Basophils are important for development of allergic skin inflammation

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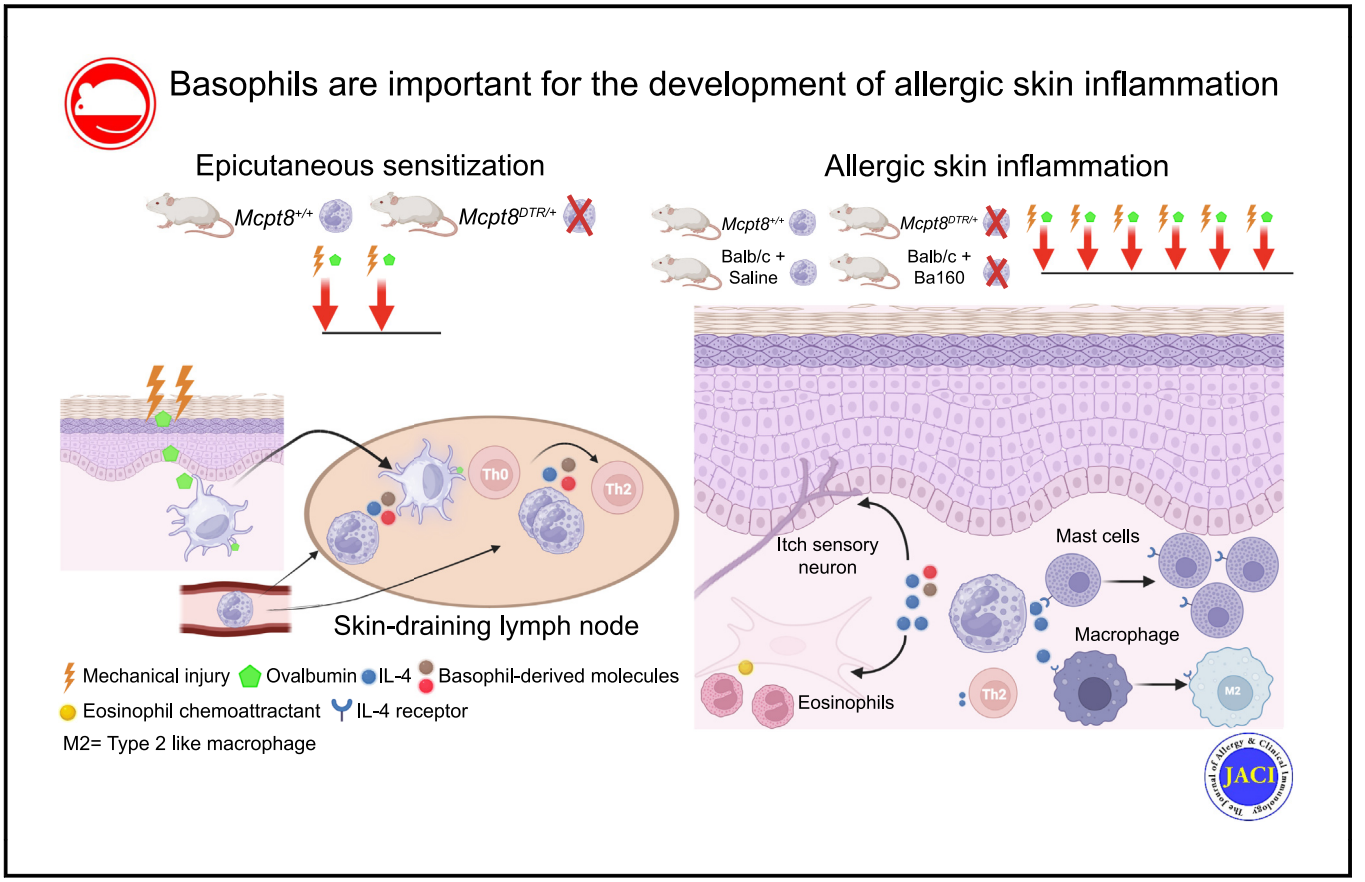
GRAPHICAL ABSTRACT



Capsule summary: Basophils play an important role in the development of antigen-driven allergic skin inflammation in mice.

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Basophils are important for development of allergic skin inflammation

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Background: Atopic dermatitis skin lesions exhibit increased infiltration by basophils. Basophils produce IL-4, which plays an important role in the pathogenesis of atopic dermatitis.
Objective: We sought to determine the role of basophils in a mouse model of antigen-driven allergic skin inflammation.
Methods: Wild-type mice, mice with selective and inducible depletion of basophils, and mice expressing *Il4*-driven enhanced green fluorescent protein were subjected to epicutaneous sensitization with ovalbumin or saline. Sensitized skin was examined by histology for epidermal thickening. Cells were analyzed for surface markers and intracellular expression of enhanced green fluorescent protein by flow cytometry. Gene expression was evaluated by real-time reverse transcription–quantitative PCR.
Results: Basophils were important for epidermal hyperplasia, dermal infiltration by $CD4^+$ T cells, mast cells, and eosinophils in ovalbumin-sensitized mouse skin and for the local and systemic T_H2 response to epicutaneous sensitization. Moreover, basophils were the major source of IL-4 in epicutaneous-sensitized mouse skin and promote the ability of dendritic cells to drive T_H2 polarization of naive T cells.
Conclusion: Basophils play an important role in the development of allergic skin inflammation induced by cutaneous exposure to antigen in mice. (J Allergy Clin Immunol 2024;153:1344-54.)

Key words: *Atopic dermatitis, basophils, T_H2*

Abbreviations used
AD: Atopic dermatitis
ARG1: Arginase 1
DC: Dendritic cell
dLN: Draining lymph node
DT: Diphtheria toxin
EC: Epicutaneous
eGFP: Enhanced green fluorescent protein
4get: $Il4^{eGFP/eGFP}$ animal model
HDM: House dust mite
iBOB: Infrared behavior observation box
iNOS: Inducible nitric oxide synthase
mAb: Monoclonal antibody
MHC-II: Major histocompatibility complex class 2
OVA: Ovalbumin

Atopic dermatitis (AD) is the most common skin inflammation in infants and young children. It is characterized by intense itching, skin barrier defects, epidermal hyperplasia, dermal infiltration by T cells and eosinophils, and type 2–dominated local immune response, evidenced by increased cutaneous expression of type 2 cytokines and eosinophilia. In addition, AD patients exhibit elevated levels of serum IgE and IgE antibodies to environmental and food allergens.[1,2]

The T_H2 cytokines IL-4 and IL-13 are responsible for many of the pathologic features of AD.[1-3] IL-4 and IL-13 drive the expression of chemoattractants for eosinophils, basophils, and T_H2 cells; inhibit keratinocyte differentiation and the production of antimicrobial peptides; impair skin barrier function; promote lipid abnormalities in the epidermis; increase endogenous protease activity; drive skin remodeling; and promote pruritus.[2,4-9] The essential role of IL-4 and IL-13 in allergic skin inflammation has been demonstrated in several mouse models[10-12] and is strongly supported by the beneficial effect of anti–IL-4Rα monoclonal antibody in AD.[5,13-15] A recent study using single cell RNA sequencing analysis suggests that innate cells, rather than $CD4^+$ T cells, are the major source of IL-4 and IL-13 in allergic skin inflammation elicited by epicutaneous (EC) sensitization of mice.[16] However, the major individual cellular sources of these cytokines in allergic skin inflammation remains controversial.

Basophils account for less than 1% of peripheral blood leukocytes. Basophils produce IL-4 and IL-13 and have been identified as important players in type 2 immune responses against allergens and parasitic infection.[17] Basophils contribute to the priming of T_H2 responses through the skin. Basophils migrating in the skin-draining lymph nodes (dLNs) provide cytokines that promote T_H2 polarization and could possibly present antigens.[18-24] In addition, mouse models show that basophils play important roles in the severity of skin inflammation induced by chemical compounds.[25-28] In healthy human skin, basophils are not present; however, they are detected in lesional skin of diverse cutaneous inflammatory diseases.[29] In particular, basophils are detected in AD lesional skin and produce IL-4.[28,30] In addition, blood basophils from AD patients exhibit increased expression of CD203c, indicating an activated phenotype.[31]

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These data, taken together, suggest that basophils may play an important role in AD.

We have taken advantage of our well-established mouse model of acute allergic skin inflammation induced by EC sensitization with antigen[32] and selective and inducible depletion of basophils using Mcpt8^{DTR} mice[33] or intravenous injection of anti-CD200R3 Ba160 antibody[34] to define the role of basophils in a mouse model of allergic skin inflammation that shares many characteristics with AD. Our results support a role for basophils in antigen-driven allergic skin inflammation and reveal an important role of basophils in promoting the ability of dendritic cells (DCs) to drive T_H2 polarization of naive T cells after cutaneous encounter with antigen.

METHODS

Mice

The Mcpt8$^{DTR/+}$, Il4$^{eGFP/eGFP}$ (4get) mice we used, which were bred on a BALB/c background, have been previously described.[33,35] BALB/c mice were purchased from Charles River Laboratories. All mice were kept in a pathogen-free environment and fed a diet free of ovalbumin (OVA). All procedures were performed in accordance with the Animal Care and Use Committee of the Boston Children's Hospital.

EC sensitization

Female mice 6 to 8 weeks old were epicutaneously sensitized for 10 days, as described previously.[32] Briefly, for the first cycle of tape stripping, mice were tape stripped 6 times; then the area was covered with a film dressing (Tegaderm, 3M). For each of the other cycles, at days 2, 4, 6, 8, and 10, mice were tape stripped only twice. Antigen sensitization consisted of applying 200 μg OVA (Sigma-Aldrich) after each tape-stripping cycle. Saline application was used as control. Analyses were done at day 12.

Basophil depletion

Mcpt8$^{DTR/+}$ mice or *Mcpt8$^{+/+}$* littermates (BALB/c genetic background) received an intraperitoneal injection of diphtheria toxin (DT) (750 ng per 20 g body weight) at 2 days before the initiation of the experiments or every 5 days during EC sensitization. Basophils were depleted 2 days before the initiation of the experiments or every 5 days during EC sensitization by intravenous injection of 50 μg of anti-CD200R3 antibody (clone Ba160).

Histology and measurement of epidermal thickness

Skin specimens were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin. Microphotographs were captured on an EVOS M700 imaging system, and epidermal thickness was analyzed as previously described by ImageJ software (imagej.nih.gov/ij).[36]

Mouse tissue cell preparation and flow cytometry

Cell isolation from the back skin or the skin dLNs was performed as previously described.[37,38] Cells were preincubated with FcγR-specific blocking monoclonal antibody (mAb) (2.4G2) and washed before staining with the following mAbs: B220 (RA3-6B2), CD3 (17A2), CD4 (GK1.5), CD11c (N418), CD19 (1D3), CD45 (30F11), CD90.2 (53-2.1), Gr1 (RB6-8C5), δ-TCR (ebioGL3), major histocompatibility complex class 2 (MHC-II) (M5/114.15.2), CD80 (16-10A1), CD40 (1C10), CD86 (GL1), CD11c (N418), Ly6c (HK1.4), Ly6g (1A8), inducible nitric oxide synthase (iNOS; CXNFT), and arginase 1 (ARG1; A1exF5) from eBioscience, CD11b (M1/70), F4/80 (BM8), and CD117 (2B8) from BioLegend, and anti-IgE (R35-72) from BD Biosciences. Streptavidin (BV605) from BioLegend was used to detect biotinylated antibodies. For intracellular staining, cells were fixed and permeabilized (BD Biosciences Cytofix/Cytoperm) and stained in permeabilization solution with antibodies against ARG1 and iNOS. For cytokine staining, cells were stimulated with ionomycin (0.5 μg/mL; Sigma-Aldrich), phorbol 12,13-dibutyrate (1 μg/mL; Sigma-Aldrich), brefeldin A (eBioscience), and monensin (eBioscience) in complete RPMI 1640 medium for 4 hours before surface staining. Subsequently, cells were fixed and permeabilized (BD Biosciences Cytofix/Cytoperm) and stained in permeabilization solution with antibodies against IL-4 and IL-13 from eBioscience. Cells were analyzed by flow cytometry with an LSRFortessa machine (BD Biosciences). The data were analyzed by FlowJo software (Becton Dickinson).

mRNA expression analyses

Total skin RNA extraction and measurement of cytokines were performed and analyzed as previously described.[39] RNA was extracted from mouse skin using RNeasy plus mini kit (Qiagen). cDNA was prepared with the iScript cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR was done with the TaqMan gene expression assay, universal PCR master mix, and Quantstudio 5 Real Time PCR system (Thermo Fisher Scientific). Fold induction was calculated using $\Delta\Delta C_t$ with normalization to the internal control β_2 microglobulin.

Cell culture and *in vitro* cytokine expression

Single cell suspensions of splenocytes were cultured and stimulated with OVA, and their supernatants were analyzed for cytokines by ELISA as previously described.[39]

Isolation and functional analysis of mouse skin dLN DCs

For *in vivo* DC priming experiment, OVA or DQ-OVA (1 mg in 100 μL saline) was epicutaneously applied to shaved, tape-stripped skin of mice, and 24 hours after sensitization, DCs were quantified and analyzed as previously described.[38]

Measurement of spontaneous itch

At day 10, EC-sensitized mice were habituated to the infrared behavior observation box (iBOB). The day after, mice were placed in iBOB for 60 minutes of video recording. Bouts of scratching were counted by observers unaware of the treatment groups.

Statistical analysis

Statistical significance was determined by the 2-tailed Student *t* test with 1-way or 2-way ANOVA.

RESULTS

DT-injected *Mcpt8*$^{DTR/+}$ mice exhibit decreased allergic skin inflammation and systemic T_H2 response after EC sensitization with OVA. We examined the effect of basophil depletion on the development of allergic skin inflammation elicited by EC sensitization with OVA antigen. *Mcpt8*$^{DTR/+}$ mice and *Mcpt8*$^{+/+}$ controls were injected with DT during EC sensitization, as depicted in Fig 1, *A*. As previously reported,[33] DT injection in unmanipulated *Mcpt8*$^{DTR/+}$ mice efficiently depleted CD45$^+$CD3$^-$IgE$^+$CD117$^-$ basophils from blood and spleen but had no significant effect on the number of CD45$^+$CD3$^-$IgE$^+$CD117$^+$ mast cells in ear skin (Fig 1, *B,* and see Fig E1, *A* and *B,* in this article's Online Repository available at www.jacionline.org). However, DT-injected *Mcpt8*$^{DTR/+}$ mice subjected to EC sensitization with OVA demonstrated significantly diminished epidermal thickening and dermal infiltration by CD45$^+$ cells, CD4$^+$ T cells, eosinophils, and mast cells compared to DT-injected *Mcpt8*$^{+/+}$ controls (Fig 1, *C-G*). EC sensitization with OVA induced an increase in the accumulation of basophils in the skin of DT-injected *Mcpt8*$^{+/+}$ controls, which, as expected, was virtually abolished in the skin of DT-injected *Mcpt8*$^{DTR/+}$ mice (Fig 1, *H*). Upregulation of *Il4* and *Il13* expression in OVA-sensitized skin was significantly decreased in DT-injected *Mcpt8*$^{DTR/+}$ mice compared to controls, whereas *Il17a* and *Ifng* mRNA levels were unaffected (Fig 1, *I*).

The systemic T_H2 immune response to EC sensitization with OVA was impaired in DT-injected *Mcpt8*$^{DTR/+}$ mice compared to controls. This was evidenced by significantly decreased serum levels of OVA-specific IgE and IgG_1, but not IgG_2a, antibody levels and significantly diminished secretion of IL-4 and IL-13, but not IL-17A or IFN-γ, by splenocytes in response to *in vitro* stimulation with OVA (Fig 1, *J* and *K,* and see Fig E2, *A* and *B,* in the Online Repository available at www.jacionline.org). These findings demonstrate that basophils play an important role in the development of acute allergic skin inflammation and in the T_H2 systemic response to EC sensitization with OVA.

Basophil depletion by intravenous injection of Ba160 antibody decreases allergic skin inflammation elicited by EC sensitization with OVA. In addition to depleting basophils, injection of a high dose of DT in *Mcpt8*$^{DTR/+}$ mice ablates granulocyte-macrophage progenitors in bone marrow.[40] We therefore examined the effect of basophil depletion using an alternative method of Ba160 antibody administration.[34] As expected, mice injected with Ba160 antibody during EC sensitization, as depicted in Fig 2, *A,* showed decreased percentages of basophils in the circulation (Fig 2, *B*).

Ba160-injected BALB/c mice subjected to EC sensitization with OVA demonstrated significantly diminished epidermal thickening and dermal infiltration by CD45$^+$ cells, CD4$^+$ T cells, and eosinophils, but not mast cells, compared to mice EC sensitized with OVA and injected with saline vehicle (Fig 2, *C-G*). As expected, Ba160-injected BALB/c mice exhibited reduced percentages of basophils in EC-sensitized skin with OVA (Fig 2, *H*). Ba160-injected BALB/c mice exhibited decreased *Il4* and *Il13*, but not *Il17a* or *Ifng,* mRNA levels in OVA-sensitized skin compared to controls (Fig 2, *I*). They also exhibited comparable serum levels of OVA-specific IgE and reduced IL-4, but not IL-13, IL-17A, and IFN-γ, secretion by splenocytes in response to *in vitro* stimulation with OVA compared to controls (Fig 2, *J* and *K,* and data not shown).

EC sensitization with OVA resulted in a significant increase in percentages of CD45$^+$F4/80$^-$Ly6c$^+$ monocytes and CD45$^+$CD11b$^+$F4/80$^+$Ly6g$^-$Ly6c$^-$CD11c$^+$MHC-II$^{low/+}$ macrophages, including Arg1$^+$ M2-like macrophages and inducible nitric oxide synthase (iNOS)-positive M1-like macrophage, but not in the percentages of CD45$^+$CD11b$^+$F4/80$^+$Ly6c$^+$CD11c$^{+/high}$MHC-II$^{+/high}$ monocyte-derived DCs or CD45$^+$CD11b$^+$F4/80$^+$Ly6g$^-$Ly6c$^-$CD11c$^{+/high}$MHC-II$^{+/high}$ myeloid DCs compared to EC sensitization with saline (see Fig E3 in the Online Repository available at www.jacionline.org). Ba160-injected BALB/c mice exhibited diminished percentages of macrophages in OVA-sensitized skin (Fig E3, *E*). Remarkably, Ba160-injected BALB/c mice exhibited a reduction in total as well as ARG1$^+$ M2-like macrophages, but not in iNOS$^+$ M1-like macrophages (Fig E3, *F* and *G*). Ba160 injection had no significant effect on the accumulation of monocytes or DCs in OVA-sensitized skin (Fig E3, *B-D*).

Itching is one of the main symptoms experienced by AD patients.[1,2] We examined whether basophils play a role in itching induced by allergic skin inflammation in EC-sensitized mice. Spontaneous itch behavior was assessed on day 11 by placing the mice in an iBOB and recording the animals' spontaneous activity over 60 minutes. Videos were watched by observers unaware of the animals' group status, who quantified bouts of scratching. Mice EC sensitized with OVA exhibited significantly increased scratching behavior compared to mice EC sensitized with saline (Fig 2, *L*). Basophil depletion by Ba160 antibody injection significantly decreased scratching behavior in EC-sensitized mice with OVA (Fig 2, *L*). These findings confirmed that basophils play an important role in the development of acute allergic skin inflammation and its associated itching.

Basophil depletion by intravenous injection of Ba160 antibody decreases allergic skin inflammation elicited by EC sensitization with house dust mite (HDM). To examine whether the diminished allergic skin inflammation after basophil depletion in EC sensitization is antigen specific, BALB/c mice injected with Ba160 antibody and controls were EC sensitized with HDM, as depicted in Fig E4, *A,* in this article's Online Repository available at www.jacionline.org. Ba160-injected BALB/c mice EC sensitized with HDM exhibited decreased allergic skin inflammation, as evidenced by decreased epidermal hyperplasia, skin infiltration by CD45$^+$ cells, CD4$^+$ T cells, eosinophils, and ARG1$^+$ M2-like macrophages, but not mast cells, in OVA-sensitized skin compared to controls. Ba160 antibody–injected mice also had lower cutaneous *Il4* and *Il13,* but not *Il17a* or *Ifng,* mRNA expression (Fig E4, *B-E*). HDM-specific IgE was comparable between Ba160-injected mice and controls (Fig E4, *F*). In addition, splenocytes from Ba160-injected BALB/c mice secreted less IL-13, but not IL-4, IL-17A, or IFN-γ, after HDM stimulation (Fig E4, *G*). These results suggest that the role of basophils in allergic skin inflammation is not restricted to OVA.

Basophils promote the T_H2 response to EC sensitization. To investigate the role of basophils in the T_H2 response to EC sensitization, we first determined the kinetics of this response using 4get mice. Very few IL-4–producing CD4$^+$GFP$^+$ T cells were present in the skin dLNs of unmanipulated *Il4*$^{eGFPe/GFP}$ mice. After EC sensitization with OVA, their numbers remained the same at day 1, increased modestly at day 3, and substantially increased at day 5 (Fig 3, *A*). There were very few basophils in the skin dLNs of unmanipulated 4get mice (Fig 3, *B*). Notably, virtually all these basophils were positive for enhanced green fluorescent protein (eGFP; see Fig E5 in the Online Repository available at

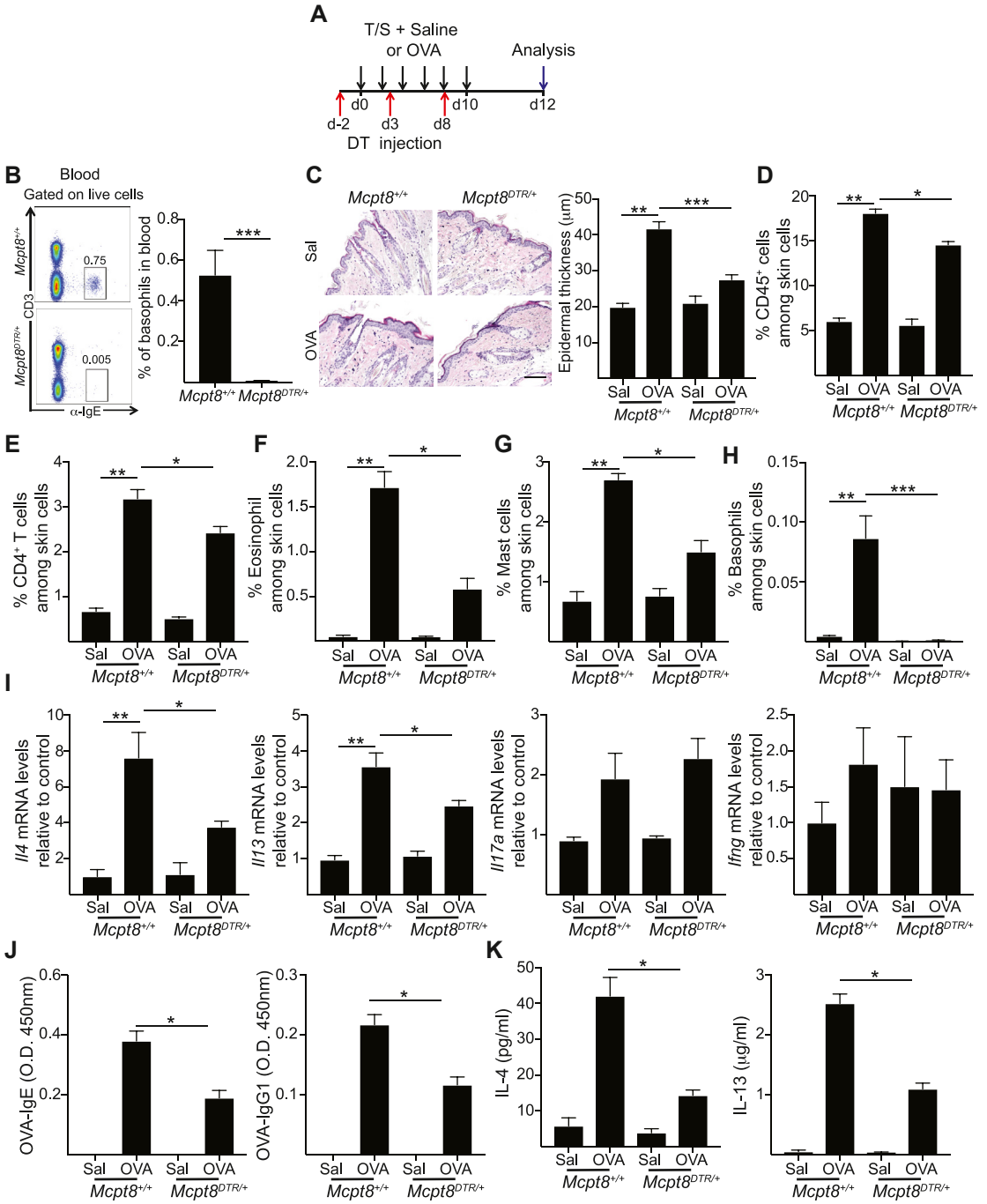

FIG 1. Basophils are required for acute allergic skin inflammation. **A,** Experimental protocol. **B,** Representative flow cytometry plot *(left)* and quantitation *(right)* of basophils in blood of DT-treated *Mcpt8*$^{DTR/+}$ mice and *Mcpt8*$^{+/+}$ controls. **C-K,** Representative hematoxylin and eosin staining *(C, left)* and epidermal thickness *(C, right)*, percentage of skin CD45$^+$ T cells *(D)*, CD4$^+$ T cells *(E)*, eosinophils *(F)*, mast cells *(G)*, and basophils *(H)*, mRNA levels of *Il4, Il13, Il17a,* and *Ifng* in skin expressed relative to mean of saline-sensitized wild-type controls *(I)*, serum OVA-specific IgE and IgG$_1$ *(J)*, and IL-4 and IL-13 secretion by OVA-restimulated splenocytes *(K)* in saline-sensitized and OVA-sensitized DT-injected *Mcpt8*$^{DTR/+}$ mice and *Mcpt8*$^{+/+}$ controls. *Bars* and *error bars* represent means ± SEMs. Results in *(B-K)* are representative of 2 independent experiments with 4-5 mice per group. **P* < .05, ***P* < .005.

www.jacionline.org), consistent with previous observations in 4get mice.[35] After EC sensitization with OVA, basophil numbers increased modestly on day 1, peaked on day 3, and diminished by day 5 (Fig 3, *B*)—and again, virtually all were eGFP$^+$ (Fig E5).

The kinetics of accumulation of IL-4$^+$ (eGFP$^+$) T cells and basophils suggested that basophils could play a role upstream of the T_H2 response to EC sensitization. To investigate this, we analyzed the number of CD3$^+$CD4$^+$IL-4$^+$IL-13$^+$ T_H2 cells in the skin

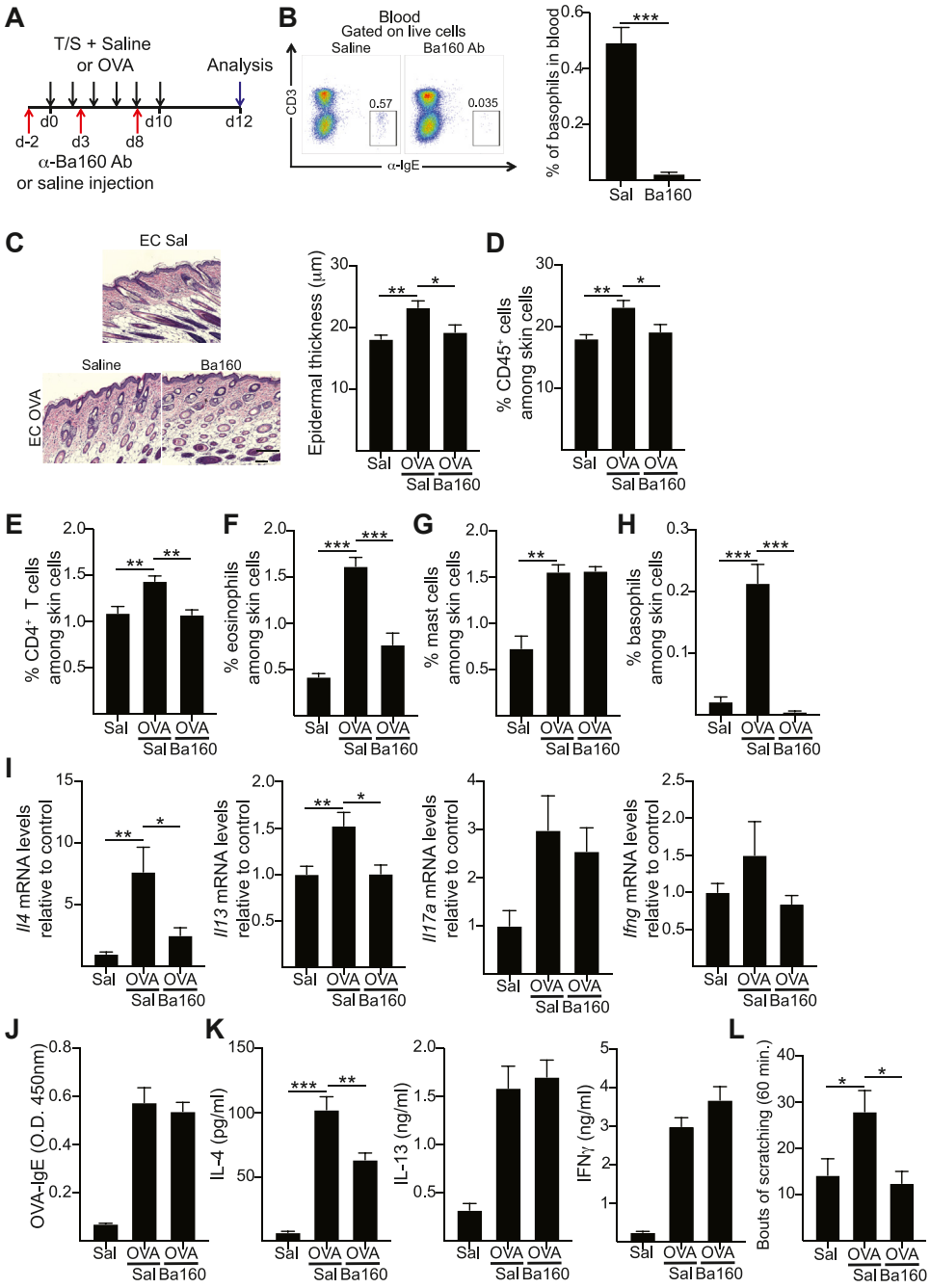

FIG 2. Basophils are required for acute allergic skin inflammation. **A,** Experimental protocol. **B,** Representative flow cytometry plot *(left)* and quantitation *(right)* of basophils in blood of saline (Sal)- or Ba160-injected wild-type (WT) mice. **C-L,** Representative hematoxylin and eosin staining *(C, left)* and epidermal thickness *(C, right),* percentage of skin $CD45^+$ T cells *(D),* $CD4^+$ T cells *(E),* eosinophils *(F),* mast cells *(G),* and basophils *(H),* and mRNA levels of *Il4, Il13, Il17a,* and *Ifng* in skin expressed relative to mean of Sal-sensitized WT controls *(I),* serum OVA-specific IgE *(J),* and IL-4, IL-13, and IL-17A secretion by OVA-restimulated splenocytes *(K)* and quantitation of spontaneous itch *(L)* in Sal-sensitized and OVA-sensitized WT mice injected with Ba160 antibody or vehicle (Sal). *Bars* and *error bars* represent means ± SEMs. Results in *(B-L)* are pool of 2 independent experiments with 4 mice per group. $*P < .05$, $**P < .005$, $**P < .001$.

dLNs of DT-injected $Mcpt8^{DTR/+}$ mice and DT-injected $Mcpt8^{+/+}$ controls. DT injection 2 days before EC sensitization efficiently depleted basophils from the skin dLNs of $Mcpt8^{DTR/+}$ mice at day 3 after EC sensitization (Fig 3, *C*). Importantly, this caused a significant reduction in the number of total $CD4^+$ T cells and $CD3^+CD4^+IL\text{-}4^+IL\text{-}13^+$ T_H2 cells in the skin dLNs at day 5 after EC sensitization (Fig 3, *D* and *E*). These results suggest that basophils play a role in the accumulation of

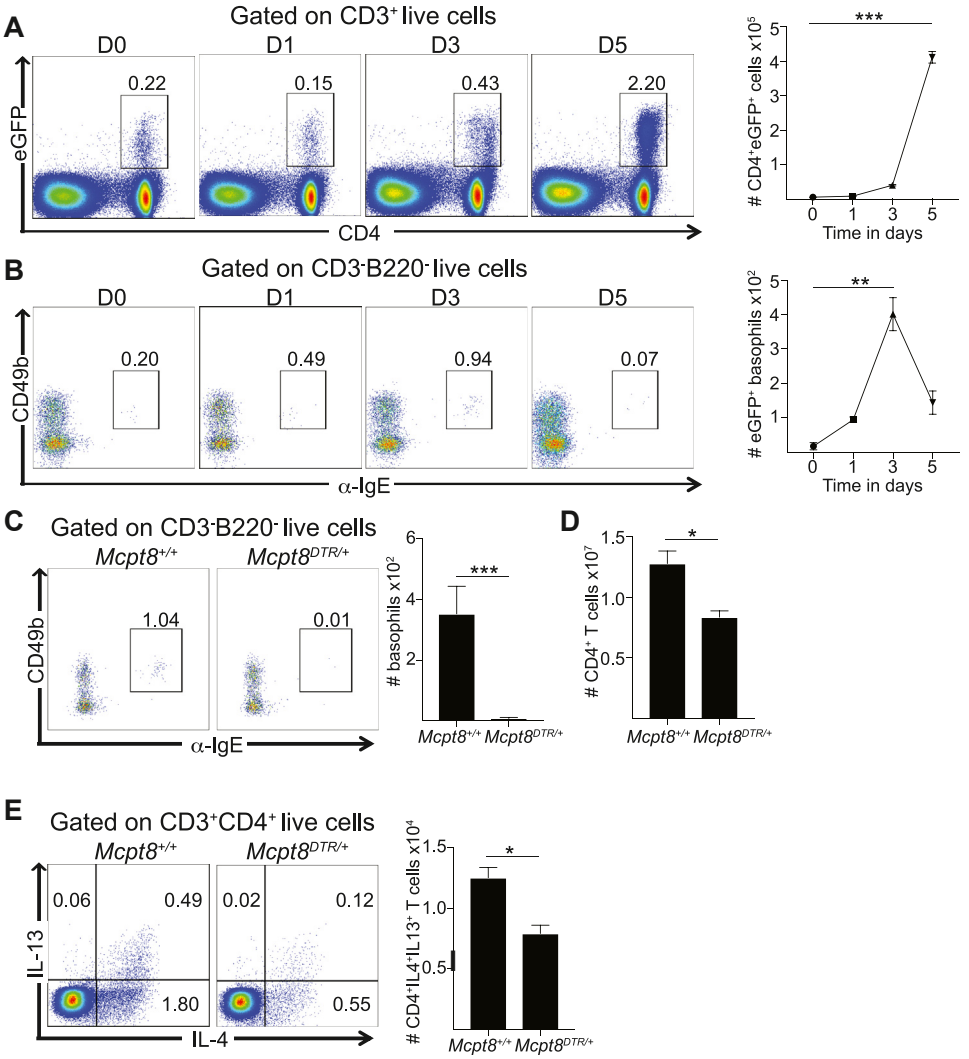

FIG 3. Basophils play an important role in T_H2 polarization induced by EC sensitization. **A** and **B**, Representative flow cytometry plot *(left)* and numbers *(right)* of $CD4^+eGFP^+$ T cells *(A)* and $eGFP^+$ basophils *(B)* in skin dLNs of 4get mice at different time points after EC sensitization with OVA. **C-E,** Representative flow cytometry plot and number of basophils *(C),* number of $CD4^+$ T cells *(D),* and representative flow cytometry plot and number of $CD4^+IL\text{-}4^+IL\text{-}13^+$ T_H2 cells *(E)* in skin dLNs of DT-injected *Mcpt8*$^{DTR/+}$ mice and *Mcpt8*$^{+/+}$ controls at day 5 of EC sensitization with OVA. *Bars* and *error bars* represent means ± SEMs. Results in *(A-E)* are representative of 2 independent experiments with 4-5 mice per group. **P* < .05, ***P* < .005.

$CD4^+$ T cells and T_H2 cells in lymph nodes that drain antigen-sensitized skin.

Basophils promote the ability of DCs from dLNs of OVA-sensitized skin to polarize antigen-specific naive T cells to a T_H2 phenotype. After cutaneous exposure to antigen, DCs capture antigen, upregulate surface activation markers, and migrate to the skin dLNs, where they activate and polarize recirculating naive antigen-specific T cells. DCs that express high levels of MHC-II antigens in the skin dLNs represent recent skin emigrants.[41] To determine whether basophils affect the function of skin-derived DCs, we first examined the effect of basophil depletion on antigen uptake by skin-derived DCs, their migration to dLNs, and their activation. DT-injected *Mcpt8*$^{DTR/+}$ mice and DT-injected *Mcpt8*$^{+/+}$ controls were EC sensitized with DQ-OVA, a self-quenched conjugated of OVA and fluorescent dye. On proteolytic degradation of DQ-OVA in their lysosomes, DCs exhibit a strong green fluorescence signal. Twenty-four hours later, after application of DQ-OVA to tape-stripped skin, skin dLNs were analyzed for $CD11c^+MHC\text{-}II^{high}$ recent skin emigrants that carry DQ-OVA. The numbers of $CD11c^+MHC\text{-}II^{high}DQ\text{-}OVA^+$ DCs in skin dLNs, as well as the intensity of DQ-OVA fluorescence in these cells, were comparable in DT-injected *Mcpt8*$^{DTR/+}$ mice and DT-injected *Mcpt8*$^{+/+}$ controls (Fig 4, *A* and *B*). In addition, expression of the activation markers CD40, CD80, and CD86 by $CD11c^+MHC\text{-}II^{high}DQ\text{-}OVA^+$ cells in skin dLNs was comparable between the 2 mouse strains (Fig 4, *C*). These findings indicate that antigen uptake/processing, migration, and activation of skin-derived DCs after EC sensitization is independent of basophils.

We next investigated the role of basophils in modulating the ability of skin-derived DCs to cause proliferation and polarization of naive antigen-specific $CD4^+$ T cells. $CD11c^+$ DCs were purified from skin dLNs 24 hours after OVA sensitization of DT-

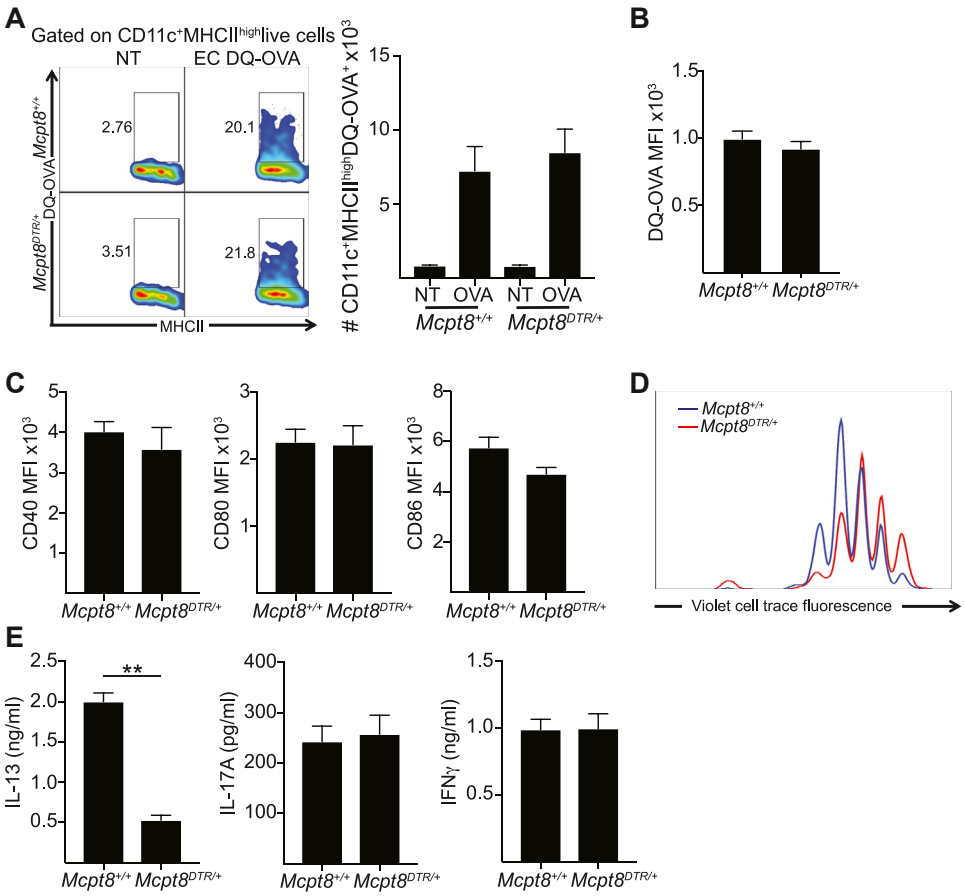

FIG 4. Basophils promote ability of skin-derived DCs to drive T_H2 polarization. **A,** Representative flow cytometry plot *(left)* and number *(right)* of $CD11c^+MHC\text{-}II^{high}DQ\text{-}OVA^+$ DCs in skin dLNs in unmanipulated (NT) DT-injected *Mcpt8*$^{DTR/+}$ mice and *Mcpt8*$^{+/+}$ controls sensitized with DQ-OVA 24 hours before analysis. **B** and **C**, Mean fluorescence intensity (MFI) of DQ-OVA fluorescence *(B)* and CD40, CD80, and CD86 expression *(C)* by $CD11c^+MHC\text{-}II^{high}DQ\text{-}OVA^+$ DCs in skin dLNs of DT-injected *Mcpt8*$^{DTR/+}$ mice and *Mcpt8*$^{+/+}$ controls sensitized with DQ-OVA 24 hours before analysis. **D** and **E**, Representative flow cytometry histogram of proliferation of $CD4^+$ T cells *(D)* and IL-13, IL-17A, and IFN-γ secretion *(E)* by naive CellTrace Violet (CTV)-loaded $CD4^+$ T cells from DO11.10 mice cocultured with DCs from skin dLNs from DT-injected *Mcpt8*$^{DTR/+}$ mice and *Mcpt8*$^{+/+}$ controls EC sensitized with OVA 24 hours before analysis. *Bars* and *error bars* represent means ± SEMs. Results in *(A-E)* are representative of 2 independent experiments with 4-5 mice per group. **P* < .05, ***P* < .005.

treated *Mcpt8*$^{DTR/+}$ mice and DT-treated *Mcpt8*$^{+/+}$ controls and cocultured with splenic naive $CD4^+$ T cells from DO.11.10 mice, which express in T cells a transgenic T-cell receptor specific for an $OVA_{323\text{-}339}$ peptide. No exogenous OVA was added to the cultures. DCs from DT-injected *Mcpt8*$^{DTR/+}$ mice caused less proliferation and markedly less production of IL-13 by naive $CD4^+$ T cells compared to DCs from DT-injected *Mcpt8*$^{+/+}$ littermates (Fig 4, *D* and *E*). No difference was observed in the ability of DCs from the 2 strains to drive IL-17A and IFN-γ production by naive $CD4^+$ T cells (Fig 4, *E*). Our results indicate that basophils play an important role in causing skin-derived DCs to promote T_H2 polarization, and therefore the induction of a T_H2 response to EC sensitization.

Basophils are a major source of cutaneous IL-4 at later time points in allergic skin inflammation after EC sensitization. To investigate the contribution of basophils to IL-4 production in antigen-sensitized skin, we subjected 4get mice to EC sensitization with OVA and examined their skin on day 12. Skin cells were isolated and gated for eGFP (IL-4) expression; then $eGFP^+$ cells were analyzed for cellular markers. As expected, all $eGFP^+$ cells were in the $CD45^+$ cell population and accounted for 23.8 ± 3.1% of $CD45^+$ cells (n = 4 mice) (Fig 5, *A*). $CD3^+$ T cells accounted for 2 ± 0.5% of $eGFP^+$ cells, and virtually all $CD3^+eGFP^+$T cells (95%) were $CD4^+$ (Fig 5, *B*). Eosinophils ($CD45^+CD4^-GR1^+SiglecF^+$) and mast cells ($CD45^+CD4^-\alpha\text{-}IgE^+CD117^+$) were represented almost equally in the $eGFP^+$ population at 37.2 ± 1.2% and 41.0 ± 14.5%, respectively. Basophils ($CD45^+CD4^-\alpha\text{-}IgE^+CD117^-$) represented 12.5 ± 0.5% of $eGFP^+$ cells (Fig 5, *A* and *B*).

Innate immune cells that potentially produce IL-4 in mice include basophils, eosinophils, and mast cells, as indicated by their constitutive expression of eGFP in 4get mice.[35] However, not all of these cells transcribe *Il4*.[42] To assess the potential contribution of different cell types to *Il4* expression in OVA-sensitized skin, subpopulations of $CD45^+$ cells were sorted from OVA-sensitized skin and analyzed for *Il4* expression by real-time reverse transcription–quantitative PCR. *Il4* mRNA levels were highest in basophils and exceeded by ~100-fold those found

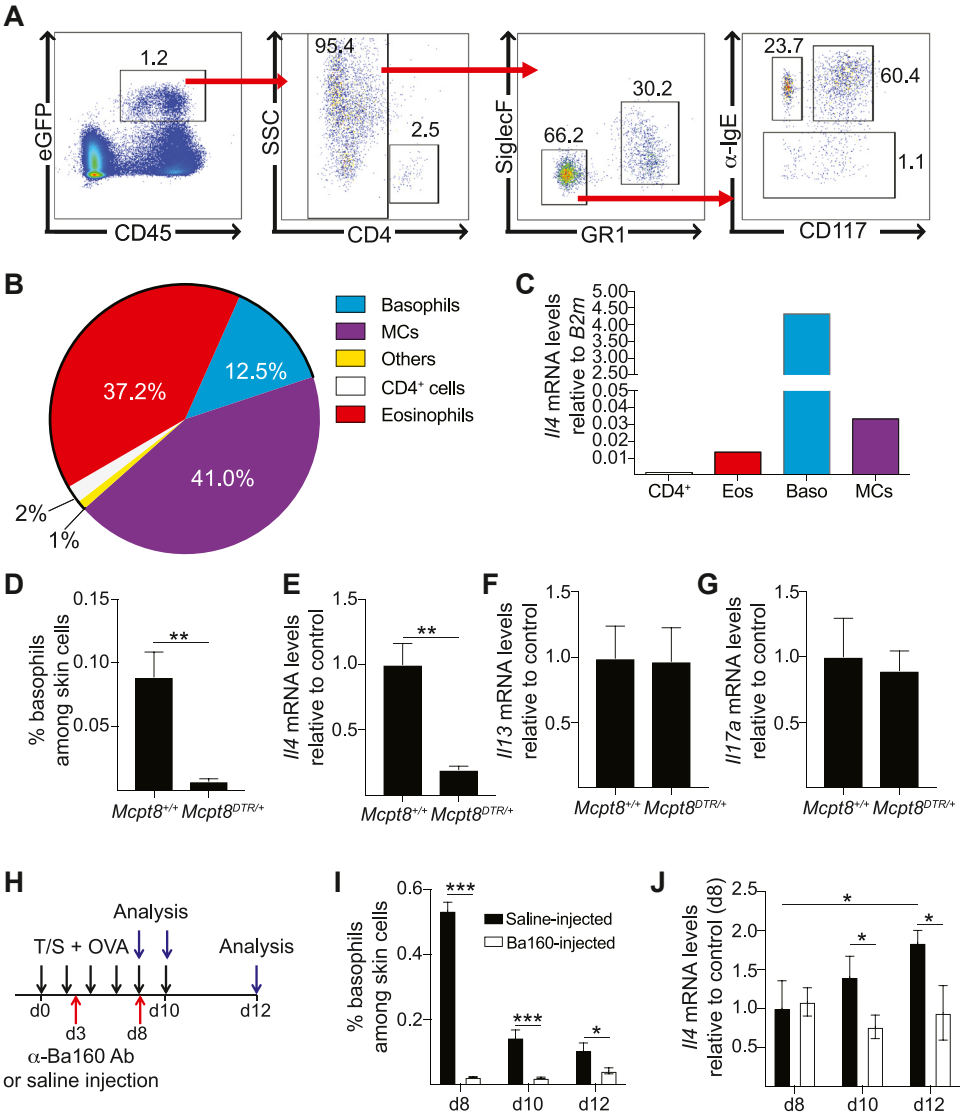

FIG 5. Basophils are a major source of IL-4 in acute allergic skin inflammation. **A,** Gating strategy for fluorescence-activated cell sorting analysis and sorting of $CD4^+$ T cells, eosinophils, mast cells, and basophils expressing IL-4 (eGFP). **B,** Pie graph of distribution of $eGFP^+$ cells in OVA-sensitized skin from 4get mice. **C,** *Il4* mRNA levels in sorted cells from OVA-sensitized skin of wild-type (WT) mice. *Baso,* Basophils; *Eos,* eosinophils; *MC,* mast cells. **D-G,** Percentage of basophils among skin cells *(D)* and mRNA levels of *Il4 (E), Il13 (F),* and *Il17a (G)* in OVA-sensitized skin of $Mcpt8^{DTR/+}$ mice and $Mcpt8^{+/+}$ controls injected with DT at day 7 and analyzed at day 12. **H,** Experimental protocol. **I** and **J,** Percentage of basophils among skin cells *(I)* and mRNA levels of *Il4 (J)* at different time points in OVA-sensitized skin of OVA-sensitized WT mice injected with Ba160 antibody or vehicle (saline). *Bars* and *error bars* represent means ± SEMs. Results in *(B)* and *(D-G)* are representative of 2 independent experiments with 4-5 mice per group. Results in *(I)* and *(J)* are representative of 1 experiment with 4 mice per group. $^{**}P < .005$.

in mast cells and eosinophils (Fig 5, *C*). *Il4* expression in $CD4^+$ T cells was negligible at this time point (Fig 5, *C*).

To examine the contribution of basophils to *Il4* expression in OVA-sensitized skin, we depleted basophils at a late stage of EC sensitization. $Mcpt8^{DTR/+}$ and $Mcpt8^{+/+}$ mice were injected with DT at day 7 of EC sensitization with OVA, and the basophil numbers and *Il4* mRNA levels were evaluated at day 12. As expected, basophils were almost undetectable in DT-injected $Mcpt8^{DTR/+}$ mice EC sensitized with OVA (Fig 5, *D*). Basophil depletion on day 7 resulted in a drastic reduction in *Il4,* but not *Il13* or *Il17a,* mRNA levels in OVA-sensitized skin (Fig 5, *E-G*).

We performed kinetic analysis to determine the contribution of basophils to cutaneous *Il4* expression during the later phase of EC sensitization on days 8, 10, and 12. In these experiments, basophil depletion was performed by injecting wild-type mice with Ba160 antibody on days 3 and 8 of the 10-day EC sensitization protocol (Fig 5, *H*). Efficient depletion of basophils in skin was noted at all 3 time points (Fig 5, *I*). *Il4* mRNA expression in OVA-sensitized skin of control mice increased from day 8 until day 12 (Fig 5, *J*). Basophil depletion did not affect *Il4* mRNA expression at day 8, but it significantly impaired *Il4* upregulation at later time points (Fig 5, *J*). This result suggests that basophils are required for

cutaneous *Il4* induction and are likely the major source of this cytokine at later time points in our antigen-driven model of allergic skin inflammation.

DISCUSSION

Our study demonstrates that basophils are important for the development of allergic skin inflammation.

Using 2 different strategies for basophil depletion, basophil depletion significantly reduced allergic skin inflammation driven by cutaneous sensitization with antigen. This is in line with the important role that basophils play in skin inflammation induced by repeated cutaneous injection of hapten protein conjugate in recipients of IgE–anti-trinitrophenyl, or by repeated topical application of oxazolone or OVA peptide.[43-45] However, this contrasts with the redundant role of basophils in chronic EC sensitization induced by repeated OVA application to tape-stripped skin for 7 weeks.[44] Thus, basophils are essential during the acute phase of allergic skin inflammation, whereas their role in chronic allergic skin inflammation could be circumvented by other mechanisms, such as accumulation of allergen-specific T_H2 cells and activation of mast cells by allergen-specific IgE.

Our results indicate that basophils play an important role in the itch induced by short-term EC sensitization with OVA. This is in line with a recent report highlighting the key role of basophils in promoting itch, especially during flares of inflammation in AD patients.[31] Basophils that infiltrate AD lesional skin have the potential to produce several molecules involved in itch (eg, the cytokines IL-4, IL-13, and IL-31), histamine, prostaglandins, cysteinyl leukotrienes, and substance P.[46] The molecular mechanisms that underlie basophil-dependent itching in our model remain to be evaluated.

Our results indicate that basophils are required for an optimal T_H2 response to short-term EC sensitization. Importantly, our results uncover an interesting and novel role for basophils in promoting T_H2 polarization of naive T cells by skin-derived DCs. These findings are consistent with the demonstrated role of basophils in the T_H2 response in mouse models of allergic skin inflammation induced by subcutaneous injection of cysteine proteases plus antigen or topical application of MC903.[18,47] In contrast, basophils are dispensable for the T_H2 response in experimental asthma induced by repeated inhalation of HDMs.[48]

In addition to their role in driving the T_H2 response to EC sensitization, our results indicate that basophils are the major source of IL-4 at cutaneous sites of allergic skin inflammation later in the response to EC sensitization with antigen (days 10-12), whereas other innate cells are likely sources of cutaneous IL-4 earlier (day 8) in the course of this response. The role of basophils as a major source of IL-4 later in the course of allergic skin inflammation agrees with our recent findings using single cell RNA sequencing analysis of OVA-sensitized mouse skin on day 12 after sensitization.[16] It is also consistent with the pathogenic role of basophils and IL-4 in allergic skin inflammation induced by repeated application of oxazolone and MC903.[25-28] Importantly, basophils infiltrate AD lesional skin and have the capacity to produce IL-4; further, their numbers correlate with infiltration by $CD4^+$ T cells.[28,29,49] Whether basophils are a major source of IL-4 in the lesional skin of patients with AD and what potential role basophil derived IL-4 plays in disease severity need further study.

Limitations of this study include the potential of target effects of DT injection in *Mcpt8*$^{DTR/+}$ mice,[50] which include the possible deletion of granulocyte-macrophage progenitors and mature cells (eosinophils and neutrophils),[40] as well as the potential activation of mast cells by injection of CD200R3 (Ba160) antibody.[51]

DISCLOSURE STATEMENT

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Key messages

- **Basophils are important for the development of allergic skin inflammation induced by EC sensitization in mice.**
- **Basophils are the major source of IL-4 in EC-sensitized mouse skin.**
- **Basophils promote DC-driven T_H2 polarization of naive $CD4^+$ T cells.**

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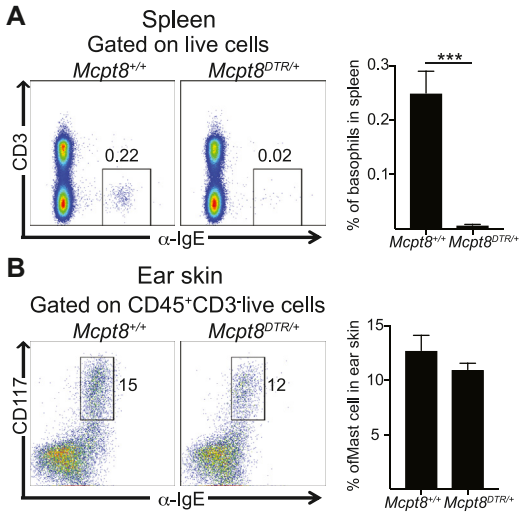

FIG E1. DT treatment in *Mcpt8*$^{DTR/+}$ mice effectively deplete spleen basophils without affecting dermal mast cells. Representative flow cytometry plot *(left)* and quantitation *(right)* of spleen basophils **(A)** and dermal mast cells **(B)** of DT-treated *Mcpt8*$^{DTR/+}$ mice and *Mcpt8*$^{+/+}$ controls. *Bars* and *error bars* represent means ± SEMs. Results are representative of 2 independent experiments with 4-5 mice per group. ***$P < .001$.

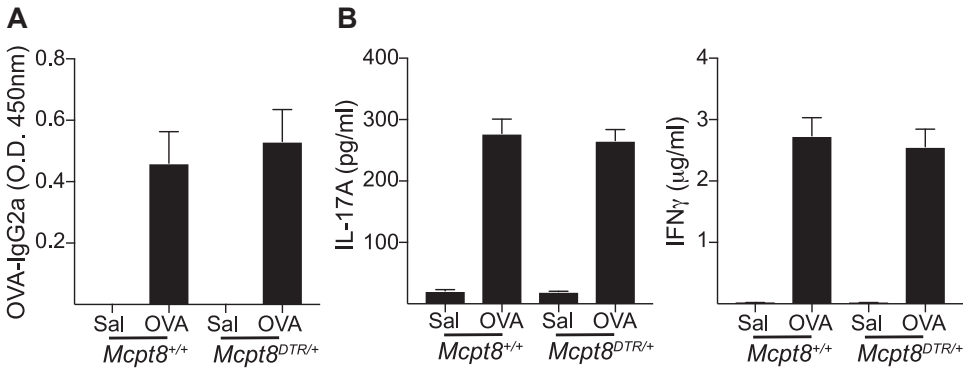

FIG E2. Basophil depletion does not affect serum IgG_2a or secretion of IL-17A and IFN-γ induced by EC sensitization. **A** and **B**, OVA-specific IgG_2a *(A)* and IL-17A and IFN-γ secretion by OVA-restimulated splenocytes *(B)* in saline-sensitized and OVA-sensitized skin of DT-injected *Mcpt8*$^{DTR/+}$ mice and *Mcpt8*$^{+/+}$ controls. *Bars* and *error bars* represent means ± SEMs. Results in *(B-K)* are representative of 2 independent experiments with 4-5 mice per group.

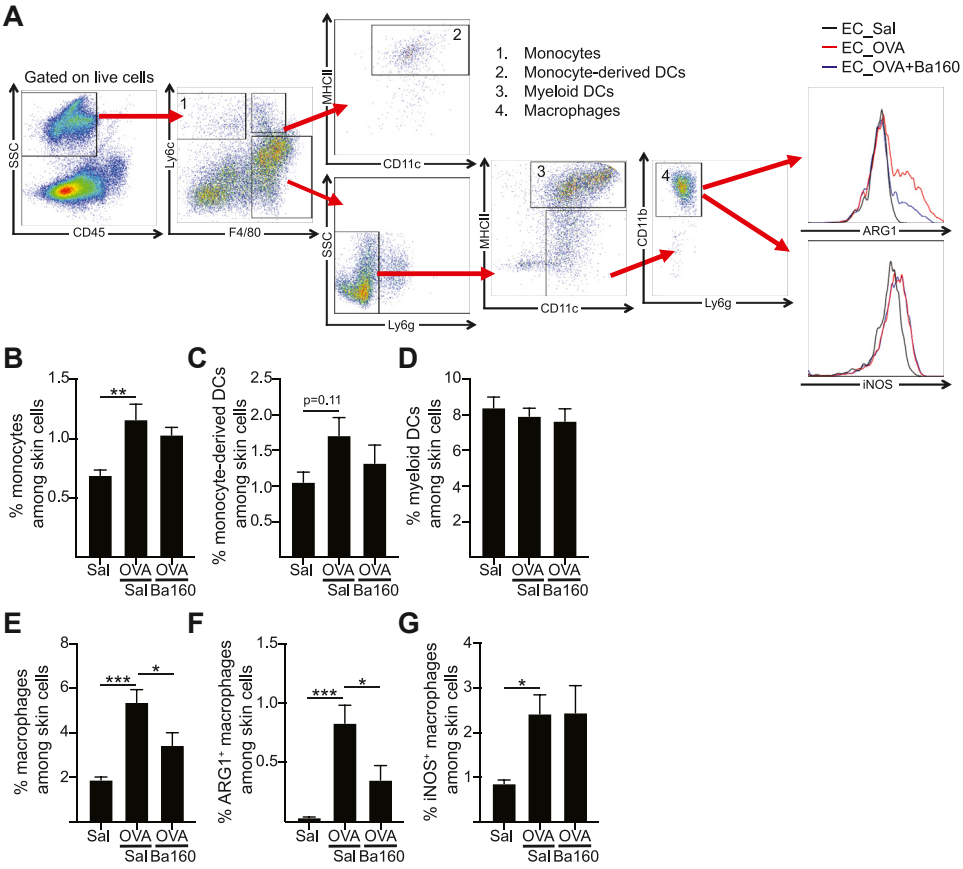

FIG E3. Basophil depletion decreased accumulation of ARG1^{+} macrophages without affecting other myeloid cells. **A,** Representative flow cytometry plots showing gating strategy for CD45^{+}F4/80^{-}Ly6c^{+} monocytes *(1),* CD45^{+}F4/80^{+}Ly6c^{+}CD11c$^{+/high}$MHC-II$^{+/high}$ monocyte-derived DCs *(2),* CD45^{+}CD11b^{+}F4/80^{+}Ly6g^{-}Ly6c^{-}CD11c$^{+/high}$MHC-II$^{+/high}$ myeloid DCs *(3),* and CD45^{+}CD11b^{+}F4/80^{+}Ly6g^{-}Ly6c^{-}CD11c^{+}MHC-II$^{low/+}$macrophages *(4),* as well as representative histograms showing expression of ARG1 and iNOS in macrophages. **B-G,** Percentage of monocytes *(B),* monocyte-derived DCs *(C),* myeloid DCs *(D),* macrophages *(E),* ARG1^{+} macrophages *(F),* and iNOS^{+} macrophages *(G)* in saline (Sal)-sensitized and OVA-sensitized skin of wild-type (WT) mice injected with Ba160 antibody or vehicle (Sal). *Bars* and *error bars* represent means ± SEMs. Results are pool of 2 independent experiments with 4 mice per group. **P* < .05, ***P* < .005, ***P* < .001.

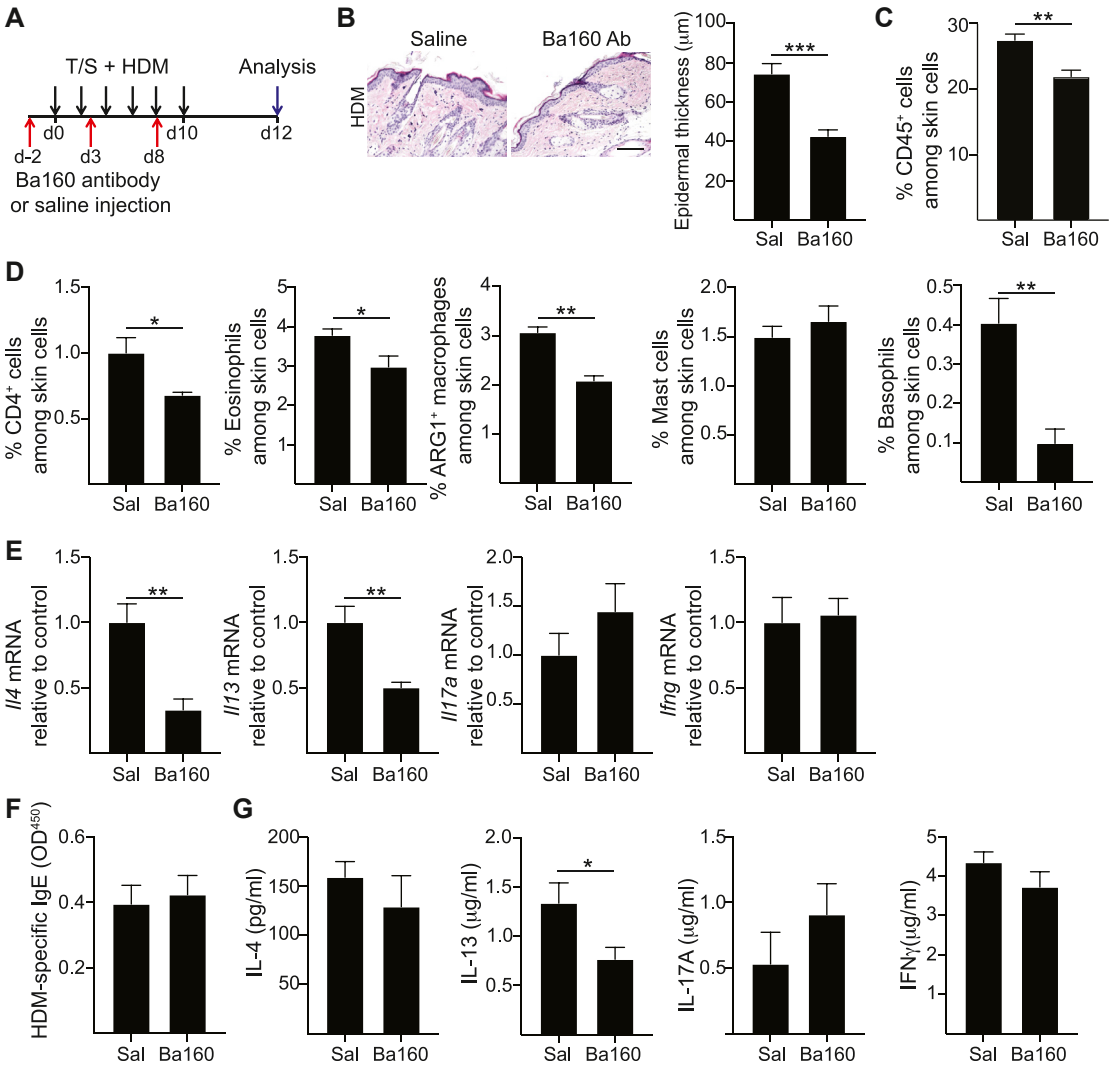

FIG E4. Basophils are required for acute allergic skin inflammation elicited by EC sensitization with HDM. **A,** Experimental protocol. **B-G,** Representative hematoxylin and eosin staining *(B, left)* and epidermal thickness *(B, right),* percentage of skin CD45$^+$ T cells *(C),* CD4$^+$ T cells, eosinophils, ARG1$^+$ macrophages, mast cells, and basophils *(D),* mRNA levels of *Il4, Il13, Il17a,* and *Ifng* in skin expressed relative to mean of saline (Sal)-sensitized wild-type (WT) controls *(E),* serum HDM-specific IgE *(F),* and cytokine secretion by OVA-restimulated splenocytes *(G)* in Sal-sensitized and OVA-sensitized skin of WT mice injected with Ba160 antibody or Sal. *Bars* and *error bars* represent means ± SEMs. Results are from 1 experiment with 5 mice per group. **P* < .05, ***P* < .005, ***P* < .001.

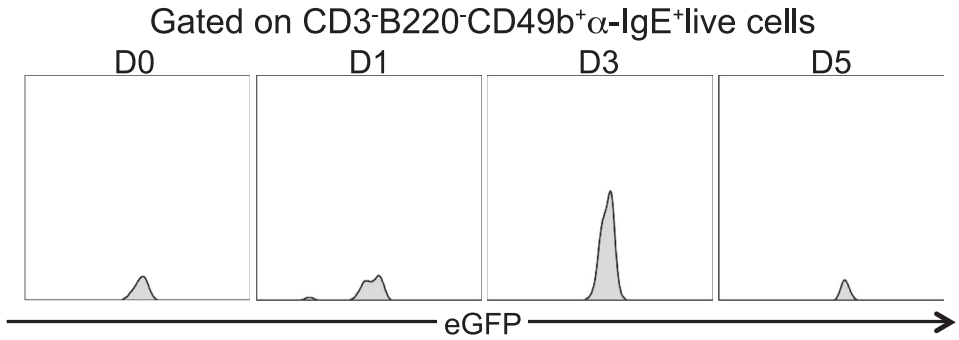

FIG E5. Basophils infiltrating skin dLNs during EC sensitization with OVA express IL-4 (eGFP). Representative flow cytometry histograms of IL-4 (eGFP) expression in basophils in skin dLNs of 4get mice at different time points after EC sensitization with OVA.