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Basophils Promote Innate Lymphoid Cell Responses in Inflamed Skin

Brian S. Kim,*†‡§†‡§‡, Kelvin Wang,*†‡§‡, Mark C. Siracusa,*†‡§‡, Steven A. Saenz,*†‡§, Jonathan R. Brestoff,*†‡ Laurel A. Monticelli,*†‡ Mario Noti,*†‡ Elia D. Tait Wojno,*†‡ Thomas C. Fung,*†‡ Masato Kubo,*†‡ and David Artis,*†‡

Type 2 inflammation underlies allergic diseases such as atopic dermatitis, which is characterized by the accumulation of basophils and group 2 innate lymphoid cells (ILC2s) in inflamed skin lesions. Although murine studies have demonstrated that cutaneous basophil and ILC2 responses are dependent on thymic stromal lymphopoietin, whether these cell populations interact to regulate the development of cutaneous type 2 inflammation is poorly defined. In this study, we identify that basophils and ILC2s significantly accumulate in inflamed human and murine skin and form clusters not observed in control skin. We demonstrate that murine basophil responses precede ILC2 responses and that basophils are the dominant IL-4–enhanced GFP-expressing cell type in inflamed skin. Furthermore, basophils and IL-4 were necessary for the optimal accumulation of ILC2s and induction of atopic dermatitis–like disease. We show that ILC2s express IL-4Rα and proliferate in an IL-4–dependent manner. Additionally, basophil-derived IL-4 was required for cutaneous ILC2 responses in vivo and directly regulated ILC2 proliferation ex vivo. Collectively, these data reveal a previously unrecognized role for basophil-derived IL-4 in promoting ILC2 responses during cutaneous inflammation.

a significant accumulation of these clusters in AD-associated skin in comparison with healthy control skin. Temporal analyses revealed that the accumulation of basophils in murine skin precedes that of ILC2s in the context of AD-like inflammation. Furthermore, loss- and gain-of-function studies demonstrated that basophils are required to promote cutaneous ILC2 responses in vivo. Strikingly, employing IL-4–enhanced GFP (eGFP) (4get) reporter mice, we found that basophils were the dominant source of IL-4–eGFP expression in AD-like skin. Both basophils and IL-4 were necessary for the optimal accumulation of ILC2s and induction of AD-like disease. Further examination of ILC2s revealed cell surface expression of IL-4Rα and their dependence on IL-4 for proliferation in AD-like inflammation. Basophil-derived IL-4 was required for cutaneous ILC2 responses in vivo and directly regulated ILC2 proliferation ex vivo. Collectively, these studies identify that basophils are early regulators of ILC2 responses in the context of cutaneous type 2 inflammation and AD-like disease.

Materials and Methods

Patients and samples

Human skin samples were assessed as previously described for flow cytometry, and basophils were defined as CD123+FcεR1α+ cells negative for expression of CD3, CD3ε, CD19, CD19, and c-KIt, whereas ILC2s were defined as CD25+IL-33R+ cells negative for expression of lineage (CD3, CD1c, CD19, CD56, and FcεR1α) markers (10, 20). Experiments using human skin samples were approved by the University of Pennsylvania Institutional Review Board (protocol 814945).

Mice and samples

C57BL/6 wild-type (WT), BALB/c WT, 4get, Rag1−/−, and Il4−/− mice were purchased from The Jackson Laboratory. H4Rα−/− mice were purchased from Taconic. BaS-TREck (BaS) mice were provided by Dr. M. Kubo (Tokyo). All mice were treated with MC903 as previously described (9, 20, 27). Murine skin samples were assessed as previously described for flow cytometry, and basophils were defined as CD123+FcεR1α+ cells negative for expression of CD3, CD3ε, CD1c, CD19, NK1.1, and c-KIt, whereas ILC2s were defined as CD25+IL-33R+ cells negative for expression of lineage (CD3, CD5, CD11b, CD11c, B220, NK1.1, and FcεR1α) markers (10, 20). Splenic basophils were sort-purified from TSLP cDNA plasmid-treated WT or Il4−/− mice using a BD FACSAria cell sorter. ILC2s were sort-purified from pooled skin-draining lymph nodes, mesenteric lymph nodes, and peritoneal cavity and adipose tissue as previously described (20, 28). Annexin V, 7-aminoactinomycin D (7-AAD), killer-cell lectin-like receptor G1 (KLRG1), CD45, and Ki67 staining of ILC2s was performed as previously described (29–31). Experiments were performed according to the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee.

Histology

For all human and murine immunofluorescence (IF) microscopy, paraffin-embedded 5-μm skin sections were rehydrated and incubated with primary Ab at 4°C overnight, followed by incubation with secondary Abs at 37°C for 30 min. For human samples, primary Abs against 2D7 (1:250, BioLegend, Ab mouse IgG1), IL-33R (1:250, MD Bioproducts, biotin-conjugated mouse IgG1), or CD3 (1:50, Dako, rabbit IgG) and secondary Abs to mouse IgG conjugated with PE-Cy5 (1:300), streptavidin-Cy3 (1:300), or rabbit IgG conjugated with Cy2 (1:300), respectively, were used. Murine samples were stained with H&E or primary Abs against Mcp8 (1:500, TUG8, BioLegend, rat IgG2) or IL-33R (1:300, D8, MD Bioproducts, biotin-conjugated rat IgG1) and secondary Abs to rat IgG conjugated with PE-Cy5 (1:300) and streptavidin-Cy3 (1:300), respectively. Sections were placed in mounting medium with DAPI (Vectorshield).

Histology score and ear thickness measurements

Histology score was determined by the following formula: (total number of lymphocytes per high-power field [HPF] + thickness of the epidermis measured in micrometers from the basement membrane to the top of the stratum corneum)/100. The ear thickness was measured on day 7 following treatment by measuring the entire width of the ear at the widest point in micrometers from the top of one stratum corneum to the top of the stratum corneum on the opposite side of the ear. All measurements were performed using the National Institutes of Health ImageJ program.

In vitro/ex vivo cultures

Sort-purified basophils from the spleens of TSLP cDNA plasmid-treated WT and Il4−/− mice were cultured for 24 h at 37°C in supplemented DMEM with mIL-3 (10 ng/ml, R&D Systems) as previously described (9). CFSE-labeled ILC2s (20,000 cells) from mIL-33–treated mice were then cultured in 200 μl of WT and Il4−/− basophil-conditioned media for 4 d at 37°C with mIL-7 (R&D Systems) as previously described (20), and cell division was assessed by FACS analysis of CFSE dye dilution as previously described (32).

Basophil–ILC2, basophil–T cell, and ILC2–T cell cluster analysis

Basophil–ILC2 (yellow circle), basophil–T cell (green circles), and ILC2–T cell (red circle) clusters were analyzed by drawing 25-μm colored circles around the cell clusters per HPF.

Statistical analysis

Data are means ± SEM unless indicated otherwise. Statistical significance was determined by an unpaired Student t test or one-way ANOVA for multiple comparisons using GraphPad Prism 6 software. The p values are denoted for each analysis.

Results

Basophils and ILC2s are enriched and found in close proximity to each other in the dermis of human AD lesions

We recently reported that murine basophils and ILC2s are elicited in a TSLP-dependent manner during AD-like inflammation (9, 20). Consistent with this, basophils have been identified in skin lesions from AD patients, and we recently showed that ILC2s are enriched in the inflamed skin of patients (20, 33). However, whether basophils and ILC2s accumulate in the same lesions and whether these cell types colocalize in inflamed human skin have not been examined. To address these issues, 4-mm punch biopsies of normal and diseased human skin were obtained from healthy control subjects and AD patients. Strikingly, frequencies of CD123+FcεR1α+ basophils (Fig. 1A) and lineage (Lin−) CD25+IL-33R+ ILC2s (Fig. 1B) were markedly elevated in lesional human AD skin, indicating that basophil and ILC2 responses occur simultaneously in the same inflamed lesion.

To visualize the distribution of cutaneous basophils and ILC2s, IF microscopy was employed. Basophils were identified by staining with an Ab against the basophil-specific granule marker 2D7 (34). Consistent with the flow cytometric analyses (Fig. 1A), 2D7+ basophils were undetectable in healthy control skin (Supplemental Fig. 1A) but were readily identified in lesional AD skin (Fig. 1C, Supplemental Fig. 1B). To visualize ILC2s and identify non–T cell populations that express the IL-33R, sections were co-stained with anti-CD3 Ab and anti–IL-33R Ab. Although IL-33R+ cells and CD3+ T cells were rarely identified in healthy control skin (Supplemental Fig. 1C), both were enriched in lesional AD skin and accumulated in a perivascular distribution in the dermis (Fig. 1D, Supplemental Fig. 1D). Most IL-33R+ cells were negative for CD3 (Fig. 1D, white arrows), suggesting that these IL-33R+ cells are ILC2s. Finally, 2D7+ basophils and IL-33R+ ILC2s accumulated in close proximity to each other in the papillary dermatitis in lesional human AD skin compared with healthy control skin (Fig. 1E–G, Supplemental Fig. 1E, 1F). Although our analyses demon-
strated that some basophils express both 2D7 and IL-33R (data not shown), the vast majority of basophils were single-positive (2D7+ IL-33R2) by IF (Fig. 1E [white arrows], Supplemental Fig. 1F). Taken together, analyses of the flow cytometry data and microscopy images indicate that basophils and ILC2s are enriched in the dermis of lesional skin from AD patients and accumulate in close proximity in inflamed lesions.

To quantify basophil–ILC2 clusters in the skin, 2D7+ cell–IL-33R+ cell groupings were counted in both healthy control and lesional AD skin (Fig. 1F–H). Enumeration of these groupings identified a marked and significant increase of basophil–ILC2 clusters in the inflamed skin (Fig. 1H). Additional analyses of CD3+ T cells and their clustering with basophils and ILC2s identified a significant increase of both basophil–T cell clusters (Fig. 1I) as well as ILC2–T cell clusters (Fig. 1J). A three-way comparison of basophil–ILC2, basophil–T cell, and ILC2–T cell clusters demonstrated that basophil–ILC2 and ILC2–T cell clusters are more numerous than basophil–T cell clusters (Fig. 1K). Taken together, these findings provoke the hypothesis that basophils, ILC2s, and T cells interact in vivo.

**Cutaneous basophil responses precede ILC2 responses in AD-like inflammation**

To test whether murine basophils and ILC2s also accumulate in murine AD-like skin lesions, a previously established mouse model of AD-like disease was employed (19, 20) in which WT mice were treated topically with vehicle (EtOH) or a vitamin D analog (MC903) (20). On day 7 of treatment, flow cytometric analysis revealed that CD49b+FceRIα+ basophils (Fig. 2A) and Lin–CD25+IL-33R+ ILC2s (Fig. 2B) were significantly enriched in AD-like skin lesions compared with healthy control skin. Similar to results obtained using human samples, IF microscopy revealed that basophils (identified by staining with anti-Mcpt8 murine basophil-specific marker Ab) could not be detected and ILC2s (identified by staining with anti–IL-33R Ab) were rare in control EtOH-treated healthy murine skin (Fig. 2C). In contrast, Mcpt8+ basophils and IL-33R+ innate cells accumulated and colocalized in clusters within the papillary dermis in AD-like lesional skin from MC903-treated lymphocyte-deficient Rag12/− mice (Fig. 2D, 2E). Collectively, these findings suggest that basophils and ILC2s reside in close proximity in inflamed human and murine skin.
Whereas basophils and ILC2s accumulate in close proximity to each other in the inflamed skin of humans and mice, whether functional interactions or cross-regulation occurs between these cell populations is unknown. To examine this, we performed temporal analysis of cutaneous basophil and ILC2 responses following elicitation of AD-like disease. By day 4 after MC903 treatment, cutaneous basophil responses were markedly elevated (Fig. 2F, 2G). In contrast, frequencies and absolute numbers of ILC2s were not significantly increased in MC903-treated mice at this early time point relative to controls (Fig. 2F, 2H) and did not increase in the skin until day 7 posttreatment (Fig. 2F), indicating that cutaneous basophil responses precede ILC2 responses.

ILC2 responses are dependent on basophils in the context of skin inflammation

To test whether early cutaneous basophil responses influence ILC2 responses in vivo, we employed BaS mice, which harbor basophils that selectively express the simian diphtheria toxin receptor (35). Treatment of BaS mice with diphtheria toxin resulted in selective depletion of cutaneous basophils during inflammation (9, 10). Treatment of BaS mice with both MC903 and diphtheria toxin resulted in effective depletion of basophils in the inflamed skin (Fig. 3A, 3B), which was associated with a reduction in skin ILC2 responses (Fig. 3C, 3D), CD4+ T cell responses (Supplemental Fig. 2), histological findings of epidermal hyperplasia (acanthosis), thickening of the stratum corneum (hyperkeratosis) and dermal inflammation (Fig. 3E, 3F), and ear thickness (Fig. 3G). These data suggest that basophil responses precede, and are necessary for, ILC2 responses in the context of skin inflammation.

To test whether basophils are sufficient to promote skin-associated ILC2 responses and AD-like inflammation, gain-of-function studies were performed. Basophils were sort-purified from WT mice and adoptively transferred by i.d. injection into the skin of naive WT mice. Flow cytometric analysis of the skin of basophil-injected mice revealed elevated frequencies of ILC2s (Fig. 3H, 3I) and the induction of AD-like disease, histologically characterized by a mixed dermal inflammatory infiltrate associated with acanthosis and hyperkeratosis (Fig. 3J). These findings indicate that basophils are sufficient for the induction of ILC2 responses and AD-like skin inflammation.

Multiple studies indicate that basophils promote type 2 inflammation via production of IL-4 (25, 36–38). Employing IL-4–eGFP reporter (4get) mice, we observed elevated IL-4–eGFP+ cells following MC903 treatment (Fig. 4A), and ~70% of IL-4–eGFP+ cells were basophils (Fig. 4B), whereas only ~12% were T (CD3+CD5+) and B (CD19+) cells (data not shown, Fig. 4C). These data provoked the hypothesis that IL-4 may be a key factor through which basophils influence ILC2 responses in the context of skin inflammation. To directly test this, WT or Il42/2 mice were treated topically daily with MC903, and the skin was examined by flow cytometry on day 7. Basophil accumulation was unim-
paired in Il4−/− mice compared with WT mice (Fig. 4D, 4E), but skin ILC2 responses were significantly reduced following induction of AD-like inflammation (Fig. 4F, 4G). Flow cytometric analysis revealed that cutaneous ILC2s from WT mice expressed the IL-4Rα (Fig. 5A), suggesting that ILC2s are capable of responding to basophil-derived IL-4. Finally, to test whether basophil-derived IL-4 was required to induce cutaneous ILC2 responses, basophils were sort-purified from WT or Il4−/− mice and injected i.d. into the skin of naive WT mice. On day 4 following transfer of basophils, WT basophils induced local skin ILC2 responses whereas IL-4–deficient basophils did not (Fig. 5B, 5C). In association with decreased ILC2 responses in the skin, WT mice injected with Il4−/− basophils exhibited reduced inflammation as determined by histology (Fig. 5D, 5E) and ear thickness (Fig. 5F). Collectively, these findings indicate that skin ILC2 responses and AD-like disease are dependent on basophil-derived IL-4.

Although IL-4 critically promotes basophil-mediated cutaneous ILC2 responses and AD-like disease (Fig. 5), how IL-4 influences ILC2 function remains unclear. To test this, we employed Il4−/− mice that lack endogenous IL-4 and examined ILC2 development, survival, and proliferation. Examination of the bone marrow (BM) and the skin of WT versus Il4−/− mice in the steady-state revealed no significant differences in the frequencies of ILC2s between WT and Il4−/− mice (Fig. 6A, 6B). Additionally, consistent with a progenitor phenotype, ILC2s in the BM of both WT and Il4−/− mice lacked expression of KLRG1 (30), whereas skin ILC2s expressed KLRG1, indicating a mature phenotype (Fig. 6C). Taken together, these studies suggest that there are no defects in ILC progenitors in the BM or the skin between WT and Il4−/− mice. To test whether there was differential survival of ILC2s in WT versus Il4−/− mice, ILC2s in the skin were stained with 7-AAD and annexin V to assess for apoptosis. In contrast to small populations of 7-AAD+annexin V+ cells in the CD45+ pan-leukocyte compartment...
in both WT and $I^4^{-/-}$ mice, both 7-AAD and annexin V were not detected on ILC2s (Fig. 6D). These studies indicate that there is no enhancement of ILC2 apoptosis in $I^4^{-/-}$ mice.

To examine whether there were any differences in ILC2 proliferation, both WT and $I^4^{-/-}$ mice were treated with topical MC903 for 7 d, and proliferation was assessed by Ki67 staining.

**FIGURE 4.** Skin ILC2 responses are dependent on IL-4. (A) Skin IL-4–eGFP+ cells and (B) basophils as a percentage of IL-4–eGFP+ cells in MC903-treated 4get mice. (C) Frequencies of skin basophils and T and B cells as a percentage of IL-4–eGFP+ cells. (D) Skin basophils and associated (E) frequencies on day 7 in WT mice treated with EtOH or MC903 and $I^4^{-/-}$ mice treated with MC903. (F) Skin ILC2s and associated (G) frequencies on day 7 in WT mice treated with EtOH or MC903 and $I^4^{-/-}$ mice treated with MC903. Data are representative of three independent experiments; $n = 3–4$ mice/group/experiment. The $p$ values are indicated.

**FIGURE 5.** Basophil-derived IL-4 is necessary for the induction of skin ILC2 responses and AD-like disease. (A) Expression of IL-4Ra on ILC2s from WT and $I^{4ra^{-/-}}$ mice and CD11c+ DCs from WT mice. Data are representative of five individual mice. (B) Skin ILC2s in WT mice on day 4 following i.d. injection of PBS, or sort-purified basophils from TSLP cDNA-injected WT or $I^4^{-/-}$ mice and associated (C) frequencies, (D) histopathology, (E) histology score, and (F) ear thickness. Scale bars, 50 μm. Cell frequencies are noted as a percentage of Lin− cells. Data are representative of three independent experiments; $n = 4$ mice/group/experiment. The $p$ values are indicated.
Examination of the skin revealed proliferation of ILC2s in WT mice that was significantly diminished in Il4−/− compared with WT mice (Fig. 6E, 6F), suggesting that IL-4 supports ILC2 proliferation. Finally, to test whether basophil-derived IL-4 is necessary for the proliferation of ILC2s, TSLP-elicited basophils were sort-purified from WT and Il4−/− mice and stimulated in vitro with rmIL-3. Sort-purified CFSE-labeled ILC2s were then cultured in supernatants from either WT or Il4−/− basophils for 4 d. Strikingly, supernatants from WT basophils promoted the proliferation of ILC2s as measured by CFSE dilution whereas supernatants from Il4−/− basophils did not (Fig. 6G). Collectively, these studies indicate that although there are no detectable defects in ILC2 progenitor capacity or cell survival in the steady-state, basophil-derived IL-4 is critical for ILC2 proliferation in the context of skin inflammation.

**Discussion**

Recent studies demonstrated that ILC2s critically promote AD-like inflammation and significantly accumulate in lesional skin of AD patients (20, 39, 40). Additionally, basophils have also been shown to contribute to type 2 inflammation in the skin and accumulate in skin lesions of AD patients (9, 33). We have previously shown that TSLP elicits both basophils (9) and ILC2s (20) early in the context of AD-like disease. Despite these associations, whether functional interactions or cross-regulation occurs between these cell lineages has never been tested.

The present study provides three conceptual advances that broaden our understanding of the function and regulation of basophils and ILC2s. First, we show that both basophil and ILC2 populations accumulate in close proximity in human and murine inflamed skin. Second, we demonstrate a previously unrecognized role for basophils in promoting ILC2 responses in the skin. Third, we identify that basophil-derived IL-4 is necessary for the induction of ILC2 responses, optimal proliferation of ILC2s, and AD-like inflammation. Collectively, these findings indicate that coordinated interactions between basophils and ILC2s regulate skin inflammation.

Basophils have been implicated in human AD and have been shown to be initiators of chronic allergic dermatitis in mice (22, 33, 41). Furthermore, we have shown that TSLP-elicited basophils promote local Th2 cell responses in the context of AD-like disease and promote epicutaneous sensitization to food allergens (9, 10, 41). However, the mechanisms by which basophils contribute to type 2 cytokine–associated inflammation in the skin has been poorly defined. In the present study, we identify a previously unrecognized basophil–ILC2 axis in the elicitation of skin inflammation.

Emerging evidence indicates that ILC2s can interact with multiple cell populations to influence type 2 inflammation (3). We have previously shown that adoptive transfer of ILC2s can induce local Th2 cell responses and AD-like disease (20). Recent studies have also demonstrated that this process can be mediated by ILC2-
derived IL-13 influencing DC migration and/or contact-dependent stimulation of T cells by MHC class II or other factors (32, 42). Taken together, these studies highlight a role for ILC2s in promoting innate and adaptive immune responses. Furthermore, Roediger et al. (43) employed two-photon intravital microscopy in the skin to show that ILC2s suppress the function of other granulocytes such as mast cells in vivo. However, whether other upstream innate cellular mechanisms influence ILC2 function in the context of AD was not known. We now show that basophils can directly influence ILC2 function through the production of IL-4. Therefore, although ILC2s can be activated directly by epithelial cell–derived cytokines (3), it appears that hematopoietic cell–derived cytokines can also influence ILC2 function in the context of inflammation.

It has been appreciated that IL-4 is highly expressed in lesional AD skin and contributes to the pathogenesis of AD (13, 44, 45). Furthermore, other common γ-chain cytokines, such as IL-2 and IL-7, have shown to be critical for the development and survival of ILCs (43, 46). However, we found that IL-4 was dispensable for these processes but essential to promote ILC2 proliferation both in vitro and in vivo. This is consistent with the role of IL-4 in supporting the growth and proliferation of multiple other cell types such as T cells and B cells (47). However, our studies do not preclude other mechanisms by which basophils and IL-4 can directly promote ILC2 function. For example, basophil-derived IL-4 may influence the responsiveness of ILC2s to epithelial cell–derived cytokines such as IL-25, IL-33, and TSLP (3), or influence the activation and/or recruitment of ILC2s into inflamed skin. Motomura et al. (48) recently demonstrated a role for basophils in regulating ILC2 activation in the context of protease–induced airway inflammation. These findings, coupled with the present study, suggest that the capacity of basophils to promote ILC2 responses may be a conserved pathway at multiple barrier surfaces.

In addition to IL-4, basophils produce a variety of factors such as proinflammatory eicosanoids that can influence the function of other immune cells that promote type 2 inflammation (21). Therefore, other endogenous factors derived from basophils may also act in concert with IL-4 to modulate ILC2 function. In support of this, recent studies have shown that both murine and human ILC2s directly respond to leukotrienes (49, 50). Moreover, both IL-33 and TSLP are known to differentially regulate the production of cytokines and chemokines from basophils (9). However, how the differential regulation of basophils influences ILC2 function and type 2 inflammation remains to be fully defined. Notwithstanding this, the present study demonstrates that TSLP–elicited basophils and IL-4 production promote cutaneous ILC2 responses and proliferation, suggesting that selective targeting of TSLP and TSLP–dependent basophils could prove to be a beneficial strategy in the treatment of AD and related allergic diseases.

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Disclosure

The authors have no financial conflicts of interest.


