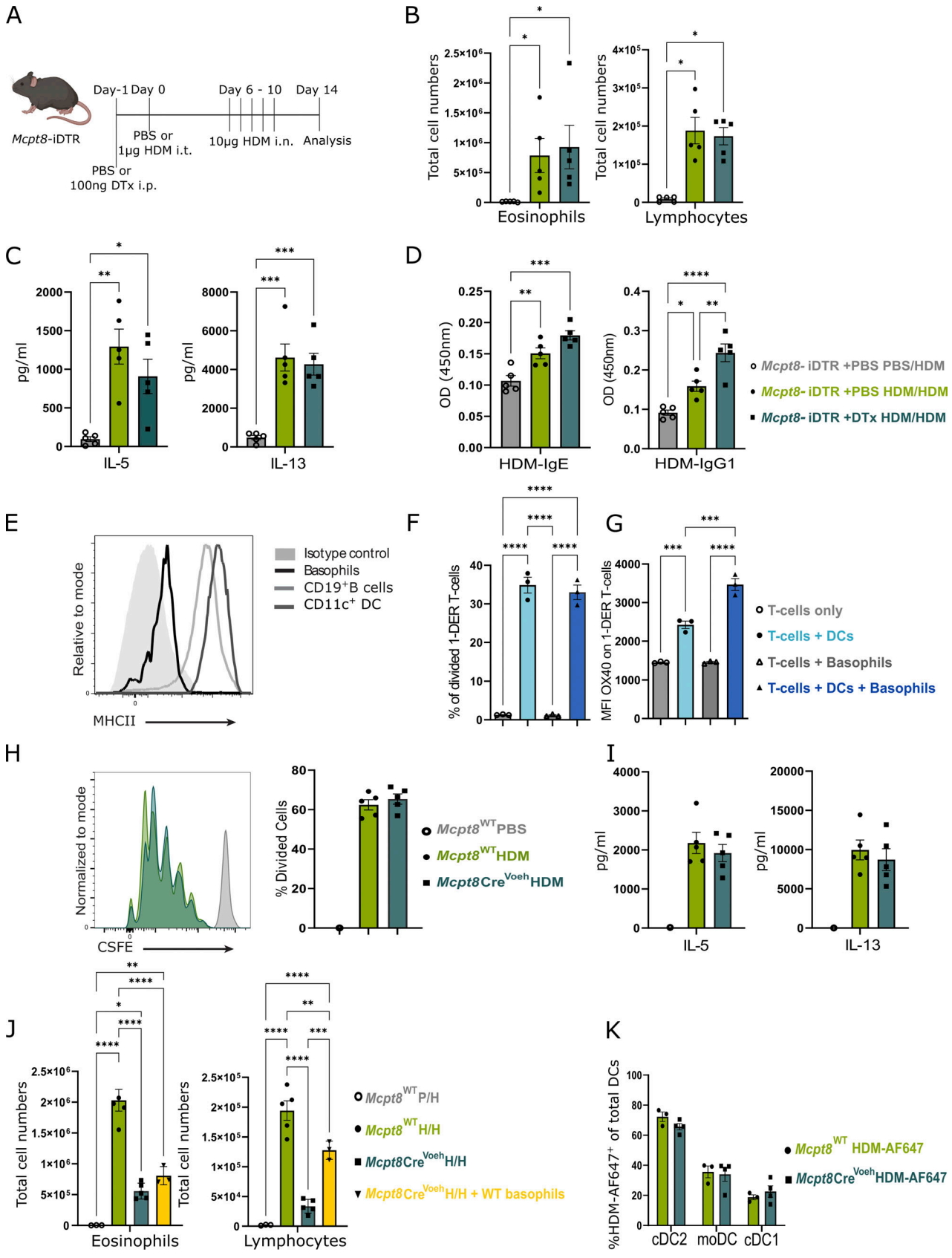

Figure 2. **Basophils are redundant for Th2 priming to inhaled HDM allergen. (A)** *Mcpt8*-iDTR mice received PBS or 100 ng DTx i.p. 1 day before sensitization with 1 µg HDM extract and were subsequently challenged i.n. with 10 µg HDM extract daily from day 6 till 10 and sacrificed on day 14. **(B)** Eosinophil and lymphocyte numbers in BAL at day 14. **(C and D)** Graphs show levels of IL-5 and IL-13 in supernatant of restimulated MLN cells and serum concentration of

HDM-specific IgE and IgG1, respectively, as measured by ELISA. **(E)** MHCII expression by basophils compared with CD19⁺ B cells and CD11c⁺ DCs, as measured by flow cytometry. **(F and G)** In vitro co-culture of 1-DER T cells with sorted basophils and DCs, graphs show 1-DER proliferation (F) by analysis of CFSE content and OX40 expression levels (G) after 3 days of culture. **(H)** Proliferation of adoptively transferred 1-DER T cells labeled with CFSE, measured 3 days after single HDM (10 µg) injection in *Mcpt8*Cre^{Voeh} or littermate controls. **(I)** Cytokine production from MLNs of mice receiving 1-DER T cells, collected 3 days after HDM injection. **(J)** Graphs show differential eosinophil and lymphocyte cell count in BAL after adoptive transfer of in vitro cultured WT BM basophils into *Mcpt8*Cre^{Voeh} at the time of sensitization. **(K)** Percentage of HDM-AF647 positive DCs within the MLN 24 h after HDM-AF647 i.n. instillation. One representative of three independent experiments, all of which gave similar results, with three to five mice per group is shown. Data are means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Ordinary ANOVA (B–D, F, G, and J).

induce Th2 inflammation without the need for sensitization and DC-mediated expansion of Th2 cells, we generated 1-DER Th2 T cells in vitro using a combination of IL-4 and blocking antibodies to IFNγ and IL-12 (Sekiya and Yoshimura, 2016) (Fig. S3 C). We adoptively transferred these 1-DER Th2 T cells intravenously into WT or basophil deficient *Mcpt8*Cre^{Voeh} animals, followed by HDM challenges (Fig. 3 G). The absence of basophils significantly curtailed the magnitude of the subsequent eosinophil infiltrate 3 days after the last HDM challenge (Fig. 3 H), concomitant with reduced Th2 cytokines in the MLN (Fig. 3 I). We next investigated why the 1-DER Th2 cells in the basophil-deficient mice were unable to induce airway eosinophilia. Therefore, we verified the presence of adoptively transferred 1-DER Th2 cells in the lungs of HDM-challenged mice and detected significantly fewer cells in the lungs of basophil-depleted animals (Fig. 3 J). Interestingly, the recovered Th2 cells within the depleted animals showed a lower expression of CD44 and a similar expression of ST2 compared with those recovered from basophil-sufficient mice (Fig. 3 J). Together, these results indicate that basophils drive the ongoing immune response to HDM and that they directly or indirectly provide signals required for CD4⁺ T cell function and recruitment.

IL-33–activated basophils producing IL-4 control type 2 immunity during HDM challenge

Since basophils were required for the development of airway inflammation to HDM during the challenge phase, we considered which signals would drive basophil activation during ongoing allergic responses. In mice and humans, the high-affinity IgE receptor FcεRIα is constitutively expressed on basophils, which can acquire serum IgE by probing the vascular space and where they can alter vascular function by the release of vasoactive mediators and can produce chemokines that attract CD4⁺ T cells and eosinophils (Cheng et al., 2013; Chhiba et al., 2017). In line, IgE-activated basophils could promote allergic inflammation in the skin (Cheng et al., 2015). To examine whether IgE-dependent basophil activation was necessary to induce airway eosinophilia after the HDM inhalation challenge, we generated *Fcer1*$^{-/-}$ → WT bone-marrow chimeras to obtain either full hematopoietic FcεRIα deficiency or 50:50 *Fcer1*$^{-/-}$: *Mcpt8*Cre^{Voeh} → WT chimeras to create basophil-restricted FcεRIα deficiency. Surprisingly, lack of FcεRIα expression on all hematopoietic cells or only on basophils did not significantly reduce eosinophil influx after HDM challenge and was unable to recapitulate the reduced type 2 phenotype observed in basophil-deficient animals, suggesting that IgE is not the major contributor of basophil activation in this model (Fig. 4 A). Next to IgE crosslinking, cytokines can also potently activate basophils (Schneider et al., 2009; Chhiba et al., 2017). We first measured the cytokine milieu in the lungs after the HDM challenge and found increased concentrations of epithelial cytokines thymic stromal lymphopoietin (TSLP), IL-33, and GM-CSF (Fig. 4 B). Previous research by others has demonstrated that TSLPR expression on basophils is not required for HDM-driven asthma (Kabata et al., 2020). Basophils, however, constitutively express the IL-33 receptor T1/ST2 (Fig. S4 A) and IL-33–dependent basophil activation leads to rapid release of IL-4 and IL-13, of which the first is sufficient to drive eosinophilic skin inflammation (Chan et al., 2001; Schneider et al., 2009; Chhiba et al., 2017). To test the importance of IL-33–dependent activation in our model of HDM-induced allergic airway inflammation, mice were treated intratracheally with recombinant soluble ST2 (sST2), a decoy receptor for IL-33–ST2 signaling, during the challenge phase of the response (Holgado et al., 2019). As compared with non-treated animals, mice locally exposed to sST2 had a significant attenuation of BAL eosinophils after the challenge (Fig. 4 C). The effect was less than that observed in basophil-deficient or conditionally depleted mice (Fig. 1 G and Fig. 3 B). Blockade of ST2 signaling lowered the levels of the cytokines IL-5 and IL-13 within the lung (Fig. 4 D), consistent with lower eosinophil numbers, but did not affect serum immunoglobulin levels (Fig. 4 E). Intratracheal administration of sST2 further led to a reduced number of basophils infiltrating the lungs of HDM-challenged mice (Fig. 4 F), but did not alter the number of mast cells or ILC2s in the lung (Fig. S4 B).

To verify that basophil-intrinsic IL-33–ST2 signaling was the main signal for basophil activation required for airway eosinophilia in HDM-challenged mice, we conditionally depleted basophils during the challenge phase in *Mcpt8*-iDTR mice and reintroduced in vitro bone marrow-cultured basophils lacking key components of the IgE or cytokine receptor signaling complexes (Fig. S4 C). Similar to previous experiments (Fig. 3 E), WT basophils could almost completely restore BAL eosinophilia, as well as DC and T cell infiltration, and this was also the case when *Fcer1a*$^{-/-}$ basophils were used to reconstitute, illustrating that IgE receptor crosslinking was not crucial. However, the addback of *Il1rl1*$^{-/-}$ basophils lacking T1/ST2 expression was unable to restore BAL eosinophilia after the HDM challenge (Fig. 4 G). In several experiments, IL-33 has been shown to elicit IL-4 production from basophils, and IL-4 was identified as a major effector cytokine of recruited basophils in the skin and lung (Voehringer et al., 2004; Min et al., 2004; Khodoun et al., 2004; Phillips et al., 2003; Luccioli et al., 2002). Indeed, when we exposed activated in vitro cultured basophils to IL-33, we observed a strong induction of IL-4 transcript, which was largely dependent on IL-33 signaling (Fig. 4 H). When *Mcpt8*-iDTR mice

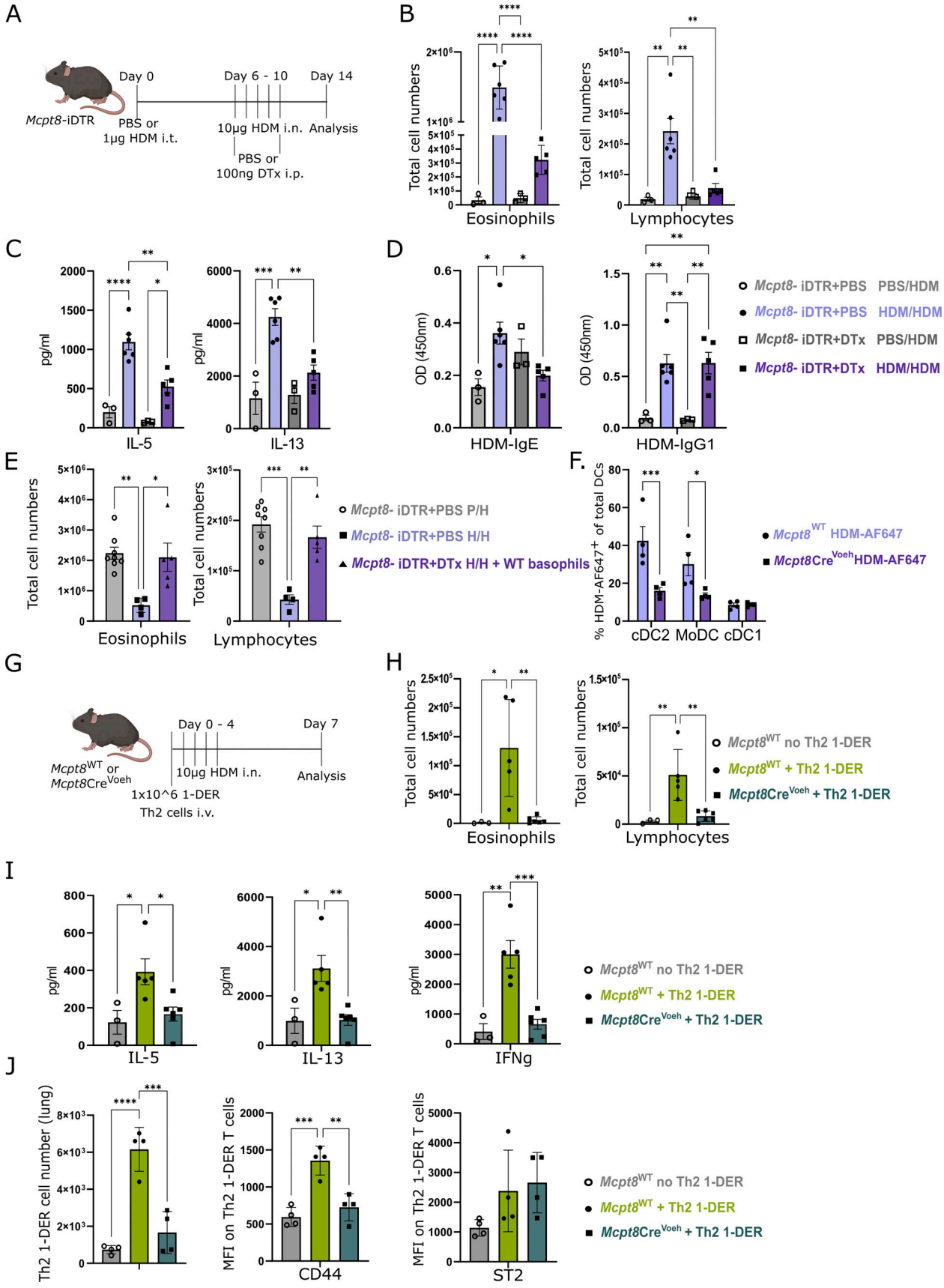

Figure 3. **Basophils exert their main function during lung allergen challenge. (A)** *Mcpt8*-iDTR mice were administered with PBS or 100 ng DTx every other day selectively during the HDM challenge. **(B)** Numbers of eosinophils and lymphocytes from BAL were measured at day 14. **(C and D)** Graphs show IL-5 and IL-

13 levels in supernatant of restimulated MLN cells and concentration of HDM-specific IgE and IgG1 in serum, respectively. **(E)** BAL cell analysis after adoptive transfer of ex vivo cultured WT BM basophils into *Mcpt8*-iDTR mice just before challenge. **(F)** Percentage of HDM-AF647 positive DCs in MLN 24 h after challenge. **(G)** *Mcpt8*Cre^{Voeh} or littermate controls received 1 × 10^6 Th2-polarized 1-DER T cells 1 day before 10 µg HDM i.n. daily challenge between day 0 and 4. **(H)** Differential cell counts of *Mcpt8*Cre^{Voeh} or littermate controls that received Th2-polarized 1-DER T cells and HDM challenge. **(I)** Cytokine production from restimulated MLN cell supernatant of mice that received Th2-polarized 1-DER T cells. **(J)** Flow cytometry analysis of 1-DER Th2 cells in the lungs of HDM-challenged mice and the expression of CD44 and ST2. One representative of three independent experiments, all of which gave similar results, with three to eight mice per group is shown. Data are means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Ordinary ANOVA (B–F and H–J) and two-way ANOVA (F).

were reconstituted with *Il4*$^{-/-}$ basophils during challenge, HDM-induced airway eosinophilia was hampered (Fig. 4 G). Similarly, both IL-5 and IL-13 levels in the lungs of reconstituted mice were significantly decreased in mice receiving ST2-deficient or IL-4–deficient basophils (Fig. 4 I).

We previously reported that basophil-derived IL-4 could enhance endothelial intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) expression on skin blood vessels (Cheng et al., 2015), and similar findings were reported on western blot analysis of lung extracts of allergen-challenged mice (Luccioli et al., 2002). As both these cell adhesion molecules play an important role in leukocyte recruitment and migration to inflammatory areas (Singh et al., 2023), we checked VCAM-1 and ICAM-1 expression on CD31$^+$ lung endothelial cells. In WT mice, the HDM challenge of sensitized mice was able to upregulate VCAM-1, but not ICAM-1, expression on endothelial cells (Fig. 4 J). At the same time, both VCAM-1 and ICAM-1 levels were significantly lower on endothelial cells from basophil-deficient *Mcpt8*Cre^{Voeh} mice after HDM challenge (Fig. 4 J). Microscopic analysis revealed that VCAM-1 expression on von Willebrand factor (VWF) positive endothelial cells showed a patchy distribution in WT mice, which was totally absent in *Mcpt8*Cre^{Voeh} mice after HDM challenge (Fig. 4 K). Since recruitment of Th2 CD4$^+$ T lymphocytes to the lung is critical for the orchestration of airway eosinophilia during HDM-induced allergic airway inflammation (Rahimi et al., 2020), we investigated whether basophil deficiency and reduced levels of VCAM-1 and ICAM-1 would impair 1-DER Th2 cell migration (independent of prior active HDM sensitization). Therefore, we generated 1-DER Th2 cells in vitro and adoptively transferred them into WT or basophil-deficient *Mcpt8*Cre^{Voeh} animals and analyzed the extravasation of 1-DER cells using CD45 i.v. labeling, selectively gating on lung recruited and resident Th2 cells that were excluded from i.v. labeling (Fig. S4 D). We observed that in WT mice, HDM challenge increased the number of extravasated resident HDM-specific 1-DER Th2 T cells in the lung tissues (Fig. 4 L). Ultimately, both basophil deficiency, as well as the absence of IL-33 signaling in all immune cells (*Il1rl1*$^{-/-}$ -> WT chimera) or selectively in basophils (50:50 *Il1rl1*$^{-/-}$: *Mcpt8*Cre^{Voeh} -> WT chimera) resulted in a significantly reduced lung extravasation of 1-DER Th2 T cells after HDM challenge (Fig. 4 L) and reduced chemokine levels in BAL (Fig. 4 M). Although recruitment of 1-DER Th2 T cells to the lung was affected, 1-DER Th2 T cell levels in the MLN were not significantly altered (Fig. 4 L). Together, these data show that ST2-dependent basophil activation and IL-4 from basophils is required for optimal extravasation of T lymphocytes into the lungs of asthmatic mice and subsequent orchestration of airway eosinophilia after HDM challenge.

Uncontrolled activation of basophils exacerbates mild asthma

Basophils are known to be hyperactivated in human chronic asthma, and several signatures of activated basophils track with severity of airway disease in human eosinophilic asthma, but underlying mechanisms are unclear (Gordon et al., 2016b; Li et al., 2022; Winter et al., 2021; Brooks et al., 2017). In one scenario, basophil activation might be genetically favored through gain-of-function polymorphisms in the *FCER1A* gene that encodes the high affinity IgE receptor expressed on basophils; in another, cytokine-mediated hyperactivation of basophils might be caused by activating polymorphisms in *IL33* or *IL1RL1* or loss of downstream regulators of cytokine signaling (Li et al., 2012). To further model and explore the role of hyperactivated basophils in HDM-induced allergic airway inflammation, we designed mice specifically lacking TNFAIP3 (also known as A20) in the basophil lineage by crossing *Tnfaip3*$^{fl/fl}$ mice to the *Mcpt8*$^{Cre/YFP}$ mice (*Mcpt8*^{Cre} × *Tnfaip3*$^{fl/fl}$) (Fig. S5). Loss of function polymorphisms of *TNFAIP3* have been previously linked to increased allergy and asthma susceptibility in epidemiological studies (Schuijs et al., 2015; Stein et al., 2016; Li et al., 2012). Deletion of this ubiquitin-modifying protein renders cells unable to shutdown NF-κB signaling after activation in response to TLR, TNFR, or IL-1R family signaling, including the IL-33 receptor T1/ST2 (Holgado et al., 2023). This would predict a hyperactivated phenotype of basophils, akin to the situation in mast cells that lack *Tnfaip3* expression (Heger et al., 2014). To better capture the impact of increased basophil activation in vivo, we lowered the amount of HDM in the challenge phase by 90% (Fig. 5 A). We then exposed *Mcpt8*^{Cre} × *Tnfaip3*$^{fl/fl}$ mice to low doses of HDM, and basophils in the MLN showed elevated expression of the basophil activation markers CD200R3 and T1/ST2, irrespective of active sensitization (Fig. 5 B). Similarly, lung basophils showed higher mean fluorescence intensity values for CD200R3, T1/ST2, and the degranulation marker CD63 (Fig. 5 C) and were present in higher numbers in the lungs of *Mcpt8*^{Cre} × *Tnfaip3*$^{fl/fl}$ mice after low-dose HDM exposure (Fig. 5 D). Whereas actively sensitized WT mice could now only mount very mild airway eosinophilia in response to low-dose HDM challenge, the *Mcpt8*^{Cre} × *Tnfaip3*$^{fl/fl}$ mice showed significantly higher numbers of eosinophils in the BAL (Fig. 5 E), enhanced MLN Th2 cytokine production (Fig. 5 F), and increased serum HDM-specific immunoglobulins (Fig. 5 G). To further investigate the dependency of this phenotype on IL-33 signaling, we treated *Mcpt8*^{Cre} × *Tnfaip3*$^{fl/fl}$ mice with sST2 during the challenge phase. As compared with non-treated *Mcpt8*^{Cre} ×

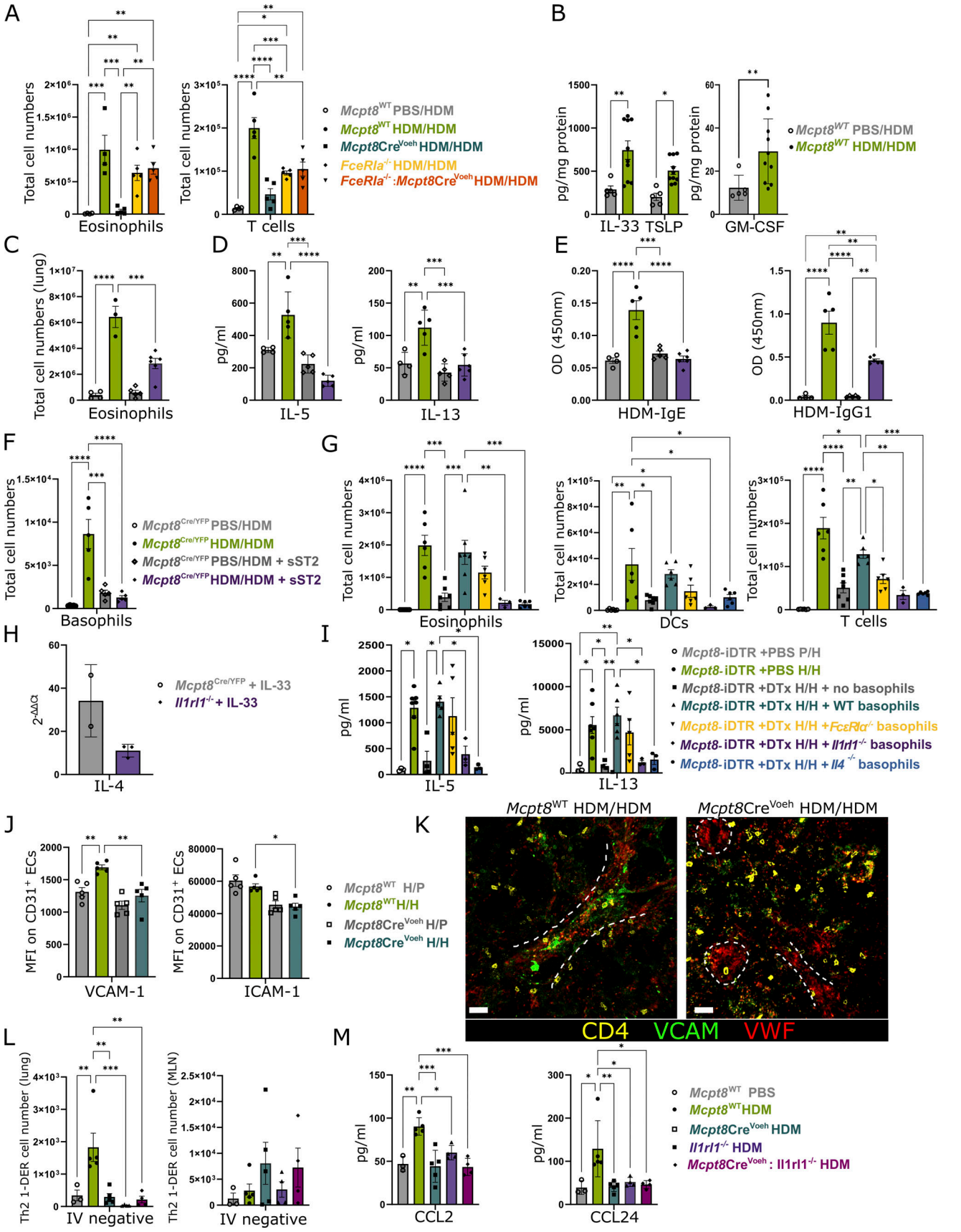

Figure 4. **IL-33–activated basophils producing IL-4 control type 2 immunity during HDM challenge. (A)** Analysis of BAL eosinophil and T cell numbers in BM chimeric mice with selective FcεRIα deficiency. **(B)** Level of epithelial alarmins in lung homogenates after HDM stimulation. **(C–E)** Mice were treated i.n.

with 10 µg sST2 during HDM exposure and eosinophil numbers in BAL were measured at day 14 (C). **(D)** Graphs show the levels of IL-5 and IL-13 in lung homogenates, as measured by ELISA. **(E and F)** (E) Serum concentrations of HDM-specific immunoglobulins and (F) basophil numbers in the lungs. **(G)** Reconstitution of *Mcpt8*-iDTR basophil depleted mice during challenge, with various in vitro cultured BM basophils from different genotypes at challenge; the graphs show cell numbers of eosinophils, DCs, and T cells in BAL after HDM sensitization and challenge. **(H)** IL-4 expression levels from in vitro cultured *Mcpt8*$^{Cre/YFP}$ or *Il1rl1*$^{-/-}$ basophils stimulated with PBS or Il-33 for 24 h, as determined by RT-qPCR. **(I)** Cytokine production by MLN cells restimulated with HDM for 3 days ex vivo. **(J)** Flow cytometric analysis of VCAM and ICAM expression on CD31^{+} endothelial cells in *Mcpt8*Cre^{Voeh} or littermate controls. **(K)** Confocal image shows VCAM expression on vessels of *Mcpt8*Cre^{Voeh} or littermate controls. **(L)** Enumeration of CD45iv negative 1-DER Th2 cells in the lungs and MLN of various BM chimeric mice after HDM challenge. **(M)** Graphs show chemokines CCL2 and CCL24 measurement in BAL of various BM chimeric mice after HDM challenge. One representative of three independent experiments, all of which gave similar results (A and C–M) with two to seven mice per group or pooled data of two independent experiments (B) with five to ten mice per group is shown. Data are means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Ordinary ANOVA (A, C–G, I, J, L, and M) and two-way ANOVA (B).

Tnfaip3$^{fl/fl}$ mice, mice treated with sST2 had attenuated BAL eosinophilia and lymphocytosis after challenge (Fig. 5 H). Similarly, lung basophil numbers were reduced, as well as their activation state (Fig. 5 I). Moreover, mice treated with sST2 showed reduced BAL chemokine levels (Fig. 5 J). Serum immunoglobulin levels were unaffected (Fig. 5 K). Together, these results indicate that *Tnfaip3*-deficient hyperactivated basophils can exacerbate mild HDM-induced allergic airway inflammation, which is in part mediated by enhanced IL-33 responsiveness in this model.

Discussion

Although it is established that basophils can contribute to systemic anaphylaxis and type 2–mediated skin disease (Karasuyama et al., 2017), their precise contribution to pulmonary type 2 inflammation has long been enigmatic. Recent clinical data from asthmatics have revealed clear signs of basophil activation, tracking with the severity of eosinophilic asthma, likely driven by cytokine activation (Gordon et al., 2016b; Li et al., 2022; Winter et al., 2021; Brooks et al., 2017). Unraveling the precise role of basophils has been difficult due to the lack of specific tools to deplete or target basophils and due to the inherent functional overlap with various innate type 2 immune cells like mast cells and ILC2s that all express receptors for epithelial cytokines like IL-33, TSLP, and IL-25. These cytokines have all become drug targets for intervention in chronic asthma (Lambrecht et al., 2019), and even basophils can be directly targeted in the clinic through the administration of the cytotoxic antibody benralizumab that kills IL-5Rα expressing basophils and is now widely used to deplete eosinophils in severe eosinophilic asthma (Lommatzsch et al., 2020). How much of the clinical benefit of benralizumab is caused by eosinophil versus basophil depletion is currently unknown.

Therefore, it was important to unravel the precise role of basophils in asthma and uncover the upstream pathway leading to their expansion and/or activation. Using different depletion and reconstitution experiments, we demonstrate in an HDM-driven mouse asthma model that IL-33/ST2-dependent activation of basophils plays a non-redundant role during the effector immune response to allergens by facilitating the recruitment of adaptive immune system Th2 cells to the site of allergen challenge. By controlling a major pathogenic adaptive immune cell, innate basophils thus contribute crucially to the establishment of eosinophilic airway inflammation, mucus overproduction, and bronchial hyperreactivity. The cytokine IL-33 non-redundantly activated basophils to secrete IL-4, which activated integrin expression and allowed extravasation of lung Th2 cells, the orchestrators of type 2 immunity. In human eosinophilic asthma, a similar pathway seems operative, driven by genetically determined alternative splicing of IL-33, leading to basophil overactivation and secretion of type 2 cytokines as also seen in our murine model (Gordon et al., 2016a, 2016b).

Using adoptive transfer models of in vitro BM-cultured basophils, as well as different chimeric models, we observed an important role for cell-intrinsic IL-33–dependent basophil activation on airway eosinophilia. This is in line with observations within experimental models of eosinophilic esophagitis, where basophil-intrinsic IL-33/ST2 signaling was also key (Venturelli et al., 2016). This is remarkable since IL-33 not only stimulates basophil IL-4 and IL-13 production (Siracusa et al., 2013); it is also a well-known amplifier of other immune cells like ILC2s, mast cells, DCs, and Th2 cells immunity (Llop-Guevara et al., 2014; Kamijo et al., 2013; Oboki et al., 2010). Interestingly, our model disrupting IL-33/ST2 signaling specifically in basophils fully mimicked basophil depletion, suggesting IL-33 to be the most crucial cytokine for optimal basophil activation during the effector phase of HDM-induced allergy, even when other cytokines made in asthmatic airways like IL-18 and TSLP can also activate basophils. In line with this, recent experiments in basophil-intrinsic *Tslpr*-deficient mice showed that the expansion of basophils in an OVA-driven or papain-driven model does not depend on direct effects of this cytokine on basophils, but rather via indirect effects on DCs, Th2 cells, and ILC2s that promote activation and survival signals for basophils, one likely candidate being IL-3 (Kabata et al., 2020).

Despite the well-known effector responses of basophils mediated through IgE crosslinking and despite the effect that we observed increased HDM-specific IgE binding even to basophils, we observed only mild reductions in airway inflammation when basophils selectively lacked FcεRI. This is in marked contrast to findings in a skin inflammation model, where IgE-induced basophil activation was identified as the main regulator of eosinophil entry into the skin (Cheng et al., 2015). Also in models of helminthic infection (*H. Polygyrus* and *N. Brasiliensis*), it was demonstrated that FcεRI expression on basophils and subsequent release of IL-4/IL-13 was required for protective immunity and worm expulsion (Schwartz et al., 2014). As such, we do not completely rule out a role for the activating FcεRIα on basophils in asthma, especially since it has been suggested that IgE-mediated basophil activation could enhance cytokine production

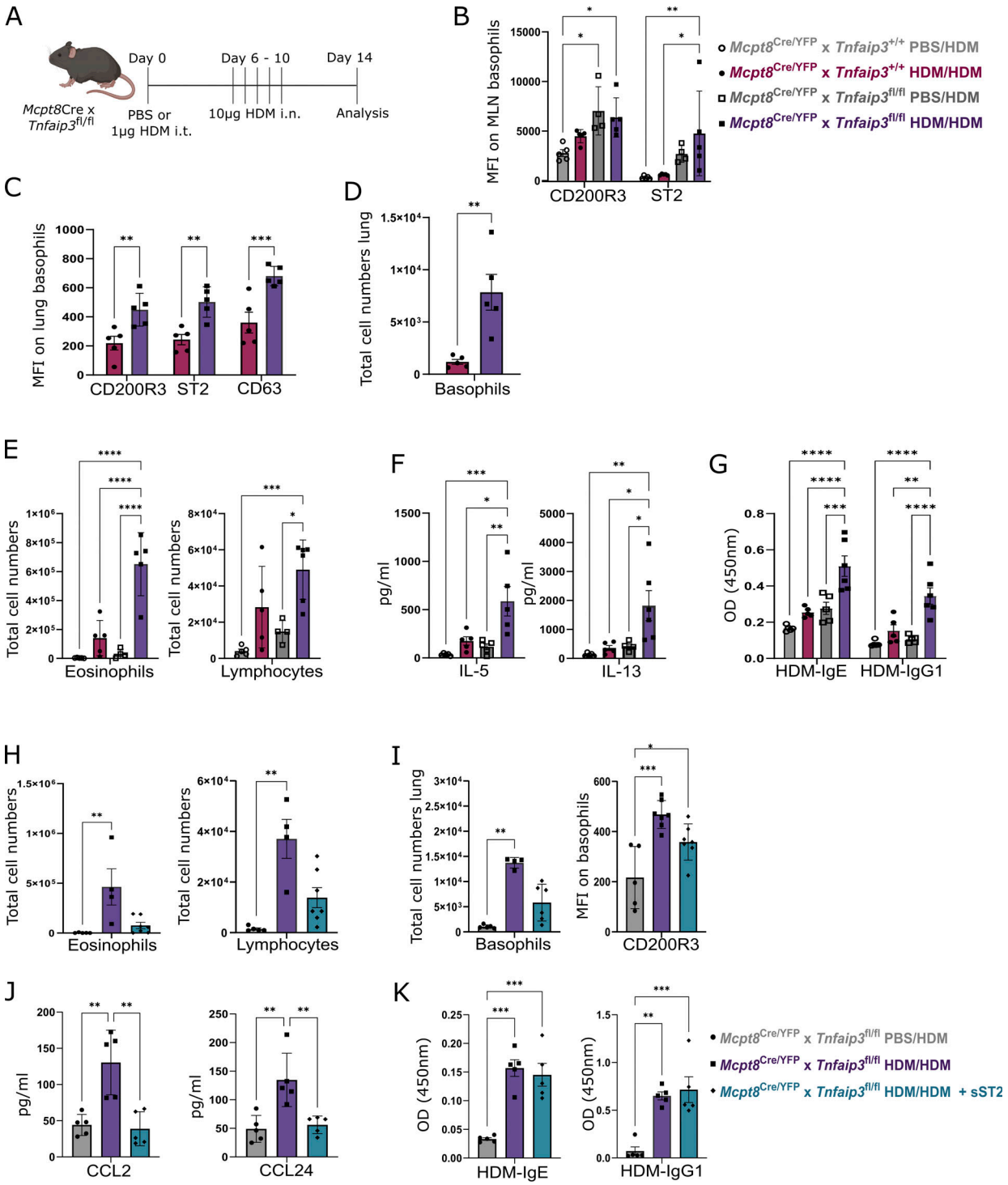

Figure 5. **Uncontrolled activation of basophils exacerbates mild asthma. (A)** Schematic representation of *Mcpt8*^{Cre} × *Tnfaip3*$^{fl/fl}$ treatment with 1ug HDM during challenge. **(B and C)** (B) Flow cytometric analysis of CD200R3 and ST2 expression on MLN basophils and (C) CD200R3, ST2, and CD63 on lung basophils after low-dose HDM challenge. **(D)** Representative flow cytometric analysis of lung basophil numbers in *Mcpt8*^{Cre} × *Tnfaip3*$^{fl/fl}$ or littermate controls. **(E–G)** Graphs show differential cell count of eosinophils and lymphocytes in BAL (E), cytokine production in the supernatant of ex vivo restimulated MLN cells (F), and levels of serum HDM-specific IgE and IgG1 (G). **(H)** *Mcpt8*^{Cre} × *Tnfaip3*$^{fl/fl}$ were treated i.n. with 10 µg sST2 during HDM exposure and eosinophil and lymphocyte numbers in BAL were measured at day 14. **(I)** Flow cytometric analysis of total basophils on the lung and their expression of CD200R3. **(J and K)** Graphs show chemokine levels in BAL at day 14 (J) or concentration of immunoglobulins in serum (K). One representative of three independent experiments, all of which gave similar results, with four to six mice per group is shown. Data are means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Student's *t* test (D), ordinary ANOVA (E, F, and H–K) and two-way ANOVA (B, C, and G).

by human basophils, particularly when cocultured with lung epithelial cells (Schroeder and Bieneman, 2017). In omalizumab-treated asthmatic children, the reduced occupancy of IgE on the FcεRI is associated with a reduction in basophil number, concurrent with improvement in clinical condition (Eckman et al., 2010; Hill et al., 2014). It could be that the way that our asthma model was set up, the importance of IgE was minimal, and largely taken over by IgG1, another well-known immunoglobulin class that can activate basophils (Tsujimura et al., 2008). In accordance with previous reports showing that eosinophilic airway inflammation to HDM does not depend on IgE or FcεRI, we found that in mice lacking FcεRI on all hematopoietic cells, eosinophilic airway inflammation was unaffected (Dullaers et al., 2017; McKnight et al., 2017). A specific role for allergen-specific IgG1 in murine airway basophil biology remains to be uncovered.

A number of models have previously described the amplificatory role of basophils in allergic asthma (Motomura et al., 2014; Ohnmacht et al., 2010; Wakahara et al., 2013; Pulendran et al., 2010), but most studies highlighted their contribution as key components of the innate effector response or as early innate producers of IL-4, which could then bridge innate and adaptive immunity and play a role in the licensing of DCs (Motomura et al., 2014; Halim et al., 2016; Kim et al., 2014). It was proposed that basophils might serve as the source of polarizing IL-4, and at the same time act as antigen-presenting cells for naïve T cells to polarize along the Th2 pathway, but we have previously already shown using depleting antibodies that basophils are neither necessary nor sufficient as antigen-presenting cells for the development of Th2 immunity to HDM (Hammad et al., 2010). Here, using more specific genetic tools to deplete basophils in a time-controlled manner, as well as precise TCR transgenic read-out systems to probe for naïve T cell activation, we again found that selective depletion of basophils during HDM-sensitization did not affect Th2 polarization nor subsequent airway eosinophilia after HDM exposure. In line with the relatively low presence of MHCII on the basophil surface, our coculture data indicate that basophils were unable to induce 1-DER TCR-transgenic proliferation. In line with our observations, Th2 cell polarization was found to be unaffected in gene-based basophil-KO mice during primary infection with different helminths (Schwartz et al., 2014; Ohnmacht et al., 2010; Sullivan et al., 2011). We favor a model where conventional DCs are the drivers of Th2 polarization after antigen encounter in the lung (Hammad et al., 2010) and that previous studies did not fully exclude inadvertent DC targeting when basophils were depleted (Motomura et al., 2014; Tang et al., 2010).

By comparing full basophil-deficient mice with mice in which basophils were depleted only during the challenge, we found that the main function of basophils is in mediating the extravasation of primed Th2 cells to the lungs. For adequate secondary type 2 immune responses to HDM allergen to occur, it is imperative that primed Th2 cells get recruited to sites of allergen challenge where they can take residence as resident memory T cells in defined niches or control vascular recruitment of eosinophils (Rahimi et al., 2020). The details of such extravasation of Th2 cells are poorly studied for the lung vasculature, but likely involves integrin-mediated entrapment, arrest, and adhesion driven by cytokines derived from basophils. In line, we found that *Il1rl1*-deficient basophils were unable to recruit Th2 cells to the lungs and that basophils were required for allergen-induced VCAM1 and ICAM1 integrin upregulation on the vasculature (Horitani et al., 2023). We have not formally shown however that the extravasation of Th2 cells indeed depends on the expression of these integrins on endothelial cells of the lungs. We also yet have to analyze if the recruitment of Th2 cells to the lungs indeed depends exclusively on basophil-derived IL-4, a known inducer of VCAM1 expression (Luccioli et al., 2002), a scenario described for vessel-lining basophils regulating the endothelial expression of integrins that allow eosinophil exit into the skin through IL-4 (Cheng et al., 2015).

Several asthma susceptibility loci center around basophil biology, such as polymorphisms in *FCER1A*, *IL33*, *IL1RL1*, and the ubiquitin modifying enzyme *TNFAIP3*, a negative regulator of TLR, TNFR, and IL1R family signaling (Schuijs et al., 2015; Stein et al., 2016; Li et al., 2012). We have previously shown that deficiency of *Tnfaip3* in macrophages renders these cells hypersensitive to IL-33 stimulation (Holgado et al., 2023) and now report that basophil deficiency of this asthma susceptibility gene increases basophil activation, numbers, and subsequent eosinophilic airway inflammation. In light of the other findings of our study, it is likely that this is also due to increased reactivity to IL-33 since part of the enhancement was abolished by sST2 blockade. Potentially residual basophil activation is mediated via cell-intrinsic TLR4, IL18R, or IL1R, which are all crucial for the HDM model. We have previously reported that deficiency of *Tnfaip3* selectively in yolk sac–derived connective tissue *Mcpt5*$^{+}$ mast cells alter other key aspects of ovalbumin-driven asthma (Heger et al., 2014), such as eosinophilia, Th2 cytokine production, and IgE synthesis, through enhanced signaling of IL-33, pinpointing a remarkable parallel in the function of *Tnfaip3* in mast cells and basophils. Mucosal mast cells do not express *Mcpt5*, yet can expand massively during allergen exposure and express *Mcpt8* (Derakhshan et al., 2021). Since we used *Mcpt8*Cre to genetically target *Tnfaip3*, it is possible that part of the phenotype of these mice is due to hyperactivation of these pro-inflammatory mast cells, in addition to clear effects on basophils.

Taken together, these data highlight a prominent role for IL-33 activated basophils during the effector phase of HDM-induced allergic airway inflammation, pinpointing an important bridging function for these innate type 2 cells in the recruitment of adaptive type 2 immune cells. Moreover, our observations on the temporal importance of basophils during the asthmatic response could have important implications for the use of basophil-directed treatments during asthma exacerbations.

Materials and methods

Mice

WT C57Bl/6J mice were obtained from Harlan, B6.129S4-*Mcpt8*$^{tm1(cre)Lky}$/J mice were initially described (Sullivan et al., 2011) obtained from the Jackson Laboratory (JAX: 017578). Tg(Mcpt8-cre)1Voeh were initially described (Ohnmacht et al.,

2010) and *Il1rl1*$^{-/-}$ mice provided by Dr. P. Fallon (Trinity College Dublin, Dublin, Ireland). 1-DER TCR transgenic mice were initially described (Coquet et al., 2015) and maintained on a *Rag2*$^{-/-}$ background. Other strains used include B6.129S2(Cg)-*Fcer1a*^{tm1Knt}/J, *Tnfaip3*$^{fl/fl}$, B6-Gt(ROSA)26Sor$^{tm1(HBEGF)Awai}$/J, and *Il4*$^{4get/4get}$ and these were backcrossed to C57BL/6J for at least 10 generations and maintained in the VIB-UGent specific pathogen–free animal facility in accordance with local regulation. Experiments were approved by the animal ethical committee of Ghent University (ECD2014-008).

In vivo challenges

To induce allergic airway inflammation, mice were sensitized i.t. with 1 µg crude HDM extract (Greer Laboratories) on day 0 and subsequently challenged with 10 µg HDM extract i.n. on days 6–10. In some experiments, the challenge dose was lowered to 1 µg daily. Control mice received sham sensitization with PBS. 4 days after the last challenge, mice were sacrificed and organs were collected for analysis. Blood was taken to screen for serum antibodies (BD Biosciences). For basophil depletion by DTx, *Mcpt8*-iDTR mice were treated with 100 ng of DTx (Sigma-Aldrich) i.p., as described. Recombinant sST2 (r-sST2) was made in-house (Willart et al., 2012). In neutralization experiments, IL-33 was blocked during the effector phase by use of r-sST2 (10 µg/mouse) in PBS, injected i.t. on the first, third, and fifth day of the challenge phase. To label all immune cells in circulation some experiments make use of i.v. injection of CD45 antibody (1 µg/mouse) 3 min before euthanasia.

Lung preparation

For BAL collection, lungs were instilled with 3 × 1 ml of PBS containing EDTA. The lavage was centrifuged and the supernatant was stored for cytokine analysis. The pellet was used for flow cytometry analysis. For flow cytometry or sorting of basophils, lung lobes were excised and chopped followed by shaking incubation for 30 min at 37°C, 200 rpm in RPMI-1640 (Gibco; Thermo Fisher Scientific) containing 20 µg/ml liberase TM (Roche), 10 U/ml DNase I (Roche), and 10% of FCS (Bodinco). Following tissue dissociation, cell suspensions were filtered through a 70-µm nylon mesh, washed, and treated with ammonium chloride buffer (10 mM KHCO3, 155 mM NH4Cl, 0.1 mM EDTA in milliQ water) for erythrocyte lysis before final suspension in PBS. Cells were stained for flow cytometry or sorting. For some experiments mice were injected i.v. with anti-CD45 monoclonal antibody before euthanasia, to label all circulating immune cells. Pictures were obtained with AnalySIS getIT (Olympus Soft Imaging Solutions GmbH).

Cell culture

To analyze Th2 cytokine secretion, 2 × 10^5 MLN cells/well were restimulated in vitro with 15 µg/ml HDM for 3 days. The supernatant was collected and assayed for cytokine levels (Ready-Set-Go Kits; eBioscience) or chemokine levels (Duo-set; R&D Systems) by ELISA.

Whole bone marrow cells from WT, *St2*$^{-/-}$, *Il4*$^{-/-}$, and *FcεRI*$^{-/-}$ mice were isolated from femurs and tibias of 8–12-wk-old mice and cultured (37°C, 5% CO_2) at 2.5 × 10^6 cells/ml in 10 ml of penicillin and streptomycin, 2 mM *L*-glutamine, 50 µM β-mercaptoethanol, 10% fetal bovine serum–containing RPMI 1640 medium supplemented with recombinant mouse IL-3 (10 ng/ml) for 9 days with medium changed every 3 days. Subsequently, basophil-enriched populations (c-Kit$^-$ FcεRI$^+$CD49b$^+$) were sort-purified using a BD FACS Aria III flow cytometer. Sorted basophils were ∼98% c-Kit$^-$ FcεRI$^+$ upon reanalysis and were injected i.v. (1 × 10^5/mouse) in recipient mice and challenged with HDM as described. For the passive transfer of Th2-skewed 1-DER T cells, 1-DER T cells and DCs were isolated from spleen and lymph nodes and purified using Magnisort kits (eBioscience). 1-DER T cells and DCs were cultured at 1:5 ratio in a culture medium supplemented with mIL-2 (10 µg/ml; R&D systems), mIL-4 (20 ng/ml; R&D systems), anti-IFNγ (20 µg/ml, in house production), and anti-IL-12p40 (20 µg/ml, in house production) for 5 days. Naïve or primed congenic 1-DER T cells were isolated as above, labeled with Cell Trace Violet (Invitrogen), and injected into recipients via the tail vein (1 × 10^6/mouse). Following the transfer, mice were inoculated with HDM as described. For the quantification of IL-4 gene expression in basophils, WT and Il1rl1$^{-/-}$ bone marrow–derived basophils were cultured for 24 h with 10 ng/µl of mIl-33 (R&D systems). Cells were lysed with RLT plus buffer (Qiagen).

RNA extraction and RT-PCR

For RNA extraction, the accessory lung lobe was collected in TriPure isolation reagent (Roche) and RNA was isolated according to the manufacturer's instructions. RNA was reverse-transcribed with a Transcriptor High Fidelity cDNA Synthesis Kit (Roche) and samples were analyzed by SYBR green-based RT-PCR. PCR primers for Muc5ac and the reference genes Rpl13a, Hprt, and Sdha were as follows: Muc5ac: forward 5′-GAG GCTCCCACATGTCCA-3′, reverse 5′-TGAAGGCATTACTGTCAC AGGG-3′; Rpl13a: forward 5′-CCTGCTGCTCTCAAGGTTGTT-3′, reverse 5′-TGGTTGTCACTGCCTGGTACTT-3′; Hprt: forward 5′-TCCTCCTCAGACCGCTTT-3′, reverse 5′-CCTGGTTCATCATCG CTAATC-3′; and Sdha: forward 5′-TTTCAGAGACGGCCATGA TCT-3′, reverse 5′-TGGGAATCCCACCCATGTT-3′. For basophil RNA extraction RNeasy Plus Micro Kit was used (Qiagen) using the manufacturer's instructions. For cDNA generation SensiFAST cDNA synthesis kit (Bioline) was used. For RT-PCR SensiFAST SYBR No-Rox kit was used following the manufacturer's instructions. Primers for Il4 (forward: 5′-CTCATGGAGCTGCAG AGACTCTT-3′, reverse: 5′-CATTCATGGTGCAGCTTATCGA-3′) and Actb as housekeeping gene (forward: 5′-GCTTCTAGGCGG ACTGTTACTGA-3′, reverse: 5′-GCCATGCCAATGTTGTCTCTT AT-3′) were used. RT-PCR was performed with a LightCycler 480 system (Roche)

Bone marrow chimeras

For the generation of bone marrow chimeras, C57BL/6J congenic mice were lethally irradiated with 9 Gy γ-irradiation. The following day, mice were reconstituted i.v. with bone marrow cells. Mice were used in experiments 6–8 wk after irradiation.

Flowcytometry and sorting

Monoclonal, murine-specific antibodies from eBiosciences included anti-CD3 (145-2C11), anti-CD4 (RM4-5), anti-CD11b (M1/70), anti-CD11c (N418), anti-CD19 (1D3), anti-CD31 (390), anti-CD45 (30-F11), anti-CD45.2 (104), anti-CD49b (HMa2), anti-CD63 (NVG-2), anti-CD90.2 (30-H12), anti-CD103 (2E7), anti-CD117 (2B8), anti-B220 (RA3-6B2), anti-FcεRIa (MAR-1), anti-Gr-1 (RB6-8C5), anti-Ly6G (1A8), anti-F4/80 (BM8), anti-GATA-3 (TWAJ), and anti-TCR-γδ (GL-3). Antibodies from BD Biosciences included anti-CD4 (RPA-T4), anti-CD8a (RPA-T8), anti-CD11b (M1/70), anti-CD64 (X54-5/7.1), anti-IgE (R35-72), anti-Ly6C (AL21), anti-Siglec-F (E50-2440), anti-CD45.1 (A20). Antibodies from Biolegend including anti-CD161 (PK136) and anti-CD200R3 (Ba13) were used. For i.v. labeling anti-CD45 (30-F11) was injected via the tail vein prior to the section. All stainings were performed at 4°C for 30–45 min. Prior to antibody staining, cells were incubated with 2.4G2 Fc receptor antibody (L. Boon, JJP Biologics, Poland) to reduce non-specific binding, together with fixable live/dead marker (eBioscience) in PBS. Fluorescence minus one control was taken along to allow for proper gating. Murine basophils were identified as live $CD45^+Lin^-CD117^-CD49b^+$ $Fc\varepsilon RIa^+$ and in some experiments including MCPT8-YFP^+ or IgE^+.

Flexivent

For the measurement of airway hyperresponsiveness, mice were anesthetized with urethane, paralyzed with D-tubocurarine, and tracheotomized, followed by mechanical ventilation on a flexiVent machine (SCIREQ). Increasing concentrations of methacholine were administered through nebulization (0–400 mg/ml). Dynamic resistance and compliance were recorded after a standardized inhalation manoeuvre given every 10 s for 2 min. Baseline resistance was restored before administering the subsequent doses of methacholine.

Histology

Lung left lobes were fixed with 4% PFA overnight and embedded in paraffin, 5-µm sections were cut with a microtome, sections were stained with periodic acid-Schiff stain to visualize mucus production, and images were acquired using a Zeiss Axioscan Z1 slide scanner. For immunofluorescence images lungs were perfused via the right ventricle with 1 ml PBS, lungs were inflated with O.C.T (Sakura Finetek), frozen in liquid nitrogen, embedded in O.C.T, and stored at −80°C. Samples were cut into 5-µm sections using a cryostat. Sections were rehydrated with PBS, fixed with 4% PFA for 2 min, and blocked with 2% BSA plus donkey serum. Antibodies against GFP-AF488 (FM264G; BioLegend) and CD49b-APC (HMa2; BD Biosciences) were stained overnight at 4°C. DAPI (Life Technologies) was used for nuclear staining. For thicker sections, lungs were fixed with 2% PFA overnight and embedded in 3% normal-melting agarose, and samples were cut into 200-µm sections using a Leica VT1200 S vibratome. Sections were blocked with 0.3% triton X-100, 4% BSA, and 5% donkey or goat normal serum. Antibodies against GFP-AF488 (FM264G; BioLegend), VCAM (429; eBioscience) + AF594 (Thermo Fisher Scientific), and VWF (Thermo Fisher Scientific) + DL755 for overnight staining at 4°C. DAPI (Life Technologies) was used for nuclear staining. Slides were mounted with Polyvinyl alcohol with DABCO (Sigma-Aldrich). All images were generated using a Leica STELLARIS 8 confocal microscope (Leica Microsystems) and visualized with IMARIS software (Oxford Instruments).

Statistical analysis

All data are represented as mean ± SEMs. Statistical significance between groups was calculated with one-way, or two-way ANOVA with multiple comparison analysis, Mann–Whitney *U* testing, or Student's *t* test where applicable, using Graphpad Prism software (GraphPad software). P values are shown as follows: *P = 0.01–0.05; **P = 0.001–0.01; ***P < 0.001; ****P < 0.0001.

Online supplemental material

Fig. S1 shows type 2 cytokine in MLN, T cell attracting chemokines in BAL, and HDM-specific immunoglobulin levels in circulation, confocal microscopic identification of lung basophils, and flow cytometry gating strategy for lung basophils, including CD45 i.v. labeling. Fig. S2 shows basophil depletion efficiency in lung, MLN, and spleen in $Mcpt8^{Cre/YFP}$ mice and pulmonary basophil numbers at challenge after deletion during sensitization. Fig. S3 shows basophil depletion efficiency during the challenge phase and the recovery efficiency of transferred cultured WT basophils after the challenge. Fig. S3 also shows flow cytometry data on the phenotype of Th2-skewed 1-DER T cells after in vitro culture. Fig. S4 shows basophils ST2 expression in comparison to ILC2s and $CD4^+$ T cells, as well as the total cell number of pulmonary mast cells and ILC2s after IL-33 blockade with sST2. Fig. S4 further shows gating strategies for adoptively transferred basophils, including recovery efficiency, and Th2-skewed 1-DER T cells with CD45 i.v. labeling. Fig. S5 shows the genetic analysis of $Mcpt8^{Cre/YFP}$ × $Tnfaip3^{fl/fl}$ mice.

Data availability

The data are available from the corresponding author upon reasonable request.

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Supplemental material

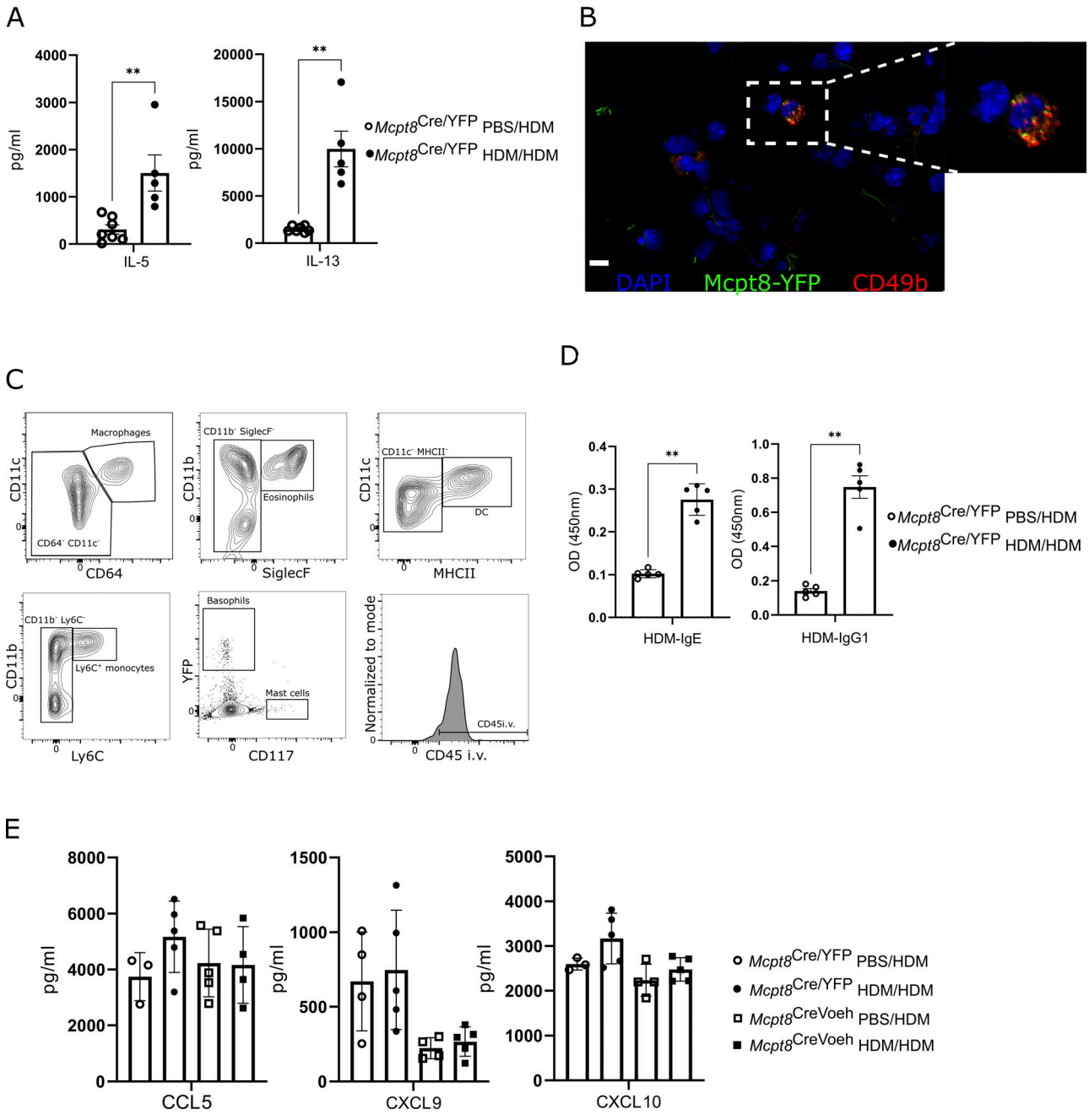

Figure S1. **Analysis of basophils in a house dust mite model of asthma. (A)** IL-5 and IL-13 levels in the supernatant of restimulated MLN cells, as measured by ELISA. **(B)** Confocal microscopy image of the lung basophils with staining for Mcpt8-YFP (green), CD49b (red), and nuclear stain (blue). Scale bar = 10 µm. **(C)** Flow cytometric gating strategy for lung basophils using the *Mcpt8*$^{Cre/YFP}$ reporter mouse and intravascular CD45 labeling. **(D)** HDM-specific IgG1 and IgE concentration in serum, as measured by ELISA. **(E)** Chemokine levels as measured in BAL. One representative of three independent experiments, all of which gave similar results, with five to seven mice per group is shown. Data are means ± SEM. **P < 0.01. Student's *t* test (A and D) or ordinary ANOVA (E).

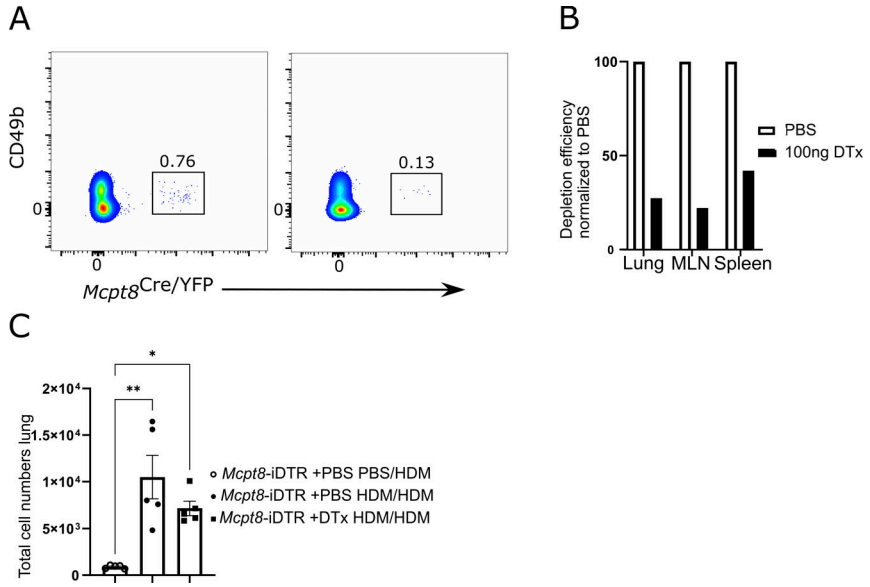

Figure S2. **Validation of basophil depletion. (A)** Representative flow plot of lung basophil depletion after DTx administration. **(B)** Basophil depletion efficiency 3 days after 100 ng DTx injection, normalized to PBS, in lung, MLN, and spleen. **(C)** Basophil numbers 14 days post depletion with 100 ng DTx during sensitization. One representative of three independent experiments, all of which gave similar results, with five mice per group is shown. Data are means ± SEM. *P < 0.05; **P < 0.01. Ordinary ANOVA (C).

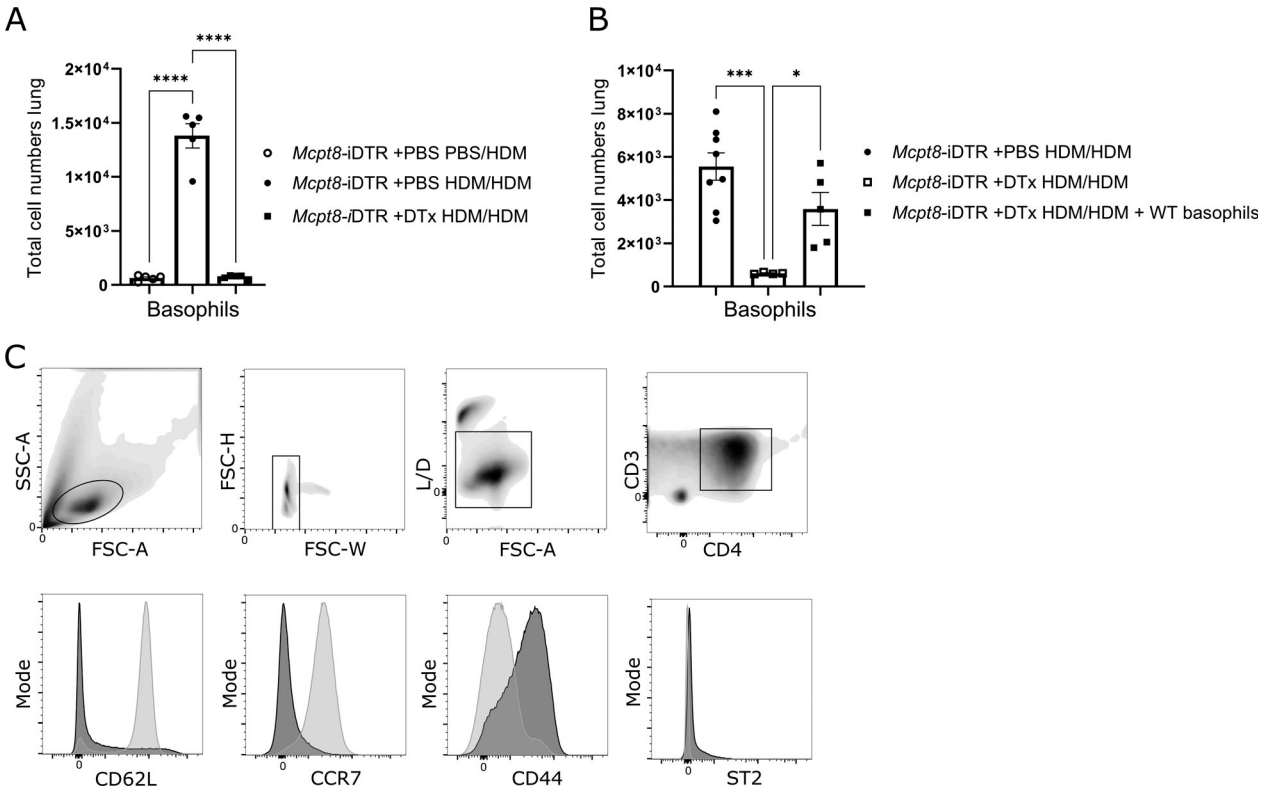

Figure S3. **Validation and kinetics of basophil depletion in conditional basophil depleter mice. (A)** Basophil numbers in the lung 4 days after depletion during the challenge phase. **(B)** Basophil numbers in the lung 8 days after transfer. **(C)** Flow cytometry gating strategy to identify and phenotype transferred Th2 1-DER T cells compared to naïve CD4+ 1-DER T cells. One representative of three independent experiments, all of which gave similar results, with five to eight mice per group is shown. Data are means ± SEM. *P < 0.05; ***P < 0.001; ****P < 0.0001. Ordinary ANOVA (A and B).

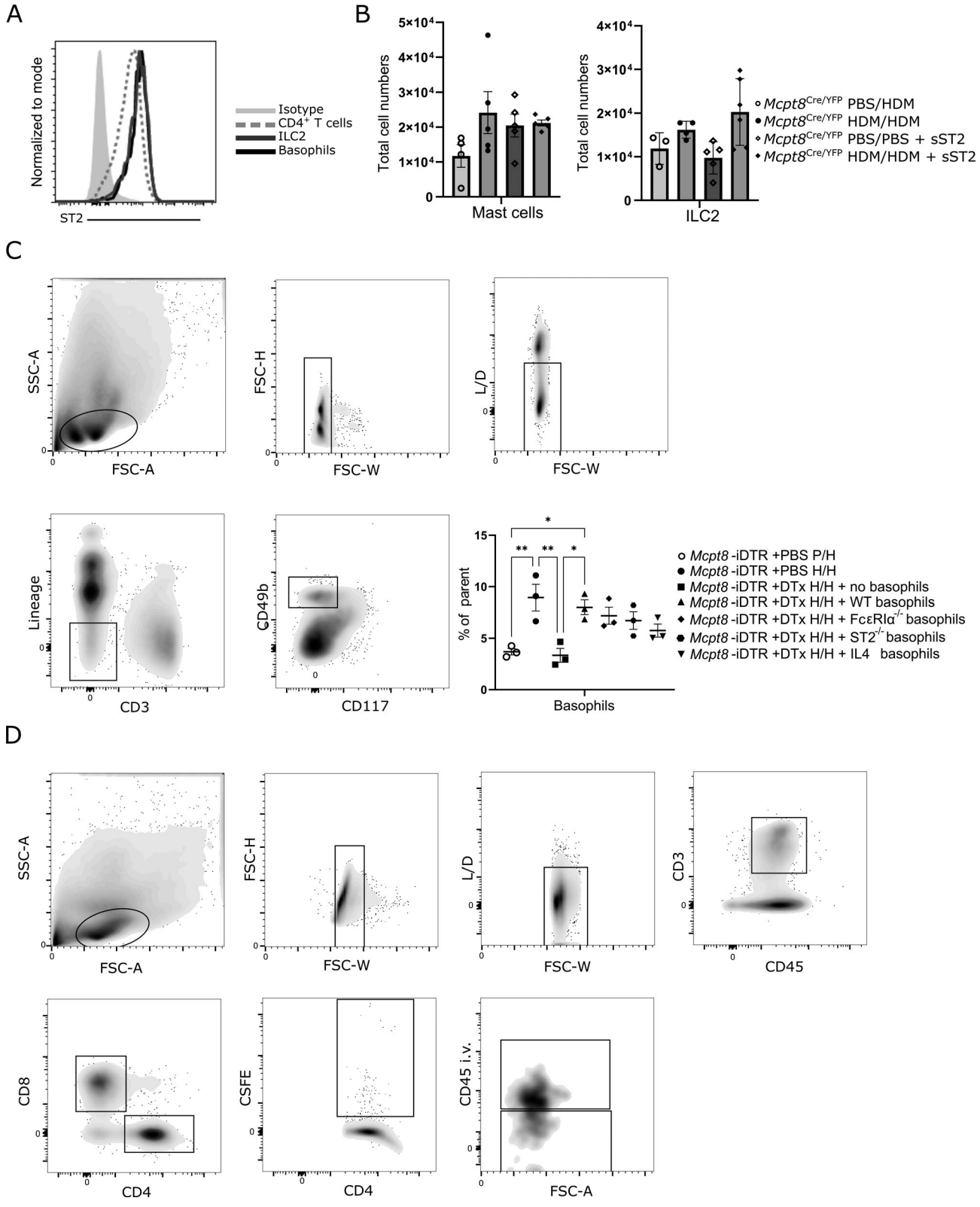

Figure S4. **Flow cytometry gating strategy to asses ST2 expression and intravascular labeling on various immune cells. (A)** Flow cytometry analysis of ST2 expression on CD4⁺ T cells, ILC2s, and basophils. **(B)** Lung mast cell and ILC2 numbers of mice treated with sST2 at day 14 after sensitization. **(C)** Flow cytometry gating strategy showing for basophil identification in lung. Graph depicts basophil numbers in lung 14 days after reconstitution. **(D)** Flow cytometry gating strategy of Th2 CD4⁺ T cells with CD45 intravascular staining. One representative of three independent experiments, all of which gave similar results, with three to five mice per group is shown. Data are means ± SEM. *P < 0.05; **P < 0.01.

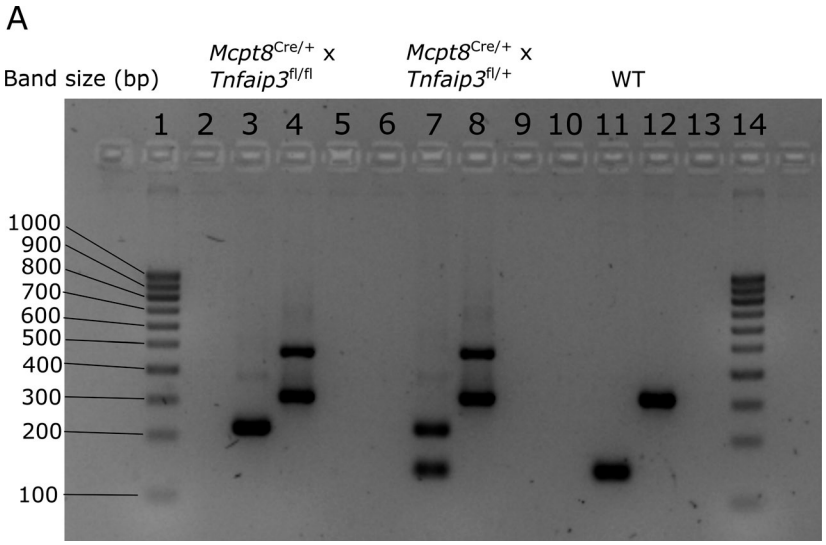

Figure S5. **PCR-based analysis of *Mcpt8*$^{Cre/YFP}$ and *Tnfaip3*$^{fl/fl}$ genotype in KO, Hz, and WT mice, respectively. (A)** *Mcpt8*-WT band size of 314 base pairs (bp) and *Mcpt8*-Cre band size of 478 bp. *Tnfaip3*-WT band size of 160 bp and *Tnfaip3*-floxed band size of 250 bp. Source data are available for this figure: SourceData FS5.